HYPHAL HETEROGENEITY IN *ASPERGILLUS NIGER*

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HYPHAL HETEROGENEITY IN *ASPERGILLUS NIGER*

Hyfe heterogeniteit in *Aspergillus niger*
(met een samenvatting in het Nederlands)

Proefschrift

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door
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geboren op 19 januari 1983
te Veghel

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CONTENTS


CHAPTER 1

GENERAL INTRODUCTION

Aspergillus niger

The genus *Aspergillus* comprises about 175 species that are among the most abundant fungi worldwide. Soil and decaying material are the natural habitats of Aspergilli and they are considered important food spoilage fungi (Pitt & Hocking, 1997). Although most Aspergilli normally exhibit a saprophytic life style, many of them (in particular *Aspergillus fumigatus* but including *Aspergillus niger*) can infect immuno-compromised animals and humans (de Hoog *et al.*, 2000).

A. niger is one of the most common *Aspergillus* species. It can be found all over the world and is particularly abundant in autumn and winter in the Northern hemisphere. *A. niger* can be seen with the naked eye as a fluffy layer on molded bread or fruit. This fluffy layer consists of aerial hyphae and conidiophores that carry black a-sexual conidiospores (Figure 1). These spores are dispersed by wind and insects and can give rise to a new mycelium. Germination of spores results in the formation of hyphae that extend at their apices. Sub-apical branching and hyphal fusion results in a mycelium, which consists of a dense network of interconnected hyphae. The cytoplasm in this mycelium is continuous due to highly porous septa that divide hyphae in compartments. These septa allow streaming of water, nutrients and even organelles from one compartment to the other. This is particularly functional when the mycelium colonizes a substrate in which nutrients and water are not uniformly distributed (Jennings *et al.*, 1974).

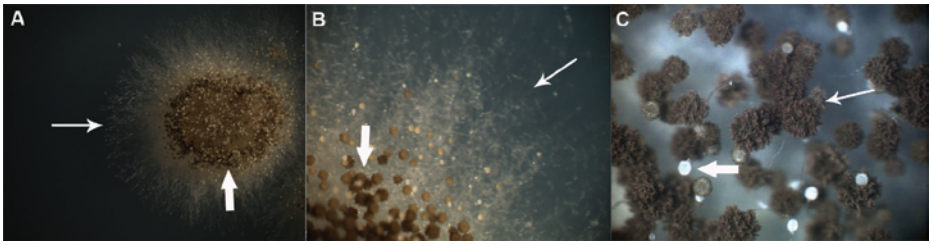

Figure 1 *A. niger* colony grown on solid medium in a Petri dish. (A) gives an overview of a growing colony. (B) zooms in on the periphery of the colony showing both aerial conidiophores and exploring hyphae. In these pictures exploring hyphae are indicated using thin arrows and conidiophores using thick arrows. In (C) mature conidiophores (thin arrowhead) can be seen which carry black spores as well as immature conidiophores (thick arrowhead) that were just formed and that have not yet formed pigments.

A. niger, like other fungi, secretes a wide variety and large amounts of enzymes. These enzymes degrade dead and living organic material into molecules that can be taken up to serve as nutrients. Protein secretion is thus an essential step in nutrient uptake and might therefore explain the exceptionally high secretion capacity of filamentous fungi in general and *A. niger* in particular. Apart from enzymes, *A. niger* secretes large amounts of organic acids such as citric acid (Nout, 2000). *A. niger* does not produce mycotoxins under the industrial conditions that are being used. Its fermentation products are therefore generally recognized as safe. This and the enormous secretion capacity make *A. niger* one of the most important industrial cell factories (Wösten *et al.*, 2007; Punt *et al.*, 2002). Its products are applied in different processes varying from the production of food, feed and beverages to paper and pharmaceuticals (for an example see Table 1). Commercial citric acid production with the use of *A. niger* was already initiated at the beginning of the twentieth century (Bennett, 1998). Production of glucoamylase is another example of the use of *A. niger.* Strains have been described that secrete up to 30 grams of this protein per liter (Finkelstein *et al.*, 1989). This starch degrading enzyme is used in the production of glucose syrups.

Table 1. Examples of enzymes of *A. niger* and their application in the food and feed industry (Wösten *et al.*, 2007).

Enzyme	Application(s)
α-Amylase	Preparation of starch syrup and dextrose; preparation of alcohol and beer.
Catalase	Preservation of color, texture, flavor, taste and aroma of frozen foods.
Cellulase	Brewing and baking; wine and juice production; improvement of digestibility of feed.
Glucoamylase	Saccharification of steamed rice and potato; preparation of glucose syrup.
Glucose oxidase	Removal of residual glucose or oxygen to increase shelf life; flavor and color stability; reduction of alcohol percentage in wine.
Lipase	Manufacturing of cheese, cheese flavors and other dairy products.
Proteases	Softening of dough; improvement of texture, elasticity and volume of bread; brewing; production of miso and tofu; flavor development in cheese; improving digestibility of animal feeds; preparation of soy bean milk and dehydrated soups; clarification of wine.
Xylanases	Production of food-additives; improvement of digestibility of feed; preparation of baking products; clarification of fruit juices.

Methods to culture *Aspergillus niger*

In nature, most filamentous fungi grow on solid substrates. Yet, in industrial settings, they are mainly cultivated in liquid media in large scale bioreactors. This industrial way of growth results in dispersed growth or in sphere-like micro-colonies, also called pellets. Growth and protein secretion within the mycelium are not easily studied in liquid cultures. Therefore, a two-dimensional way of growth on solid medium was developed (Wösten *et al.*, 1991). In this so called sandwiched culture, the colony is grown in a thin agarose layer between two perforated polycarbonate membranes. Gas, water, nutrients and proteins can freely diffuse through the membranes. In contrast, hyphae cannot grow through the pores of the membranes. This prevents growth of hyphae into the air or into the medium. The near two-dimensional growth of the sandwiched culture allows microscopic examination of individual hyphae. Moreover, colonies can be easily transferred to another agar medium or to a ring plate (Wösten *et al.*, 1991; Levin *et al.*, 2007a). The ring plate can be used to study zonal differences in protein secretion. It consists of a polycarbonate disc with concentric wells. The wells are 0.5 cm wide and 0.5 cm deep and can be filled with liquid medium.

Heterogeneity within a fungal mycelium

Zonal heterogeneity

A. niger forms a colony of several centimeters in diameter when it is grown for a week as a sandwiched culture on solid minimal medium. Approximately 50% of the 14,000 genes of *A. niger* (Pel *et al.*, 2007) were expressed in these sandwiched colonies when maltose and/or xylose were used as a carbon source (Levin *et al.*, 2007b). To assess whether different parts of the mycelium have a different expression profile, RNA was extracted from 5 concentric zones of the colony (Levin *et al.*, 2007b). 60% of the expressed genes were expressed on both carbon sources in all 5 concentric zones. This means that a considerable number of genes were expressed in particular zones or on a specific carbon source. 9% of the active genes were expressed in only one of five concentric zones on both xylose and maltose, whereas 3% of the genes were uniquely expressed in a single zone on one of the carbon sources. Moreover, more than 25% of the active genes showed at least a two-fold difference in expression between the outer and innermost zone of the colony.

A principal component analysis of the expression profiles of the zones of the xylose and maltose grown colonies showed that 37% of the variation in the gene expression could be attributed to the carbon source in the medium (Levin *et al.*, 2007b). Interestingly, 55% of the variation was related to the position of the zone in the colony. It was argued that this zonal variation could also be due to differences in the nutrient composition. Due to radial extension of the mycelial network the center represents the oldest part of the colony. Here, nutrients may have become limited or even completely exhausted. On the other hand, the periphery represents the youngest part of the mycelium and nutrients are expected to still be present in the underlying medium. Indeed, sandwiched *A. niger* colonies that had been grown for 7 days on minimal medium with 25 mM maltose or xylose as a carbon source had exhausted the

carbon source in the medium underlying the central and middle parts of the colony. In contrast, the carbon source was still available at the periphery. The spatial distribution of the phosphor, sulphur and nitrogen source was hardly affected by the growth of *A. niger* (Levin *et al.*, 2007b).

To study the effect of the carbon source limitation on the expression profiles in the centre of sandwiched colonies, xylose grown sandwiched colonies were either or not transferred for 24 h to fresh medium after 6 days of growth. By comparing the expression profiles of peripheral and central zones of transferred and non-transferred colonies it was shown that 50% of the variation in gene expression was medium independent.

Other studies have also implicated heterogeneity in the fungal vegetative mycelium. Two growth zones were identified in sandwiched colonies of *A. niger* (Figure 2A) and *Phanerochaete chrysosporium* (Wösten *et al.*, 1991; Moukha *et al.*, 1993). Secretion of proteins was confined to these two growth zones that are located at the periphery and the centre of the colony (Figure 2B). Interestingly, glucoamylase was shown to be secreted at the outer growth zone of colonies of *A. niger* (Wösten *et al.*, 1991) (Figure 2C). In contrast, lignin peroxidase was secreted in the central growth zone of *P. chrysosporium* colonies (Moukha *et al.*, 1993). Secretion and RNA profiles were also shown to be different in concentric zones of *Neurospora crassa* colonies (Kasuga & Glass, 2008). It is thus concluded that a fungal vegetative mycelium represents a heterogenic body, which results in zonal differences in growth, secretion and gene expression. This is an intriguing observation considering the phenomena of cytoplasmic continuity and cytoplasmic streaming.

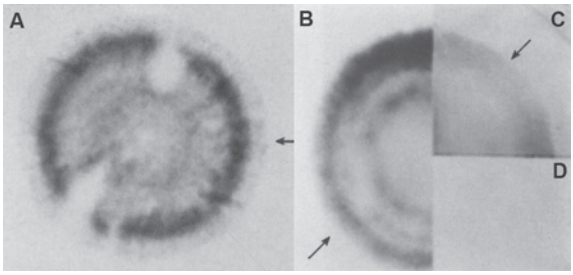

Figure 2 Localization of growth (A) and protein secretion (B, C) in sandwiched colonies of *A. niger*. Growth (A) was localized by autoradiography after labeling colonies with ^{14}C-labelled N-acetylglucosamine. Secretion was localized on PVDF membranes that had been placed under the colony by labeling with ^{14}C-labelled amino acids (B) or with an antiserum raised against glucoamylase (C). As a negative control a PVDF membrane treated with normal rabbit serum is shown (D). The arrow indicates the edge of the colony. The Figure is taken from (Wösten *et al.*, 1991).

Hyphal heterogeneity

Studies in *P. chrysosporium* showed that hyphae that secrete lignin peroxidase are much thinner when compared to the surrounding non-secreting hyphae (Moukha *et al.*, 1993). Morphological differences within a zone of a colony were also found in *N. crassa* between fusion-competent and fusion-incompetent substrate hyphae (Hickey *et al.*, 2002). Although hyphae at the periphery of *A. niger* colonies are similar in morphology, they do appear to be heterogenic. Immunolabeling indicated that neighboring hyphae

within the peripheral zone of a sandwiched colony do not all secrete glucoamylase (Figure 3A) (Wösten *et al.*, 1991). This was a surprising finding considering the fact that all these hyphae encountered similar environmental conditions. To investigate this phenomenon, expression of the *glaA* gene was studied at the periphery of an *A. niger* colony. To this end, the promoter of the AmyR regulated *glaA* gene was fused to *GFP*. GFP fluorescence of peripheral hyphae was studied after inducing the *glaA* promoter by transferring the sandwiched colony for several hours to a medium containing maltose (Vinck *et al.*, 2005). Although all hyphae exhibited green fluorescence, clear differences in the intensity of the signal were observed (Figure 3B). Statistical analysis of these fluorescence intensities showed that the deviations did not follow a normal distribution. Using the bootstrap re-sampling technique and mathematical modeling it became clear that the fluorescence deviations could be explained by a bimodal distribution. In other words, these hyphae do not comprise one population, but at least two populations (Figure 3C). The most simple explanation would be the presence of two populations; one population of hyphae lowly expressing *glaA* and one population of highly expressing ones. When *GFP* was directly fused to the *glaA* promoter, only 27% of the peripheral hyphae belonged to the lowly expressing type after 8 hours of induction. However, in these experiments the cytoplasmic GFP streamed intercellularly, which affected the analysis. By targeting GFP to the endoplasmic reticulum (ER) streaming was minimized and the number of hyphae expressing *glaA* at a low level increased to 50% (Vinck *et al.*, 2005). These findings show that neighboring hyphae are heterogenic despite the fact that they experience identical environmental conditions.

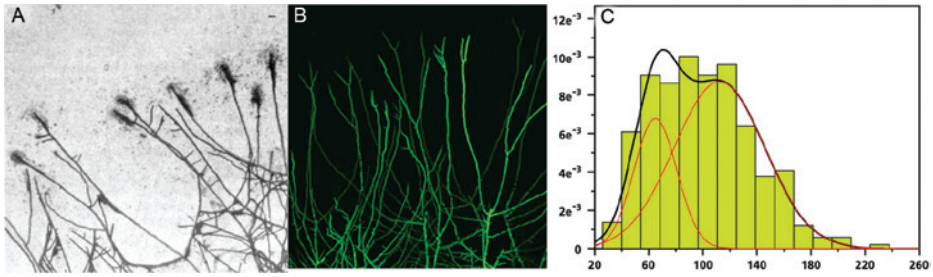

Figure 3 Heterogeneity at the periphery of an *A. niger* colony. A) Localization of glucoamylase by immunogold labeling shows that some hyphae secrete glucoamylase, whereas others do not B). Confocal microscopy of GFP fluorescence resulting from expression of the encoding gene from the *glaA* promoter. C) The fluorescence intensity distribution of hyphae depicted in (B) can be fitted assuming two populations of hyphae; one highly expressing the *glaA* gene, the other lowly expressing this gene. Figure A is taken from (Wösten *et al.*, 1991) and Figures B and C from (Vinck *et al.*, 2005).

Mechanisms behind hyphal heterogeneity

The mechanism(s) underlying hyphal heterogeneity are not yet elucidated. They could be caused by stochastic and/or epigenetic processes.

Stochasticity

Stochastic processes are often regarded as undesirable and unpredictable. However, living systems are inherently noisy and are optimized to function in the presence of stochastic fluctuations (McAdams & Arkin, 1999). It has been shown that organisms use stochastic fluctuations to introduce diversity in a population. This occurs for instance during competence development in *Bacillus subtilis* (Smits *et al.*, 2005). An important source of stochasticity is the random noise in transcription and translation. This can result in different rates of production of particular proteins in genetically identical cells that are exposed to identical environments (Elowitz *et al.*, 2002; Ozbudak *et al.*, 2002; Blake *et al.*, 2003). Noise is especially effective in the case of transcription factors that are lowly abundant in the cell. The effect of noise can even be amplified when the transcription factor stimulates its own expression (Ferrell Jr, 2002). As a result, two populations of cells emerge; those with and those without a significant number of molecules of a particular transcription factor. Subsequently, only in part of the cells target genes will be activated and this leads to heterogeneity. Taken together, heterogeneity in *glaA* expression in *A. niger* may be due to stochastic variation in expression of its regulator *amyR*.

Epigenetics

An epigenetic event is defined as the structural adaptation of chromosomal regions to register, signal or perpetuate altered activity states (Bird, 2007). It has been shown that epigenetic mechanisms are essential for development, cell differentiation and the integration of endogenous and environmental signals during the life of a cell or an organism (Jaenisch & Bird, 2003). It adds diversity and complexity to the genome of organisms. Epigenetic regulation is one of the crucial mechanisms of clonal expansion of cells to diverse cell types. Many epigenomes can be distinguished in an embryo, which are associated with a multitude of cell fates during development (Chinnusamy & Zhu, 2009). Even gene expression of individual adult stem cells varies widely, despite its phenotypic homogeneity (Ramos *et al.*, 2006). Epigenetic regulation is also found in the "lower eukaryotes". For instance, the dimorphic yeast *Candida albicans* creates biofilms that harbor heterogenic populations of cells as a consequence of epigenetic phenomena (Nobile & Mitchell, 2007). Possibly, epigenetic processes might also underlie hyphal heterogeneity in *A. niger*.

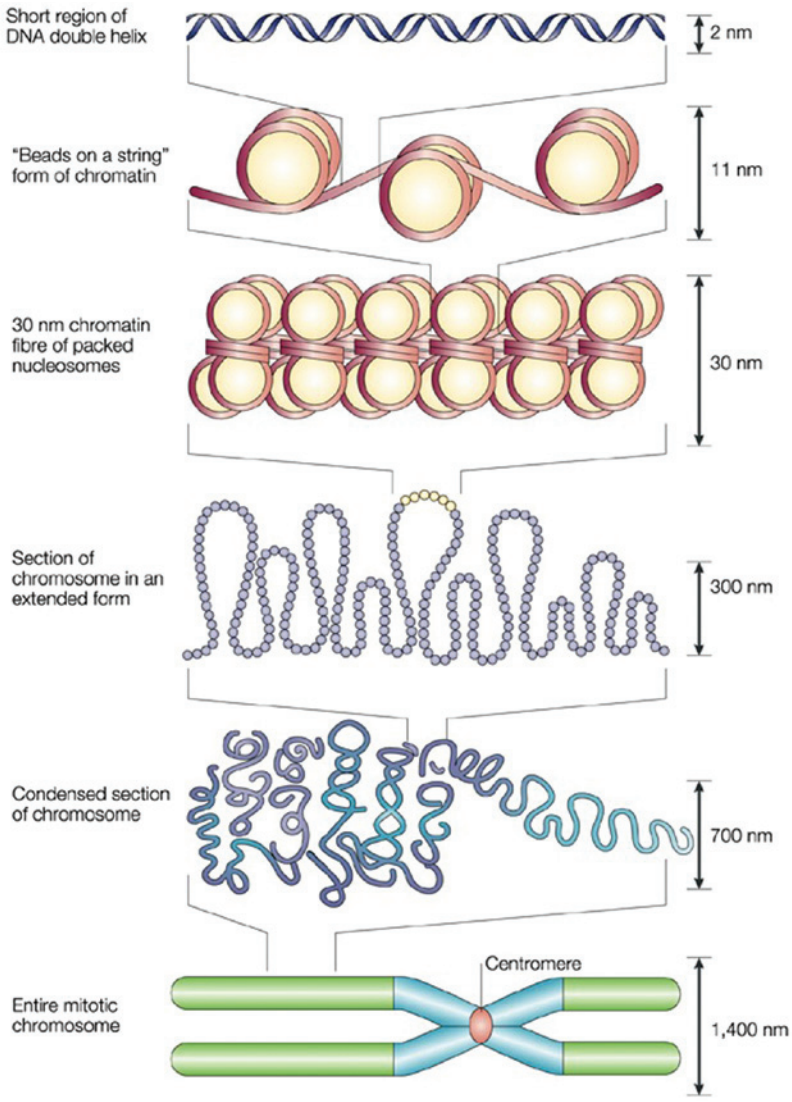

Figure 4 Chromatin structure. The double stranded helical structure of DNA is wrapped 1.65 times around 8 histone proteins, which together form a nucleosome. These nucleosomes fold up to produce a 30 nm fiber which again form loops averaging 300 nm in length. These 300 nm fibers are subsequently compressed and folded into 700 nm long fibers that are finally tightly coiled and form the chromatid of a chromosome. This Figure is taken from (Schlissel, 2003).

Chromatin is an organized structure of DNA and histone proteins (Figure 4). The basic unit of chromatin is the nucleosome, which consists of a histone core complex (H2A, H2B, H3 and H4), a piece of the DNA which is wrapped around it, and the histone linker (H1). The N-terminal tails of core histones undergo post-translational modifications such as (de)acetylation, methylation, phosphorylation and glycosylation. Moreover,

cytosine within the DNA undergoes methylation and demethylation. These histone and DNA modifications are critical in chromatin folding and thus determine the functional state of chromatin. A lightly packed structure allows active transcription and is called euchromatin. Heterochromatin, a tightly packed structure, does not permit transcription (Khorasanizadeh, 2004). Among the modifications, acetylation and methylation are by far the most extensively studied (Brosch *et al.*, 2008). Because these modifications are reversible, genes can be easily switched from an active to an inactive state and vice versa. Chromatin remodeling has been studied to some extent in Aspergilli. However, it hasn't been studied in *A. niger*. Its genome, however, does contain genes predicted to be involved in DNA methylation, histone methylation, histone deacetylation and histone acetylation (Table 2).

Table 2 Predicted DNA methyltransferase, histone methyltransferase, histone deacetylase and histone acetylase genes in the genome of *A. niger*.

Gene	JGI Function	Similarity (NCBI)
An04g01770	DNA methyltransferase 1 – associated protein	Strong similarity to DNMT1 associated DMAP1 – *Homo Sapiens*
An08g03380	Histone H3(lys9) methyltransferase SUV39 H1/Clr4	Strong similarity to clr4p – *Schizosaccharomyces pombe*
An01g00140	Histone H3 (Lys4) methyltransferase complex, subunit CPS60/ASH2/BRE2	Similarity to trithorax related protein Bre2 - *Saccharomyces cerevisiae*
An14g06850	Histone H3 (Lys4) methyltransferase complex and RNA cleavage factor II complex, subunit SWD2	Strong similarity to hypothetical WD-repeat protein - *Ajellomyces capsulatus*
An18g0684	Histone H3 (Lys4) methyltransferase complex, subunit SET1 and related methyltransferases	Similarity to trithorax protein All1 – *Homo sapiens*
An07g07850	Histone deacetylase complex, catalytic component RPD3	Strong similarity to histone deacteylase rpd3A – *Aspergillus nidulans*
An07g08380	Histone deacetylase complex, catalytic component RPD3	Strong similarity to histone deacetylase Hos2 – *Saccharomyces cerevisiae*
An16g01840	Histone deacetylase complex, catalytic component HDA1	Strong similarity to histone deacetylase – *Saccharomyces cerevisiae*
An14g00560	Histone deacetylase complex, catalytic component HDA1	Similarity to hypothetical protein – *Saccharomyces cerevisiae*
An02g14390	NAD-dependent histone deacetylases and class I sirtuins (SIR2 family)	Strong similarity to regulator protein Sir2 -*Saccharomyces cerevisiae*
An02g04100	Histone acetyltransferase type b catalytic subunit	Strong similarity to histone acetyltransferase Hat1 - *Saccharomyces cerevisiae*
An04g05760	Histone acetyltransferase (MYST family)	Similarity to histone acetyltransferase HBO1 - *Homo sapiens*
An16g03110	Histone acetyltransferase (MYST family)	Strong similarity to histone acetyl transferase hMOF - *Homo sapiens*
An02g13870	Histone acetyltransferase (MYST family)	similarity to transcription silencing protein Sas2 - *Saccharomyces cerevisiae*
An14g05810	Histone acetyltransferase SAGA/ADA, catalytic subunit PCAF/GCN5 and related proteins	Strong similarity to histone acetyltransferase Gcn5 - *Saccharomyces cerevisiae*

So far, there is no proof for DNA methylation in Aspergilli (Lee *et al.*, 2008). However, sexual development in *A. nidulans* was affected when a predicted DNA methyltransferase gene, called *dmtA*, was deleted. From these results it was concluded that *A. nidulans* has a low, undetectable, methylation activity or that the gene has another unknown function (Lee *et al.*, 2008). There is also relatively little known about histone methylation in filamentous fungi. In many eukaryotes, methylation of lysine 9 of histone 3 (H3K9) is a hallmark of heterochromatin formation and subsequent gene silencing. The H3K9 methyltransferase of *Schizosaccharomyces pombe* is Clr4. Inactivation of the *clr4* homologue in *A. fumigatus* resulted in reduction in radial growth. Moreover, the number of conidia that were produced was reduced and their formation was delayed (Palmer *et al.*, 2008). The latter was caused by a delayed expression of *brlA*, which is the master regulator of conidiophore development.
The degree of acetylation of histone proteins is the result of histone acetyl transferases (HATs) and histone deacetylases (HDACs). Generally, histone acetylation is associated with gene activation. However, there are also examples of activation of genes as a result of deacetylation (Brosch *et al.*, 2008). Filamentous fungi, among which the Aspergilli, contain various genes encoding HATs. These redundant enzymes have hardly been studied. In contrast, gene deletions and the use of inhibitors have shown that HDACs affect diverse processes in fungal growth and development. Gene *hdaA* of *A. fumigatus* was shown to be involved in germination and secondary metabolite production (Lee *et al.*, 2009), whereas *hdaA* of *A. nidulans* was shown to function in secondary metabolite production and growth under oxidative stress (Tribus *et al.*, 2005; Shwab *et al.*, 2007). HDACs have also been shown to be involved in regulation of genes involved in primary metabolism. Treating *A. nidulans* with the histone deacetylase inhibitor trichostatin A resulted in activation of promoters of genes involved in proline metabolism (García *et al.*, 2004). A similar but stronger effect was observed in mutants affected in the catabolite repressor CreA. The authors concluded that the effect of CreA depends on the acetylation state of the chromatin. Conversely, the histone deacetylase HDC1 of the maize pathogen *Cochliobolus carbonum* seems to influence expression of metabolic genes independently from CreA (Baidyaroy *et al.*, 2001). This was based on the observation that inactivation of *hdc1* of *C. carbonum* resulted in reduced expression of both *creA* and genes encoding enzymes involved in degradation of the host cell wall.

Outline of the thesis
The aim of this PhD thesis was to explore the extent of heterogeneity in the mycelium of *A. niger* and to study the mechanism underlying this phenomenon. Understanding this mechanism is of interest not only from a fundamental but also from an applied point of view. Heterogeneity within a culture could be a target to improve protein secretion in fungal cell factories.

An efficient transformation protocol is required to study mechanisms involved in growth and development in *A. niger.* Novozyme 234 was traditionally used to make protoplasts for transformation. However, this enzyme cocktail is no longer on the market. **Chapter 2** describes a new enzyme cocktail to protoplast *A. niger*. The cocktail consists of lysing enzymes from *Trichoderma harzianum*, chitinase from *Streptomyces griseus* and

β-glucuronidase from *Helix pomatia*. The generated protoplasts were used to efficiently transform *A. niger*.

Previously, it has been shown that zones of macro-colonies grown on solid medium are heterogenic with respect to gene expression and protein secretion. In **Chapter 3** it was assessed whether heterogeneity can also be found between and within micro-colonies of a liquid shaken culture. To study heterogeneity between micro-colonies, *A. niger* strains were used that express *GFP* from the promoter of the amylolytic *glaA* gene or the promoter of the xylanolytic *faeA* gene. Colonies formed in a liquid culture were sorted on basis of diameter and fluorescence using a particle sorter (COPAS technique). Both the distribution of fluorescence intensity and micro-colony diameter were not normally distributed in the *GFP* expressing strains. This indicates that at least two populations of micro-colonies are present within a liquid shaken culture with respect to size and gene expression.

Central and peripheral parts of the mycelium of the strain expressing *GFP* from the *glaA* promoter were isolated by laser micro-dissection and pressure catapulting to assess heterogeneity within a micro-colony (**Chapter 3**). QPCR showed that similar amounts of *18S* rRNA and RNA of the *actin*, *glaA* and *faeA* genes had been isolated from both zones. However, the RNA from the centre was derived from 50 times more hyphal material than that from the periphery of the micro-colony.

Chapter 4 demonstrates that not only expression of the amylolytic gene *glaA* is heterogenic at the periphery of macro-colonies, but also that of the amylolytic gene *aamA* and the xylanolytic genes *aguA* and *faeA*. Co-expression studies, using GFP and dTomato as reporters, showed that hyphae highly expressing one of these genes, also highly expressed the other genes encoding secreted proteins. Moreover, expression levels correlated with that of the amylolytic regulatory gene *amyR* and the constitutively expressed gene *gpdA*. *In situ* hybridization demonstrated that hyphae highly expressing the selected genes also have a high rRNA content. It is proposed that two types of hyphae can be distinguished at the periphery of colonies of *A. niger;* those with a high and those with a low transcriptional and translational activity.

The *A. niger* strain expressing *GFP* from the *glaA* promoter was treated with butyrate and 5-azacytidine to explore whether epigenetic mechanisms underly the hyphal heterogeneity at the periphery of the colony (**Chapter 4**). These chemicals create a euchromatin structure by inhibiting deacetylases and (DNA) methyltransferases, respectively. Using confocal microscopy and statistical analysis it was demonstrated that butyrate and 5-azacytidine affect the bimodal *glaA* distribution. This indicates that chromatin modification plays a role in hyphal heterogeneity.

Chapter 5 describes a protocol for isolation and amplification of small amounts of RNA from selected (sub-cellular parts of) hyphae. This protocol was used to compare the RNA profile of the tips of the most outer hyphae of the colony with that of the periphery of the colony as a whole. To this end, *A. niger* Affymetrix arrays were hybridized with cDNA amplified from RNA of 500 hyphal tips and from an equivalent amount of RNA from the periphery of the mycelium. Several functional gene categories, such as transport

facilitation, transcription, cell rescue, defense, and virulence, and regulation of or interaction with cellular environment, were found to have a more significant contribution to the gene expression in hyphal apices. These data imply that hyphal tips of exploring hyphae are adapted to grow actively and to anticipate to the uncolonized environment they encounter.

Differences in gene expression between 5 neighboring single hyphal tips was assessed in **Chapter 6**. The cDNA resulting from amplification of 1 picogram of RNA from these individual hyphae was used for QPCR and microarray analysis. QPCR showed that levels of *18S* rRNA, and of RNA of the *actin* gene and the glucoamylase gene *glaA* were heterogenic between the neighboring hyphae. Heterogeneity was much less, if present at all, when cDNA was used from pools of 100 hyphal tips or from mycelium of the whole periphery. Microarray analysis resulted in a present call for 4-7% of the *A. niger* genes from which 12% showed heterogenic RNA levels. These genes belonged to a wide range of functional gene categories, which indicates that heterogeneity in exploring hyphae is caused by stochastic gene expression and / or by epigenetic processes.

The results are summarized and discussed in **Chapter 7**.

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CHAPTER 2

ABSTRACT

Filamentous fungi are commonly genetically modified using protoplast transformation. Novozym 234 has been used for many years to prepare protoplasts but is no longer on the market. Therefore, a new enzyme cocktail was formulated to protoplast *Aspergillus niger*. A 20 ml incubation volume with 2.5 grams wet weight mycelium resulted in 15-40 million protoplasts when 5 mg ml^{-1} lysing enzymes from *T. harzianum* were combined with 0.15 units ml^{-1} chitinase from *Streptomyces griseus*, and 460 units ml^{-1} β-glucuronidase from *Helix pomatia.* 50% and 30% of the protoplasts, respectively, regenerated when they had been plated directly or had first been stored at -80 °C using a Nalgene Mr Frosty. In both cases, 0.02% of the regenerating protoplasts could be transformed.

AN ENZYME COCKTAIL FOR EFFICIENT PROTOPLAST FORMATION IN *ASPERGILLUS NIGER*

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INTRODUCTION

Filamentous fungi can be genetically transformed by biolistics methods, by electroporation, by using *Agrobacterium tumefaciens* and by treating protoplasts with polyethylene glycol. Electroporation and biolistics have been shown to be inefficient in *Aspergillus* (Meyer *et al.*, 2003). In contrast, protoplast transformation and *A. tumefaciens*-mediated transformation (ATMT) result in high numbers of transformants in this genus (Meyer *et al.*, 2003; Goosen *et al.*, 1987; Sugui et al., 2005; Michielse *et al.*, 2005; Gouka *et al.*, 1999; Dawe *et al.*, 2000; Punt *et al.*, 1987). Both methods can be used in *Aspergillus* for gene inactivation by homologous recombination. However, the copy number of introduced DNA is generally lower with ATMT, which may hamper over-expression of genes.

The enzyme cocktail Novozyme 234 (Novo Industries) was commonly used to generate protoplasts of *A. niger*. However, this cocktail is no longer on the market. We here report an enzyme cocktail that results in high protoplast numbers. Regeneration efficiency is reduced only by 40% when protoplasts have been stored at -80 °C after being frozen using a Nalgene Mr Frosty. Both fresh and stored protoplasts could be efficiently transformed.

MATERIAL AND METHODS

Protoplast formation

Strain N593 of *A. niger* (*pyrA*$^{-}$, *cspA*), which is derived from N402 (Goosen *et al.*, 1987), was grown at 30 °C in 1 L Erlenmeyer flasks with 250 ml transformation medium (Kusters-van Someren *et al.*, 1991). After growing for 16 h at 250 rpm, mycelium was harvested by filtration over a Büchner funnel with nylon gauze. After washing with

0.9% NaCl (w/v) (Peraza *et al.*, 2003), 0.3 grams of mycelium (25 mg dry weight) was resuspended in 20 ml stabilization buffer (SB; 0.2 M phosphate buffer (pH 6.0), 0.8 M sorbitol). 5 mg ml^{-1} lysing enzymes from *T. harzianum*, 0.15 units ml^{-1} chitinase from *Streptomyces griseus* and 460 units ml^{-1} β-glucuronidase from *Helix pomatia* (all from Sigma) were added and the mixture was incubated in a 100 ml glass bottle for 2 h at 37 °C and 130 rpm. Mycelial debris was removed by filtration over sterile glass wool (superfine from Assistant). Protoplasts were washed two times in 45 ml cold STC (1.33 M sorbitol, 50 mM $CaCl_2$ and 10 mM Tris/HCl pH 7.5) (Kusters-van Someren *et al.*, 1991) with centrifugation steps at 2000 rpm at 4 °C for 10 min. Protoplast yield (protoplasts ml^{-1}) was determined by using a Neubauer haemocytometer (Marienfeld). Protoplasts were either or not preserved in STC at -80 °C using a Nalgene Mr Frosty.

Protoplast transformation

Protoplasts were transformed with pGW635 (plasmid containing *pyrA* gene; CBS 513.88) according to Kusters-van Someren *et al.* (1991). After transformation, protoplasts were taken up in 5 ml MMST (minimal medium pH 6.0, 0.95 M sucrose and 0.6% agar) and spread on selective (when transformed) or non-selective 25 ml MMS (minimal medium pH 6.0, 0.95 M sucrose and 1.2% agar) plates.

RESULTS

Addition of 5 mg ml^{-1} lysing enzymes (Sigma, St. Louis, MO, USA) to the protoplasting buffer did not result in any protoplasts of the *A. niger* strain N539. Similarly, no protoplasts were obtained when an enzyme mixture was used that was previously used to protoplast *Aspergillus* sp. FP-180 and *Aspergillus awamori* NRRL-3112 (Peraza *et al.*, 2003). In this cocktail, 2-10 mg ml^{-1} lysing enzymes from *T. harzianum* were combined with 0.008 or 0.015 units ml^{-1} chitinase from *Streptomyces griseus,* 0.03 or 0.06 units ml^{-1} hemicellulase from *A. niger* and 460 or 690 units ml^{-1} β-glucuronidase from *Helix pomatia* (all from Sigma, St. Louis, MO, USA). Increasing the concentration of β-glucuronidase or hemicellulase 2-10 fold did not improve the protoplasting (Table 1). In contrast, a 10-fold increase in the concentration of chitinase resulted in the release of 1.8×10^6 protoplasts. When the incubation volume was scaled up from 10 to 20 ml, 5-12-fold more protoplasts were obtained. Increasing the mycelial wet weight from 0.6 to 2.5 grams (*i.e.* from 50 to 200 mg dry weight) linearly increased the number of protoplasts. This was only observed when all mycelial material was present at the start of protoplasting (Table 1).

Table 1 Improvement of *A. niger* protoplasting using a starting enzyme cocktail containing 5 mg ml^{-1} lysing enzymes from *T. harzianum*, 0.015 units ml^{-1} chitinase from *S. griseus*, 0.06 units ml^{-1} hemicellulase from *A. niger* and 460 units ml^{-1} β-glucuronidase from *H. pomatia*.

Volume	Alteration enzyme cocktail	mg mycelium (dry weight)	Yield x 10^6	Yield x 10^6 prot./ml
10 ml	-	25	-	-
10 ml	2x β-glucuronidase	25	-	-
10 ml	10x β-glucuronidase	25	-	-
10 ml	2x chitinase	25	-	-
10 ml	10x chitinase	25	1.8	4.3
10 ml	10x chitinase	100	7.3	12.8
20 ml	10x chitinase	50	8.6	16.0
10 ml	10x chitinase	25+25 after 1h	2.0	4.0
10 ml	10x chitinase, no hemicellulase	25	1.7	3.0
10 ml	10x chitinase, no hemicellulase	100	4.8	4.8
20 ml	10x chitinase, no hemicellulase	50	22.0	22.0
20 ml	10x chitinase, no hemicellulase	200	14.4	18.0
10 ml	10x chitinase, no hemicellulase, no β-glucuronidase	25	0.3	0.8
20 ml	10x chitinase, no hemicellulase	50	32.0	32.0
20 ml	10x chitinase, no hemicellulase	200	39.5	39.5

Taken together, a 20 ml incubation volume with a 10-fold increased chitinase activity (*i.e.* 0.15 units ml^{-1}) and a 4 times increased mycelial biomass (*i.e.* 2.5 grams wet weight) resulted in 15-40 x 10^6 protoplasts. A similar number of protoplasts was obtained when the hemicellulase was discarded from the enzyme cocktail. The number of protoplasts was also not affected when β-glucuronidase was discarded from the enzyme cocktail. However, in this case large mycelial clumps were observed trapping most of the protoplasts, which hampered their harvest (Figure 1). About 50% of the protoplasts regenerated when plated on a non-selective medium (minimal medium pH 6.0, 0.95 M sucrose and 1.2% agar). Freezing in STC at -80 °C (by placing the protoplasts directly in the freezer) reduced regeneration with 90%. However, freezing at -80 °C using Nalgene Mr Frosty reduced regeneration efficiency with only 40%. Up to 0.02% of the regenerating protoplasts (either or not stored at -80 °C) could be transformed with 1 µg of plasmid pGW635, which contains the *pyrA* gene (Kusters-van Someren *et al.*, 1991). Protoplasting and transformation efficiency were similar when strain NW249 *(leuA1, pyrA1, nicA1,* Δ*argB, cspA1)* (Vondervoort and Muller, unpublished data) was used instead of N539.

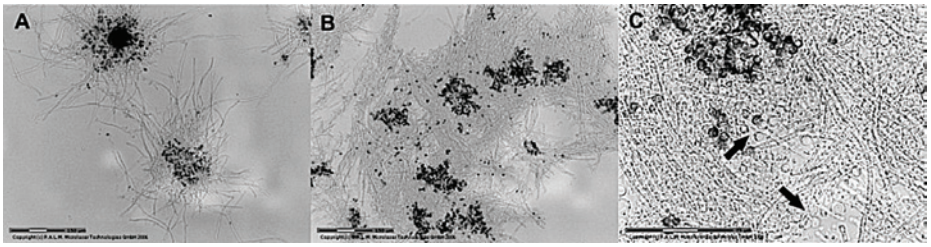

Figure 1 Incubation of *A. niger* mycelium in the presence (A) and absence (B, C) of β-glucuronidase in the lysing enzyme cocktail. C represents a magnification of clumped mycelium that traps protoplasts (indicated with arrows). Bar represents 150 (A, B) and 75 µm (C).

DISCUSSION

Since Novozym 234 (Novo Industries) is no longer on the market, a new enzyme cocktail had to be formulated to obtain protoplasts for genetic transformation of *A. niger*. Here, a cocktail mixture is described that can be used to make protoplasts of *A. niger.* These protoplasts can be efficiently transformed either or not after storage at -80 °C using a Nalgene Mr Frosty. In the cocktail mixture lysing enzymes are combined with a relatively high activity of chitinase. β-glucuronidase had no effect on the number of protoplasts. However, this enzyme is necessary to avoid clumping of cells. A similar phenomenon has been observed with *A. awamori* and *Aspergillus* strain FP-180 (unpublished data). β-glucuronidase is also used in other protoplasting protocols such as that of *Aspergillus nidulans* (Jung *et al.*, 2000) and various strains of red-pigmented yeasts (Evans and Conrad, 1987). It may be that also in these cases this enzyme somehow prevents clumping of cells.

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CHAPTER 3

ABSTRACT

The fungus *Aspergillus niger* forms (sub) millimeter micro-colonies within a liquid shaken culture. Here, it is shown that such micro-colonies are heterogenic with respect to size and gene expression. Micro-colonies of strains expressing *GFP* from the promoter of the glucoamlyase gene *glaA* or the ferulic acid esterase gene *faeA* were sorted on basis of diameter and fluorescence using the Complex Object Parametric Analyzer and Sorter (COPAS). Statistical analysis revealed that the liquid shaken culture consisted of two populations of micro-colonies that differ 90 µm in diameter. The population of small micro-colonies of strains expressing *GFP* from the *glaA* or *faeA* promoter comprised 39% and 25% of the culture, respectively. Two populations of micro-colonies could also be distinguished when expression of *GFP* in these strains was analyzed. The population lowly expressing *GFP* consisted of 68% and 44% of the whole population, respectively. It is also shown that mRNA accumulation is heterogenic within micro-colonies of *A. niger*. Central and peripheral parts of the mycelium were isolated with laser micro-dissection and pressure catapulting (LMPC) and RNA from these samples was used for quantitative PCR analysis. This showed that RNA content per hypha was about 45 times higher at the periphery than in the centre of the micro-colony. Our data imply that protein production of *A. niger* can be improved in industrial fermentations by reducing the heterogeneity within the culture.

HETEROGENEITY IN MICRO-COLONIES OF *ASPERGILLUS NIGER* IN LIQUID SHAKEN CULTURES

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INTRODUCTION

Germination of fungal spores results in the formation of hyphae that grow at their apices and that branch sub-apically. As a result, a network of interconnected cells is formed that is called a mycelium. Such a mycelium can be small (micro-colonies with a diameter in the (sub)millimeter scale) or large (macro-colonies with a diameter in the centimeter to meter scale). The cytoplasm within these mycelia is believed to be continuous. This is due to the fact that the septa that divide hyphae in compartments have large pores that allow intercellular streaming of molecules and even organelles (Jennings *et al.*, 1974).

Filamentous fungi secrete large amounts of enzymes into the environment. These enzymes degrade the substrate into small molecules that can be taken up by the fungus to serve as nutrients. *Aspergillus niger* is an example of a fungus with an enormous secretion capacity. Some strains secrete up to 30 gram of protein per liter (Finkelstein *et al.*, 1989), which makes this fungus an important production platform for industrial and pharmaceutical proteins (Conesa *et al.*, 2001; Punt *et al.*, 2002). *A. niger* forms macro-colonies on a solid substrate. Using such colonies, it was shown that proteins are only secreted by the limited number of growing hyphae within the mycelium (Wösten *et al.*, 1991; Moukha *et al.*, 1993). Over and above this, not every growing hypha secretes a particular protein. For instance, glucoamylase is secreted by growing hyphae at the periphery of a macro-colony of *A. niger* but not by the growing hyphae in the central zone (Wösten *et al.*, 1991). In contrast, lignin peroxidase is secreted in the central growth zone but not at the periphery of macro-colonies of *Phanerochaete chrysosporium* (Moukha *et al.*, 1993). Taken together, it can be concluded that a fungal macro-colony is not a mass of identical hyphae. Indeed, RNA profiles of concentric zones of macro-colonies of *A. niger* are distinct (Levin *et al.*, 2007). For instance, 9% of the genes that are active in a macro-colony are expressed in only one of five concentric zones. Moreover, more than 25% of the active genes show at least a two-fold difference in expression between the outer and innermost zone of the colony. As an

example, expression of the glucoamylase gene *glaA* was more than 3-fold higher at the periphery of maltose-grown colonies when compared to the centre of the mycelium. Similarly, the ferulic acid esterase gene *faeA* was five times higher expressed at the periphery of xylose grown colonies. The differences in gene expression within a macro-colony of *A. niger* can be explained by the availability of the carbon source and, to a similar extent, by medium independent mechanisms (Levin *et al.*, 2007). Differences in zonal expression have also been found in macro-colonies of *Neurospora crassa* (Kasuga & Glass, 2008) and *Aspergillus oryzae* (Masai *et al.*, 2006), suggesting that this is a widespread phenomenon in the fungal kingdom.

In industry, *A. niger* is grown in bioreactors. Under this condition, *A. niger* forms micro-colonies instead of macro-colonies. Here, it is addressed whether micro-colonies within a liquid shaken culture of *A. niger* are heterogenic with respect to size and gene expression. Moreover, it was assessed whether zones within individual micro-colonies are heterogenic with respect to gene expression. In this study, laser micro-dissection and pressure catapulting (LMPC) was used as well as the Complex Object Parametric Analyzer and Sorter (COPAS). In fact, COPAS was used for the first time in fungal microbiology.

MATERIALS AND METHODS

Strains

Strains used in this study are listed in Table 1.

Table 1 Strains used in this study.

Strain	Expression construct	Parental Strain	Genetic Background	Reference
N593	-		*pyrA6 cspA1*	Goosen *et al.*, 1987
AR9#2	*PglaA::GFP*	AB4.1	7 copies of the reporter construct at 4 different sites in the genome	Siedenberg *et al.*, 1999
CB-A110.5	*PaamA::dTomato*	N593	11 copies of the reporter construct at 9 different sites in the genome	This study
CB-A121.4	*PglaA::GFP* and *PaamA::dTomato*	CB-A112.1	copies of the GFP-reporter construct not determined for this strain 8 copies of the dTomato-reporter construct at 6 different sites in the genome	Vinck *et al.*, 2010; **Chapter 4**
UU-A005.4	*PfaeA::GFP*	NW249	20 copies of the reporter construct at 13 different sites in the genome	Vinck *et al.*, 2010; **Chapter 4**
CB-A109.1	*PglaA::dTomato*	N593	not determined	This study
CB-A118.24	*PfaeA::GFP* and *PglaA::dTomato*	UU-A005.4	20 copies of the GFP-reporter construct at 13 different sites in the genome 5 copies of the dTomato-reporter construct at 4 different sites in the genome	Vinck *et al.*, 2010; **Chapter 4**

Media and culture conditions

Spores of *A. niger* strains AR9#2 were isolated from minimal medium plates containing 3% xylose, whereas spores of UU-A005.4 and N593 were isolated from plates containing 2% glucose. Xylose represses expression of *glaA* (Boel *et al.*, 1984), while glucose represses *faeA* (de Vries & Visser, 1999). Liquid shaken cultures were inoculated with 7 10^8 spores ml^{-1} and incubated for 16 h at 30 °C and 250 rpm in 1 L Erlenmeyer flasks with 250 ml transformation medium (TM) (Kusters-van Someren *et al.*, 1991). Maltose (50 mM) or xylose (50 mM) was used as a carbon source. N593 was grown in the presence of uridine (0.2 g l^{-1}), while nicotinamide (1 mg l^{-1}), leucine (0.2 g l^{-1}) and arginine (0.2 g l^{-1}) were added in the case of UU-A005.4.

Flow cytometry using COPAS PLUS

Micro-colonies were harvested by filtration over a Büchner funnel with nylon gauze, washed with 50 ml PBS and fixed for 15 min at room temperature with 4% formaldehyde in PBS. The fixative was removed by washing 2 times in excess PBS using centrifugation at 3000 g for 5 min to pellet the micro-colonies. Micro-colonies were simultaneously sorted based on size (Time of Flight, TOF) and fluorescence using a COPAS PLUS profiler equipped with a 1 mm nozzle (Union Biometrica). Sorting parameters were set in such a way that clusters of colonies or debris were excluded from the analysis. GFP fluorescence was detected with a 488 nm solid state laser combined with a Green PMT 514/23 nm optical emission filter. To relate micro-colony diameter to TOF, the diameter of 20 sorted micro-colonies were determined by microscopy. These measurements showed that the relation between colony diameter and TOF can be described as Diameter (µm) = 0.46 * TOF + 250.

Laser micro-dissection and laser pressure catapulting

Laser micro-dissection and laser pressure catapulting (LMPC) was performed with the PALM CombiSystem (Carl Zeiss MicroImaging) equipped with an Axiovert 200M Zeiss inverted microscope (Carl Zeiss AG) and a 3CCD color camera (HV-D30, Hitachi Kokusai Electric Inc.). The 10x objective was routinely used. The 40x objective was used when it was impossible to catapult mycelium at once.

Micro-colonies were harvested by filtration over a Büchner funnel with nylon gauze, fixed with 70% ethanol on a 1 mm PEN membrane-covered microscope glass slide (Carl Zeiss MicroImaging) and subsequently air dried. Micro-colonies of approximately 750 µm were selected and cut using LMPC. Larger colonies can not be cut by the laser because they exceed the depth of field of the optical system and are therefore beyond the focus of the laser. Four zones within the micro-colonies were assigned using the PALM RoboSoftware (v4.0), after which the laser dissected and catapulted these zones from the centre (zone 1) to the periphery (zone 4). Laser intensity was set at 80% to cut the inner three zones, whereas 40% laser intensity was used to cut the PEN membrane in front of the outer hyphae of zone 4. The inner zone 1 could not be catapulted at once because some hyphae were still attached to the hyphae in zone 2. These hyphae were disconnected using laser pressure catapulting (LPC). The catapulting energy was set automatically. To this end, the laser focal point was defined and set at a minimum power still enabling cutting of the membrane. The micro-dissected material was catapulted into

the cap of a sample tube that had been placed above the section. The cap contained 50 µl RNA*later* (Qiagen) to enable RNA extraction.

RNA isolation

Individual micro-colonies or parts thereof were soaked in 50 µl RNA*later* (Qiagen), after which the material was snap-frozen in liquid nitrogen in a 2 ml Eppendorf tube to which 2 metal bullets (4.76 mm in diameter) were added. Subsequently, samples were ground in a Micro-Dismembrator U (B. Braun Biotech Int.) in a chilled container at 1500 rpm for 60 s. The frozen material was taken up in 250 µl Trizol Reagent (Invitrogen) by vortexing. After removing the metal bullets, 200 µl chloroform was added. After mixing well, samples were centrifuged at 10.000 g for 10 min. The water phase (usually around 200 µl) was mixed with 700 µl RLT from the RNeasy MinElute Cleanup Kit (Qiagen) to which 143 mM β–mercaptoethanol was added. RNA was purified following instructions of the kit. The purified RNA was eluted using 14 µl RNAse free water.

cDNA synthesis and Quantitative PCR analysis

cDNA was synthesized from total RNA using the QuantiTect Reverse Transcription Kit (Qiagen). QPCR was performed using the ABI Prism 7900HT SDS and SYBR Green chemistry (Applied Biosystems). Cycle threshold (Ct) levels were measured for *18S* rDNA, the *actin* gene, and for *glaA* and *feaA*. Primers were designed according to the recommendations of the PCR master-mix manufacturer (Applied Biosystems). Levels of mRNA of the *actin* gene were determined with the primers QPCRactFW1 and QPCRactRV1 and those of *18S* rDNA with the primer pair QPCR18SFW1 and QPCR18SRV1 (Table 2). These products had an amplification efficiency of 2. Complementary DNA of *glaA* and *faeA* were amplified using primer pairs QPCRglaAFW3 and QPCRglaARV3, and QPCRfaeAFW4 and QPCRfaeARV4, respectively (Table 2). *glaA* cDNA was amplified with an efficiency of 1.98 and *faeA* cDNA with an efficiency of 1.92.

Table 2 Primers used in this study.

Primer name	Primer sequence
QPCRactFW1	5′-GTTGCTGCTCTCGTCATT-3′
QPCRactRV1	5′-AACCGGCCTTGCACATA-3′
QPCR18SFW1	5′-GGCTCCTTGGTGAATCATAAT-3′
QPCR18SRV1	5′-CTCCGGAATCGAACCCTAAT-3′
QPCRglaAFW3	5′-GCACCAGTACGTCATCAA-3′
QPCRglaARV3	5′-GTAGCTGTCAGATCGAAAGT-3′
QPCRfaeAFW4	5′-GACGGCATCCCAAACTT-3′
QPCRfaeARV4	5′-CTCACAGCACTGTACTTCAT-3′

Acridine orange staining and fluorescence microscopy

A. niger micro-colonies were stained with acridine orange as described (Freudenberg *et al.*, 1996). Micro-colonies were fixed in 70% ethanol, after which 1/10th volume of 4 mM acridine orange in PBS (Becton Dickinson) was added. After staining for 5 min, the micro-colonies were washed twice with PBS. Fluorescence of acridine orange was monitored using a Zeiss Axioscope 2PLUS equipped with a HBO 100 W mercury lamp and a Leica LFC 420C camera (2592x1944 pixels) using standard FITC and TRITC filters.

Statistical analysis

Statistical significance of differences in QPCR data were tested using a t-test. Flow cytometry data were subjected to descriptive statistics using SPSS software. Normality of the data was tested using the Kolmogorov-Smirnov test (KS-test) with the Lilliefors correction. To assess whether distributions in size and fluorescence can be explained by a mixture of two normally distributed components, the data were modeled in the probability distribution (Vinck *et al.*, 2005): $\varphi : \varphi(x) = pN(x; \mu_1, \sigma_1) + (1 - p)\ N(x; \mu_2, \sigma_2)$, where $0 < p < 1$ and $x \rightarrow N(x; \mu, s)$ is the probability density of the normal distribution with parameters μ and s. This model describes a mixture of a $N(\mu_1, \sigma_1)$-distribution and a $N(\mu_2, \sigma_2)$-distribution in which the degree of participation of the component $N(\mu_1, \sigma_1)$ is p. The confidence interval for the participation frequency of component 1 was set to 95%. In the statistical analysis the five parameters in the model (p; μ_1; σ_1; μ_2; and σ_2) were fit to the empirical data by means of the maximum likelihood principle. Bootstrapping (500 replicates) was used to obtain interval estimates for the parameters. The fit procedures were implemented in Scilab software. The scripts of the Scilab functions are available at http://www.bio.uu.nl/~microbio/Microbiology/Tools.htm.

RESULTS

Fluorescence and volume of micro-colonies are not normally distributed

A. niger strains AR9#2 and UU-A005.4 express *GFP* from the *glaA* and the *faeA* promoter, respectively. These strains were grown as liquid shaken cultures in medium containing 50 mM maltose and 50 mM xylose, respectively. Maltose induces the AmyR regulated gene *glaA* (Petersen *et al.*, 1999), whereas xylose induces the XlnR regulated gene *faeA* (de Vries & Visser, 1999). After 16 h, micro-colonies were fixed and their size and fluorescence were monitored using a Complex Object Parametric Analyzer and Sorter (COPAS). The KS-test showed that size and fluorescence of the micro-colonies in the cultures of both strains did not follow a normal distribution ($p < 0.05$) (data not shown). Mathematical modeling showed that the distribution of the size of the micro-colonies within liquid shaken cultures of strains AR9#2 and UU-A005.4 can be explained by two normally distributed populations (Figure 1 and Table 3). The culture of strains AR9#2 and UU-A005.4 consisted for 61% and 75%, respectively, of micro-colonies with an average diameter of 595 µm, whereas 39% and 25% of the culture measured on average 505 µm. Mathematical modeling also showed that the fluorescence distribution of GFP of micro-colonies within liquid shaken cultures of strains AR9#2 and UU-A005.4

can be explained by two normally distributed populations (Figure 1 and Table 4). Highly fluorescent micro-colonies comprised 56% of the culture of strain UU-A005.4, whereas 44% of the micro-colonies made up the lowly fluorescent population. In contrast, the highly fluorescent population of strain AR9#2 consisted of 32% of the micro-colonies, while the lowly fluorescent population comprised 68% of the micro-colonies (Figure 1 and Table 4). Volume and GFP expression of micro-colonies was also heterogenic in other strains tested (Tables 3 and 4). These data show that heterogeneity is not the result of the copy number and the site of integration.

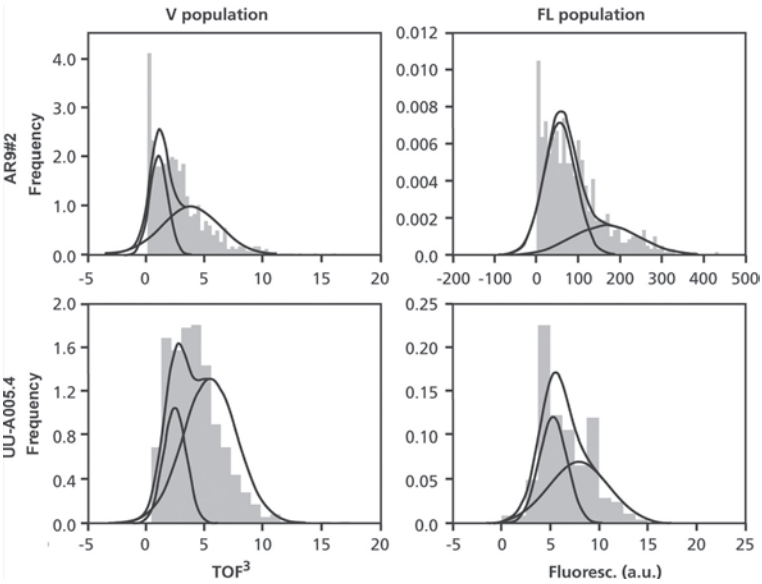

Figure 1 Heterogeneity of micro-colonies of *A. niger* in liquid shaken cultures with respect to size and gene expression. The dark grey areas represent the distribution of volume (V population) and *glaA* and *faeA* expression (Fl population) of micro-colonies within liquid cultures. These distributions can be explained by assuming the existence of two populations of micro-colonies (indicated by the curves). Strains AR9#2 and UU-A005.4 express *GFP* from the *glaA* and the *faeA* promoter, respectively.

Table 3 The volume distribution of micro-colonies of *A. niger* strains in liquid shaken cultures as assessed by COPAS. Strains expressed *GFP* and/or *dTomato* from the promoters of the glucoamylase gene *glaA*, the acid amylase gene *aamA* or the ferulic esterase gene *faeA*. N593 is not transformed with a reporter construct. Statistical analysis showed that the volume distribution can be explained by the existence of two populations of micro-colonies. N = number of analyzed micro-colonies; $\mu_{1,2}$ = mean volume of population 1 and 2, respectively; $\sigma_{1,2}$ = standard deviation of population 1 and 2, respectively; pf = participation frequency of population 1; CI = 95% confidence interval of the participation frequency of population 1.

Strain	Expression construct	N	μ_1	μ_2	σ_1	σ_2	pf	CI	
N593	-	264	1.4	2.5	0.3	1.0	0.25	0.25	0.25
AR9#2	*PglaA::GFP*	662	1.0	3.8	0.8	2.5	0.39	0.25	0.43
CB-A110.5	*PaamA::dTomato*	453	3.8	8.9	2.3	4.0	0.25	0.25	0.46
CB-A121.4	*PglaA::GFP* and *PaamA::dTomato*	247	6.5	9.3	3.9	5.7	0.25	0.25	0.43
UU-A005.4	*PfaeA::GFP*	522	2.3	5.4	0.9	2.3	0.25	0.25	0.25
CB-A109.1	*PglaA::dTomato*	1300	3.1	5.2	0.7	2.1	0.25	0.25	0.25
CB-A118.24	*PfaeA::GFP* and *PglaA::dTomato*	361	4.4	8.0	1.4	2.1	0.25	0.25	0.25

Table 4 The fluorescence distribution of micro-colonies of *A. niger* strains in liquid shaken cultures as assessed by COPAS. Strains expressed *GFP* and/or *dTomato* from the promoters of the glucoamylase gene *glaA*, the acid amylase gene *aamA* or the ferulic esterase gene *faeA*. Statistical analysis showed that the fluorescence distribution can be explained by the existence of two populations of micro-colonies. N = number of analyzed micro-colonies; $\mu_{1,2}$ = mean fluorescence of population 1 and 2, respectively; $\sigma_{1,2}$ = standard deviation of population 1 and 2, respectively; pf = participation frequency of population 1; CI = 95% confidence interval of the participation frequency of population 1. G = data concerning GFP fluorescence; R = data concerning dTomato fluorescence.

Strain	Expression construct	N	μ_1	μ_2	σ_1	σ_2	pf	CI	
AR9#2	*PglaA::GFP*	662	55.2	167.5	38.0	79.1	0.68	0.31	0.78
CB-A110.5	*PaamA::dTomato*	453	55.4	124.5	28.8	32.9	0.85	0.62	0.94
CB-A121.4	*PglaA::GFP* and *PaamA::dTomato*	247	G: 1.1 R: 2.8	G: 19.6 R: 42.9	G: 0.8 R: 2.0	G: 13.6 R: 32.2	G: 0.42 R: 0.42	G: 0.35 R: 0.34	G: 0.49 R: 0.50
UU-A005.4	*PfaeA::GFP*	522	5.3	8.0	1.5	3.2	0.44	0.30	0.99
CB-A109.1	*PglaA::dTomato*	1300	16.7	31.1	5.5	12.3	0.64	0.55	0.78
CB-A118.24	*PfaeA::GFP* and *PglaA::dTomato*	361	G: 14.6 R: 24.3	G: 40.3 R: 54.5	G: 7.2 R: 9.4	G: 21.1 R: 23.6	G: 0.52 R: 0.52	G: 0.38 R: 0.37	G: 0.81 R: 0.69

Heterogenic RNA distribution within micro-colonies

QPCR was used to determine *glaA* and *faeA* expression in individual micro-colonies of strain AR9#2. Expression of these genes was related to *18S* rRNA and mRNA of the actin gene. RNA was isolated from individual micro-colonies with a diameter of 750 to 800 µm that had been formed in liquid shaken cultures containing either 50 mM maltose, 50 mM xylose or 110 mM glucose as a carbon source. QPCR showed that accumulation of *faeA* mRNA was 3000-fold higher in xylose-grown micro-colonies

when compared to maltose- and glucose-grown micro-colonies. Gene *glaA* was highly expressed on maltose, whereas expression was 2.4 and 20 times less on glucose and xylose, respectively. These data agree with Northern analysis of RNA from whole liquid shaken cultures (de Vries & Visser, 1999; Boel *et al.*, 1984) and show that the RNA extraction and QPCR procedures that were developed to assess gene expression in individual micro-colonies (see Material and Methods for details) are reliable and reproducible.

In the next step, RNA levels of *glaA* and *faeA* were determined in the centre and periphery of individual micro-colonies. To this end, ethanol fixed micro-colonies of approximately 750 µm were cut using laser micro dissection and pressure catapulting (LMPC). Four zones were distinguished in the micro-colonies. Zone 1 represents the inner 100 µm and consists of aggregated spores. The central zone 2 represents the hyphae that are present between 100 and 300 µm. This zone is separated from the peripheral zone 4 by zone 3, which extends to 350 µm (Figure 2A). To isolate RNA from zones 2 and 4, the central zone 1 was first dissected and catapulted to remove it from the rest of the micro-colony (Figure 2B). This was followed by cutting zone 2 and by catapulting the hyphal material of this zone into a cap containing 50 µl of RNA*later* (Figure 2C). After removing zone 3 with the laser, zone 4 was cut. Hyphae of this zone were also catapulted in RNA*later* (Figure 2D).

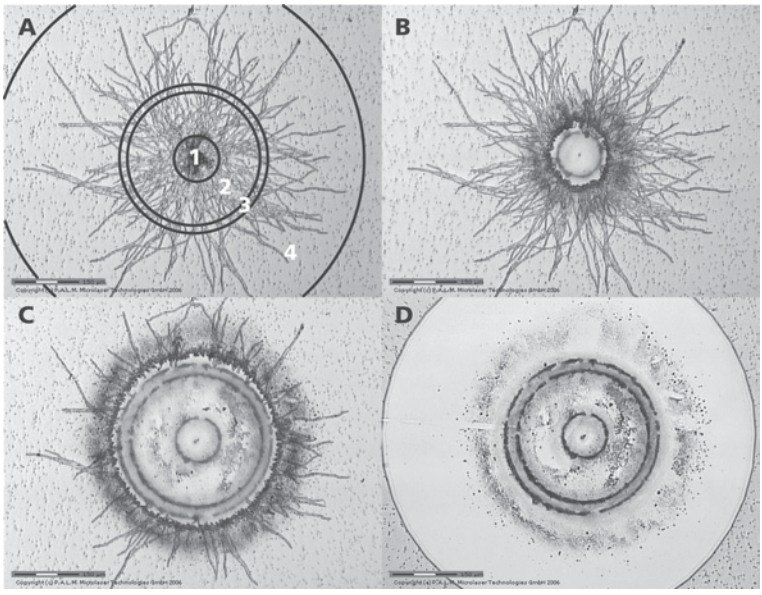

Figure 2 Laser dissection and pressure catapulting of micro-colonies of *A. niger* grown in a liquid shaken culture. Four zones are distinguished in a micro-colony (A). First, the central zone 1 was cut (B). This was followed by cutting zone 2 (C). After cutting zone 3, the outer zone 4 was dissected (D). Hyphal material of each of the zones can be isolated by catapulting.

Levels of *18S* rRNA, *actin*, *glaA* and *faeA* in zones 2 and 4 of individual micro-colonies were determined by QPCR. Accumulation of these RNAs was similar in the central zone 2 and the peripheral zone 4 ($p \geq 0.95$; Table 5). However, the RNA from the centre was derived from 45 times more hyphal material than from the periphery, which contained approximately 100 hyphae only. This indicates that RNA levels of these genes per unit mass of hypha are much higher at the periphery than in the centre. In agreement, acridine orange staining showed that the periphery of micro-colonies of *A. niger* is rich in RNA, while the centre is not (El-Enshasy *et al.*, 2006). Acridine orange binds to single- and double-stranded nucleic acids, resulting in red and green fluorescence, respectively. This experiment was repeated in the experimental set up of this study. Indeed, the periphery of the micro-colonies was red fluorescent, while green fluorescence was prominent in the centre of the micro-colony (Figure 3).

Table 5 Accumulation of transcripts in the central zone 2 and the peripheral zone 4 of micro-colonies of *A. niger* as expressed by Ct values obtained with QPCR. QPCR was performed on the total RNA extracted from the 4500 hyphae of zone 2 and the 100 hyphae of zone 4. Biological triplicates were used to calculate the average Ct and the standard deviation. *Act* encodes actin, *glaA* glucoamylase, and *faeA* ferulic acid esterase.

	***18S* rRNA**	***act***	***glaA***	***faeA***
Centre (4500 hyphae)	16.31 (± 1.79)	29.73 (± 1.61)	28.81 (± 2.42)	33.86 (± 2.25)
Periphery (100 hyphae)	16.42 (± 1.13)	28.02 (± 2.33)	27.14 (± 1.75)	31.46 (± 2.30)

Figure 3 Heterogenic distribution of RNA in micro-colonies of a liquid shaken culture of *A. niger*. Acridine orange staining shows that double stranded DNA is mainly present in the centre of a micro-colony (A), whereas single stranded RNA is predominantly observed at the periphery (B). (C) represents an overlay of (A) and (B). Zones within the colony are indicted by vertical lines.

DISCUSSION

Previously, it has been shown that macro-colonies of *A. niger* are heterogenic with respect to gene expression and protein secretion (Wösten *et al.*, 1991; Vinck *et al.*, 2005; Levin *et al.*, 2007). In industrial bioreactors, however, micro-colonies are formed rather than macro-colonies. Therefore, in this study it was investigated whether heterogeneity also exists within and between (sub)milimeter scale colonies grown in liquid shaken cultures.

800-µm-wide micro-colonies were subjected to laser micro-dissection and laser pressure catapulting to assess heterogeneity within micro-colonies. In this way, RNA could be isolated from the periphery and the central part of individual 800-µm-wide micro-colonies. QPCR did not show differences in the levels of *18S* rRNA, and of RNA of the actin gene and of *glaA* and *faeA*. However, the RNA in the centre originated from 45 times more hyphae compared to the periphery. Acridine orange staining confirmed that the hyphae in the centre of the micro-colony contained less RNA, while staining with propidium iodide showed that ≥ 99% of the hyphae were alive (our results, unpublished). The huge difference in RNA content at the periphery compared to the centre was not observed in macro-colonies grown on a solid medium (our results, unpublished). The reason for this is not yet clear. The size and open structure of the micro-colonies suggest that the centre was not affected in uptake of nutrients and transfer of gases.

The COPAS technique was used to assess whether micro-colonies in a liquid shaken culture of *A. niger* are heterogenic with respect to volume and expression of *glaA* and *faeA*. Statistical analyses showed that 16 h-old cultures consist of two populations of pellets that can be distinguished on basis of their size. The small pellets generally consist of about 25% of the population. Two populations of micro-colonies could also be distinguished in the case of expression of *glaA* and *faeA*. Interestingly, the population of micro-colonies lowly expressing *glaA* and *faeA* was larger than the population of small micro-colonies. This indicates that heterogeneity in *glaA* and *faeA* expression in a liquid shaken culture of *A. niger* is only partially caused by the heterogeneity in the size of the micro-colonies and thus also depends on an unknown other factor. This also seems to be the case for expression of the acid amylase gene *aamA*, which is like *glaA* regulated by AmyR (see Table 4).

The fact that micro-colonies within liquid cultures are heterogenic with respect to size and gene expression has implications how analysis of RNA, proteins and metabolites from whole cultures should be interpreted. By using the whole culture, the average composition or activity of the micro-colonies is determined. This average may by far not reflect the composition or activity of each of the populations within the liquid culture. Therefore, individual populations should be studied to understand mechanisms underlying biological processes. COPAS enables rapid sorting of populations of living or fixed micro-colonies and is thus an important tool to study gene expression and protein production in different populations. Such studies are expected to provide targets to reduce heterogeneity of the micro-colonies. This could result in improved protein production.

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CHAPTER 4

ABSTRACT

Colonization of a substrate by fungi starts with the invasion of exploring hyphae. These hyphae secrete enzymes that degrade the organic material into small molecules that can be taken up by the fungus to serve as nutrients. We previously showed that only part of the exploring hyphae of *Aspergillus niger* highly express the glucoamylase gene *glaA*. This was an unexpected finding since all exploring hyphae are exposed to the same environmental conditions. Using GFP as a reporter, we here demonstrate that the acid amylase gene *aamA*, the α-glucuronidase gene *aguA*, and the feruloyl esterase gene *faeA* are also subject to heterogenic expression within the exploring mycelium of *A. niger*. Co-expression studies using GFP and dTomato as reporters showed that hyphae that highly express one of these genes also highly express the other genes encoding secreted proteins. Moreover, these hyphae also highly express the amylolytic regulatory gene *amyR*, and the glyceraldehyde-3-phosphate dehydrogenase gene *gpdA*. *In situ* hybridization demonstrated that the high expressers are characterized by a high *18S* rRNA content. Taken together, it is concluded that two subpopulations of hyphae can be distinguished within the exploring mycelium of *A. niger* that differ in their transcriptional and translational activity. Notably, heterogenic *glaA* expression in the exploring hyphae was affected by chromatin modifying chemicals. From this it is concluded that the subpopulations of exploring hyphae are the result, at least partly, of regulation at the chromatin modification level.

HETEROGENIC EXPRESSION OF GENES ENCODING SECRETED PROTEINS AT THE PERIPHERY OF *ASPERGILLUS NIGER* COLONIES

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INTRODUCTION

Filamentous fungi colonize both dead and living substrates by forming a mycelium. Such a mycelium consists of a network of hyphae that grow at their apices and that branch sub-apically. The mycelium secretes large amounts of enzymes into the substrate to degrade organic polymers into small molecules that can be taken up by the fungus to serve as nutrients. Previously, it was shown that only growing hyphae within the colony secrete proteins (Wösten *et al.*, 1991; Moukha *et al.*, 1993). Notably, not every growing hypha secretes the same proteins. For instance, glucoamylase is secreted by growing hyphae at the periphery of a mycelium of *Aspergillus niger* but not by the growing hyphae in the central zone (Wösten *et al.*, 1991). In contrast, lignin peroxidase is secreted in the central growth zone but not at the periphery of colonies of *Phanerochaete chrysosporium* (Moukha *et al.*, 1993). Taken together, it can be concluded that a fungal macro-colony is not a mass of identical hyphae. Indeed, RNA profiles of outer and inner zones of colonies of *A. niger* are distinct (Levin *et al.*, 2007). For instance, 9% of the genes that are active in the mycelium are expressed in only one of five concentric zones. In addition, more than 25% of the active genes show at least a two-fold difference in expression between the outer and innermost zone of the colony. These differences in gene expression are caused by the availability of the carbon source and, to a similar extent, by medium independent mechanisms (Levin *et al.*, 2007). Differences in zonal expression have also been found in colonies of *Neurospora crassa* (Kasuga & Glass, 2008) and *Aspergillus oryzae* (Masai *et al.*, 2006) suggesting that this is a common phenomenon in the fungal kingdom.

Hyphal heterogeneity can even be observed within a specific zone of a mycelium. Glucoamylase (GlaA) was found to be secreted by a subset of hyphae at the periphery of the *A. niger* colony (Wösten *et al.*, 1991). This heterogeneity was explained by differences in the expression of *glaA* (Vinck *et al.*, 2005). Two types of hyphae were distinguished; those that highly and those that lowly express *glaA*, each making up

about 50% of the hyphal population. This was a remarkable finding considering the fact that the highly and lowly active hyphae in the outer zone of the *A. niger* colony experienced similar environmental conditions.

Heterogeneity of cells has also been found in other organisms. For instance, adult stem cells vary widely in their gene expression (Ramos *et al.*, 2006), and also cells within biofilms of *Candida albicans* exhibit heterogeneity (Nobile and Mitchell, 2007). It is assumed that heterogeneity in these organisms is a result of epigenetic processes. Epigenetic regulation has previously been shown to be one of the crucial mechanisms of clonal expansion of cells to diverse cell types. As such, it is essential for development, cell differentiation and the integration of endogenous and environmental signals (Jaenisch and Bird, 2003). It is tempting to speculate that epigenetic processes, which involve chromatin structure formation and alterations, also underlie hyphal heterogeneity in *A. niger*.

Here, evidence is presented that hyphae highly expressing *glaA* also highly express other genes encoding secreted proteins. In fact, the data indicate that the high expressers are hyphae with a high transcriptional and translational activity. Evidence supports the hypothesis that hyphal heterogeneity is the result of epigenetic regulation.

MATERIAL AND METHODS

Strains and plasmids

GFP and dTomato reporter constructs were introduced in *A. niger* strains AB4.1 (*pyrG*, *cspA1*) (van Hartingsveldt *et al.*, 1987), NW249 (Δ*argB*, *pyrA6*, *nicA1*, *leuA1*, *cspA1*) (P.J.I. van de Vondervoort and Y. Muller, unpublished data), N593 (*pyrA*, *cspA1*) (Goosen *et al.*, 1987), UU-A005.4 and CB-A112.11 (Table 1). All strains are derived from N402 (*cspA1*) (Bos *et al.*, 1988). *Escherichia coli* DH5α was used for cloning purposes.

Medium composition

A. niger was grown on minimal medium (MM) (de Vries *et al.*, 2004) with xylose, glucose or maltose as a carbon source. By growing on 200 mM D-xylose, expression of *aamA* and *glaA* was repressed, whereas these genes were induced on 25 mM D-maltose. The *aguA* and *faeA* genes were repressed and induced, respectively, by growing on 50 mM D-glucose and 25 mM D-xylose. CB-A112.11, UU-A005.4 and UU-A005.6 were grown in the presence of leucine (0.2 mg ml^{-1}), arginine (0.2 mg ml^{-1}) and nicotinamide (0.001 mg ml^{-1}). The dual-reporter strains AV112d.7, AV112d.8, CB-A114.2, CB-A114.22, CB-A115.3, CB-A115.9, CB-A116.2, CB-A116.11, CB-A117.1, CB-A117.5, CB-A118.24, CB-A118.28, CB-A121.4 and CB-A121.7 (Table 1) were grown in the presence of leucine (0.2 mg ml^{-1}) and nicotinamide (0.001 mg ml^{-1}). *E. coli* was grown at 37 °C in Luria-Bertani medium either or not supplemented with ampicillin (50 µg ml^{-1}) and agar (1.5%). Liquid cultures were shaken at 250 rpm.

Table 1 Strains used in this study.

Strain	recipient strain	transforming construct	strain description
AR9#2	AB4.1	pAN52-10S65TGFPn/s	*sGFP*(S65T) under regulation of the *glaA* promoter of *A. niger* (Siedenberg *et al.*, 1999)
AR19#1	AB4.1	P*GPD*GFP	*sGFP*(S65T) under control of the *gpdA* promoter of *A. nidulans* (Lagopodi *et al.*, 2002)
AV11#3 & AV11#4	AB4.1	P*aguA*_sGFP+	*sGFP*(S65T) under regulation of the *aguA* promoter of *A. niger*
AW001.08 & AW001.10	N593	pAW003	*sGFP*(S65T) under regulation of the *aamA* promoter of *A. niger*
UU-A005.4 & UU-A005.6	NW249	pHB68-28	*sGFP*(S65T) under regulation of the *faeA* promoter of *A. niger*
CB-A112.11	NW249	pCB020	*sGFP*(S65T) under regulation of the *glaA* promoter of *A. niger*
AV112d.7 & AV112d.8	CB-A112.11	pAV150	*sGFP*(S65T) under regulation of the *glaA* promoter of *A. niger* and *dTomato* under regulation of the *amyR* promoter of *A. niger*
CB-A114.2 & CB-A114.22	UU-A005.4	pCB009 (**A**)	*sGFP*(S65T) under regulation of the *faeA* promoter of *A. niger* and *dTomato* under regulation of the *faeA* promoter of *A. niger*
CB-A115.3 & CB-A115.9	UU-A005.4	pCB012	As (**A**) but with *dTomato* under regulation of the *aguA* promoter of *A. niger*
CB-A116.2 & CB-A116.11	UU-A005.4	pCB025	As (**A**) but with *dTomato* under regulation of the *gpdA* promoter of *A. nidulans*
CB-A117.1 & CB-A117.5	UU-A005.4	pCB027	As (**A**) but with *dTomato* under regulation of the *aamA* promoter of *A. niger*
CB-A118.24 & CB-A118.28	UU-A005.4	pCB021	As (**A**) but with *dTomato* under regulation of the *glaA* promoter of *A. niger*
CB-A121.4 & CB-A121.7	CB-A112.11	pCB027	As (**A**) but with *dTomato* under regulation of the *aamA* promoter of *A. niger*
CB-A109.1 & CB-A109.2	N593	pCB021	*dTomato* under regulation of the *glaA* promoter of *A. niger*

Sandwiched colonies of *A. niger*

A. niger was grown as sandwiched colonies (Wösten *et al.*, 1991) at 30 °C. To this end, *A. niger* was grown in a 0.2 mm thin layer of 1.25% agarose (D-1 agarose, type low EEO, Hispanagar) between two perforated polycarbonate membranes (diameter 76 mm, pore size 0.1 µm; Osmonics, GE Water Technologies) placed on top of solidified (1.5% agar) minimal medium (MM) (see below). The sandwiched cultures were routinely inoculated with small mycelial plugs from the periphery of pre-grown sandwiched cultures. Alternatively, cultures were inoculated with a micro-colony originating from a single conidium or a single hypha. In the former case, sandwiched colonies were inoculated with 50 µl of a spore solution containing 20 spores ml^{-1}. Resulting 3-day-old micro-colonies were used as an inoculum for fresh sandwiched colonies. To obtain micro-colonies originated from a single hypha, sandwiched colonies were grown with the modification that there was no agarose layer between the two membranes and that the upper PC-membrane was replaced by a Lumox membrane. The latter membrane

was taken from a Lumox dish (Greiner Bio-One) and the hydrophobic side was placed such that it faced the PC membrane. After 5 days of growth, the Lumox membrane was removed and parts of the periphery of the colony, still sticking to the PC membrane, were transferred to a 1.0 PEN MembraneSlide (Carl Zeiss MicroImaging GmbH). Subsequently, individual hyphae were dissected using a PALM system that was operated with PALM RoboSoftware V4.0 (Carl Zeiss MicroImaging GmbH). The apical parts (75 to 150 µm) of the hyphae at the periphery of the colony were dissected. These hyphal fragments that had a diameter of 4 to 6 µm were catapulted into the lid of a 0.5 ml Eppendorf tube that contained 50 µl MM with 25 mM glucose as a carbon source. The medium containing the hypha was then transferred to a 2 ml Eppendorf tube containing 0.4 ml MM with 25 mM glucose as a carbon source. After 3 days of growth at 150 rpm a small pellet had formed that was used to inoculate sandwiched colonies.

Construction of GFP reporter constructs

The primers used for PCR are presented in Table 2. The *A. niger aguA* promoter was amplified by PCR from plasmid pIM3243 (de Vries *et al.*, 2002) with primers FPraguABam and RPraguANco. The resulting 550 bp *Bam*HI-*Nco*I fragment was cloned in pAN52-10S65TGFPn/s (Siedenberg *et al.*, 1999). To this end, pAN52-10S65TGFPn/s was partially digested with *Not*I and the overhangs were blunted with Klenow. This was followed by digestion with *Nco*I to remove the 4.1 kb *glaA* promoter. The 550 bp *Bam*HI-*Nco*I fragment of the *aguA* promoter of which the *Bam*HI site was blunted with Klenow was then inserted. This resulted in construct P*aguA*_sGFP+ that contains the *sGFP*(S65T) gene (Chiu *et al.*, 1996) under the regulation of the *aguA* promoter of *A. niger*.

A 3.5 kb genomic *Sma*I fragment from pIM3207 (de Vries *et al.*, 1997) containing the *faeA* open reading frame and 2000 and 300 bp 5' and 3' flanking sequences, respectively, was cloned in pGEM7 resulting in construct pRV421. The construct pHB65-16 was derived from pRV421 by removing the open reading frame of *faeA* by outward PCR using primers pFAEANcoNot and tFAEANotPst thus introducing *Nco*I and *Not*I sites. The *Nco*I-*Not*I fragment containing the coding sequence of *sGFP*(S65T) was introduced in the respective sites of pHB65-16, resulting in construct pHB68-28.

The *A. niger aamA* promoter was amplified from N402 chromosomal DNA with primers paamANotIFW and paamANcoIRV. The resulting 1430 promoter fragment was used to replace the NotI/NcoI promoter fragment of *GPD* in PGPDGFP, resulting in pAW003. Similarly, the 820 bp *glaA* promoter was amplified with primers pglaANotIFW and pglaARV, and cloned into PGPDGFP resulting in pCB020.

Table 2 Primers used in this study. The restriction sites are underlined. A second restriction site present in the primer is indicated in bold.

Primer	sequence (5′ – 3′)
FPraguABam	GGATCCAATATATCGATACTTCTTGCC
RPraguANco	CCATGGTGGCGGGTTCCTTCTGG
F-PamyRNotI	GCGGCCGCCGACTAGTAGATCACCAC
R-PamyRNcoI	CCATGGGTATGCGGAGACAAGTGTG
pFAEANcoNot	GGGGGCGGCCGC**CCATGG**TGAAATATGTGCGACAGTGAGTG
tFAEANotPst	GGGGCGGCCGC**CTGCAG**ATTATACAAAGAAGAATAAACCTC
tomNcoIFW2	AATCCATGGTGAGCAAGGGCGAGG
tomHindIIIRV2	AAGCTTACTTGTACAGCTCGTCCATGC
pfaeANotIFW	TATTGCGGCCGCTGGTATGAGGATTGGG
pfaeANcoIRV	GCCCATGGCATCCTGAAATATGTGCGAC
paguANotIFW	GGCAGCGGCCGCAATATATCGATACTTC
paguANcoIRV2	CCATGGTGGCGGGTTCCTTCTGG
pglaANotIFW	AAAGCGGCCGCGGATCCGAACTCCAACC
pglaARV	CCATGGCTGAGGTGTAATGATGC
paamANotIFW	AAGCGGCCGCAACAGGAGGTTACTTC
paamANcoIRV	GCCCATGGCTGCGAATTATG
pgpdAFW	TAACCATGGCTCCCAAGGTCGG
pgdARV	GGTAAGCTTCTACTGGGCATCAACCTTGG

Construction of dTomato reporter constructs

The primers used for PCR are presented in Table 2. The *dTomato* (Shaner *et al*., 2004) coding region was amplified by PCR from plasmid pRO020 with primers tomNcoIFW2 and tomHindIIIRV2. Plasmid pRO020 contains the *dTomato* gene in which the internal *NcoI*-site was removed for cloning purposes (Ohm, 2010). The resulting 705 bp *Nco*I-*Hind*III fragment was used to replace the *GFP Nco*I-*Hind*III fragment of pGPDGFP (Lagopodi *et al*., 2002) resulting in pCB025.

The *A. niger faeA* promoter was amplified by PCR from *A. niger* genomic DNA with primers pfaeANotIFW and pfaeANcoIRV. The resulting 657 bp *NotI-NcoI* fragment was used to replace the *GPD NotI-NcoI* promoter fragment of pCB025, resulting in pCB009. Similarly, a 557 bp *aguA* promoter fragment, an 820 bp *glaA* promoter fragment, and a 1430 bp *aamA* promoter fragment were cloned in pCB025, resulting in constructs pCB012, pCB021 and pCB027. The *A. niger aguA* promoter was amplified from N402 chromosomal DNA with primers paguANotIFW and paguANcoIRV2. The *glaA* and *aamA* promoters were amplified as described above.

The *A. niger amyR* promoter was amplified by PCR from genomic DNA of strain N402 with primers F-PamyRNotI and R-PamyRNcoI. The resulting 1620 bp *Not*I-

*Nco*I fragment was cloned in pAN5210dTom that had been digested with the same enzymes, which resulted in construct pAV150. The plasmid pAN5210dTom is a derivative of pAN52-10*Not*I (Siedenberg *et al.*, 1999) in which a Klenow blunted *Nco*I-*Bam*HI fragment consisting of the dTomato coding sequence (of which the internal *Nco*I-site was removed by site-directed mutagenesis) has been inserted in the *Nco*I/*Eco*RV sites.

Transformation of *A. niger*

Protoplast preparation and transformation were performed as previously described (de Bekker *et al.*, 2009; **Chapter 2**). Transformants were selected by co-transforming with pAB4.1 (van Hartingsveldt *et al.*, 1986) or pGW635 (Kusters-van Someren *et al.*, 1991) that both contain the *A. niger pyr* gene and/or pIM2104 (vanKuyk *et al.*, 2004) that contains the *A. niger arg* gene. Transformants were purified by repeated streaking of conidia.

***In situ* hybridisation with PNA probes**

The distribution of *18S* rRNA in individual hyphae was visualized by *in situ* hybridizations using the eukaryotic EuUni peptide nucleic acid (PNA) probe (5'- ACCAGACTTGCCCTC – 3') (Perry-O'Keefe *et al.*, 2001), which was (5') N-terminally labelled with fluorescein. Hybridizations were performed as described by Teerstra *et al.* (2004). Specificity of the rRNA hybridisation was checked by incubating rehydrated fixed cultures for 1 hour at 37 °C in 0.5x PBS (pH 7.4) in the absence or presence of 0.5 mg ml^{-1} RNase A and 83 U ml^{-1} RNase I.

Microscopy

GFP fluorescence at the colony level was monitored with a Leica GFP2 filter set using a Leica MZ16 FA fluorescence stereo-microscope equipped with a mercury lamp and a Leica DFC420 C digital camera. Images were handled with Leica Application Suite software (version 2.8.1).

For detection of fluorescence at the hyphal level, the top PC membrane of sandwiched colonies was removed. Small pieces (~ 8 mm x 8 mm) of the periphery of the colony were excised, placed on a cover slip (24 x 50 mm) and topped with a block (~ 1 x 1 x 0.5 cm) of inducing agar medium. This prevents drying out of the samples. GFP fluorescence at the hyphal level was studied by confocal laser scanning microscopy (CLSM). The laser intensity was kept to a minimum to reduce photobleaching and phototoxic effects. For the single-reporter transformants an inverted Leica TCS SPII system equipped with a PL FLUOTAR 16x/0.5 Imm plan apochromatic objective lens was used. GFP was excited with the 488 nm laser line and fluorescence was detected at 500-550 nm band pass (BP). Images were captured as *z*-series of optical sections (~1 µm). The data sets were displayed as maximum intensity projections (1024 x 1024 pixels) using Leica LSM software.

Fluorescence of the dual-reporter strains and of strains CB-A109.1 and CB-A109.2 hybridized with the EuUni PNA probe was imaged with an inverted Zeiss LSM 5 system using a Plan-Neofluar 16x/0.5 Imm objective lens. GFP and fluorescein were excited with the 488 nm laser line, whereas a 543 nm laser line was used to excite dTomato. GFP and fluorescein fluorescence was detected at 505-530 nm BP, whereas

dTomato fluorescence was monitored at 560 nm long pass. Images were captured as *z*-series of optical sections (optimal interval 2.02 µm; 4x line average; 8 bit scan depth) using the multi-track scanning mode. The data sets were displayed as maximum intensity projections (1024 x 1024 pixels) using Zeiss software.

Image and data analysis

The intensity of fluorescence was quantified by measuring the mean pixel values of hyphae in geometrically calibrated images using a program based on KS400 software (Version 3.0; Carl Zeiss Vision). Hyphae were selected by thresholding on the basis of grey value. The average grey value of the background was determined by the inverse of a dilated hyphal image. Fluorescence was quantified as the sum grey value in the hypha minus the sum background value in an equivalent area (Vinck *et al.*, 2005). Quantification of fluorescence in strains expressing both GFP and dTomato or in strains expressing dTomato that were hybridized with the EuUni PNA probe was performed by copying a mask containing the hyphal selection obtained in the green channel onto the red channel. In this way GFP / FITC and dTomato fluorescence of the same hyphae was quantified as described above. The data were modeled as described by Vinck *et al.*, (2005) to examine whether the distribution of fluorescence intensities of the hyphae can be explained by assuming that they are composed of a weighted mixture of two normally distributed components.

A custom Python script was used to correlate intensity of GFP, dTomato and fluorescein fluorescence within single hyphae that had a minimal surface area of 100 µm^2. For each picture each green and red fluorescence signal was normalized by dividing it by the total green or red fluorescence for that picture, respectively. The normalized data was used to calculate the Pearson correlation coefficient between green (GFP or fluorescein) and red fluorescence (dTomato).

Statistical analyses were performed using SPSS 16.0 (SPSS). The Pearson correlation technique was used to examine inter-relationships between expression of genes and *18S* rRNA distribution.

RESULTS

***A. niger* strains expressing *GFP* from the *gpdA, glaA, aamA, aguA* and *faeA* promoter**

Previously, *A. niger* strains have been described that express the *sGFP*(S65T) gene from the *gpdA* or the *glaA* promoter (Lagopodi *et al.*, 2002; Siedenberg *et al.*, 1999). Here, *A. niger* was transformed with constructs containing *sGFP*(S65T) under control of the promoter of *aguA* (de Vries *et al.*, 2002), *faeA* (de Vries *et al.*, 1997) or *aamA* (Boel *et al.*, 1990). The xylanolytic genes *aguA* and *faeA* are repressed by glucose and induced by xylose (de Vries *et al.*, 1999). In contrast, the amylolytic genes *aamA* and *glaA* are repressed by xylose and induced by maltose (Fowler *et al.*, 1990). In all cases, fluorescent transformants carried multiple copies of the transforming construct. Single integrations did not result in fluorescence strong enough for fluorescence microscopy.

Therefore, two representative strains of each transformation were selected for further analysis: AV11.3 and AV11.4 (P*aguA_sGFP*); UU-A005.4 and UU-A005.6 (P*faeA_sGFP*) and AW001.08 and AW001.10 (P*aamA_sGFP*) (Table 1).

Expression of *gpdA, glaA, aamA, aguA* and *faeA* at the colony level

Expression of *sGFP*(S65T) under control of the *gpdA, aamA, glaA, aguA* and *faeA* promoter was analysed at the colony level in 5-days-old sandwiched cultures. The *gpdA*-driven expression resulted in bright GFP fluorescence throughout the mycelium irrespective of the carbon source (data not shown; Vinck *et al.*, 2005). Similar results were obtained with the *aamA, glaA, aguA* and *faeA* promoter when these strains were grown on the inducing carbon source for 5 days. In contrast, a weak fluorescent signal was observed during growth on the repressing carbon source (data not shown). An 8 h transfer of these colonies to the inducing medium resulted in an evenly distributed fluorescence in the case *sGFP*(S65T) was expressed from the *aguA* promoter (Figure 1). In contrast, fluorescence was most intense at the periphery of the colony in the case of *aamA*- or *glaA*- driven expression, whereas *faeA*-driven expression resulted in the most intense fluorescence within the colony centre.

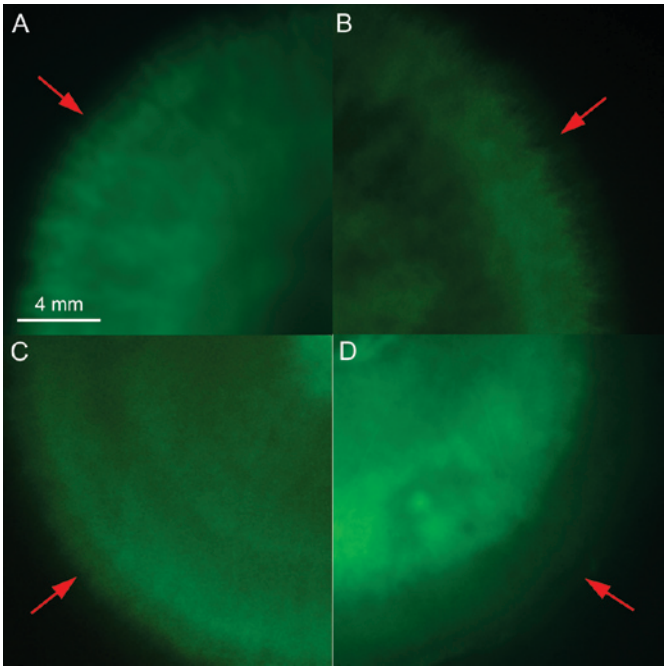

Figure 1 Expression of *glaA* (A) *aamA* (B) *aguA* (C) and *faeA* (D) in 5-days-old sandwiched colonies of *A. niger* that had been transferred for 8 hrs from a repressing to inducing medium. Expression was monitored by using sGFP as a reporter. Scale bar represents 4 mm. Arrows indicate edge of the colony.

Expression of *gpdA, glaA, aamA, aguA* and *faeA* of *A. niger* at the hyphal level

Strains expressing *sGFP*(S65T) from the *gpdA*, *glaA*, *aamA*, *aguA* or *faeA* promoter were grown for 5 days on inducing medium or were transferred to this medium for 8 hours after growth for 5 days on the repressing medium. In all cases, all hyphae within the outer zone of the colonies were fluorescent (for an example see Figure 2AB). The fluorescence of the individual hyphae was quantified, normalized and subjected to statistic analyses. The Kolmogorov-Smirnov test (henceforth KS-test) showed that fluorescence intensity was normally distributed in the strain that expresses *sGFP*(S65T) from the *gpdA* promoter (Table 3). Similar results were obtained with one of the strains that expressed *GFP* from the *faeA* promoter when it had been grown for 5 days on inducing medium. In all other cases, the distribution of fluorescence intensities of the exploring hyphae deviated from normality (Table 3). The fluorescence intensity distributions could be explained by assuming that they are composed of a weighted mixture of two normally distributed components, representing a population that highly expresses the reporter gene and a population that lowly expresses the reporter gene (Table 3; Figure 2CD). For instance, 84% and 74% of the hyphae were part of the population that expresses *aamA* at a low level when the recombinant strains AW001.08 and AW001.10 were grown continuously on the inducing maltose medium. When these transformants were induced for 8 hours 55% and 65% of the hyphae belonged to this population, whereas 45% and 35% were expressing *aamA* at a high level.

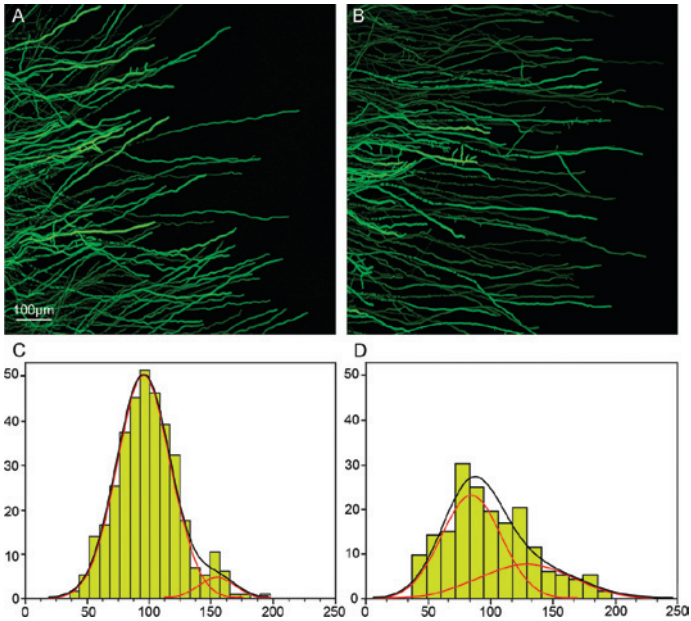

Figure 2 CLSM images (A, B) and the respective fluorescence intensity distributions (C, D) of hyphae in the exploration zone of colonies of strain AV11#3 and AW001.10 that express s*GFP* from the *aguA* and *aamA* promoter, respectively. The strains were grown for 5 days on 50 mM glucose followed by 8 hrs on 25 mM xylose (A, C) or for 5 dys on 200 mM xylose followed by 8 hrs on 25 mM maltose (B, D). The optimal fit of the fluorescence distributions of strains AV11#3 and AW001.10 can be described as a weighted mixture of two normal distributions. These normal distributions are indicated in red.

Table 3 Descriptive statistics of the fluorescence intensities of hyphae in the exploring mycelium of *A. niger*. N = sample size; KS = *p*-value of Kolmogorov-Smirnov-test (with Lilliefors significance correction); $\mu_{1,2}$ = mean of component 1 and 2, respectively; $SD_{1,2}$ = standard deviation of component 1 and 2, respectively; pf_1 = participation frequency of component 1; CI = 95% confidence interval of pf1; n.a. = not applicable.

strain & growth condition	Promoter regulating *GFP* expression	N	KS	μ_1	μ_2	SD_1	SD_2	pf_1	CI	
AR9#2; 5dys maltose	*glaA*	191	< 0.001	92.19	173.10	25.22	20.77	0.91	0.63	0.96
AR9#2; 8hrs maltose	*glaA*	156	< 0.013	88.98	146.45	21.57	16.99	0.82	0.23	0.92
AW001.08; 5dys maltose	*aamA*	114	< 0.017	88.23	163.32	27.57	33.31	0.84	0.24	0.97
AW001.08; 8hrs maltose	*aamA*	229	< 0.010	78.59	126.22	20.90	31.13	0.55	0.34	0.89
AW001.10; 5dys maltose	*aamA*	98	< 0.001	86.21	139.65	22.88	44.37	0.74	0.23	0.98
AW001.10; 8hrs maltose	*aamA*	210	< 0.002	84.60	128.85	24.04	38.55	0.65	0.31	0.96
AV11#3; 5dys xylose	*aguA*	245	< 0.009	89.47	115.35	15.58	24.81	0.61	0.28	0.98
AV11#3; 8hrs xylose	*aguA*	417	< 0.007	95.97	155.12	21.90	15.00	0.94	0.55	0.97
AV11#4; 5dys xylose	*aguA*	347	< 0.042	84.83	110.14	14.68	20.75	0.42	0.11	0.97
AV11#4; 8hrs xylose	*aguA*	274	< 0.050	72.91	108.42	11.36	24.35	0.25	0.10	0.88
UU-A005_4; 5dys xylose	*faeA*	84	< 0.005	89.48	142.66	20.98	43.09	0.80	0.15	0.97
UU-A005_4; 8hrs xylose	*faeA*	284	< 0.004	92.33	141.80	23.17	38.94	0.84	0.24	0.97
UU-A005_6; 5dys xylose	*faeA*	70	< 0.065	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
UU-A005_6; 8hrs xylose	*faeA*	238	< 0.002	84.37	140.98	24.88	39.91	0.72	0.23	0.95
AR19#1; 8hrs maltose	*gpdA*	235	> 0.200*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

* This is a lower bound of the true significance.

Correlation between expression of *gpdA, glaA, aamA, aguA* and *faeA* at the hyphal level

Strain UU-A005.4 that expresses s*GFP*(S65T) from the *faeA* promoter was transformed with constructs expressing *dTomato* from the *gpdA*, *glaA*, *aamA*, *aguA* or *faeA* promoter. Moreover, strain CB-A112.11 that expresses s*GFP*(S65T) from the *glaA* promoter was transformed with a construct containing the *dTomato* gene fused to the *aamA* promoter (Table 1). In all cases, fluorescent strains contained multiple copies of both expression constructs. Therefore, two representative strains of each transformation were selected for further study. Sandwiched colonies were either grown for 5 days on 50 mM glucose medium, after which they were transferred to a medium containing 25 mM xylose (in the case one or both of the reporters were controlled by a XlnR-regulated promoter), or grown for 5 days on 200 mM xylose medium after which they were transferred to a medium containing 25 mM maltose (in the case *glaA* and *aamA* expression were co-localized). Eight hours after transfer, GFP and dTomato fluorescence was observed in all the hyphae at the periphery of the colony of all the investigated strains (for an example see Figure 3). The relative intensity of GFP and dTomato fluorescence of individual hyphae within the periphery of the colony was determined and from these data the Pearson correlation coefficients were calculated. A strong positive correlation between the fluorescence of the reporters was obtained when co-expression of XlnR-regulated genes or co-expression of AmyR-regulated genes was assessed (0.70 – 0.80; Table 4). A similar correlation was observed between expression of the *gpdA* and *faeA* promoter (0.8-0.87; Table 4). A lower, but still significant, correlation was observed when expression of the amylolytic genes *aamA* and *glaA* was co-localized with the xylanolytic gene *faeA* (0.35 - 0.52; Table 4).

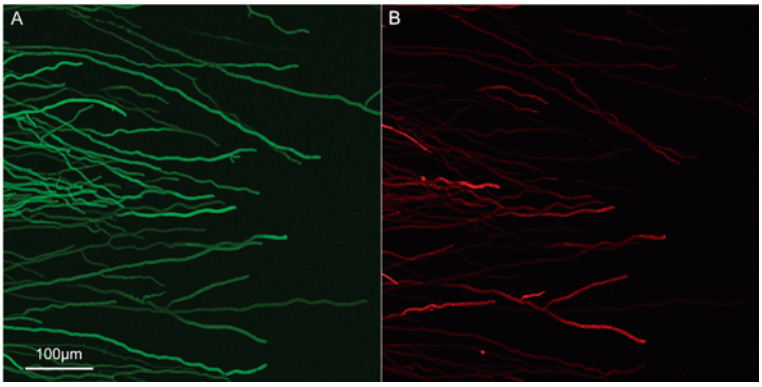

Figure 3 CLSM images of hyphae in the exploration zone of colonies of strain CB-115.9 that expresses s*GFP*(S65T) behind the *faeA* promoter (A) and *dTomato* behind the a*guA* promoter (B).

Table 4 Pearson correlation coefficient (*r*) with standard error of the mean (sem) between the intensity of fluorescence of (1) GFP and dTomato and (2) fluorescein and dTomato in hyphae at the periphery of 5-day-old *A. niger* colonies.

Strain	Growth condition	promoter regulating *dTomato* expression	promoter regulating *sGFP*(S65T) expression	*r* ± sem
CB-A114.2	8 h 25 mM xylose	*faeA*	*faeA*	0.70 ± 0.05
CB-A 114.22	8 h 25 mM xylose	*faeA*	*faeA*	0.72 ± 0.05
CB-A 115.3	8 h 25 mM xylose	*aguA*	*faeA*	0.73 ± 0.04
CB-A 115.9	8 h 25 mM xylose	*aguA*	*faeA*	0.77 ± 0.02
CB-A 121.4	8 h 25 mM maltose	*aamA*	*glaA*	0.80 ± 0.05
CB-A 121.7	8 h 25 mM maltose	*aamA*	*glaA*	0.78 ± 0.05
AV112d.7	8 h 25 mM maltose	*amyR*	*glaA*	0.73 ± 0.05
AV112d.8	8 h 25 mM maltose	*amyR*	*glaA*	0.86 ± 0.08
CB-A 116.2	8 h 25 mM xylose	*gpdA*	*faeA*	0.87 ± 0.02
CB-A 116.11	8 h 25 mM xylose	*gpdA*	*faeA*	0.80 ± 0.02
CB-A 117.1	8 h 25 mM xylose	*aamA*	*faeA*	0.46 ± 0.02
CB-A 117.5	8 h 25 mM xylose	*aamA*	*faeA*	0.52 ± 0.04
CB-A 118.24	8 h 25 mM xylose	*glaA*	*faeA*	0.35 ± 0.09
CB-A 118.28	8 h 25 mM xylose	*glaA*	*faeA*	0.46 ± 0.00
		promoter regulating *dTomato* expression	***In situ* hybridization probe**	
CB-A109.1	8 h 25 mM maltose	*glaA*	*18S* rRNA	0.81 ± 0.04
CB-A109.2	8 h 25 mM maltose	*glaA*	*18S* rRNA	0.85 ± 0.03

Correlation between expression of *glaA* and its regulator *amyR* and between expression of *glaA* and distribution of *18S* rRNA

Strain CB-A112.11 that expresses *sGFP*(S65T) from the *glaA* promoter was transformed with a construct containing the *dTomato* gene under regulation of the amylolytic regulatory gene *amyR* (Petersen *et al.*, 1999). The intensity of GFP and dTomato fluorescence was determined in hyphae within the exploration zone of the colonies that had been transferred to the inducing medium for 8 hours. A strong positive linear correlation (0.73-0.86; Table 4) was found between *glaA* and *amyR* expression. A similar approach to correlate expression of *aguA* and *faeA* with their regulator *xlnR* (van Peij *et al.*, 1998) did not succeed. This was due to the fact that the expression from the *xlnR* promoter was below detection level.

Strains CB-A109.1 and CB-A109.2 that express the *dTomato* gene under regulation of the *glaA* promoter of *A. niger* were hybridized with an *18S* rRNA PNA probe. The distribution of dTomato and *18S* rRNA was analyzed using confocal laser scanning microscopy (CLSM) (Figure 4). Image analysis showed that *glaA* expression and *18S* rRNA distribution exhibit a strong positive correlation (0.81-0.85; Table 4). In general, fluorescein fluorescence, indicative for rRNA distribution, decreased near the extreme tip. As a consequence, tips only showed the red fluorescence of dTomato (Figure 4C).

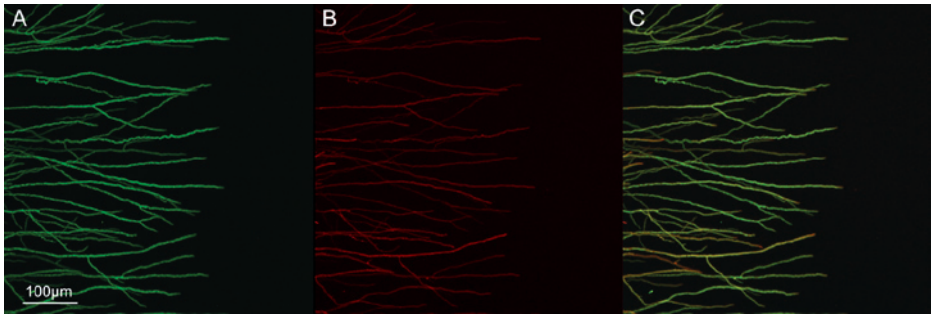

Figure 4 *In situ* hybridisation of *18S* rRNA using a fluorescein labeled PNA probe (A) in colonies of strain CB-A109#2 that expresses *dTomato* behind the *glaA* promoter (B). (C) represents an overlay of the fluorescein and dTomato signals.

Mechanisms underlying hyphal heterogeneity

Sandwiched cultures are routinely inoculated with 1.5 µl of a 1.10^8 spores ml^{-1} solution. Cultures of strain AR9#2 were inoculated with micro-colonies originating from a single spore or a single hypha (see Material and Methods) to address whether heterogeneity in gene expression is caused by heterogeneity within the inoculum. The KS-test showed that fluorescence intensity of hyphae at the periphery of sandwiched colonies deviated from normality when cultures had been inoculated with numerous spores or with micro-colonies originating from a single spore or a single hypha (Table 5). These experiments show that hyphae become heterogenic during formation of the mycelium and that heterogeneity does not depend on the inoculum used.

Table 5 Descriptive statistics of the fluorescence intensities of hyphae in the exploring mycelium of *A. niger* strain AR9#2. Sandwiched colonies were grown from a plug inoculum or from micro-colonies originating from a single spore or a single hypha. Each experiment was carried out twice. N = sample size; KS = *p*-value of Kolmogorov-Smirnov-test (with Lilliefors significance correction); $\mu_{1,2}$ = mean of component 1 and 2, respectively; $SD_{1,2}$ = standard deviation of component 1 and 2, respectively; pf_1 = participation frequency of component 1; CI = 95% confidence interval of pf1.

	N	KS	μ_1	μ_2	SD_1	SD_2	pf_1	CI	
Plug 1	508	< 0.006	65.15	112.19	16.11	33.53	0.27	0.12	0.57
Plug 2	156	< 0.013	88.98	146.45	21.57	16.99	0.82	0.23	0.92
Single spore (1)	193	< 0.011	75.17	129.72	22.21	40.41	0.54	0.24	0.94
Single spore (2)	127	< 0.001	71.94	121.36	15.34	38.42	0.50	0.27	0.81
Single hypha (1)	231	< 0.001	77.16	124.66	16.96	36.96	0.52	0.38	0.75
Single hypha (2)	222	< 0.001	69.91	139.24	19.63	43.80	0.57	0.46	0.71

In the next set of experiments the role of chromatin structure in heterogenic *glaA* expression was assessed using 5'-azacytidine (AZC) and sodium-butyrate (SB). AZC inhibits histone and DNA methylation (Jones, 1985), whereas SB causes hyperacetylation of histones (Sealy and Chalkley, 1978; Boffa *et al.*, 1978). Sandwiched

colonies of strain AR9#2 were grown for 5 days on *glaA* repressing medium containing 0, 100, 500 or 750 µM AZC or SB (these concentrations did not inhibit growth and sporulation of the colony). Subsequently, the sandwiched colonies were transferred for 6 h to *glaA* inducing medium with the same concentration of AZC or SB. The fluorescence of the individual hyphae was monitored using CSLM, quantified and normalized, and subjected to statistic analyses. The KS-test showed that fluorescence intensity did not follow a normal distribution in any of the conditions (Table 6). However, the fluorescence distribution of the colonies that were grown in the presence of AZC or SB appeared skewed to the right as a result of relatively few hyphae with a high fluorescence (Figure 5C and E). To distinguish between a skewed normal distribution and a bimodal distribution a log-transformation was performed. The log-transformed distributions of the colonies that were grown in the absence of AZC or SB still deviated from normality (Figure 5B). However, normal distributions were obtained for colonies that were grown in the presence of AZC or SB (Table 6 and Figure 5D and F).

Table 6 Descriptive statistics of the fluorescence intensities of hyphae in the exploring mycelium of *A. niger*. Colonies were grown in the absence or presence of AZC or SB. N = number of hyphae analyzed; KS = *p*-value of Kolmogorov-Smirnov-test (with Lilliefors significance correction) before and after log-transformation.

Treatment	N	KS	KS log-transformation
Control	154	> 0.000	> 0.001
	232	> 0.001	> 0.009
100 µM AZC	190	> 0.004	< 0.200
	232	> 0.000	< 0.200
500 µM AZC	339	> 0.000	< 0.200
	241	> 0.000	< 0.200
750 µM AZC	467	> 0.000	> 0.036
	230	> 0.001	< 0.200
100 µM SB	247	> 0.001	< 0.200
	192	> 0.000	< 0.200
500 µM SB	224	> 0.000	< 0.200
	227	> 0.000	< 0.200
750 µM SB	187	> 0.003	< 0.007
	277	> 0.039	> 0.200

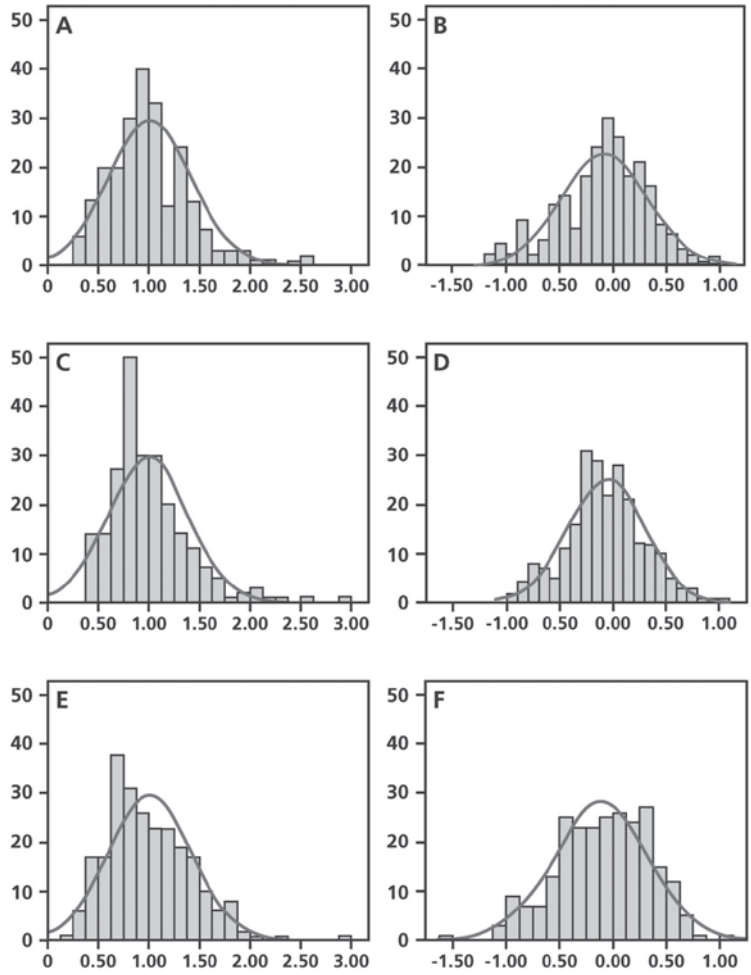

Figure 5 Fluorescence intensity distributions of hyphae in the exploration zone of colonies of strain AR9#2 that expresses *sGFP* from the *glaA* promoter before (A, C, E) and after (B, D, F) log-transformation. Sandwiched colonies were grown for 5 days on 200 mM xylose followed by an 8-hour transfer to an inducing medium containing 25 mM maltose. The media contained no AZC or SB (A, B), 100 µM AZC (C, D) and 100 µM butyrate (E, F) throughout culturing.

DISCUSSION

Previously, it was shown that the AmyR regulated genes *glaA* and *aamA* and the XlnR regulated genes *aguA* and *faeA* are most highly expressed at the periphery of colonies of *A. niger* (Levin *et al.*, 2007). We here monitored expression of these genes using sGFP(S65T) as a reporter. Colonies expressing *sGFP(S65T)* from the *glaA, aamA, aguA* or *faeA* promoter were fluorescent throughout the mycelium when they had been grown for 5 days on inducing medium. The apparent discrepancy between mRNA accumulation (Levin *et al.*, 2007) and GFP fluorescence in sandwiched cultures grown for 5 days on an inducing medium can be explained by the extreme stability of GFP (Cubitt *et al.*, 1995). GFP fluorescence in the centre of a 5-day-old colony is largely due to expression of the construct when this zone represented the periphery of the colony (data not shown). To overcome this, colonies were grown for 5 days on repressing medium, after which they were transferred to inducing medium for 8 hours. Fluorescence of sGFP(S65T) was highest at the periphery of such colonies when its encoding gene was expressed from the *glaA* or *aamA* promoter. In contrast, expression of *aguA* was more evenly distributed in *A. niger* colonies, whereas expression of *faeA* was found to be highest in the centre. The difference in the expression pattern of the amylolytic genes *glaA* and *aamA* and the xylanolytic genes *aguA* and *faeA* can be explained by the spatial expression of their activators *amyR* (Petersen *et al.*, 1999) and *xlnR* (van Peij *et al.*, 1998), respectively. The gene *amyR* is mainly expressed at the periphery of maltose-grown colonies. In contrast, *xlnR* is expressed throughout the colony (Levin *et al.*, 2007). We have no explanation why *faeA* and *aguA* show different spatial expression profiles in xylose grown colonies. Our data suggest that one of the promoters is also regulated by another transcriptional regulator.

Expression of *glaA* on inducing maltose medium was not only heterogenic at the colony level but also within the outer zone of the colony (Vinck *et al.*, 2005). This was a surprising finding considering the fact that all hyphae were exposed to a fresh medium and thus experienced similar environmental conditions. Here, it was shown that expression of *aamA*, *faeA* and *aguA* is also heterogenic at the outer part of colonies of *A. niger*. In fact, hyphae that highly express one of these genes, also highly express the other genes as was concluded from reporter studies using GFP and dTomato. Correlation coefficients were between 0.73 and 0.86 when expression of the amylolytic genes was co-localized (*glaA* with *aamA* and *glaA* with *amyR*) on an inducing maltose medium. Similarly, correlation coefficients were between 0.73 and 0.77 when expression of the xylanolytic genes *faeA* and *aguA* were co-localized on inducing xylose medium. They were lower, but still strongly significant, when expression of the XlnR regulated gene *faeA* was correlated to the AmyR regulated genes *glaA* and *aamA*. It should be noted that these studies were performed on a medium containing 25 mM xylose. This induces *faeA* but *glaA* and *aamA* are not induced. Adding 25 mM maltose to the xylose medium did not increase *glaA* and *aamA* expression, whereas replacing xylose for maltose induced the amylolytic genes but completely repressed *faeA* expression (data not shown). Thus, we could not co-localize expression of amylolytic and xylanolytic genes when both are induced. We also could not correlate expression

of *aguA* and *faeA* with their regulatory gene *xlnR*. Gene *xlnR* is too lowly expressed to result in detectable levels of fluorescence of the reporter dTomato.

The high correlation coefficient between *glaA* and *amyR* and between *glaA* and *aamA* suggests a causative role of AmyR in heterogenic expression of the amylolytic genes at the periphery of the *A. niger* colony. However, the correlation between expression of the amylolytic genes and the xylanolytic genes suggests a higher level of regulation. This is strengthened by the correlation of the expression of xylanolytic gene *faeA* and constitutively expressed gene *gpdA* and by the strong correlation between expression of *glaA* and the abundance of *18S* rRNA. We propose that at least two populations of hyphae exist at the periphery of the *A. niger* colony that can be discriminated by their transcriptional and translational activity. Intuitively, one would assume that a lower transcriptional and translational activity would result in a lower growth rate. Yet, this is not the case. The reporter proteins that were monitored were formed in an 8-h period. During this time the outer zone had expanded 0.6 mm. In our analysis, the apical zones (100 µm) of the outermost hyphae were visualized. Therefore, these hyphae must have had a similar growth rate. Taking this into account, we assume that the transcriptional and translational activity in the low-expressing hyphal population is sufficient to support growth. The higher activity in the high-expressing hyphal population may result in a higher secretory activity. It is known that filamentous fungi secrete a significant part of their proteins into their environment. Considering the fact that this is executed by a minority of the hyphae in the colony, one would indeed expect increased cellular activity in the secreting hyphae.

What could be the mechanism underlying hyphal heterogeneity? First, it was explored whether heterogeneity in gene expression is the result of heterogeneity within the inoculum. In the study of Vinck *et al.* (2005) colonies were inoculated with multiple spores. Here it was shown that heterogenic *glaA* expression also occurs at the periphery of colonies that originate from a single spore or a single hypha. This strongly indicates that the hyphae that highly and lowly express *glaA* arise during vegetative growth and are not due to heterogeneity in spores produced in the conidiophores. In the next step, it was assessed whether epigenetic regulation may be involved in heterogenic gene expression. Epigenetic regulation concerns processes involved in altering and maintaining the chromatin structure of the DNA. The role of this type of regulation was assessed using AZC and SB. AZC alters the chromatin structure by inhibiting methylase activity and by incorporating into the DNA by replacing cytidine. This inhibits histone and DNA methylation, respectively (Jones 1985). SB causes hyperacetylation of histones by inhibiting histone deacetylases (Sealy and Chalkley, 1978; Boffa *et al.*, 1978). Both AZC and SB result in a more lightly packed DNA structure, which would allow a more efficient transcription. The presence of AZC and SB resulted in a normal distribution of fluorescence intensity at the periphery of the colony. These results indicate that epigenetics might very well be involved in establishing hyphal heterogeneity.

Future studies will be devoted to assess gene expression in high and low expressing hyphae at the periphery of the colony. This should provide further insight into the mechanism of heterogeneity. Moreover, it may reveal why colonies send out exploring hyphae that are heterogenic with respect to gene expression.

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CHAPTER 5

ABSTRACT

Mycelia of filamentous fungi, such as *Aspergillus niger*, are exposed to a heterogenic environment. This heterogeneity is reflected in the different RNA profiles at the centre and the periphery of the colony. Here, the RNA profile of the periphery of the colony was compared to that of the tips of the most outer hyphae of this zone. To this end, a protocol was developed to isolate and amplify the small amounts of RNA from selected (sub-cellular parts of) hyphae. A 3-4 µm wide and 100 µm long hyphal tip of *A. niger* was shown to contain 1 picogram of total RNA. *A. niger* Affymetrix arrays were hybridized with cDNA amplified from RNA of 500 hyphal tips and from an equivalent amount of RNA from the periphery of the mycelium. The functional gene categories transport facilitation, transcription, cell rescue, defense, and virulence, and regulation of or interaction with cellular environment were among the categories that have a more significant contribution to the top 10% of the most abundant RNAs within the hyphal apices. Notably, 3 out of 4 rRNA genes belonged to the top 100 of genes that have a relatively high RNA abundance in the hyphal tip. This agrees with the finding that the most outer part of the colony contains more RNA per hyphal unit when compared to the more inner part of the peripheral zone. Taken together, the RNA composition of the hyphal tip of exploring hyphae seems to be adapted to grow actively and to anticipate to the uncolonized environment they encounter.

SUBCELLULAR RNA PROFILING IN *ASPERGILLUS NIGER*

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Parts of this chapter have been submitted.

INTRODUCTION

Filamentous fungi colonize substrates by means of hyphae that grow at their apices and that branch sub-apically. This results in mycelia that consist of interconnected hyphae. The cytoplasm within a mycelium is assumed to be continuous due to highly porous septa that compartmentalize the hyphae. Such continuity would facilitate colonization of a heterogenic environment by enabling intra-mycelial translocation of water and nutrients (Jennings *et al.*, 1974). Colonies grown on a solid agar medium are exposed to such a heterogenic environment. The periphery of the colony experiences an unexplored, fresh substrate, whereas the center is confronted with a substrate that has been utilized. Previous studies have shown that this heterogeneity of the substrate is reflected in heterogenic protein secretion and gene expression within the colony. RNA profiles at the centre and the periphery of colonies of *Aspergillus niger* (Levin *et al.*, 2007b), *Aspergillus oryzae* (Masai *et al.*, 2006) and *Neurospora crassa* (Kasuga & Glass, 2008) were shown to be highly different. In the case of *A. niger* it was shown that these differences in gene expression were caused by medium dependent as well as medium independent mechanisms (Levin *et al.*, 2007a). As a result of heterogenic gene expression, enzymes are secreted within particular zones in the colony. For instance, the enzyme glucocamylase was shown to be secreted at the periphery of colonies of *A. niger* (Wösten *et al.*, 1991), whereas lignin peroxidase was found to be secreted at the center of colonies of *Phanerochaete chrysosporium* (Moukha *et al.*, 1993).

Here, the RNA profile of the periphery of the colony was compared to that of the tips of the most outer hyphae within this zone of the colony (*i.e.* the exploring hyphae). To this end, a protocol was developed to isolate and amplify picogram amounts of RNA from individual hyphae. Using this protocol it is shown by microarray analysis that hyphal tips are enriched in ribosomal RNA as well as RNA from several gene categories including the categories of transport facilitation, transcription, cell rescue, defense, and virulence, and regulation of or interaction with cellular environment.

MATERIAL AND METHODS

Strain and culture conditions

Experiments were done with strain AR9#2. This strain was made by transforming *A. niger* strain AB4.1 (*pyrG*, *cspA1*) (van Hartingsveldt *et al.*, 1987) with construct pAN52-10S65TGGPn/s (Siedenberg *et al.*, 1999). This construct contains *sGFP*(S65T) under the regulation of the promoter of the glucoamylase gene *glaA* of *A. niger*. The *A. niger* strain was cultured as a sandwiched colony at 30 °C in the light. To this end, the fungus was grown between a perforated polycarbonate (PC) membrane (diameter 76 mm, pore size 0.1 µm; Osmonics, GE Water Technologies) and a Lumox membrane (diameter 76 mm; Greiner Bio-One) (Vinck *et al.*, 2010; **Chapter 4**). The PC membrane was placed on top of solidified (1.5% agar) minimal medium (MM) (de Vries *et al.*, 2004) containing 25 mM maltose as a carbon source. Freshly harvested spores (1.5 µl of a solution of 0.8% NaCl and 0.005% Tween-80 containing 10^8 spores ml^{-1}) were placed in the center of the PC membrane. The droplet was allowed to dry, after which the Lumox membrane was placed on top of the PC membrane with its hydrophobic side facing the inoculum. After 7 days of growth, the Lumox membrane was removed. The mycelium and the underlying PC membrane were cut with a scalpel and parts of the periphery of the colony were placed upside down onto a nucleotide and RNAse free glass slide. The PC membrane that was now facing the air was removed and the mycelium was fixed with 70% ethanol and air-dried.

Laser micro-dissection and pressure catapulting

Hyphae or parts thereof were isolated using laser pressure catapulting (LPC) with the PALM CombiSystem (Carl Zeiss MicroImaging). This system was equipped with an Axiovert 200M Zeiss inverted microscope (Carl Zeiss AG) and a 3CCD color camera (HV-D30, Hitachi Kokusai Electric Inc.). The PALM CombiSystem was operated with PALM RoboSoftware V4.0 (Carl Zeiss MicroImaging GmbH) from which the autoLPC option was routinely used in combination with a 40x objective. Hyphal material was catapulted into lids of 0.5 ml Eppendorf tubes that contained 50 µl RNA*later* (Qiagen) to preserve the RNA within the dissected hyphae.

RNA isolation and amplification

Mycelium was snap-frozen in liquid nitrogen and homogenized in a 2 ml Eppendorf tube using two pre-cooled metal balls (4.7 mm in diameter), a chilled container and a Micro-Dismembrator U (B. Braun Biotech Int.). RNA was isolated with TRIzol reagent (Invitrogen) according to the instructions of the manufacturer and was purified using Nucleobond columns (Machery-Nagel). RNA concentration was measured using a Nanodrop (Nanodrop Technologies Inc.).

RNA from hyphal material was also isolated using TRIzol reagent. To this end, the material that had been collected in 50 µl RNA*later* (see above) was transferred to a 2 ml Eppendorf tube. The cap of the 0.5 ml Eppendorf centrifuge tube was taped on top of the 2 ml tube, which was followed by centrifugation at 10.000 g for 5 s. After snap-freezing in liquid nitrogen, 2 pre-cooled metal bullets (4.76 mm in diameter) were

added and samples were ground in a Micro-Dismembrator U (B. Braun Biotech Int.) in a chilled container at 1500 rpm for 60 s. The frozen material was taken up in 250 µl Trizol Reagent (Invitrogen) by vortexing. After removing the metal bullets, 200 µl chloroform was added. After mixing well, samples were centrifuged at 10.000 g for 10 min. The water phase (approximately 200 µl) was mixed with 700 µl RLT from the RNeasy MinElute Cleanup Kit (Qiagen) to which 143 mM β–mercaptoethanol was added. RNA was purified following instructions of the manufacturer and eluted using 12 µl RNAse free water.

RNA samples were amplified using the Ovation Pico WTA System (Nugen). According to the supplier, input amounts of 500 pg to 50 ng of total RNA will result in 6 to 10 µg cDNA product of about 50-1500 bases in length. The quality and quantity of the resulting cDNA samples were checked using a Bioanalyzer (Agilent Technologies) and Nanodrop (Nanodrop Technologies Inc.), respectively.

Quantification of RNA in exploring hyphae

RNA from 1000 tips of exploring hyphae (3-4 µm in width and 100 µm in length) was spotted onto a Roti-Nylon plus membrane (Roth) together with a series of RNA with known concentration. After cross-linking with UV-light, the RNA was hybridized overnight at 42 °C (de Vries *et al.*, 2002) with α-^{32}P-CTP labeled random primed probe of *18S* rDNA. The blot was exposed to X-OMAT Blue XB films (Kodak) in a BioMax cassette (Kodak) with a BioMax TranScreen-HE (Kodak) at -80 °C.

Microarray analysis

Five µg of amplified cDNA (see above) was fragmented via combined chemical and enzymatic fragmentation using the Encore Biotin Module (Nugen). The fragments were biotin-labeled to the 3-hydroxyl end using the same Module according to the instructions of the manufacturer. The labeled cDNA was hybridized to Affymetrix GeneChip *A. niger* Genome Arrays. The GeneChip Hybridization, Wash and Stain Kit (#900720, Affymetrix) was used for hybridizations according to manufacturers protocol with the modification that the hybridization cocktail was prepared according to the Encore Biotin Module. The MAS5.0 algorithm (Affymetrix, 2002) was used for quality control of the hybridized arrays. This algorithm analyzes each array independently and compares the overall fluorescence intensities of the arrays. The MAS5.0 algorithm uses the Tukey's biweight estimator to provide a robust mean signal value and the Wilcoxon's rank test to calculate both significance and detection call for each probe set. Summarized expression values of the pheriphery and hyphal tip samples were calculated separately using the log-scale robust multi-array analysis (RMA) (Irizarry *et al.*, 2003). For further analyses, technical duplos were averaged. The top 10% highest signal values per biological sample were tested for over-representation of FunCats (Ruepp *et al.*, 2004) and non-FunCat categories with all 14455 probe sets as background using a hyper-geometrical test (Draghici *et al.*, 2003). Classification of the *A. niger* genes in FunCat categories has been described (Pel *et al.*, 2007).

Acridine orange, calcufluor white and DAPI staining

Parts of the periphery of *A. niger* sandwiched colonies were cut with a scalpel, transferred to a glass slide, fixed with 70% ethanol, and dried at room temperature. Mycelium was stained for 5 min with 0.4 mM acridine orange (Becton Dickinson, Franklin Lakes, NJ) in PBS. Fluorescence of acridine orange was monitored using a Leica MZ16 FA fluorescence stereomicroscope equipped with a mercury lamp and a Leica DFC420 C digital camera. Green fluorescence (indicative for DNA) was monitored with the Leica GFP3 filter set, whereas red fluorescence (indicative for RNA) was monitored with the Leica dsRed filter set. Calcofluor white (CFW) staining was done using PBS containing 0.01% Fluorescent Brightener 28 (Sigma F-3543), whereas DAPI staining was performed using 5 ng μl^{-1} DAPI (4', 6-diamidino-2-phenylindole; Sigma D-9542) in vector shield (Vector Laboratories). In both cases, samples were washed once with PBS after staining for 1 min. A Zeiss Axioscope 2PLUS equipped with a HBO 100 W mercury lamp, a Leica LFC 420C camera (2592x1944 pixels) and a standard DAPI filter was used to monitor fluorescence. Images were handled with Leica Application Suite software (version 2.8.1).

RESULTS

Hyphal architecture at the periphery of a sandwiched colony

Distribution of septa, nuclei, RNA and DNA was monitored at the periphery of sandwiched colonies. Septa, visualized with CFW, were not detected within the first 400 µm from the tip (Figure 1A). After the first septum, septa were separated by 50 to 100 µm. Nuclei, visualized by DAPI, were found throughout the hypha, except for the region 10-20 µm from the tip (Figure 1B-D). Acridine orange staining was used to localize DNA and RNA at the periphery of the colony. Acridine orange binds to single- and double-stranded nucleic acids, resulting in red and green fluorescence, respectively (Freudenberg *et al.*, 1996). The RNA/DNA fluorescence ratio at the outer 1 mm of the periphery was higher than that of the more inner part of the periphery (Figure 1E-G). In fact, RNA was most abundant in the apical 100-300 µm of the most outer hyphae of the colony.

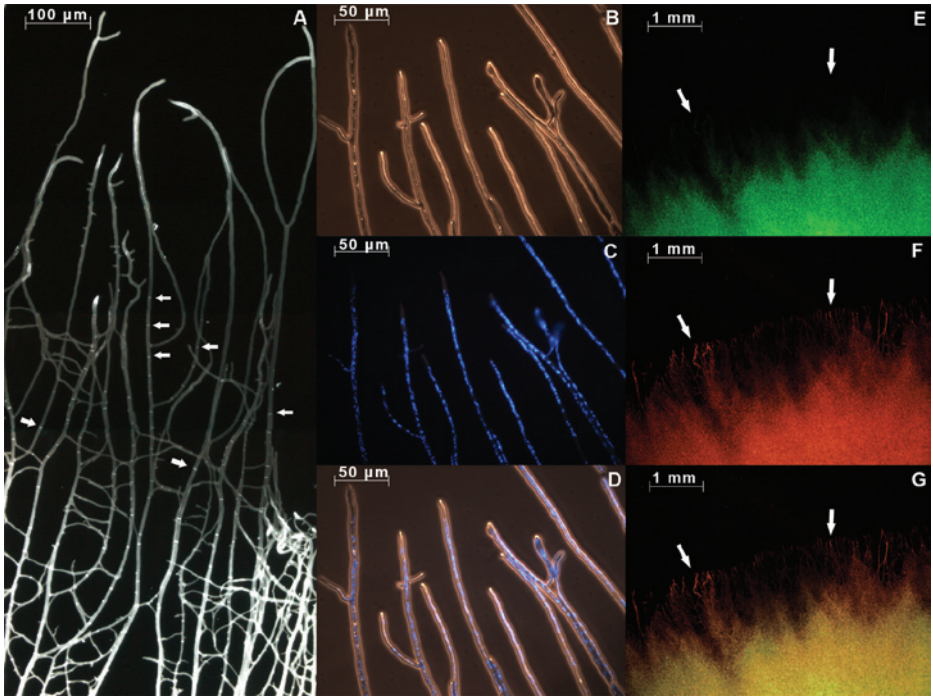

Figure 1 Distribution of septa, nuclei, RNA and DNA at the periphery of a sandwiched colony. (A) CFW staining visualizing the septa (indicated by arrows). The first septum is positioned 400 µm from the apex of the hyphae. (B-D) DAPI staining visualizing the nuclei within the hyphae; (B) brightfield, (C) DAPI fluorescence and (D) overlay of (B) and (C). Nuclei can be observed throughout the hyphae, except for the region 10-20 µm from the apex. (E-G) Acridine orange staining visualizing double stranded DNA (E) and total RNA (F). The overlay of DNA and RNA fluorescence (G) shows that the RNA/DNA ratio increases towards the periphery of the sandwiched colony. RNA is particularly abundant in the apices of the outermost hyphae of the colony (indicated by arrows).

RNA profiling of hyphal tips

A reproducible RNA extraction and amplification protocol was developed to enable analysis of transcript profiles of selected (parts of) hyphae within a mycelium. This protocol includes growth conditions, fixation, laser dissection, homogenization of the fungal material, RNA isolation, amplification and labeling of the amplified material (Appendix 1). The protocol was used to isolate RNA from 1000 hyphal tips (with a width of 3-4 µm and a length of 100 µm) from the outer periphery of sandwiched colonies of *A. niger* strain AR9#2. The RNA was spotted onto a nylon membrane and hybridized with an *18S* rDNA probe. The hybridization signal was compared to that of samples with a known RNA concentration. From this it was concluded that the 1000 hyphal tips contain 1 ng of RNA (Figure 2). This amount of RNA had to be amplified to micrograms of cDNA to enable hybridization of Affymetrix *A. niger* gene chips. The Ovation Pico WTA System was used for the amplification step. RNA from 500 hyphal tips with a length of 100 µm, *i.e.* 500 pg hyphal tip RNA, was amplified to 5.1 – 5.4 µg total cDNA. Similarly,

amplification of 500 pg of RNA isolated from the whole periphery of the colony (*i.e.* the outer most 3 mm of the mycelium) yielded 7.8 – 8.9 µg total cDNA. Bioanalyser analysis showed that the cDNA of the periphery samples mainly consisted of amplicons of 600 to 1500 nucleotides in size (Figure 3A). The amplicons of the hyphal tips were mainly 200-300 and 600-950 nucleotides in length (Figure 3B). The profiles were reproducible for each sample type.

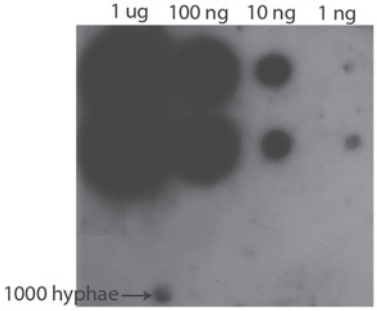

Figure 2 *18S* rRNA hybridization of a dot blot containing total RNA of 1000 hyphal tips and RNA samples with known quantity. Hybridization shows that 1000 hyphal tips contain about 1 ng total RNA.

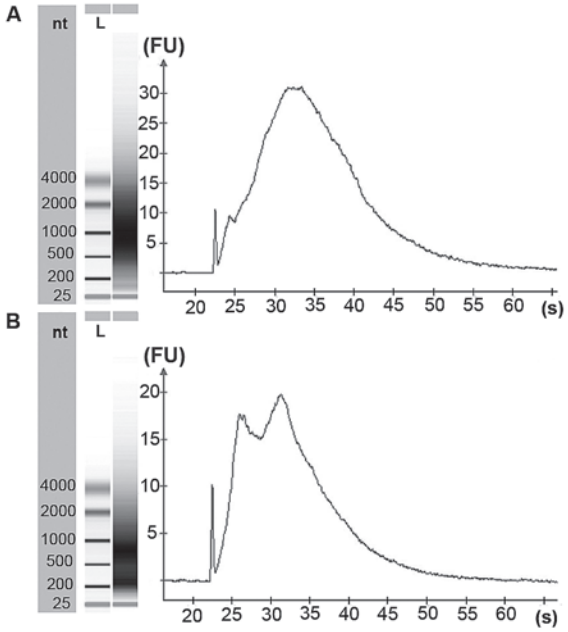

Figure 3 Bioanalyser analysis of cDNA amplified from 500 pg RNA from the periphery of a sandwiched colony (A) or from RNA derived from 500 hyphal tips of 100 µm in length (B). The electrophoresis gel image (nt, nucleotides; L, ladder) and the electropherogram (y-axis represents fluorescence units (FU); x-axis represents runtime in seconds (s)) for both sample types are given.

After fragmentation, the amplified samples were biotin labeled and hybridized to Affymetrix *A. niger* gene chips. For each sample type, two technical duplos of two biological duplos were analyzed. Based on MAS5.0 detection calls, transcripts of 71.7% and 76.9% of the genes were found to be present within the two periphery samples. This was only 38.1% and 49.8% for the hyphal tip samples. Genes with an absent call in the hyphal tip samples had generally low levels of RNA in the periphery samples (Figure 4). PCA plots showed that the RNA profiles of the technical and biological duplos were very similar (Figure 5A). However, the profiles from the periphery and the hyphal tip did not cluster (Figure 5A). This difference between the sample types was also illustrated by box plots. The dispersion and skewness of expression were very similar within the sample type but different between them (Figure 5B). Moreover, the scale factors of the sample types differed. They were between 0.49 and 0.63 for the periphery samples, and between 2.57 and 4.10 for the hyphal tip samples. This is a >3 fold difference, implying that these samples cannot be normalized and analyzed as a whole. Therefore, normalization and statistics were performed separately for each sample type. This was followed by indirect comparison of the profiles of the periphery and the hyphal tips.

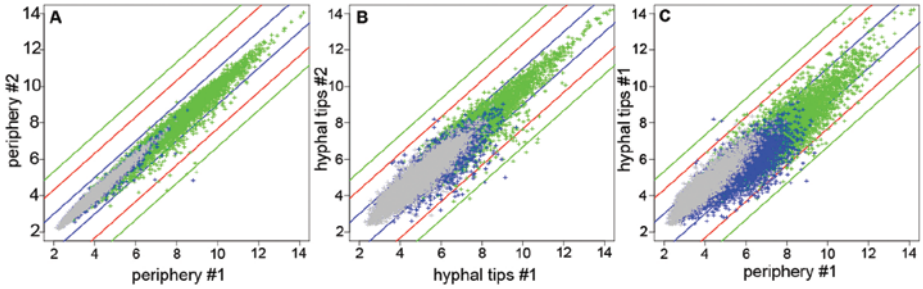

Figure 4 Scatter plots depicting the present and absent calls of all probe sets for the biological duplos of the periphery samples (A), the biological duplos of the hyphal tip samples (B) and for a sample from the periphery and the hyphal tip (C). These plots show that probe sets that are classified as absent in the hyphal tip are generally lowly expressed within the periphery. Green = present call in both samples, Blue = present call in one of the samples, and Grey = absent call in both samples.

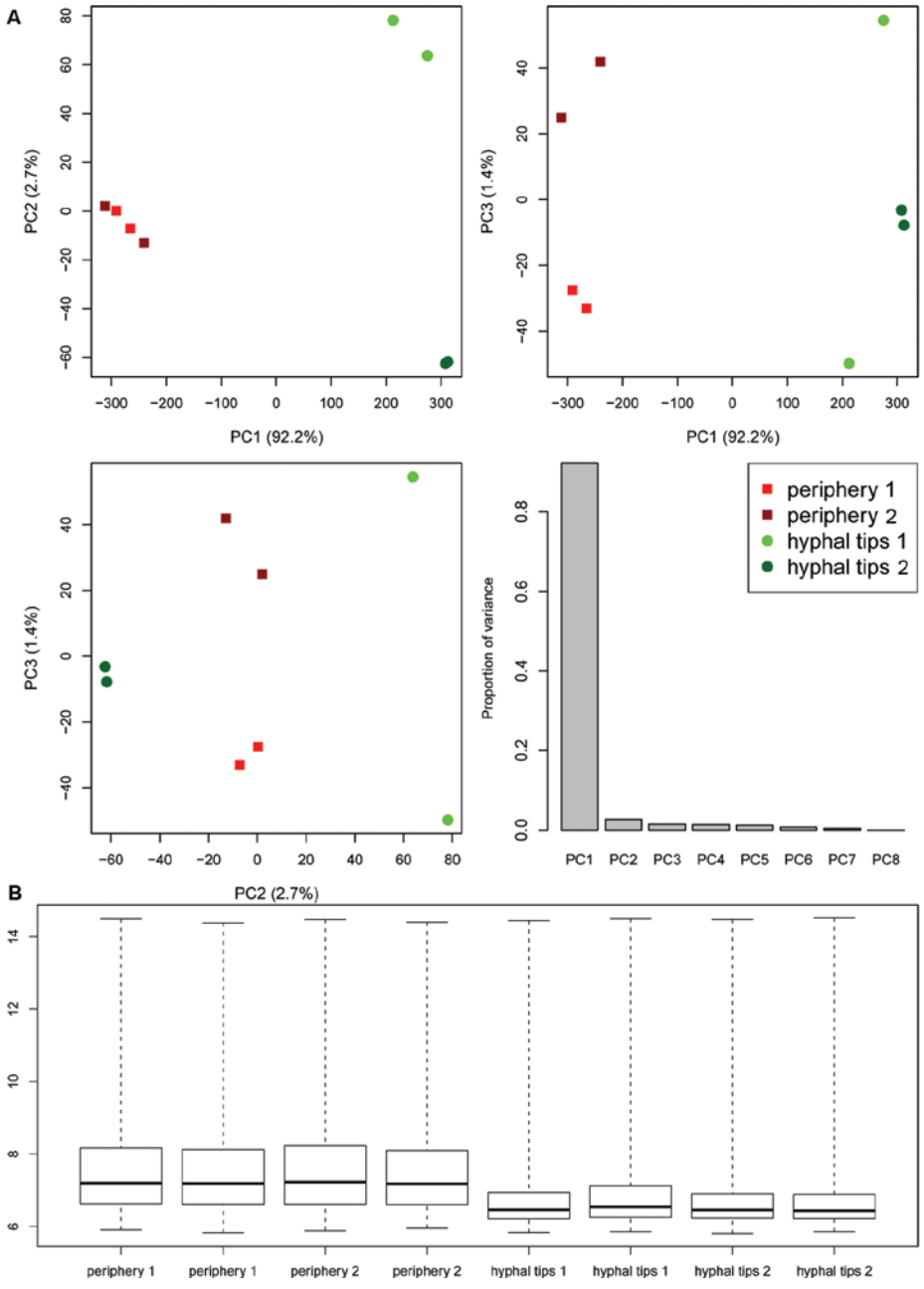

Figure 5 PCA plots (A) and boxplots (B) of the RNA profiles from the periphery and from the hyphal tips of the exploring hyphae of sandwiched colonies of *A. niger*. The plots show that the expression profiles of the periphery and the apices are different from each other. In contrast, technical and biological duplos are very similar. PC1 explains the largest variance, followed by PC2 and PC3.

Funcat analysis

The *A. niger* genes have been classified into 443 unique functional categories (FunCats) (Pel *et al.*, 2007). These categories consist of 1 to 7690 members. For over-representation testing, FunCats were selected that comprise 5-250 genes. Two additional non-FunCat categories (*i.e.* rRNA and tRNA) were added to the 301 selected FunCat categories. These categories were tested for their contribution to the top 10% of genes with the most intense hybridization signals within a sample type. Out of the 303 selected categories, 77 were found to significantly ($p \leq 0.01$) contribute to this top 10% in at least one of the biological replicates of the two sample types (Suppl. Table 1). These categories included 75 FunCats as well as the rRNA and tRNA categories. Of these 77 categories, 8 and 3 categories were found to only contribute to the top 10% of genes of the periphery and the hyphal tip, respectively (Table 1). Notably, 5 out of these 8 "peripheral" FunCats deal with protein fate.

Table 1 Functional gene categories that contribute significantly ($p \leq 0.01$) to the top 10% of genes with the most intense hybridization signal in either the periphery or the hyphal tip sample only. The number of probe sets within the category and the p-value for each analysis is given.

Categories periphery	Number of probe sets	Periphery 1	Periphery 2	Hyphal tips 1	Hyphal tips 2
01.01.01.07.06 biosynthesis of lysine	18	0.0064	0.0012	0.2663	0.2663
01.07.01 biosynthesis of vitamins, co-factors, and prosthetic groups	103	0.0062	0.0062	0.1464	0.0264
04.07 RNA transport	19	0.0086	0.0086	0.2947	0.1150
06.07.02 modification with sugar residues (e.g. glycosylation)	65	0.0044	0.0016	0.0111	0.0262
06.07.05 modification by ubiquitination, deubiquitination	56	0.0083	0.0083	0.1027	0.0492
06.07.11 protein processing (proteolytic)	31	0.0095	0.0026	0.1932	0.0305
06.13.04.02 vacuolar degradation	20	0.0004	0.0004	0.0112	0.0112
06.13.99 other proteolytic degradation	39	0.0001	0.0003	0.0893	0.0365
Categories hyphal tips	**Number of probe sets**	**Periphery 1**	**Periphery 2**	**Hyphal tips 1**	**Hyphal tips 2**
01.01.01.01.02 biosynthesis of the glutamate group (proline, hydroxyproline, arginine, glutamine, glutamate)	41	0.0180	0.0475	0.0060	0.0060
02.07.01 pentose-phosphate pathway oxidative branch	11	0.0895	0.0185	0.0002	0.0027
13.01.01 homeostasis of cations	147	0.0202	0.0109	0.0006	0.0013

In the next analysis, the 303 gene categories were ranked based on the p-value of the over-representation analysis (Supplementary Table 2). The average rank position of each of the categories within the hyphal tip sample was subtracted from that of the periphery sample. The categories that differed at least 50 positions in rank between the two samples are given in Table 2. The gene categories that have a more significant contribution in the top 10% of the genes with the highest RNA abundance at the periphery relate to the FunCat 1 classes metabolism (12 categories), cell cycle and DNA processing (2), protein fate (2), cellular transport and transport mechanisms (2), control of cellular organization (2), sub-cellular localization (2), cell fate (1), transport facilitation (1) and transposable elements viral, and plasmid proteins (1). On the other hand, FunCat 1 classes that have a more significant contribution within the hyphal apices relate to metabolism (8 categories), transport facilitation (4), transcription (4), cell rescue, defense, and virulence (3), regulation of or interaction with cellular environment (3), cell fate (2), sub-cellular localization (2), cellular communication or signal transduction mechanism (2), protein fate (1) and protein with binding function or co-factor requirement (1).

Table 2 Ranking of functional gene categories based on their p-value of over-representation within the top 10% of the most abundant RNAs at the periphery of the colony and of the hyphal tips of the most outer hyphae within this zone. Categories are listed that differed at least 50 positions when the ranking of the periphery and the hyphal tips is compared.

Categories higher ranked in periphery samples	Rank position periphery	Rank position hyphal tips	Δ rank
30.03 cytoplasm	93	256	-163
30.09 intracellular transport vesicles	76	212	-136
01.01.01.07.06.03 aminoadipic acid pathway	47	176	-129
01.01.10.04.03 degradation of leucine	154	257	-103
06.07.99 other protein modifications	107	198	-91
08.01 nuclear transport	88	172	-84
01.06.07 lipid, fatty-acid and isoprenoid utilization	100	179	-79
01.01.01.07.06 biosynthesis of lysine	45	117	-72
01.01.10.04 degradation of amino acids of the pyruvate family	160	231	-71
01.06.01.03 glycolipid biosynthesis	187	258	-71
40.08 golgi	83	149	-66
06.07.01 modification with fatty acids (e.g. myristylation, palmitylation, farnesylation)	97	161	-64
01.01.01.11.09 biosynthesis of phenylalanine	151	213	-62
01.20.13 biosynthesis of derivatives of homoisopentenyl pyrophosphate	198	259	-61
03.01.03 DNA synthesis and replication	85	145	-60
08.16.99 other export and secretion systems	158	216	-58
67.50 transport mechanism	150	207	-57
01.01.01.15.04 biosynthesis of valine	90	146	-56
29.07 proteins necessary for the integration or inhibition of transposon movement	204	260	-56

Table 2 (continued)

Categories higher ranked in periphery samples	Rank position periphery	Rank position hyphal tips	Δ rank
01.01.10 amino acid degradation (catabolism)	183	238	-55
01.06.13 lipid and fatty-acid transport	129	184	-55
01.01.99 other amino acid metabolism activities	207	261	-54
40.04 cytoskeleton	37	90	-53
14.01.03.99 other morphogenetic activities	174	226	-52
03.01 DNA processing	116	167	-51
67.01.01 ion channels	126	177	-51
Categories higher ranked in hyphal tip samples	**Rank position periphery**	**Rank position hyphal tips**	**Δ rank**
04.05.05.03 5'-end processing	216	93	123
10.01.09 second messenger mediated signal transduction	220	97	123
40.05 centrosome	292	175	117
63.03 nucleic acid binding	294	183	111
14.10 dell death	242	136	106
40.19 peroxisome	159	57	102
01.01.01.01.02.03 biosynthesis of arginine	180	79	101
01.06.99 other lipid, fatty-acid and isoprenoid metabolism activities	219	125	94
04.99 other transcription activities	286	192	94
13.01.01.99 homeostasis of other cations	177	88	89
67.11 peptide transporters	153	67	86
11.07.01 detoxification involving cytochrome P450	233	148	85
01.01.01.03.01 urea cycle	128	52	76
67.01.99 other channels and pores forming proteins	138	62	76
14.20 cell aging	191	116	75
01.01.01.03 urea cycle, biosynthesis of polyamines and creatine	133	59	74
11.05.99 other disease and defense related proteins	288	217	71
01.03 nucleotide metabolism	214	147	67
13.01.01.01 homeostasis of metal ions (NA, K, CA etc.)	194	128	66
67.04.01.01 heavy metal ion transporters (CU, FE, etc.)	223	157	66
01.07.04 utilization of vitamins, co-factors, and prosthetic groups	137	72	65
11.05.03 defense related proteins	203	140	63
04.03 tRNA transcription	239	181	58
67.99 other transport facilitators	208	151	57
04.01 rRNA transcription	118	66	52
06.07.04 modification by acetylation, deactylation	287	235	52
10.05.99 other transmembrane signal transduction mechanism	105	53	52
13.11.03.01 perception of nutrients and nutritional adaptation	165	113	52
01.05.01.01.05C-1 compound catabolism	215	164	51
01.05.01.03.04 aminosaccharide biosynthesis	271	220	51

Analysis of expression of individual genes

The log2 hybridization value of each of the 14455 genes of *A. niger* was related to the mean of the log2 hybridization signal of all genes. The ratio of each gene in the hyphal tip and periphery samples were compared (Suppl. Table 3), resulting in a top 100 of genes with the highest fold changes in RNA abundance at the periphery and the hyphal tip (Tables 3 and 4). Most of the genes (47) within the top 100 of the periphery encode unclassified proteins. Of the 53 classified genes, 20 belong to the FunCat 1 class related to metabolism. From these genes 7 are involved in C-compound and carbohydrate utilization. The remainder of genes with a relatively high RNA abundance belong to the FunCat 1 classes cell cycle and DNA processing (12 genes), protein fate (7), transcription (4), cell fate (2), sub-cellular localization (2), energy (1), cellular transport and transport mechanisms (1), cellular communication or signal transduction mechanism (1), cell rescue, defense and virulence (1), regulation of or interaction with cellular environment (1) and control of cellular organization (1) (Table 3). Most genes of the top 100 of the hyphal tips also encode unclassified proteins (56). Of the 44 classified genes a third relates to metabolism (15). Next to that, 5 tRNAs out of 137 genes encoding tRNAs (*i.e.* 5 out of 144 probe sets from this category), and 3 out of the 4 genes encoding rRNAs (*i.e.* 4 out of 8 probe sets) were found in the top 100 of genes of the hyphal tip (Table 4). The FunCat 1 classes protein synthesis and cellular transport and transport mechanisms were represented by 4 genes. The remainder of the relatively higher expressed genes in the hyphal tips belong to the FunCat 1 classes cell rescue, defense and virulence (3), sub-cellular localization (3), protein fate (2), cell cycle and DNA processing (1), transcription (1), sub-cellular localization (1) and development (1).

Table 3 Top 100 of genes with the highest ratio difference in RNA abundance between the periphery and the hyphal tip. Out of these 100 genes, 47 have not been classified (Pel *et al.*, 2007) and they have not been listed in this table. Δratio is the ratio of the hyphal tips subtracted from the ratio of periphery.

Gene number	Gene description	Δratio	Categories
An12g03150	strong similarity to multidrug resistance protein atrD - *Aspergillus nidulans*	3.17	08.16 extracellular transport, exocytosis and secretion
An02g04290	strong similarity to replication factor C chain RFC1 - *Saccharomyces cerevisiae*	2.95	03.03.01 mitotic cell cycle and cell cycle control
An04g03840	similarity to microtubule binding protein D-CLIP-190 - *Drosophila melanogaster*	2.89	40.04 cytoskeleton
An11g09520	strong similarity to D-lactate dehydrogenase KIDLD - *Kluyveromyces lactis*	2.72	02.16.03 lactate fermentation
An02g13980	strong similarity to trichodiene oxygenase cytochrome P450 CYP58 - *Fusarium sporotrichioides*	2.63	01.06.01.07.05 sesquiterpenes biosynthesis
An11g10260	strong similarity to alphaN-acetylglucosamine transferase - *Kluyveromyces lactis*	2.61	06.07.02 modification with sugar residues (e.g. glycosylation)
An07g05100	strong similarity to heterokaryon incompatibility protein het-6 - *Neurospora crassa*	2.59	14.04.03.03 pheromone response, mating-type determination, sex-specific proteins
An03g02920	strong similarity to molybdopterin cofactor biosynthetic protein cnxABC - *Aspergillus nidulans*	2.58	01.07.01 biosynthesis of vitamins, cofactors, and prosthetic groups

Table 3 (continued)

Gene number	Gene description	Δratio	Categories
An14g03420	strong similarity to CAAX prenyl protein protease RCE1 - *Homo sapiens*	2.55	06.13 proteolytic degradation
An02g07770	strong similarity to trehalose synthase TSase - *Grifola frondosa*	2.52	01.05.01.03 C-compound, carbohydrate anabolism
An08g03610	similarity to acid sphingomyelinase phosphodiesterase ASM1 - *Homo sapiens*	2.52	01.06.04 breakdown of lipids, fatty acids and isoprenoids
An11g07290	similarity to spectinomycin adenylyltransferase spc - Staphylococcus aureus	2.51	11.07.03 detoxification by modification
An08g03480	strong similarity to the mitochondrial heat shock protein Hsp78p - *Saccharomyces cerevisiae*	2.51	06.01 protein folding and stabilization
An09g05480	strong similarity to the ubiquitin-specific protease HAUSP - *Homo sapiens*	2.50	06.07.05 modification by ubiquitination, deubiquitination
An07g06490	strong similarity to insulin-degrading enzyme IDE - *Rattus norvegicus*	2.49	06.13.01 cytoplasmic and nuclear degradation
An01g11910	similarity to cut17 protein - *Schizosaccharomyces pombe*	2.49	03.03.01.03 cell cycle checkpoints (checkpoints of morphogenesis, DNA-damage,-replication, mitotic phase and spindle)
An01g13650	similarity to the cytadherence accessory protein HMW1 - *Mycoplasma pneumoniae*	2.47	40.04 cytoskeleton
An01g03540	similarity to DNA double-strand break repair or V(D)J recombination protein artemis - *Homo sapiens*	2.44	03.01.05 DNA recombination and DNA repair
An07g05900	strong similarity to fructosyl amino acid oxidase faoA - *Aspergillus terreus*	2.43	01.20.50 catabolism of secondary metabolites
An04g05870	strong similarity to E3 ubiquitin ligase TOM1 - *Saccharomyces cerevisiae*	2.42	03.03.01 mitotic cell cycle and cell cycle control
An08g10440	similarity to meiosis-specific protein HOP1 - *Kluyveromyces lactis*	2.42	03.03.02 meiosis
An09g03890	strong similarity to hydroxic acid dehydrogenase-like protein encoded by ORF1 patent DE19912706-A1 - *Staphylococcus aureus*	2.42	01.05.01 C-compound and carbohydrate utilization
An08g00070	strong similarity to chromosome region maintenance protein 1 crm1 - *Schizosaccharomyces pombe*	2.36	04.07 RNA transport
An02g06230	strong similarity to transcription initiation factor TFIIB - *Kluyveromyces lactis*	2.35	04.05.01.01 general transcription activities
An07g04390	similarity to 6-Hydroxy-D-nicotine oxidase 6-HDNO - *Arthrobacter oxidans*	2.34	01.05.01.01 C-compound, carbohydrate catabolism
An07g09800	strong similarity to putative polyphosphate synthetase VTC3 - *Saccharomyces cerevisiae*	2.33	01.04 phosphate metabolism
An02g11720	strong similarity to alpha-mannosidase msd2 – *Aspergillus nidulans*	2.33	01.05.01 C-compound and carbohydrate utilization
An03g06930	similarity to protein involved in cell cycle control rad17 - *Schizosaccharomyces pombe*	2.31	03.03.01 mitotic cell cycle and cell cycle control
An09g01240	strong similarity to phospholipase B - *Penicillium notatum*	2.30	01.06.07 lipid, fatty-acid and isoprenoid utilization
An11g09590	similarity to transcription factor Gal4 – *Saccharomyces cerevisiae*	2.28	01.05.04 regulation of C-compound and carbohydrate utilization

Table 3 (continued)

Gene number	Gene description	Δratio	Categories
An15g03210	strong similarity to gamma-butyrobetaine hydroxylase BBH - *Homo sapiens*	2.26	01.07.01 biosynthesis of vitamins, cofactors, and prosthetic groups
An18g01780	strong similarity to flavohemoglobin hmp - *Escherichia coli* [putative frameshift]	2.25	01.01.10.05.06 degradation of tyrosine
An11g00460	strong similarity to amyloid precursor protein-binding protein 1 APP-B1 - *Homo sapiens*	2.24	03.03.01 mitotic cell cycle and cell cycle control
An03g06480	strong similarity to Steroid monooxygenase smo - *Rhodococcus rhodochrous*	2.24	01.20.05.11 biosynthesis of polyketides
An18g06730	similarity to GTPase-activating protein RLIP76 - *Homo sapiens*	2.23	10.01.01 unspecified signal transduction
An01g05260	strong similarity to DEAH protein MPH1 – *Saccharomyces cerevisiae*	2.22	03.01.05.01 DNA repair
An15g00320	strong similarity to beta-fructofuranosidase precursor suc1 - *Aspergillus niger*	2.22	01.05.01.01.01 sugar, glucoside, polyol and carboxylate catabolism
An10g00870	pectate lyase plyA - *Aspergillus niger*	2.21	01.05.01.01 C-compound, carbohydrate catabolism
An08g10560	strong similarity to Rad54 homolog mus-25 - *Neurospora crassa*	2.21	03.01.05.01 DNA repair
An15g07510	strong similarity to peptide transport gene CaPTR2 – *Candida albicans*	2.20	01.01 amino acid metabolism
An16g01380	weak similarity to iron-deficiency-induced protein IdiA - *Synechococcus* PCC6301	2.19	13.01.01.01 homeostasis of metal ions (Na, K, Ca etc.)
An13g01310	similarity to the methyl-CpG binding protein MBD4 – *Homo sapiens*	2.18	30.10.03 organization of chromosome structure
An16g01950	strong similarity to Fe(II)-dependent sulfonate or alpha-ketoglutarate dioxygenase YLL057c - *Saccharomyces cerevisiae*	2.18	01.02.01 nitrogen and sulfur utilization
An04g05440	strong similarity to xanthine dehydrogenase XDH – *Homo sapiens*	2.18	01.03.16.03 DNA degradation
An11g11110	strong similarity to condensin complex component cnd1 - *Schizosaccharomyces pombe*	2.18	03.03.01 mitotic cell cycle and cell cycle control
An12g04830	strong similarity to coatomer protein zeta chain RET3 - *Saccharomyces cerevisiae*	2.17	06.04 protein targeting, sorting and translocation
An02g14980	strong similarity to single-stranded DNA-binding protein 68k chain ssb1(+) - *Schizosaccharomyces pombe*	2.17	03.01.03 DNA synthesis and replication
An05g00780	similarity to transcription elongation factor ela1 - *Saccharomyces cerevisiae*	2.17	04.05.01 mRNA synthesis
An07g07370	weak similarity to PC-MYB2 - *Arabidopsis thaliana*	2.16	04.05.01.04 transcriptional control
An14g03390	strong similarity to fluG - *Emericella nidulans*	2.16	14.01 cell growth or morphogenesis
An11g05340	strong similarity to fructosyl amine oxygen oxidoreductase - *Aspergillus fumigatus*	2.15	01.20.17 biosynthesis of secondary products derived from primary amino acids
An02g14830	strong similarity to DNA polymerase II subunit-B DPB2 - *Saccharomyces cerevisiae*	2.15	03.01.03 DNA synthesis and replication
An02g02880	strong similarity to ADP-ribosylation factor-like protein Arl1 - *Saccharomyces cerevisiae*	2.15	06.07 protein modification

Table 4 Top 100 of genes with the highest ratio difference in RNA abundance between the hyphal tips and the periphery. Of these 100 genes, 56 have not been classified (Pel *et al.*, 2007) and they have not been listed in this table. Δratio is the ratio of the periphery subtracted from the ratio of hyphal tips.

Gene number	Gene description	Δratio	Category
An03e03150	5.8S ribosomal RNA	4.57	rRNA
An03e00510	trnaQttg	3.46	tRNA
An03e03260	18S ribosomal RNA	3.46	rRNA
An03e03260	18S ribosomal RNA	2.78	rRNA
An14g04200	rhamnogalacturonase rhgB - *Aspergillus niger*	2.65	01.05.01.01.01 sugar, glucoside, polyol and carboxylate catabolism
An03e03180	28S ribosomal RNA	2.64	rRNA
An12e09740	trnaAcgc	2.60	tRNA
An01g07900	leucine zipper cpcA - *Aspergillus niger* [putative frameshift]	2.56	01.01.04 regulation of amino acid metabolism
An04g07440	similarity to endoplasmic reticulum membrane protein SHR3 - *Saccharomyces cerevisiae* [truncated ORF]	2.49	06.04 protein targeting, sorting and translocation
An01e08330	trnaSgct	2.37	tRNA
An14g04940	strong similarity to mRNA turnover 4 protein MRT4 - *Saccharomyces cerevisiae*	2.28	01.03.16.01 RNA degradation
An08g01800	strong similarity to hypothetical mitochondrial carrier protein AgPET8 - *Ashbya gossypii*	2.26	08.04 mitochondrial transport
An15g07550	strong similarity to neutral amino acid permease Mtr - *Neurospora crassa*	2.25	01.01.07 amino acid transport
An17g00120	strong similarity to major facilitator superfamily transporter protein mfs1 - *Botrytis cinerea*	2.23	11.07.05 detoxification by export
An08g07360	strong similarity to mitochondrial import receptor TOM20 - *Neurospora crassa*	2.18	06.04 protein targeting, sorting and translocation
An08g06850	similarity to hypothetical C2H2 zinc-finger protein SPBC1105.14 - *Schizosaccharomyces pombe*	2.12	40.10 nucleus
An05g00540	strong similarity to cytoplasmic ribosomal protein of the large subunit L19 - *Saccharomyces cerevisiae*	2.04	05.01 ribosome biogenesis
An12g04530	strong similarity to versicolorin B synthase vbs - *Aspergillus parasiticus* [putative sequencing error]	2.04	01.20.05.11 biosynthesis of polyketides
An18g04310	strong similarity to cytoplasmic ribosomal protein of the small subunit S12 - *Sus scrofa*	2.04	05.01 ribosome biogenesis
An02g00570	strong similarity to atrazine chlorocyclohydrolase AtzA - *Pseudomonas* sp.	2.01	11.07.03 detoxification by modification
An17g00650	similarity to the cercosporin resistance protein Crg1 - *Cercospora nicotianae*	1.98	11.07 detoxification
An01g13570	similarity to poly A polymerase pla1 - *Schizosaccharomyces pombe* [truncated ORF]	1.98	40.10 nucleus
An04g05730	similarity to inhibitor of endosome-lysosome fusion DotA - *Legionella pneumophila*	1.97	08.07 vesicular transport (Golgi network, etc.)
An11e07400	trnaLcaa	1.96	tRNA

Table 4 (continued)

Gene number	Gene description	Δratio	Category
An04g09380	strong similarity to proline permease prnB - *Aspergillus nidulans*	1.95	01.01.07 amino acid transport
An04g02030	strong similarity to ATP-dependent RNA helicase DED1 - *Saccharomyces cerevisiae*	1.95	04.05.05.01 splicing
An07g06650	similarity to acetoacetyl-CoA reductase phbB - *Zoogloea ramigera* [truncated ORF]	1.92	01.06.01.05 fatty acid biosynthesis
An01g15000	strong similarity to HC-toxin efflux pump TOXA - *Cochliobolus carbonum*	1.92	08.16 extracellular transport, exocytosis and secretion
An08g08280	weak similarity to homeobox protein slouch slou - *Drosophila melanogaster*	1.91	25 DEVELOPMENT (Systemic)
An03g05290	similarity to glucan 1,3-beta-glucosidase BGL2 - *Saccharomyces cerevisiae*	1.89	01.05.01 C-compound and carbohydrate utilization
An09g01860	strong similarity to polyketide synthase alb1 - *Aspergillus fumigatus*	1.85	01.20.35.01 biosynthesis of phenylpropanoids
An11e07410	trnaLcaa	1.85	tRNA
An18g02380	similarity to the glucose or galactose transporter GluP - *Brucella abortus*	1.85	01.05.07 C-compound, carbohydrate transport
An06g02030	strong similarity to monosaccharide transporter Mst-1 - *Amanita muscaria*	1.83	08.19 cellular import
An02g13850	strong similarity to cytoplasmic ribosomal protein of the large subunit URP1 - *Saccharomyces cerevisiae*	1.82	05.01 ribosome biogenesis
An03g03270	similarity to ankyrin Ank-1 - *Mus musculus*	1.79	40.04 cytoskeleton
An17g02040	strong similarity to the protein required for normal CLN1 and CLN2 G1 cyclin expression Ctr9 - *Saccharomyces cerevisiae*	1.79	03.03.01 mitotic cell cycle and cell cycle control
An18g04560	similarity to peptidoglycan GlcNAc deacetylase PgdA - *Streptococcus pneumoniae*	1.75	01.05.01 C-compound and carbohydrate utilization
An07g01150	strong similarity to phytoene dehydrogenase PDH1- *Cercospora nicotianae*	1.75	01.07.04 utilization of vitamins, cofactors, and prosthetic groups
An04g06490	strong similarity to 3,4-dihydroxy-5-hexaprenylbenzoate methyltransferase - *Rattus norvegicus*	1.74	01.07.01 biosynthesis of vitamins, cofactors, and prosthetic groups
An16g05350	strong similarity to bifunctional folic acid synthesis protein precursor - *Pisum sativum* [putative sequencing error ORF]	1.73	01.07.01 biosynthesis of vitamins, cofactors, and prosthetic groups
An18g03920	strong similarity to the defender against apoptotic cell death DAD1 - *Homo sapiens*	1.73	14.10 cell death
An14g02900	weak similarity to cellobiose dehydrogenase CDH - *Trametes versicolor*	1.72	01.20.50 catabolism of secondary metabolites
An01g04430	strong similarity to translation initiation factor eIF-3 subunit - *Schizosaccharomyces pombe*	1.71	05.04.01 initiation

DISCUSSION

Colonies of *A. niger* are highly heterogenic. Gene expression and protein secretion vary between zones within the mycelium (Wösten *et al.*, 1991; Masai *et al.*, 2006; Levin *et al.*, 2007b; Kasuga & Glass, 2008). Notably, even within a zone heterogeneity is observed. Studies on protein secretion in *A. niger* showed that glucoamylase is only secreted by part of the growing hyphae at the periphery (Wösten *et al.*, 1991). Reporter studies indicated that this heterogenic glucoamylase secretion is caused by heterogenic expression of its encoding gene *glaA*. Two populations of hyphae were distinguished, those that highly and those that lowly expressed this gene (Vinck *et al.*, 2005). Follow up studies showed that hyphae that highly express *glaA* also highly express other genes encoding secreted proteins. Moreover, these hyphae highly express the glyceraldehyde-3-phosphate dehydrogenase gene *gpdA* and contain relatively high levels of *18S* rRNA (Vinck *et al.*, 2010; **Chapter 4**). From this it was concluded that part of the hyphae at the periphery of *A. niger* colonies show a high transcriptional and translational activity, whereas others show a low activity (Vinck *et al.*, 2010; **Chapter 4**). Here, it is shown that the RNA composition of tips of the most outer hyphae of the colony differ from the RNA composition of the periphery as a whole. A difference in composition was not expected a priori considering the fact that septa of fungi such as *A. niger* contain large pores. These pores allow intra- and intercellular streaming of water, nutrients, metabolites, and even organelles (Jennings *et al.*, 1974).

An RNA extraction and amplification protocol was developed to enable RNA profiling of selected hyphae or parts thereof. Using this protocol, it was established that the most apical 100 µm of the hypha contains 1 pg of RNA. It is known that a typical mammalian cell contains about 10-30 pg total RNA (Alberts *et al.*, 1994), whereas the smaller *Escherichia coli* cells contain about 5.6 fg RNA (Pang & Winkler, 1994; Pang & Winkler, 1994; Pang & Winkler, 1994; Pang & Winkler, 1994). The amount of RNA in fungal hyphae or yeast cells was not yet established. *S. cerevisae* has been reported to contain 60.000 mRNAs per cell (Zenklusen *et al.*, 2008). Assuming that these mRNAs comprise 5% of the total RNA (Alberts *et al.*, 1994; Neidhardt & Umbarger, 1996) and that the average RNA length is 2500 nucleotides (Alberts *et al.*, 1994), *S. cerevisiae* would contain 2.5 pg total RNA per cell. This amount is well in line with the 1 pg of RNA that has been extracted from the hyphal apex of *A. niger.*

RNA profiles were determined for the 3 mm wide peripheral zone of sandwiched colonies of *A. niger* and for the apices (*i.e.* the first 100 µm) of the most outer hyphae within this zone. The selected apical region of the exploring hyphae only represents part of the first compartment since the first septum is formed 400 µm from the tip. The more subapical septa were found to be separated by about 50-100 µm. This has also been found in *Aspergillus fumigates* (Hickey & Read, 2009). Nuclei were observed throughout the first compartment of the exploring hyphae of *A. niger*, except for the absolute apex, which comprised 10-20 µm. This is similar to what has been observed in *N. crassa* (Freitag *et al.*, 2004). The outer 1 mm of the periphery contained relatively more RNA than DNA when compared to the more inner part of this zone of the colony. In fact, RNA was most abundant within the first 100-300 µm of the first compartment of

the exploring hyphae. This finding is supported by *in situ* hybridization using *18S* rRNA as a probe (Teertstra *et al.*, 2004; Vinck *et al.*, 2010; **Chapter 4**). These data indicate that the apices of the exploring hyphae of *A. niger* are the parts within the periphery, and even within the whole colony, that are most rich in RNA. A high concentration of RNA has been associated with a high growth rate in bacteria (De Vries *et al.*, 2004; Pérez-Osorio *et al.*, 2010) and *S. cerevisae* (Waldron & Lacroute, 1975). Rapid changes in rRNA due to changing growth rates were found in *E. coli*, *S. cerevisiae*, and *N. crassa*. This was associated with a change in ribosomal protein levels and the number of ribosomes (Sturani *et al.*, 1973; Dennis & Bremer, 1974; Waldron & Lacroute, 1975; Sturani *et al.*, 1976; Donovan & Pearson, 1986; Herruer *et al.*, 1987). A high amount of RNA at the most outer part of the hypha would make sense considering the fact that hyphal extension takes place at the tip region and not in subapical parts.

RNA of 3 out of 4 rRNA genes was found to be enriched in the growing hyphal tips when compared to the periphery as a whole. This enrichment had consequences for the microarray analysis. Microarrays are hybridized with similar amounts of cDNA. In this study, cDNA was not only synthesized from the mRNA pool (*i.e.* from the polyA tail). Instead, a mixture of poly-dT and random primers was used according to the Ribo-SPIA Technology developed by Nugen. This technology has been shown to give the most reliable results when compared to other amplification protocols (Clément-Ziza *et al.*, 2009; our own unpublished results). As a consequence of the Ribo-SPIA Technology, rRNA is also amplified. Ribosomal RNAs comprise about 80% of the total RNA population (Alberts *et al.*, 1994; Neidhardt & Umbarger, 1996). Therefore, a fixed amount of cDNA with a higher relative amount of rDNA species will contain a lower number of mRNA-derived cDNAs. As a result, "expression levels" of genes other than those of rRNA will be lower and the number of absent calls after hybridization will be higher. This effect is increased by the high signals obtained for rRNA in the hybridization. Increased rRNA signals reduce the relative signal of the mRNA and thereby increase the number of absent calls. Taken together, enrichment of rRNAs in the hyphal tip precluded a direct comparison between the RNA profiles of the hyphal tips at the outer part of the colony and the periphery as a whole. To overcome this, RNA abundance of individual genes and of functional gene categories were compared indirectly by over-representation testing. Only 8 and 3 categories were found that significantly contributed to the top 10% of genes with the highest RNA abundance in only the periphery or the hyphal tip, respectively. Of the 8 periphery specific categories, 5 dealt with protein fate. Processes involved in protein fate are thus expected to be of a higher importance in the periphery than in the hyphal tip. In the next analysis, 303 functional gene categories were ranked based on the p-value of over-representation within the top 10% of genes with the highest hybridization signals. This revealed that the FunCat categories cytoplasm and intracellular transport vesicles, which both belong to the class I FunCat category control of cellular organization, are also more important in the periphery when compared to the hyphal apices. This ranking method also showed that genes with a relatively high hybridization signal in the hyphal tips are often related to interaction with the environment (*i.e.* cell rescue, defense and virulence; and regulation of and interaction with cellular environment). It makes sense that interaction with the environment is of higher importance for hyphae that explore a new substrate than for

hyphae that are located in a more established part of the mycelium. The tips of the exploring hyphae were also shown to be enriched for RNAs involved in transcription and transport facilitation. The latter would enable active uptake of molecules from the fresh substrate.

Analysis of relative RNA accumulation of individual genes revealed that the hyphal tips were not only enriched in rRNAs but also in RNAs of other genes involved in translation (tRNA and genes involved in protein synthesis). This analysis also showed that RNA of genes involved in stress response and transport facilitation are enriched in the tip of the outer hyphae of the colony. In both sample types many genes involved in metabolism were found in the top 100 of genes with a relatively high RNA accumulation. In the hyphal tips 20% of these metabolism related genes were involved in C-compound and carbohydrate utilization, while this was 35% in the rest of the periphery. Taken together, these data strongly indicate that hyphal tips of exploring hyphae have a different RNA composition when compared to the periphery as a whole. The RNA composition seems to be adapted to enable active growth and anticipate to the uncolonized environment they encounter.

This study is the first example of subcellular RNA profiling in microorganisms. The picogram amounts of RNA that can be amplified in a reproducible way enable a comparison of RNA profiles of different compartments or those of branches and tips. Moreover, processes like invasion of symbiotic, parasitic or saprotrophic fungi can be monitored at the cell level. The main advantage of this technology is that one can analyze the RNA profile of cells that are actually active in a certain process rather than using the RNA of the mycelium as a whole. Since the mycelium contains cells with different activities, differential gene expression is leveled out when the whole mycelium is used for expression analysis. This hampers the identification of genes involved in fungal growth and development and results in inaccurate expression networks that are established during these processes.

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APPENDIX 1

Protocol for (sub)cellular whole transcriptome analysis in *Aspergillus niger*

A protocol was set up to isolate and amplify the small amounts of RNA present in hyphal apices to enable whole genome gene expression. This protocol includes:

1. Colony growth and sample preparation
2. Harvesting hyphae using LPC technology
3. RNA isolation
4. RNA amplification
5. cDNA labeling

1. Colony growth and sample preparation

Hyphal material is harvested using LPC (see below). For this, colonies need to be grown in a (near) 2 dimensional way. To establish this, colonies are normally grown in a thin agarose layer in between perforated PC membranes (Wösten *et al.*, 1991). However, the agarose layer precludes dissection of the hyphae. Therefore, mycelium was grown directly in between membranes:

- Petri dishes, 9 cm in diameter, are filled with 20 ml medium containing 1.5% agarose.
  - The plate is dried (without lid) at 60 °C for 10 min after the medium has solidified.
- A sterile polycarbonate (PC) membrane (Profiltra) with 0.1 micron pores and a diameter of 76 mm is placed on top of the medium.
  - PC membranes are sterilized between wet paper in a glass Petri dish. They are dried at 60 °C before use with the lid kept on the container.
- 1.5 ul of a fresh *A. niger* spore solution (10^8 spores per ml 0.8% NaCl, 0.005% Tween-80) or a small piece of mycelium is used as an inoculum. In the case a spore solution is used, excess of water is removed by drying for 8 to 16 hours at room temperature (with lid on Petri dish) before the Lumox foil (see below) is placed on top of the inoculum.
- Sterile Lumox foil with a diameter of 76 mm (cut from a Greiner bio-one, roll 5 m x 305 mm) is placed on the PC membrane with the hydrophobic side of the foil facing the inoculum.
- Colonies are grown for 7 days at 30 °C in a water saturated growth chamber.
- The Lumox foil is removed from the colony with a pair of forceps. The colony (and the underlying PC membrane) is cut in 12 parts ("pie pieces") with a scalpel.
  - Because the hyphae harvested from these slides will be used for RNA isolation and amplification it is important that small equipment is cleaned with RNaseZAP (Ambion) and DNA-OFF (MP Biomedicals) prior to sample preparation.
  - Note that the colony will stick to the PC membrane and not to the Lumox foil.

- Clean a microscope object glass with RNaseZAP and DNA-OFF. Transfer colony pieces to these slides using the PC membrane underlying the mycelium as a carrier.
- Place sample gently upside down on the slide (*i.e.* mycelium first; the PC membrane is now positioned on top of the colony part).
- Peel the PC membrane off starting from the central part of the colony towards the periphery. In this way the peripheral hyphae will maintain their position.
- Fix the sample on the slide by wetting the mycelium with 70% ethanol (about 200-500 µl). Air-dry the sample. This fixation procedure results in the isolation of intact total RNA.

Note: PEN-membrane slides (Carl Zeiss Micro Imaging) are generally used for laser dissection. These slides, function as a sample carrier after micro-dissection. In this protocol glass slides are used. Using glass slides, hyphal tips cannot be dissected and catapulted as a whole, but have to be catapulted in pieces. Fragmentation of the hyphal material leads to improved homogenization in combination with the dismembrator (See section 3. RNA isolation) and, as a consequence, in more reproducible RNA isolation.

2. Harvesting hyphae using LPC technology

Hyphae are harvested with a PALM CombiSystem (Carl Zeiss Micro Imaging) equipped with an Axiovert 200M Zeiss inverted microscope (Carl Zeiss AG) and a 3CCD color camera (HV-D30, Hitachi Kokusai Electric Inc.). Prior to laser pressure catapulting (LPC) the slide holder and the capture devise are cleaned with RNaseZAP and DNA-OFF (these parts of the instrument will be in direct contact with the samples).

- Pipet 50 µl RNAlater (Qiagen) in the cap of a sterile 0.5 ml Eppendorf tube. This avoids RNA degradation within the catapulted hyphal material.
- Place cap in the capture device. Position cap above the slide containing the mycelial sample.
- Hyphae are catapulted into the Eppendorf cap using a 40x magnification and the auto LPC option. In order to harvest, draw an area around the hypha of choice with the draw tool. The auto LPC option will fill this area with dots. These dots mark the spots where the laser will give pulses. In this way hyphal fragments are catapulted into the Eppendorf cap.
    - Make sure the sample is in focus (adjustment of the objective) and the right percentage of the laser power is used. Adjust the laser settings using a piece of the sample you are not planning to use for harvesting. The laser power must be adjusted such that all of the mycelium is catapulted up while only a discrete dot is punctured in the glass slide underneath the sample.
- The cap containing RNAlater and the harvested hyphal material is taped on a 2 ml conical Eppendorf tube in which homogenization will take place. The RNAlater and the hyphae are transferred to the 2 ml tube by centrifugation for 5 sec in an Eppendorf centrifuge (10000 rpm)
    - Note that transferring is not done by pipetting. This is to minimize the risk that hyphal material is lost by sticking to the pipet tip.

- The cap is removed and the sample is snap frozen in the 2 ml Eppendorf tube using liquid nitrogen.

3. RNA isolation

Make sure that work space and equipment is RNAse and DNA free by cleaning with RNaseZAP and DNA-OFF. Use clean pipettes that are not used for GMO work in combination with nuclease free filter tips (Axygen, Inc.). The Qiagen RNA MinElute Cleanup kit is used for purification.

- Two new metal balls (that had been cooled with liquid nitrogen), 4.76 mm in diameter, are added to the 2 ml Eppendorf tube with frozen sample.
- Samples are ground in a Micro-Dismembrator U (B. Braun Biotech Int.) in a chilled container for 3 disposable test tubes of 2.2 ml (cat. no. 8531889, B.Braun Biotech Int.) at 1500 rpm for 60 sec.
- Add 250 µl TRIzol (Invitrogen) and vortex until sample is thawed and suspended. Remove the metal balls using a magnet and leave sample at room temperature for 5 min. Note that the metal balls are removed after the homogenized sample is dissolved in Trizol to make sure that hyphal material is not lost. Do not re-use the metal balls.
- Add 200 µl chloroform, vortex and leave sample at room temperature for 3 min.
- Centrifuge for 10 min at 10.000 g in an Eppendorf centrifuge and transfer water phase (upper phase; usually around 150-200 µl) to a new 2 ml Eppendorf tube. The water phase was well mixed with 525-700 µl RLT from the RNeasy MinElute Cleanup Kit (Qiagen) to which 143 mM β–mercaptoethanol was added (according to the protocol provided by the manufacturer).
- Add 500 µl 96-100% ethanol and mix well by pipetting. Immediately continue with the next step.
- Transfer aliquots of up to 700 µl to an RNeasy MinElute spin column placed in a 2 ml collection tube. Close the lid gently and centrifuge for 15 sec at 10.000 rpm. Discard the flow-through.
- Place the spin column in a new collection tube and wash the column membrane by adding 500 µl RPE buffer. Close the lid gently, and centrifuge for 15 sec at 10.000 rpm. Discard the flow-through.
- Add 500 µl freshly made 80% ethanol (v/v) to the spin column, close the lid gently, and centrifuge for 2 min at 10.000 rpm to wash and dry the column membrane.
- Place the spin column in a new 2 ml collection tube, open the lid and centrifuge at full speed for 5 min. This eliminates possible carry-over of ethanol and residual flow-through remains on the outside of the spin column.
- Place the spin column in a new 1.5 ml collection tube. Add 12 µl RNase-free water directly to the membrane (dead volume of the column is 2 µl). Close the lid gently and leave at room temperature for 5 min. Elute the RNA sample from the column membrane by centrifugation for 1 min at 10.000 rpm.

4. RNA amplification

Isolated RNA is amplified using the Ovation Pico WTA System (Nugen) or the WT-Ovation One-Direct RNA Amplification System (Nugen). The "pico kit" is used in case samples contain 500 pg RNA or more, while the "one-direct kit" can be used for smaller samples. In both kits double stranded cDNA is made, which is subsequently purified and amplified. The RNA sample is eluted in 10 µl. Note that only a 5 µl volume can be used for the amplification. Precipitation using a carrier prior to amplification is not recommended because the carrier might interfere with downstream reactions. Therefore, realize that only half of the eluted RNA sample can be used for amplification.

- Prior to RNA amplification, make sure workspace and equipment is RNAse and DNA free by cleaning with RNaseZAP and DNA-OFF. Use clean pipettes that are not used for GMO work in combination with filter tips (Axygen, Inc.).
- Amplification protocols provided by NuGEN are followed up to every detail.
- Amplified cDNA is purified using the QIAQuick PCR Purification kit (Qiagen) en eluted in 30 µl H2O provided in the Nugen kit.
- Concentration is measured using 2 µl sample and a Nanodrop with the ssDNA option.
- Amplification is checked using a Bioanalyser in combination with the RNA 6000 Nano Assay (Agilent Technologies) following the protocol provided by the manufacturer. Normally 25 to 500 ng of RNA is used in this assay. If micrograms of cDNA are obtained after amplification, 1 µl of sample is sufficient for the quality check with the Bioanalyzer.

5. cDNA labeling

5 ug of amplified cDNA is fragmented labeled using the Encore Biotin Module (NuGEN). This results in cDNA suitable for hybridization of Affymetrix GeneChip arrays.

CHAPTER 6

ABSTRACT

Mycelia of filamentous fungi explore new substrates by means of hyphae that extend from the periphery of the colony. Previously, it has been shown by immuno-labelling, reporter studies and *in situ* hybridization that these exploring hyphae are heterogenic with respect to protein secretion and transcription. Here, single hyphal tip RNA profiling was performed to assess the differences in RNA accumulation in 5 neighboring exploring hyphae. For this, a protocol was optimized enabling extraction and amplification of 1 picogram of RNA from individual hyphae. The resulting cDNA (5-9 µg) was used for quantitative PCR (QPCR) and microarray analysis. QPCR showed that levels of *18S* rRNA, and of RNA of the *actin* gene and the glucoamylase gene *glaA* were heterogenic between the neighboring hyphae. Standard deviations amounted 2.4, 6.1 and 4.6 cycles for *18S* rRNA, and for *actin* and *glaA* mRNA, respectively. Standard deviations were much lower when cDNA was used from pools of 100 hyphal tips or from the mycelium of the whole periphery. Microarray analysis resulted in a present call for 4-7% of the *A. niger* genes. Within this set of genes, 12% showed heterogenic RNA levels. These genes belonged to a wide range of functional gene categories, among which metabolism, rRNA, tRNA and transcription. These data indicate that heterogeneity in exploring hyphae is caused by stochastic gene expression and / or by epigenetic processes.

SINGLE TIP TRANSCRIPTOMICS OF NEIGHBORING HYPHAE OF *ASPERGILLUS NIGER*

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Parts of this chapter have been submitted.

INTRODUCTION

Cellular heterogeneity within an isogenic cell population is a widespread event in both prokaryotic and eukaryotic organisms. For instance, cells within clonal cultures of bacteria such as *Escherichia coli* and *Bacillus subtilis* exhibit phenotypic variation under defined conditions (Elowitz *et al.*, 2002; Veening *et al.*, 2008a). Heterogeneity of cells can be beneficial for the organism in many ways. For example, many cases of phenotypic variability in bacterial cultures relate to responses to environmental stress. This suggests that phenotypic variation aids in the survival of cells under adverse conditions (Veening *et al.*, 2008a; Veening *et al.*, 2008b). The existence of persistor cells in biofilms of *Candida albicans* also indicates that phenotypic variation is a strategy for survival. The persistor cells comprise a small fraction of the population and are tolerant to stress because they have a low cellular activity and therefore survive challenges that are lethal to growing cells (Nobile & Mitchell, 2007). Heterogeneity also exists within cell types in higher organisms. One example is the heterogeneity of adult hematopoietic stem cells in mice. Only up to ten percent of these cells are thought to be active in contributing to blood production at any time (Jordan & Lemischka, 1990; Keller & Snodgrass, 1990).

Recently, it has been shown that mycelia of filamentous fungi are also more complex than was generally assumed. Gene expression in colonies of *Aspergillus niger*, *Aspergillus oryzae* and *Neurospora crassa* depends on the position within the colony (Masai *et al.*, 2006; Levin *et al.*, 2007; Kasuga & Glass, 2008). These differences can be explained by the availability of carbon source and by spatial and temporal differentiation (Levin *et al.*, 2007). As a consequence of the heterogeneity in gene expression, not every growing hypha secretes a particular protein. For instance, glucoamylase is secreted at the periphery of an *A. niger* colony, but not in the central growth zone (Wösten *et al.*, 1991). In fact, neighboring hyphae at the periphery of a colony have been shown to be heterogenic in glucoamylase expression (Vinck *et al.*, 2005) and secretion (Wösten *et al.*, 1991) despite the fact that these hyphae encountered identical environmental

conditions. Recently, it has been demonstrated that the acid amylase gene *aamA*, the α-glucuronidase gene *aguA*, and the feruloyl esterase gene *faeA* of *A. niger* are also subject to heterogenic expression (Vinck *et al.*, 2010; **Chapter 4**). Co-expression studies showed that hyphae that highly express one of these genes also highly express the other genes encoding secreted proteins. Moreover, these hyphae highly express the amylolytic regulatory gene *amyR* and the glyceraldehyde-3-phosphate dehydrogenase gene *gpdA*, and are characterized by a high *18S* rRNA content. Taken together, it was concluded that two subpopulations of hyphae exist within the outer zone of the mycelium of *A. niger.* These subpopulations are characterized by a high and a low transcriptional activity, respectively (Vinck *et al.*, 2010; **Chapter 4**).
Although heterogeneity is being studied in more and more biological systems, the underlying mechanisms are far from being elucidated. Single cell analysis is an important tool to unravel such mechanisms but this technique has not been exploited widely. Here, we studied for the first time the transcriptome of single cells of a microbe. It is shown that neighboring hyphae of *A. niger* are heterogenic in expression of a wide variety of genes. This indicates that heterogeneity is the result of stochastic gene expression and /or epigenetic processes.

MATERIAL AND METHODS

Strains
Experiments were done with strain AR9#2 of *A. niger*. This strain is a derivative of strain AB4.1 (*pyrG*, *cspA1*) (van Hartingsveldt *et al.*, 1987) in which the construct pAN52-10S65TGGPn/s was introduced (Siedenberg *et al.*, 1999). This construct contains *sGFP*(S65T) under the regulation of the *glaA* promoter of *A. niger*.

Colony growth
A. niger was cultured as a sandwiched colony at 30 °C in the light. To this end, *A. niger* was grown between a perforated polycarbonate (PC) membrane (diameter 76 mm, pore size 0.1 µm; Osmonics, GE Water Technologies) and a Lumox membrane (diameter 76 mm; Greiner Bio-One) (Vinck *et al.*, 2010; **Chapter 4**). The PC membrane was placed on top of solidified (1.5% agar) minimal medium (MM) (de Vries *et al.*, 2004) containing 25 mM maltose as a carbon source. Freshly harvested spores (1.5 µl of an aqueous solution containing 0.8% NaCl, 0.005% Tween-80 and 10^8 spores ml^{-1}) were placed in the center of the PC membrane. The droplet was allowed to dry, after which the Lumox membrane was placed on top of the PC membrane with its hydrophobic side facing the inoculum. After 7 days of growth, the Lumox membrane was removed. The mycelium and the underlying PC membrane was cut with a scalpel and parts of the periphery of the colony were placed upside down onto a nucleotide and RNAse free glass slide. The PC membrane that was now facing the air was removed and the mycelium was fixed with 70% ethanol and air dried (see Appendix 1 of **Chapter 5)**.

Laser micro-dissection and pressure catapulting

Hyphae or parts thereof were isolated by laser pressure catapulting (LPC) using the PALM CombiSystem (Carl Zeiss MicroImaging) (see Appendix 1 of **Chapter 5)**. This system was equipped with an Axiovert 200M Zeiss inverted microscope (Carl Zeiss AG) and a 3CCD color camera (HV-D30, Hitachi Kokusai Electric Inc.). The PALM CombiSystem was operated with PALM RoboSoftware V4.0 (Carl Zeiss MicroImaging GmbH), from which the autoLPC option was routinely used in combination with a 40x objective. Hyphal material was catapulted into lids of 0.5 ml Eppendorf tubes that contained 50 µl RNA*later* (Qiagen).

RNA isolation and amplification

Hyphal material that was collected in 50 µl RNA*later* was transferred to a 2 ml Eppendorf tube by a quick centrifugation step (see Appendix 1 of **Chapter 5)**. After snap-freezing in liquid nitrogen, 2 pre-cooled metal bullets (4.76 mm in diameter) were added and samples were ground in a Micro-Dismembrator U (B. Braun Biotech Int.) in a chilled container at 1500 rpm for 60 s. The frozen material was taken up in 250 µl Trizol Reagent (Invitrogen) by vortexing. After removing the metal bullets, 200 µl chloroform was added. After mixing well, samples were centrifuged at 10.000 g for 10 min. The water phase (approximately 200 µl) was mixed with 700 µl RLT from the RNeasy MinElute Cleanup Kit (Qiagen) to which 143 mM β–mercaptoethanol was added. RNA was purified following instructions of the manufacturer and was eluted with 12 µl RNAse free water. RNA samples were amplified using the WT-Ovation One-Direct RNA Amplification System (Nugen). According to the supplier, 10-500 pg input RNA results in 5-10 µg of cDNA amplicons with a length of about 50 to 500 base pairs. The quality and quantity of the cDNA samples were checked using a Bioanalyzer (Agilent Technologies) and a Nanodrop (Nanodrop Technologies Inc.), respectively.

QPCR analysis on amplified samples

Quantitative PCR was performed using the ABI Prism 7900HT SDS and SYBR Green chemistry (Applied Biosystems). Cycle threshold (Ct) levels were measured for *18S* rRNA, and for mRNA of the *glaA* and *actin* gene. Primers were designed according to the recommendations of the PCR master-mix manufacturer (Applied Biosystems). Ct levels of *actin* were determined with the primers QPCRactFW1 and QPCRactRV1 and those of *18S* rRNA were determined with the primer pair QPCR18SFW1 and QPCR18SRV1 (see Table 2 of **Chapter 3**). These products had an amplification efficiency of 2. cDNA of *glaA* was amplified using primer pair QPCRglaAFW3 and QPCRglaARV3 (see Table 2 of **Chapter 3**) with an amplification efficiency of 1.98.

Microarray analysis

Five µg of amplified cDNA (see above) was fragmented via combined chemical and enzymatic fragmentation using the protocol of the Encore Biotin Module (Nugen). The fragments were biotin-labeled to the 3-hydroxyl end using the same Module following the instructions of the manufacturer. The labeled cDNA was hybridized to Affymetrix GeneChip *A. niger* Genome Arrays. The GeneChip Hybridization, Wash and Stain Kit (#900720, Affymetrix) was used for the hybridizations according to manufacturers

protocol with the modification that the hybridization cocktail was prepared according to the Encore Biotin Module and that the hybridization time was extended to 40 h as recommended by Nugen. The MAS5.0 algorithm (Affymetrix, 2002) was used for the quality control of the hybridized arrays. This algorithm analyzes each array independently and compares the overall fluorescence intensities of the arrays. The MAS5.0 algorithm uses the Tukey's biweight estimator to provide a robust mean signal value and the Wilcoxon's rank test to calculate both significance and detection call for each probe set. Summarized expression values of the single hypha samples were calculated using the log-scale robust multi-array analysis (RMA) (Irizarry *et al.*, 2003). Principal Component Analysis (PCA) and Hierarchical clustering of probe sets and samples was performed on the Z-scores derived from the log2 RMA data. Hierarchical clustering was performed in Spotfire Decision Site 7.3 software (http://spotfire.tibco.com) using Complete Linkage as clustering method and correlation as distance measure. Different subsets were tested for overrepresentation of FunCats (Ruepp *et al.*, 2004) and non-FunCats categories with all 14455 probe sets as background using a hyper-geometrical test (Draghici *et al.*, 2003). The classification of the *A. niger* genes in FunCat categories has been described (Pel *et al.*, 2007).

RESULTS

RNA isolation and amplification of single hyphal tips

In **Chapter 5** a protocol was developed to analyze RNA profiles of a few hundred *A. niger* hyphae or fragments thereof. Here, the aim was to analyze expression profiles of individual hyphae at the periphery of an *A. niger* colony. To this end, the protocol developed in **Chapter 5** was used except that the cDNA was amplified with the WT-Ovation One-Direct RNA Amplification System (Nugen). RNA was isolated from apical regions of 200 µm in length from 5 individual neighboring hyphae from the outermost region of the periphery of an *A. niger* sandwiched colony (Figure 1). Fragments of each hypha were catapulted into caps of an Eppendorf tube using the autoLPC option. After RNA isolation, cDNA was amplified from the five samples. This resulted in 5.9-10.1 µg of cDNA from 1 pg of RNA from each of the single hyphae. The amplicons of three of the samples were mainly 50-100 bp in length, while the majority of the amplicons of the other two hyphae had a length of 100-300 bp (Figure 2). Notably, the latter two samples had been amplified at another day than the former three samples.

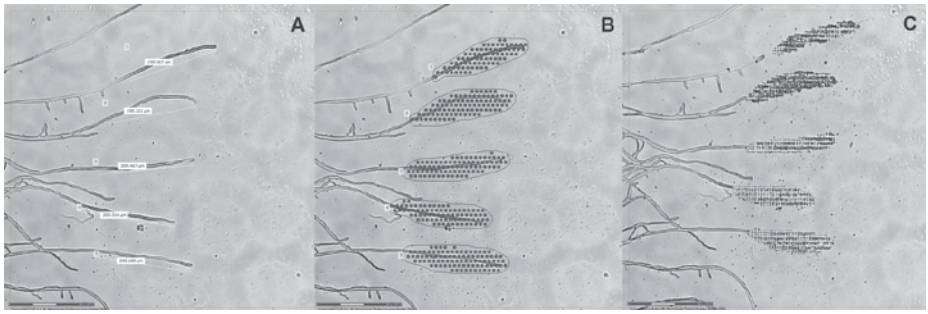

Figure 1 Isolation of fragments of single hyphae using LPC. Apical regions of 200 µm of hyphae at the periphery of sandwiched colonies were selected by using the measuring (A) and drawing (B) tool. Fragments of a single hypha were catapulted into a cap of an Eppendorf tube by using the autoLPC option (C). This was done for 5 neighboring hyphae.

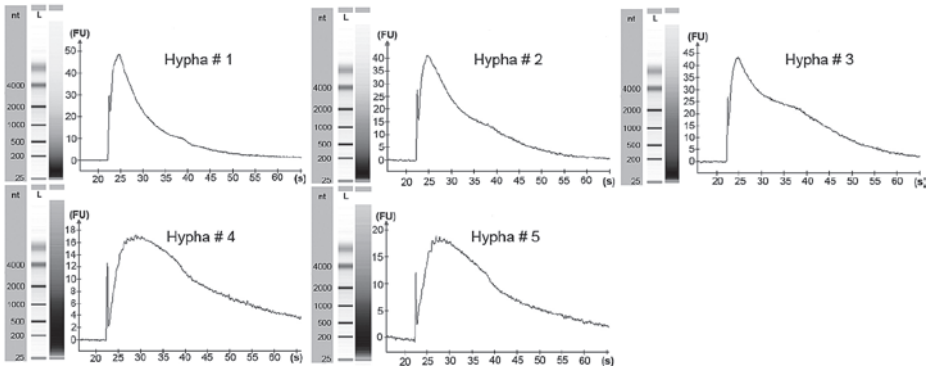

Figure 2 Bioanalyser analysis of cDNA amplified from RNA of single hyphal tips of 200 µm in length. The electrophoresis gel image (nt, nucleotides; L, ladder) and the electropherogram (y-axis represents fluorescence units (FU); x-axis represents runtime in seconds (s)) are given for the 5 samples. Amplicons of hyphae 1-3 and hyphae 4-5 were mainly 50-100 bp and 100-300 bp in length, respectively.

Hyphal heterogeneity analyzed by QPCR

The cDNA samples of the 5 single hyphae were analyzed by QPCR. As a control, amplified cDNA was used from 3 biological replicates of a pool of 100 hyphal tips and of mycelium of the whole periphery of sandwiched colonies. Ct levels were determined for *18S* rRNA, *actin*, and *glaA* using 1 ng cDNA and six technical replicates for each sample. Ct values for the RNA samples from the periphery of the colony were very similar (Table 1). In contrast, large differences in RNA levels were found between the individual hyphae (Table 1). Such differences were not observed within the technical replicates. Standard deviations found for the cDNA samples of 100 hyphal tips were between those of the single hyphal tips and the periphery (Table 1). Higher standard deviations for the single hyphal tips were still obtained after splitting the samples into a set of hyphae 1-3 and a set of hyphae 4-5 (each representing one of the amplification experiments) (Data not shown).

Table 1 QPCR analysis using 1 ng of cDNA amplified from RNA of single hyphal tips, from RNA of a pool of 100 hyphal tips and from RNA of the periphery of sandwiched colonies of *A. niger*. The average (μ) Ct's and their standard deviations (σ) are given for *18S* rDNA and the *actin* and *glaA* genes. The range in standard deviations obtained for the 6 technical replicates for each of the biological replicates is also given (σ technical).

Gene	Sample type	μ Ct	σ	σ technical
18S	1 hypha	20.23	2.40	0.53-1.26
	100 hyphae	17.39	0.42	0.26-0.43
	5 pg periphery	12.58	0.28	0.15-0.35
actin	1 hypha	30.30	6.07	0.11-1.03
	100 hyphae	28.15	4.80	0.12-1.71
	5 pg periphery	18.05	0.57	0.09-0.22
glaA	1 hypha	25.47	4.63	0.06-0.66
	100 hyphae	24.51	2.22	0.10-0.22
	5 pg periphery	18.65	0.49	0.10-0.14

Hyphal heterogeneity analyzed by microarrays

Biotin labeled amplified cDNA of the single hyphal tips were hybridized to Affymetrix *A. niger* gene chips. Based on MAS5.0 detection calls, transcripts of 4.1-6.7% of the genes had a present call in each of the single hyphae (Table 2). Genes with an absent call had generally low signal values in hybridization experiments where 500 pg RNA from the periphery or from a pool of 500 hyphal tips was used (see **Chapter 5**) (Figure 3). The scale factors of the sample types had a difference < 5-fold. Due to the low number of present calls, this difference in scale factors was considered to be low enough to normalize and analyze the samples as a whole (Table 2). In total, 2608 of the 14455 probe sets were found to be present in at least one of the samples of the single hyphae (Suppl. Table 1). These probe sets were found to comprise all 19 different Class I functional categories (FunCats) (Ruepp *et al.*, 2004)) found in *A. niger* (Pel *et al.*, 2007) as well as the non-FunCat categories tRNA and rRNA. Almost half of the detectable probe sets belonged to unclassified proteins (Table 3). Metabolism was the second largest group with 550 hybridizing probes. Categories with more than 50 probe sets with a present call comprised protein fate (148), transcription (116), cell cycle and DNA processing (106), cellular transport and transport mechanisms (76), protein synthesis (75), and cell rescue defense and virulence (52). The categories with a lower number of present calls generally comprised a small number of total probe sets. In most categories 10-30% of the probe sets had a present call. This was 50-75% for the categories rRNA, tissue localization, and protein with binding function or co-factor requirement.

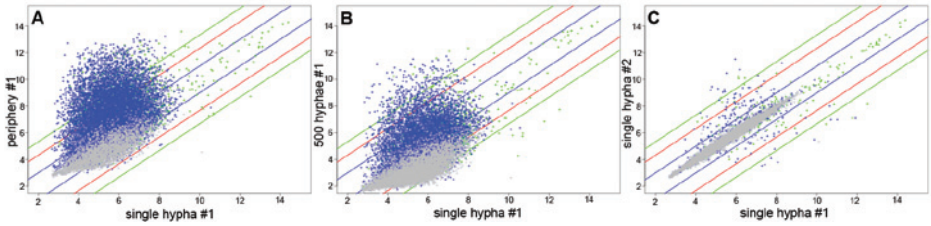

Figure 3 Scatter plots depicting present and absent calls of all probe sets of an array of a single hyphal tip and of an array of the periphery of the colony (A); an array of a single hyphal tip and of an array of a pool of 500 hyphal tips (B) and two arrays of different single hyphal tips. Probe sets with an absent call in an array of a single hyphal tip have generally low signal values in arrays of RNA from the periphery or from a pool of 500 hyphal tips. Green = present call in both samples, Blue = present call in one of the samples, and Grey = absent call in both samples.

Table 2 Affymetrix quality control checks after hybridizing amplified cDNA from single hyphal tips. RNA from hyphae 1-3 and hyphae 4-5 were amplified on different days.

Sample	Scale Factor	Percentage present calls	Number of probe sets with a present call
Hypha #1	4.7	5.8	951
Hypha #2	3.9	6.7	1101
Hypha #3	6.6	6.1	975
Hypha #4	16.9	4.1	668
Hypha #5	15.3	4.3	684

Hierarchical clustering of the hyphae was done on basis of the Z-scores of the log2 RMA signals of the 2608 probe sets that had a present call in at least one of the hyphae (Figure 4A). This revealed that hyphae 1 and 2 were most alike (correlation coefficient -0.10). The correlation between hypha 3 and hyphae 1 and 2 and between hyphae 4 and 5 was similar (-0.22 and -0.24, respectively). PCA analysis showed similar results as the hierarchal clustering (Figure 4B). In the next analysis, overrepresentation of functional gene categories was tested for all probe sets with a present call in each of the individual hyphae. This revealed that the non-FunCat categories rRNA and tRNA were overrepresented in all 5 hyphae (Table 4 and Suppl. Table 2). Ribosome biogenesis was overrepresented in 4 hyphae, whereas other export and secretion systems and proteolytic degradation were overrepresented in 2 of the hyphae.

Table 3 Classification of the 2608 probe sets with a present call in at least one of the 5 arrays of a single hypha. Classification is based on Class I FunCats as well as the non-FunCat categories tRNA and rRNA. The total number of probe sets representing each category is also listed.

Category	Number of probe sets with a present call	Total number of probe sets	% probe sets present
01 metabolism	550	3023	18.2
02 energy	25	117	21.4
03 cell cycle and DNA processing	106	476	22.3
04 transcription	116	715	16.2
05 protein synthesis	75	241	31.1
06 protein fate (folding, modification, destination)	148	616	24
08 cellular transport and transport mechanisms	76	438	17.4
10 cellular communication or signal transduction mechanism	31	179	17.3
11 cell rescue, defense and virulence	52	267	19.5
13 regulation of or interaction with cellular environment	8	73	11
14 cell fate	15	104	14.4
25 development (Systemic)	6	26	23.1
29 transposable elements, viral and plasmid proteins	12	62	19.4
30 control of cellular organization	5	29	17.2
40 sub-cellular localization	19	137	13.9
45 tissue localization	1	2	50
63 protein with binding function or co-factor requirement	1	2	50
67 transport facilitation	5	44	11.4
99 unclassified proteins	1279	7670	16.7
rRNA	6	8	75
tRNA	39	144	27.1

Table 4 Overrepresented functional gene categories within the pool of probe sets with a present call in each of the single hyphae. 1: overrepresentation of a category ($p<0.01$); 0: absence of overrepresentation ($p>0.01$). For p-values see Suppl. Table 2.

Functional gene categories	Number of probe sets	Hypha # 1	2	3	4	5
03.01.03 DNA synthesis and replication	96	1	0	0	0	0
03.01 DNA processing	240	1	0	0	0	0
05.01 ribosome biogenesis	138	1	1	1	0	1
06.13.04 lysosomal and vacuolar degradation	32	0	0	1	0	0
06.13 proteolytic degradation	198	0	1	1	0	0
08.16.99 other export and secretion systems	15	0	0	1	1	0
29.01 LTR retro-elements (retro-viral)	28	0	0	1	0	0
40.05 centrosome	9	0	0	0	1	0
67.04.01.02 other cation transporters (NA, K, CA , NH4, etc.)	33	0	0	0	1	0
RRNA	8	1	1	1	1	1
TRNA	144	1	1	1	1	1

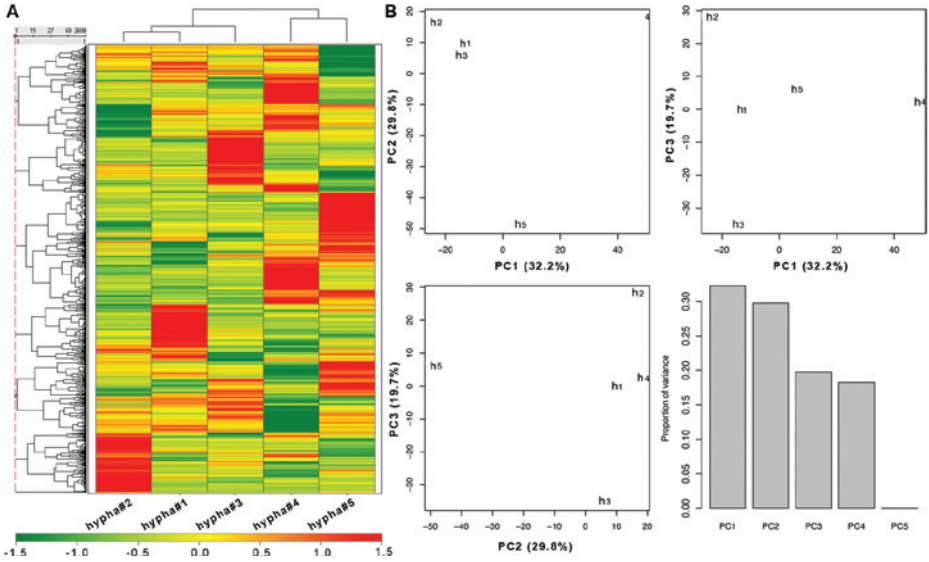

Figure 4 Hierarchal clustering (A) and principal component analysis (PCA) (B) of RNA profiles of five individual hyphae on basis of the 2608 probe sets that had a present call in at least one of the single hyphae. Clustering was done using complete linkage as clustering method with correlation as distance measure. The Z-scores of the log2 RMA signal values of the 2608 probe sets were used for clustering and PCA. The correlation of the Z-scores ranges between -1 (no similarity) to 0 (100% identity).

Within the 2608 probe sets with a present call in at least one of the five single hyphae, 308 showed a relatively high standard deviation (>0.5) between the log2 RMA signal values (Suppl. Table 3). Within these probe sets, 5 out of 19 class I FunCats are not represented. These 5 categories (regulation of or interaction with cellular environment, development, tissue localization, protein with binding function or co-factor requirement, and transport facilitation) have relatively few members. The hyphae were clustered based on the Z-scores of the signals of the 308 probe sets (Figure 5A). This revealed that hyphae 4 and 5 were most similar. Hypha 3 was more similar to hyphae 4 and 5 than to hyphae 1 and 2. PCA analysis again showed similar results as the hierarchal clustering (Figure 5B). Each hypha showed a cluster of genes with higher signals when compared to the other four hyphae (Figure 5A). These clusters were analyzed for overrepresentation of functional gene categories (Table 5 and Suppl. Table 2). This revealed that the classes ribosome biogenesis and tRNA were overrepresented in 3 of the 5 hyphae. The cluster of hypha 5 was not enriched in any functional category. In contrast, 7 FunCats were overrepresented in the cluster of hypha 2, among which 2 involved in energy.

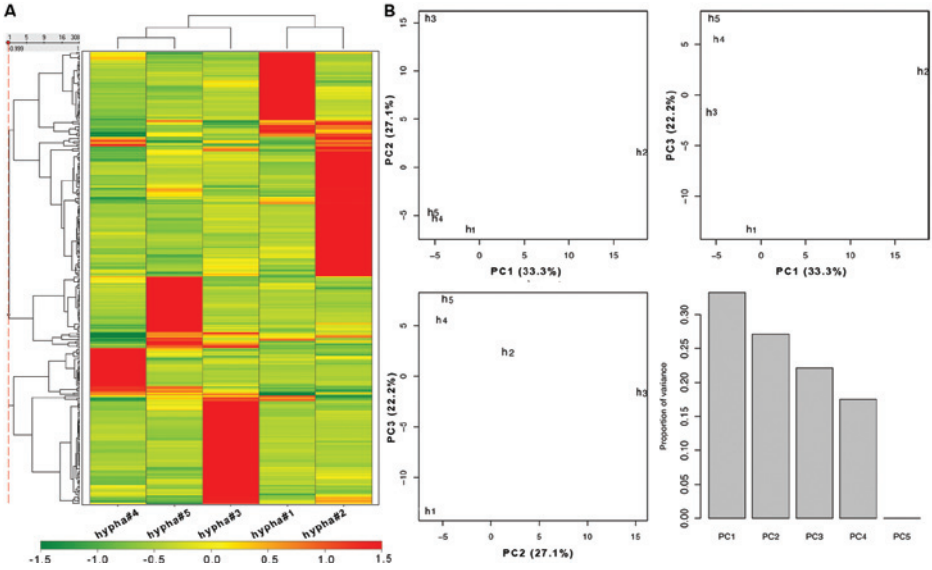

Figure 5 Hierarchal clustering (A) and principal component analysis (PCA) (B) of expression profiles of single hyphae on basis of 308 probe sets that had a present call in at least one out of 5 hyphae and that showed a standard deviation >0.5 between the signal values. Clustering was done using complete linkage as clustering method with correlation as distance measure. The Z-scores of the log2 RMA signal values of the 308 probe sets were used for clustering and PCA. The correlation of the Z-scores ranges between -1 (no similarity) to 0 (100% identity).

Table 5 Overrepresented functional gene categories within the pool of probe sets that have a signal value with a standard deviation of >0.5 between the 5 single hyphae. 1: overrepresentation of a category ($p<0.01$); 0: absence of overrepresentation ($p>0.01$). For p-values see Suppl. Table 2.

Functional gene categories	Number of probe sets	Hypha # 1	2	3	4	5
02.13.03 aerobic respiration	62	0	1	0	0	0
02.13 respiration	93	0	1	0	0	0
02.19 metabolism of energy reserves (e.g. glycogen, trehalose)	36	1	0	0	0	0
04.05.01.01general transcription activities	107	0	1	0	0	0
05.01 ribosome biogenesis	138	1	1	1	0	0
06.07.99 other protein modifications	54	0	0	0	1	0
06.13 proteolytic degradation	198	0	1	0	0	0
30.10 nucleus	55	0	1	0	0	0
67.15 electron or hydrogen carrier	48	0	1	0	0	0
TRNA	144	1	0	1	1	0

The top 100 of genes with the highest hybridization signal in each of the hyphae was selected (Suppl. Table 4). This selection comprised a total of 207 genes. Of these genes, 43 and 18 are found within the top 100 of all 5 hyphae and of 4 hyphae, respectively (Table 6). A major part of these genes (19 out of 43 and 11 out of 18) encode unidentified proteins. Examples of genes that have a predicted function are the 4 rRNAs, 4 tRNAs and 14 genes involved in metabolism. A number of 119 genes (from which 43 encoded unidentified proteins) were found in only one of the hyphae (Table 7). The glucoamylase gene *glaA*, the cellulase gene *eglb*, 7 genes encoding cytoplasmic ribosomal proteins and 13 tRNAs were among these genes. The 119 genes were randomly distributed in the top 100 of the different hyphae.

Table 6 Genes with the highest signal values that can be found in the top 100 of 2, 3, 4 or 5 out of the 5 single hyphae. Unidentified proteins are listed in Supplementary Table 4.

	In top 100 of all hyphae	
Gene number	**Description**	**Functional gene category**
An01g03480	strong similarity to sorbitol dehydrogenase gutB - *Bacillus subtilis*	01 metabolism
An04g02220	strong similarity to L-serine dehydratase CHA1 - *Saccharomyces cerevisiae*	01 metabolism
An04g02410	strong similarity to cobalamin and cobamide biosynthesis protein COB W patent WO9111518-A - *Pseudomonas denitrificans*	01 metabolism
An09g05770	strong similarity to cytosolic exopolyphosphatase PPX1 - *Saccharomyces cerevisiae*	01 metabolism
An10g00520	weak similarity to 1-aminocyclopropane-1-carboxylate deaminase - *Pseudomonas* sp. [truncated orf]	01 metabolism
An11g01390	similarity to phosphoglycerate dehydrogenase serA - *Bacillus subtilis*	01 metabolism
An11g05690	similarity to hypothetical alpha subunit of dinitrogenase reductase nifH - unidentified nitrogen-fixing bacteria	01 metabolism
An12g00670	strong similarity to cinnamyl-alcohol dehydrogenase CAD14 – *Nicotiana tabacum*	01 metabolism
An12g09340	strong similarity to ferulic acid decarboxylase FDC1 patent EP857789-A2 - *Saccharomyces cerevisiae*	01 metabolism
An15g04610	strong similarity to xylitol dehydrogenase xdh - *Galactocandida mastotermitis* [truncated ORF]	01 metabolism
An16g07090	similarity to transmembrane protein sequence of patent WO9927105-A2 - *Chlamydia pneumoniae*	01 metabolism
An17g02040	strong similarity to the protein required for normal CLN1 and CLN2 G1 cyclin expression Ctr9 - *Saccharomyces cerevisiae*	03 cell cycle and DNA processing
An02g06750	strong similarity to DEAD box protein Dbp45A - *Drosophila melanogaster*	04 transcription
An07g07270	similarity to tRNA 2 -phosphotransferase TPT1 - *Saccharomyces cerevisiae*	04 transcription
An07g09050	similarity to hypothetical transcription regulator SC5F2A.29 - *Streptomyces coelicolor*	04 transcription
An11e07520	repetetive DNA	Repeat
An13e00260	repetetive DNA	Repeat
An03e03150	5.8S ribosomal RNA	rRNA

Table 6 (continued)

	In top 100 of all hyphae	
Gene number	**Description**	**Functional gene category**
An03e03180	28S ribosomal RNA	rRNA
An03e03260	18S ribosomal RNA	rRNA
An01e11490	trnaSaga	Trna
An01e12510	trnaScga	Trna
An03e02440	trnaLtag	Trna
An08e06020	trnaSaga	Trna
	In top 100 of 4 hyphae	
Gene number	**Description**	**Functional gene category**
An12g00950	rhamnogalacturonase rhgA - *Aspergillus niger*	01 metabolism
An14g03240	strong similarity to acyl-CoA dehydrogenase (NADP+) ACDH - *Mycobacterium tuberculosis*	01 metabolism
An18g01520	strong similarity to quinic-acid utilisation gene qutH - *Aspergillus nidulans*	01 metabolism
An17g00730	strong similarity to electron transfer flavoprotein (ETF) beta chain - *Paracoccus denitrificans*	02 energy
An01g10550	strong similarity to transposase Minos-2 - *Drosophila hydei*	29 transposable elements, viral and plasmid proteins
An01e01100	5S ribosomal RNA	rRNA
An12e07440	trnaStga	tRNA
	In top 100 of 3 hyphae	
Gene number	**Description**	**Functional gene category**
An01g14740	glucose oxidase precursor goxC - *Aspergillus niger* [putative sequencing error]	01 metabolism
An12g08630	strong similarity to cytochrome P450 monooxygenase TRI11 – *Fusarium sporotrichioides*	01 metabolism
An02g06750	strong similarity to DEAD box protein Dbp45A - *Drosophila melanogaster*	04 transcription
An04g08980	strong similarity to cytoplasmic ribosomal protein of the large subunit L43A - *Saccharomyces cerevisiae*	05 protein synthesis
An02g04240	weak similarity to acinusS mRNA - *Homo sapiens*	10 cellular communication or signal transduction mechanism
An14g04060	strong similarity to chloride channel 3 clcn3 - *Mus musculus*	13 regulation of or interaction with cellular environment
An01g08120	strong similarity to ACOB protein - *Aspergillus nidulans*	25 development (Systemic)
	In top 100 of 2 hyphae	
Gene number	**Description**	**Functional gene category**
An12g03430	similarity to glucose oxidase goxC - *Aspergillus niger* [truncated ORF]	01 metabolism
An18g03470	similarity to the tRNA-specific adenosine deaminase subunit Tad1 - *Saccharomyces cerevisiae*	04 transcription
An02g13850	strong similarity to cytoplasmic ribosomal protein of the large subunit URP1 - *Saccharomyces cerevisiae*	05 protein synthesis
An08g01800	strong similarity to hypothetical mitochondrial carrier protein AgPET8 - *Ashbya gossypii*	08 cellular transport and transport mechanisms
An17g00650	similarity to the cercosporin resistance protein Crg1 - *Cercospora nicotianae*	11 cell rescue, defense and virulence

Table 7 Genes with the highest signal values that can be found in the top 100 of only 1 out of the 5 single hyphae. Unidentified proteins are listed in Supplementary Table 4.

Gene number	Description	Functional gene category
An01g10030	strong similarity to syringomycin-resistance gene SYR2 - *Saccharomyces cerevisiae*	01 metabolism
An01g11270	similarity to 2,3-dihydroxybenzoic acid decarboxylase patent WO9909048-A1- *Aspergillus niger*	01 metabolism
An02g03730	strong similarity to cobW protein - *Pseudomonas denitrificans* [truncated ORF]	01 metabolism
An03g04280	strong similarity to pyridoxine synthesis component pyroA - *Aspergillus nidulans*	01 metabolism
An03g06550	glucan 1,4-alpha-glucosidase glaA - *Aspergillus niger*	01 metabolism
An04g03530	strong similarity to NAD-dependent D-arabinitol dehydrogenase ARD - *Candida tropicalis*	01 metabolism
An04g09550	strong similarity to 4-Hydroxyacetophenone monooxygenase hapE - *Pseudomonas fluorescens*	01 metabolism
An06g00990	strong similarity to fumarate reductase FRDS - *Saccharomyces cerevisiae*	01 metabolism
An07g01960	strong similarity to stearoyl-CoA desaturase P-ole1 - *Pichia angusta*	01 metabolism
An07g04300	strong similarity to 3-methylcrotonyl-CoA carboxylase (MCC) biotin-containing alpha subunit MCCA - *Homo sapiens*	01 metabolism
An07g08360	similarity to pyrazinamidase or nicotinamidase pncA - *Escherichia coli*	01 metabolism
An07g08710	alpha, alpha-trehalose-phosphate synthase (UDP-forming) 2 (trehalose-6-phosphate UDP-glucose phosphate glucosyltransferase) tpsB - *Aspergillus niger*	01 metabolism
An08g07380	strong similarity to malonyl CoA synthetase MatB - *Rhizobium trifolii*	01 metabolism
An09g03830	strong similarity to 2,2-dialkylglycine decarboxylase structural protein dgdA - *Pseudomonas cepacia*	01 metabolism
An09g04850	strong similarity to p-sulfobenzyl alcohol dehydrogenase TsaC - *Comamonas testosteroni*	01 metabolism
An11g04370	strong similarity to cytochrome b5 - *Mortierella alpine*	01 metabolism
An11g06230	similarity to oxidoreductase from patent WO0100844 - *Corynebacterium glutamicum*	01 metabolism
An12g04590	similarity to dihydrofolate reductase DHFR - *Pneumocystis carinii*	01 metabolism
An14g02900	weak similarity to cellobiose dehydrogenase CDH - *Trametes versicolor*	01 metabolism
An14g04050	strong similarity to pyridoxamine-phosphate oxidase pdx3 - *Saccharomyces cerevisiae*	01 metabolism
An15g03770	strong similarity to diacylglycerol acyl transferase (MR1) of patent WO200001713-A2 - *Mortierella ramanniana*	01 metabolism
An16g06800	strong similarity to endoglucanase eglB - *Aspergillus niger*	01 metabolism
An16g09070	strong similarity to glucosamine-6-phosphate deaminase protein of patent WO9835047-A1 - *Escherichia coli*	01 metabolism
An17g01150	strong similarity to acyl-CoA dehydrogenase MCAD - *Rattus norvegicus*	01 metabolism

Table 7 (continued)

Gene number	Description	Functional gene category
An18g04560	similarity to peptidoglycan GlcNAc deacetylase PgdA - *Streptococcus pneumoniae*	01 metabolism
An08g06550	strong similarity to subunit VIII of ubiquinol–cytochrome c reductase - *Saccharomyces cerevisiae*	02 energy
An11g10200	strong similarity to subunit Vla of cytochrome c oxidase COX13 - *Saccharomyces cerevisiae*	02 energy
An15g00690	strong similarity to 14.8 kD subunit of NADH:ubiquinone reductase - *Neurospora crassa*	02 energy
An01g04640	strong similarity to topoisomerase I CaTOP1 - *Candida albicans*	03 cell cycle and DNA processing
An02g10450	strong similarity to GTP-binding protein VPS1 - *Saccharomyces cerevisiae*	03 cell cycle and DNA processing
An04g08710	strong similarity to protein involved in DNA repair and recombination uvsH - *Emericella nidulans*	03 cell cycle and DNA processing
An08g01090	similarity to Ada Histone acetyltransferase complex component AHC1 - *Saccharomyces cerevisiae*	03 cell cycle and DNA processing
An08g03190	strong similarity to tubulin beta chain beta-tubulin - *Aspergillus flavus*	03 cell cycle and DNA processing
An11g06990	similarity to UV damage nucleotide excision repair protein Rph16 - *Schizosaccharomyces pombe*	03 cell cycle and DNA processing
An11g11110	strong similarity to condensin complex component cnd1 - *Schizosaccharomyces pombe*	03 cell cycle and DNA processing
An14g05320	strong similarity to cell cycle regulator p21 protein, Wos2 - *Schizosaccharomyces pombe*	03 cell cycle and DNA processing
An01g07250	strong similarity to 7.7 kD subunit of DNA-directed RNA polymerase II ABC10 alpha - *Saccharomyces cerevisiae*	04 transcription
An08g06940	strong similarity to histone H4.1 - *Emericella nidulans*	04 transcription
An11g10110	similarity to protein SRB8 - *Saccharomyces cerevisiae*	04 transcription
An15g03350	strong similarity to PalC - *Emericella nidulans*	04 transcription
An16g08450	strong similarity to hypothetical transcriptional regulator SPCC417.09c - *Schizosaccharomyces pombe*	04 transcription
An02g06050	strong similarity to cytoplasmic ribosomal protein of the small subunit S16.e - *Saccharomyces cerevisiae*	05 protein synthesis
An02g13840	strong similarity to cytoplasmic ribosomal protein of the small subunit S9 - Homo sapiens	05 protein synthesis
An11g01690	strong similarity to cytoplasmic ribosomal protein of the small subunit S30 - *Saccharomyces cerevisiae*	05 protein synthesis
An11g09500	strong similarity to cytoplasmic ribosomal protein of the small subunit S4.e -*Saccharomyces cerevisiae*	05 protein synthesis
An12g04670	strong similarity to translation initiation factor eIF-5 - Saccharomyces cerevisiae	05 protein synthesis
An12g04860	strong similarity to cytoplasmic ribosomal protein of the large subunit L30 - *Saccharomyces cerevisiae*	05 protein synthesis
An13g01070	strong similarity to 40S ribosomal protein S28.e.B RPS 28B (RPS33B) - *Saccharomyces cerevisiae*	05 protein synthesis
An17g02390	strong similarity to cytoplasmic ribosomal protein of the small subunit RP10B - *Saccharomyces cerevisiae* [putative sequencing error]	05 protein synthesis

Table 7 (continued)

Gene number	Description	Functional gene category
An07g02010	strong similarity to multicatalytic endopeptidase complex chain Y7 PRE8 - *Saccharomyces cerevisiae*	06 protein fate (folding, modification, destination)
An07g03750	strong similarity to phosphatidylinositol-phosphatidylcholine transfer protein SEC14 - *Yarrowia lipolytica*	06 protein fate (folding, modification, destination)
An07g03880	serine proteinase pepC - *Aspergillus niger* [putative frameshift]	06 protein fate (folding, modification, destination)
An07g08300	cyclophilin-like peptidyl prolyl cis-trans isomerase cypH - *Aspergillus niger*	06 protein fate (folding, modification, destination)
An07g09590	strong similarity to glutathione S-transferase GST of patent US5962229-A - *Zea mays*	06 protein fate (folding, modification, destination)
An15g06470	similarity to signal sequence receptor alpha chain - *Canis lupus familiaris*	06 protein fate (folding, modification, destination)
An02g06360	similarity to arp2 or 3 complex 16kD subunit arc16 - *Homo sapiens*	08 cellular transport and transport mechanisms
An12g07720	strong similarity to ferric (and cupric) reductase FRE2 – *Saccharomyces cerevisiae*	08 cellular transport and transport mechanisms
An15g01930	similarity to integral membrane protein PTH11 - *Magnaporthe grisea*	10 cellular communication or signal transduction mechanism
An14g01840	similarity to hypothetical temperature-shock induced protein TIR3 - *Saccharomyces cerevisiae*	11 cell rescue, defense and virulence
An02g08020	strong similarity to H+-transporting ATPase lipid-binding protein vma3 - *Neurospora crassa*	13 regulation of or interaction with cellular environment
An14g00710	strong similarity to bud emergence mediator BEM1 - *Saccharomyces cerevisiae*	14 cell fate
An12g00220	weak similarity to putative NADH dehydrogenase chain precursor - *Homo sapiens*	40 sub-cellular localisation
An12g08090	weak similarity to actin filament-binding protein b-nexilin - *Rattus norvegicus*	40 sub-cellular localisation
An01e00180	trnaKctt	tRNA
An01e02580	trnaMcat	tRNA
An01e09990	trnaSaga	tRNA
An01e11980	trnaYgta	tRNA
An01e12930	trnaSaga	tRNA
An02e09400	Trnakctt	tRNA
An02e09410	trnaKttt	tRNA
An05e01950	trnaYgta	tRNA
An07e02340	trnaCgca	tRNA
An15e07410	trnaYgta	tRNA
An15e07420	trnaYgta	tRNA
An15e07430	trnaYgta	tRNA
An15e07440	trnaYgta	tRNA

DISCUSSION

A. niger colonizes substrates by means of hyphae that extend from the periphery of the mycelium. Previously, it has been shown that these exploring hyphae are heterogenic with respect to protein secretion and transcription ((Wösten *et al.*, 1991; Vinck *et al.*, 2005; Vinck *et al.*, 2010; **Chapter 4**). Furthermore, there were indications that translation was also heterogenic within these hyphae (Vinck *et al.*, 2010; **Chapter 4**). Here, heterogeneity between exploring hyphae was assessed by whole genome expression analysis of five neighboring hyphae that had experienced identical environmental conditions. This study represents the first single cell whole genome analysis of a microbe. It was shown that heterogenic gene expression can be found throughout the functional gene categories. This suggests that heterogeneity is caused by stochastic gene expression and / or by epigenetic processes.

In **Chapter 5** a protocol was developed that enabled expression analysis of 500 apical compartments of hyphae. By replacing the Ovation Pico WTA System for the WT-Ovation One-Direct RNA Amplification System sufficient cDNA could be amplified from a single hypha. This protocol was used to amplify cDNA from the total RNA of five neighboring hyphae. This was done in two amplification experiments. The cDNA amplicons of one of the experiments had a length of 100-300 bp, whereas those of the other experiments were 50-100 bp in length. Hybridization of Affymetrix GeneChip *A. niger* Genome Arrays revealed that the higher amplicon length was accompanied by a lower number of genes with a present call (5.8-6.7% versus 4.1-4.3% for the small and large amplicons, respectively). The 4.1-6.7% of the genes of *A. niger* with a present call is low when one considers that about 50% of the genes are expressed in a sandwiched colony of *A. niger* (Levin et al., 2007*)*. Genes that were lowly expressed at the periphery of the colony or within a pool of hyphal tips from exploring hyphae often had an absent call in the arrays of the single hyphae. Apparently, RNA of lowly expressed genes is not efficiently amplified when one uses the RNA of a single hypha (*i.e.* 1 pg). As a consequence of the low number of probe sets with a present call, higher scale factors are obtained when compared to hybridization experiments with RNA from the whole colony. Furthermore, scale factors are highly influenced by minimal differences in the number of present calls between the samples. Taken these facts into account, the difference in scale factors between the arrays of the single hyphae (>3-fold but <5-fold) was considered to be low enough to normalize and analyze the samples as a whole. That this is justified is indicated by the fact that the two experimental sets (hyphae 1,2 and 3, and hyphae 4 and 5) do not group *per se* in hierarchical clustering, PCA analysis, and overrepresentation studies.

In total, 2608 probe sets had a present call in at least one of the five individual hyphae. These probe sets were found to comprise tRNAs, rRNAs and all 19 Class I FunCats. For each functional gene category, at least 10-30% of the probe sets had a present call, indicating that all categories were evenly well detected. Heterogeneity between the five individual exploring hyphae was assessed by testing for overrepresentation of functional gene categories within each of the single hyphae. The test revealed that

genes encoding rRNAs and tRNAs were overrepresented in all five hyphae, while genes involved in ribosome biogenesis were overrepresented in 4 out of the 5 hyphae. The other 9 overrepresented categories (Table 4) were found in one or two of the 5 hyphae. Taken together, this indicates that heterogeneity in expression in exploring hyphae can be found in many unrelated gene classes. This is supported by the finding that 308 out of the 2608 probe sets had a relatively high standard deviation (>0.5) of the log2 RMA signal values. Apparently, at least 12% of the genes is heterogenically expressed between neighboring exploring hyphae. This set of genes comprises all functional gene categories, except for the ones that have relatively few members. Hierarchical clustering of the 308 probe sets showed that each single hypha had a cluster of genes with higher signals when compared to the other four hyphae. Ribosome biogenesis and tRNAs were overrepresented in the majority of the samples. One hypha showed no overrepresented categories, whereas another hypha showed overrepresentation for 7 of the 10 found categories (Table 5). Two of these enriched categories were involved in energy implying that this hypha might have been metabolically more active than the other hyphae.

Heterogeneity within the 5 individual hyphae was also assessed by selecting the top 100 of genes with the highest hybridization signal in each of the hyphae. A total of 207 different genes was found in this selection, of which 43 were found in all 5 hyphae and 119 were found exclusively in one of the hyphae. For instance, all 5 hyphae contained the 4 rRNAs (*5S*, *5.8S*, *18S* and *28S*), 4 tRNAs and 14 genes involved in metabolism in their top 100. In contrast, 13 tRNAs, 7 genes encoding cytoplasmic ribosomal proteins and *glaA* and *eglB* were present in the top 100 of only one of the hyphae. Gene *glaA* and *18S* rRNA were also found in the list of 308 genes that showed a standard deviation >0.5 between the log2 RMA signal values. Heterogeneity of these genes as well as the *actin* gene was confirmed by QPCR. It was shown that the standard deviation of Ct values for these genes in cDNA of the 5 single hyphae was larger than those found in biological replicates of cDNA from pools of 100 tips or from cDNA from the whole periphery.

Taken together, this research has shown that neighboring hyphae are highly heterogenic with respect to expression of genes belonging to a wide range of functional gene categories. Future studies will be devoted to improve the sensitivity of the RNA profiling. This should result in models describing single cell transcription and their relation to the average expression within a zone of the mycelium.

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CHAPTER 7

GENERAL DISCUSSION

Aspergillus niger is a filamentous fungus which colonizes both dead and living substrates by forming a mycelium. Such a mycelium consists of a network of hyphae that extend at their apices and branch subapically. Mycelia secrete a wide variety and large amounts of enzymes. These enzymes degrade organic polymers into molecules that can be taken up to serve as nutrients. Protein secretion is thus an essential step in nutrient uptake and this might explain the high secretion capacity of filamentous fungi in general and *A. niger* in particular. Because of its exceptional production of extracellular proteins, *A. niger* is one of the most important industrial cell factories (Nout, 2000; Punt *et al.*, 2002; Wösten *et al.*, 2007).

Only the growing hyphae within an *A. niger* colony secrete proteins. These growing hyphae can be found in a central zone and at the periphery of the colony. These zones do not secrete the same proteins (Wösten *et al.*, 1991; Moukha *et al.*, 1993), showing that the *A. niger* colony is heterogenic with respect to protein secretion. Heterogeneity is also manifested by the different RNA profiles of the outer and inner zones of the colony (Masai *et al.*, 2006; Levin *et al.*, 2007; Kasuga & Glass, 2008). It is thus concluded that the vegetative mycelium of *A. niger* represents a heterogenic body, which results in zonal differences in growth, secretion and gene expression. Heterogeneity within the fungal mycelium can even be found within a zone. Glucoamylase was shown to be secreted by a subset of hyphae at the periphery of an *A. niger* colony (Wösten *et al.*, 1991). This heterogeneity was explained by differences in the expression of *glaA* (Vinck *et al.*, 2005). These findings show that neighboring hyphae are heterogenic despite the fact that they experience identical environmental conditions. This Thesis aimed to explore the extent of heterogeneity in the mycelium of *A. niger* and to study the mechanism underlying this phenomenon.

The extent of hyphal heterogeneity in the mycelium of *A. niger*

Previously, heterogeneity in *A. niger* was studied in concentric zones of colonies that had been grown on a solid medium. It was found that 9% of the genes that are active in these centimeter-scale macro-colonies are expressed in only one of five zones (Levin *et al.*, 2007). Moreover, more than 25% of the active genes show at least a two-fold difference in expression between the outer and innermost zone of the colony. For instance, expression of *glaA* was more than 3-fold higher at the periphery of maltose-grown colonies when compared to the colony centre. Similarly, ferulic acid esterase (*faeA*) was five times higher expressed at the periphery of xylose-grown colonies. The zonal differences in expression were explained by both medium dependent and medium independent mechanisms (Levin *et al.*, 2007).

In industrial settings, *A. niger* is grown in liquid media. This results in a dispersed mycelium or in (sub)millimeter wide micro-colonies. In **Chapter 3** it was investigated whether heterogeneity exists between the micro-colonies of a liquid culture. To this end, strains were used that express *GFP* from the *glaA* or the *faeA* promoter. A complex object parametric analyzer and sorter (COPAS) was used to analyze the size and GFP fluorescence of micro-colonies of 16 h-old cultures that had been grown in liquid inducing medium. Statistical analysis and mathematical modeling showed that both volume and fluorescence of micro-colonies were not normally distributed but could rather be explained by two normally distributed populations. About 25% of the population consisted of small colonies, whereas 75% comprised large colonies. The average size difference of these two populations was 90 µm. In the case of gene expression, the participation level of high and low expressers differed per strain but never comprised the 75% versus 25% ratio, respectively, as was found for colony size. This indicates that the heterogeneity of *glaA* and *faeA* expression is only partly explained by the size of the micro-colony. Taken together, these data show that micro-colonies within a liquid shaken culture are heterogenic with respect to size and gene expression. This finding has implications how analysis of RNA, proteins and metabolites from whole cultures should be interpreted.

In the next set of experiments, heterogeneity was assessed within zones of a micro-colony (**Chapter 3**). To this end, the center and periphery of micro-colonies were dissected with laser micro-dissection and collected using laser pressure catapulting. RNA was isolated from these zones and RNA accumulation was examined using QPCR. Similar levels of *18S* rRNA, *actin*, *glaA* and *faeA* mRNA were found in the centre and periphery of the micro-colonies. However, the RNA from the centre originated from 45 times more hyphae when compared to the periphery. From this and acridine orange staining it is concluded that the few hyphae at the periphery contain more RNA than the hyphae in the centre. Heterogenic RNA levels were not found when the centre and the periphery of a macro-colony were compared. However, RNA levels of the most outer hyphae of a macro-colony did differ from the rest of the periphery **(Chapter 5)**. Acridine orange staining showed that the outer 1 mm of the periphery contained relatively more RNA when compared to the more inner part of this colony zone. In fact, RNA was most abundant in the apical region (*i.e.* within the first 100-300 µm) of the most outer hyphae (known as the exploring hyphae). This finding is supported by *in situ* hybridizations that have been performed in the past (Teertstra *et al.*, 2004). A high RNA content in the apex

would make sense considering the fact that hyphae extent in this region and not in sub-apical parts.

An RNA extraction and amplification protocol was developed that enables RNA profiling of selected hyphae or parts thereof (**Chapter 5)**. It was shown that the first 100 µm of the exploring hyphae of a macro-colony of *A. niger* contains only 1 pg of RNA. This 100 µm represents only part of the first hyphal compartment since the first septum is found at about 400 µm from the apex. The RNA extraction and amplification protocol was used to show that the RNA composition of tips of the exploring hyphae differs from that of the periphery as a whole. RNA of 3 out of the 4 rRNA genes was found to be enriched in the hyphal tips of the exploring hyphae. This enrichment had consequences for the microarray analysis. Similar amounts of total RNA were used for the amplification but as a consequence of the rRNA enrichment in the tips a lower amount of cDNA of mRNA was obtained. As a result "expression levels" of genes other than those of rRNA will be lower in the hyphal tip sample when compared to the periphery sample. This effect is increased by the high hybridization signals obtained for rRNA leading to a further reduction of the relative mRNA signals in hyphal tip samples. As a consequence, the RNA profiles from the hyphal tip and the periphery could not be directly compared. Therefore, profiles of the sample types were separately normalized, followed by separate statistical analyses and indirect comparison of the RNA. RNA abundance of functional gene categories was compared indirectly by overrepresentation testing. Only 8 and 3 categories were found that significantly contributed to the top 10% of genes with the highest RNA abundance in only the periphery or the hyphal tip, respectively. The periphery specific categories were mainly related to protein fate, implying that these gene categories are less important for the hyphal tips of exploring hyphae. Ranking of functional gene categories based on the p-value of overrepresentation within the top 10% of genes with the highest hybridization signals revealed that the FunCat categories cytoplasm and intracellular transport vesicles are more important in the periphery when compared to the hyphal apices. In contrast, functional gene categories related to interaction with the environment (*i.e.* cell rescue, defense and virulence; and regulation of and interaction with cellular environment) seem to be more important in the hyphal tip. It makes sense that interaction with the environment is of higher importance for hyphae that explore a new substrate than for hyphae that are located in a more established part of the mycelium. The tips of the exploring hyphae were also shown to be enriched for RNAs involved in transcription and transport facilitation. The latter was also found in an overrepresentation analysis of individual genes. Up-regulation of transport genes may facilitate uptake of molecules from the fresh substrate. Overrepresentation analysis of individual genes also revealed that the hyphal tips were enriched in rRNAs (see above), in RNAs of other genes involved in translation (tRNAs and genes involved in protein synthesis) and in RNA of genes involved in stress response. Taken together, these data indicate that the RNA composition of hyphal tips of exploring hyphae is adapted to enable active growth and to anticipate to the uncolonized environment they encounter.

The subcellular RNA profiling described in **Chapter 5** is unique for microbes. In fact, subcellular mRNA populations have only been studied in the subdomains of neuronal cells (Willis & Twiss, 2010). These cells also form filaments but are much bigger in size (dendrites 0.5-1.0 mm, cell body 0.05 mm and axon 40 mm). The isolation and

amplification protocol described in **Chapter 5** will enable a comparison of RNA profiles of different compartments or those of branches and tips. Moreover, processes like invasion of symbiotic, parasitic or saprotrophic fungi can be monitored at the cell level. The main advantage of this technology is that one can analyze the RNA profile of cells that are actually active in a certain process rather than using the RNA of the mycelium as a whole. Since the mycelium contains cells with different activities, differential gene expression is leveled out when the whole mycelium is used for RNA extraction. This hampers the identification of genes involved in fungal growth and development and results in inaccurate expression networks that are established during growth and development within particular cells. It should be noted that the current subcellular expression profiling relies on amplification of the RNA. It cannot be excluded that there is preferential amplification of RNA species and that this affects the transcriptome analysis. Direct sequencing of RNA is claimed to be more sensitive (Ozsolak *et al.*, 2009) and this is expected to circumvent the use of amplification of cDNA.

In **Chapter 4** it was assessed whether apart from *glaA* also other genes encoding secreted proteins are heterogenically expressed at the periphery of macro-colonies. GFP was used as a reporter to monitor expression of the acid amylase gene *aamA*, the α-glucuronidase gene *aguA*, and the feruloyl esterase gene *faeA*. Transformants were made using a new enzyme mixture to obtain protoplasts (**Chapter 2**). Like for *glaA*, GFP fluorescence intensity distributions could be explained by assuming that they are composed of a weighted mixture of two normally distributed components. These components represent a population that highly and a population that lowly expressed *GFP*. In all cases, the population of lowly expressing hyphae contributed a high percentage of the total number of hyphae. This indicates that the number of hyphae contributing to secretion of enzymes is low, probably far less than 1% of the total macro-colony.

In the next step, co-localization studies were done using strains expressing *GFP* and *dTomato* from the *amyR*, *glaA*, *aamA*, *faeA* or *aguA* promoters. These studies showed that hyphae that highly express one of these genes, also highly express the other genes. Significant, high correlation coefficients were found when amylolytic genes were co-localized on inducing maltose medium and when xylanolytic genes were co-localized on inducing xylose medium. Lower, but still strongly significant correlation coefficients were found when the XlnR regulated *faeA* gene was co-localized with the AmyR regulated *glaA* and *aamA* genes on a medium containing xylose. This carbon source induces *faeA* but results in a low expression of the AmyR regulated genes. Taken together, these results suggest a higher level of regulation than a mere causative role for the transcriptional regulators of the genes encoding secreted proteins. This was strengthened by the significant, high correlation of the expression of the xylanolytic gene *faeA* and the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase gene *gpdA*. Morever, a strong correlation was found between the expression of *glaA* and the abundance of *18S* rRNA. These results suggest that there are at least two populations of hyphae at the periphery of a colony that can be discriminated by their transcriptional and translational activity. Interestingly, we have no indications that the hyphae with a high transcriptional activity have a higher growth rate. Possibly, the transcriptional and translational activity in the low expressing hyphae is sufficient to support growth. A high transcriptional and translational activity would enable a high secretion activity.

Mechanisms underlying heterogeneity

Macro-colonies are routinely inoculated using multiple spores or a piece of mycelium containing multiple hyphae. Therefore, heterogeneity in gene expression could result from heterogeneity in the hyphae or spores within the inoculum. To assess this hypothesis, colonies were analyzed that resulted from single spores or single hyphae. It was shown that these cultures also exhibited heterogenic *glaA* expression **(Chapter 4)**. Apparently, variability in *glaA* expression arises during vegetative growth and is not a result of pre-programming in the spores or hyphae from which the mycelium originated.

Two approaches were followed to investigate the mechanism of heterogeneity in the hyphae of macro-colonies. The first approach was to study the effect of sodium butyrate and 5'-azacytidine **(Chapter 4)**. These compounds alter the chromatin structure by causing hyperacetylation of histones (Boffa *et al.*, 1978; Sealy & Chalkley, 1978) and by inhibiting histone and DNA methylation (Jones, 1985), respectively. Both compounds are expected to yield a more lightly packed DNA, and as such could influence heterogeneity in gene expression. Indeed, the bimodal *glaA* expression was affected in macro-colonies exposed to sodium butyrate and 5'-azacytidine. This indicates that chromatin structure across the DNA is involved in establishing hyphal heterogeneity. The different aspects of chromatin remodelling (DNA methylation, histon methylation, histone deacetylation and histone acetylation) have been studied only to some extent in Aspergilli, but not yet in *A. niger*. So far, there is no proof for DNA methylation (Lee *et al.*, 2008). However, sexual development in *Aspergillus nidulans* was affected when a predicted DNA methyltransferase gene was deleted. From these results it was concluded that *A. nidulans* might have a low, undetectable, methylation activity (Lee *et al.*, 2008). Since the *A. niger* genome contains a lowly expressed gene predicted to be involved in DNA methylation, this could very well also hold for this member of the *Aspergillus* genus. There is also relatively little known about histone methylation in filamentous fungi. In many eukaryotes, methylation of lysine 9 of histone 3 (H3K9) is a hallmark of heterochromatin formation and subsequent gene silencing. Inactivation of the homologue of the H3K9 methyltransferase of *Schizosaccharomyces pombe* in *Aspergillus fumigatus* resulted in reduction in radial growth and impaired conidia formation (Palmer *et al.*, 2008). Aspergilli also contain various genes encoding histon acetyl transferases (HATs) and histon deacetylases (HDACs). HDACs have been shown to affect diverse processes in fungal growth and development. They were shown to be involved in germination, secondary metabolite production and growth under oxidative stress (Lee *et al.*, 2009);(Tribus *et al.*, 2005; Shwab *et al.*, 2007). HDACs have also been shown to be involved in regulation of genes involved in primary metabolism. For instance, it was found in *A. nidulans* that the catabolite repressor CreA (which in turn regulates, among others, amylolytic gene expression) depends on the acetylation state of the chromatin. It could thus very well be that in *A. niger*, heterogenic *glaA* expression is indirectly regulated by the effect of the chromatin structure on CreA.

The second approach to investigate the mechanism of hyphal heterogeneity in macro-colonies was by whole transcriptome analysis of five neighboring exploring hyphae that had experienced identical environmental conditions **(Chapter 6)**. This was done by using a modified version of the protocol that had been developed to enable expression analysis of 500 apical compartments (**Chapter 5)**. RNA from five neighboring

single hyphae (comprising 1 pg of total RNA per hypha) was amplified to cDNA and hybridized to Affymetrix GeneChip *A. niger* Genome Arrays. As a result, 4-7% of the probesets yielded a present call. This is low when one considers that about 50% of the genes are expressed in a sandwiched colony (Levin et al., 2007). Genes with an absent call were shown to be generally lowly expressed at the periphery of the colony or within a pool of hyphal tips from exploring hyphae **(Chapter 5)**. From this it is concluded that lowly expressed genes were not well amplified, explaining the low number of genes with a present call in the single cell hybridization experiments. Direct sequencing of RNA may be used in the future to increase coverage of the transcriptome in the analysis.

The 2608 probe sets with a present call in at least one of the five individual hyphae were found to comprise all functional gene categories (tRNAs, rRNAs and all 19 Class I FunCats). At least 10-30% of the probe sets had a present call for each category. Overrepresentation testing of functional gene categories within each of the five individual exploring hyphae was used to assess hyphal heterogeneity. rRNAs and tRNAs were found to be overrepresented in all five hyphae, genes involved in ribosome biogenesis were overrepresented in 4 out of the 5 hyphae and the other 9 unrelated gene categories were found in one or two of the 5 hyphae. Heterogenic expression in exploring hyphae is thus found in different gene classes. This was strengthened by the finding that 12% of the genes with a present call had a relatively high standard deviation (>0.5) when expression between the 5 single hyphae was compared. These 308 "heterogenic" probe sets again comprised all functional gene categories, except for the ones that have relatively few members. Hierarchical clustering showed that each single hypha had a cluster of genes with higher signals when compared to the other four hyphae. In the majority of the clusters, ribosome biogenesis and tRNAs were overrepresented. One hypha showed no overrepresented categories, whereas another hypha showed overrepresentation for 7 of the 10 found categories. Two of these enriched categories were involved in energy implying that this hypha might have been metabolically more active when compared to the other four hyphae. The top 100 of genes with the highest hybridization signal in each of the hyphae was also analyzed as a measure of heterogeneity. These top 100s comprised 207 different genes of which 43 were found in all 5 hyphae. These 43 genes contained all 4 rRNAs, 4 tRNAs and 14 genes involved in metabolism. In contrast, 119 genes were found exclusively in one of the hyphae. This gene set comprised 13 tRNAs, 7 genes encoding cytoplasmic ribosomal proteins and *glaA* and *eglB*. Genes *glaA* and *18S* rRNA were in the list of the 207 genes that were part of the top 100 and in the list of the 308 genes that showed a standard deviation in expression >0.5 between the single hyphae. Heterogeneity of these genes was confirmed by QPCR **(Chapter 6)**. For these genes as well as the *actin* gene it was shown that the standard deviation of their Ct values in cDNA of the 5 single hyphae was larger than that found in biological replicates of cDNA from pools of 100 tips or from cDNA from the whole periphery.

Chapter 6 represents the first single cell whole genome analysis of a microbe. It was shown that neighboring hyphae are highly heterogenic with respect to gene expression. Heterogeneity can be found throughout the functional gene categories. This indicates that heterogeneity might be caused by stochastic gene expression and / or by epigenetic processes. This has also been suggested for other eukaryotes (Raj &

van Oudenaarden, 2008). Variations in mRNA and protein levels in all kinds of organisms appear to depend on both biophysical parameters governing gene expression and on gene network structure. These cell-to-cell variations are found to have important consequences for cellular function and seem to be beneficial in isogenic populations. Future studies will be devoted to improve the sensitivity of the RNA profiling. This should result in models describing single cell transcription and their relation with the average expression within a zone of the mycelium.

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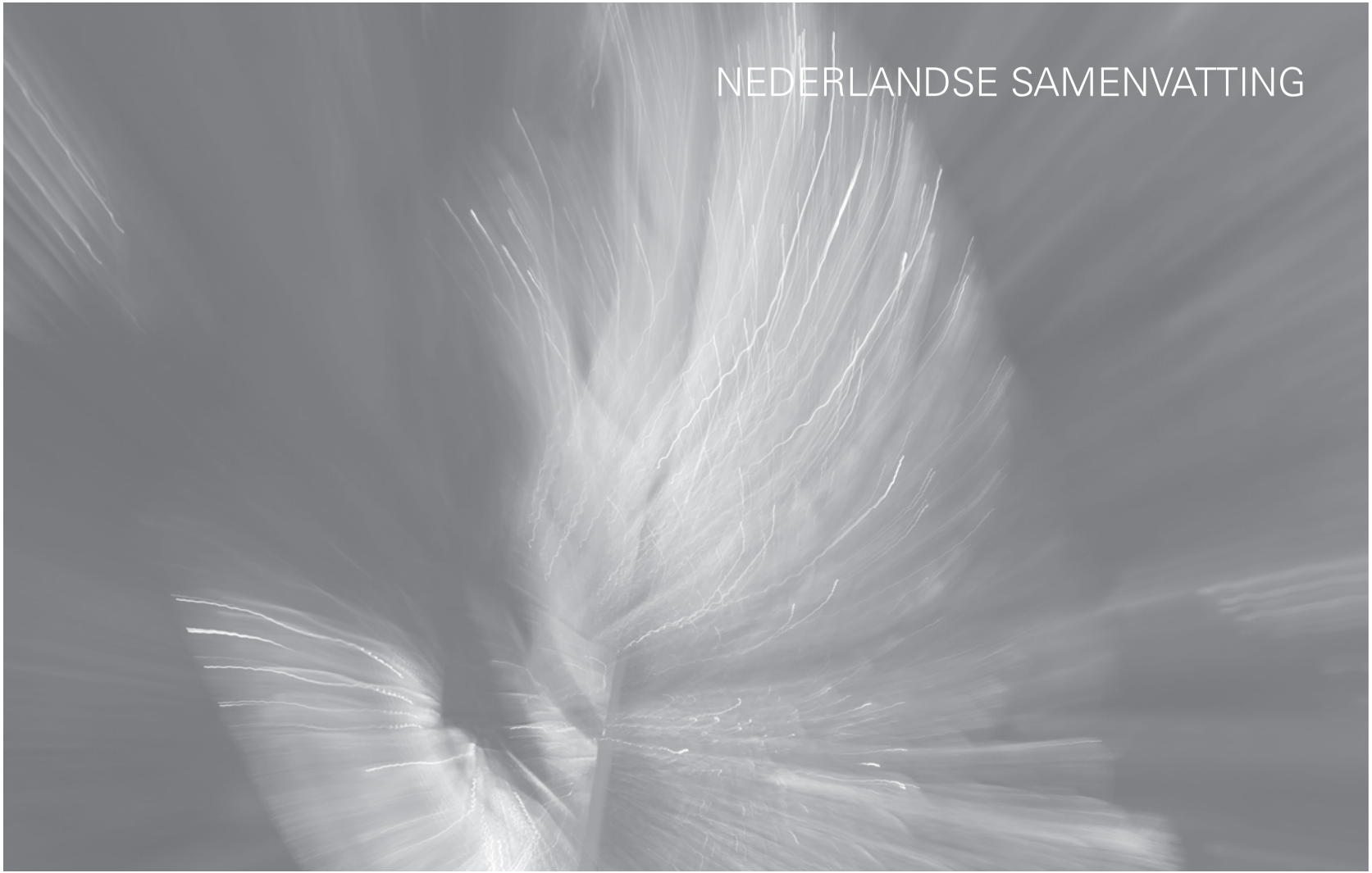

NEDERLANDSE SAMENVATTING

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Filamenteuze schimmels zoals *Aspergillus niger* kunnen zowel levende als dode substraten koloniseren middels het vormen van een mycelium. Een dergelijk mycelium, ook wel kolonie genoemd, bestaat uit een netwerk van hyfen die groeien aan hun top en die onder de top vertakken. Deze hyfen scheiden grote hoeveelheden enzymen uit die organische polymeren afbreken tot kleinere moleculen die als nutriënten kunnen worden opgenomen. Secretie van eiwitten is dus een essentiële stap in nutriëntopname en zou dus de hoge secretiecapaciteit van filamenteuze schimmels zoals *A. niger* kunnen verklaren. De productie van extracellulaire eiwitten door *A. niger* is zelfs zo hoog dat het één van de belangrijkste enzymproductiesystemen is voor de industrie.

Slechts de groeiende hyfen binnen een *A. niger* kolonie scheiden eiwitten uit. Deze groeiende hyfen bevinden zich in bepaalde zones binnen de kolonie, namelijk in het centrum en aan de periferie. Deze zones scheiden niet dezelfde eiwitten uit, wat aangeeft dat de kolonie van *A. niger* heterogeen is met betrekking tot eiwitsecretie. Heterogeniteit binnen de kolonie manifesteert zich ook in de RNA profielen die in de verschillende zones van het mycelium worden gevonden. Heterogeniteit kan zelfs binnen zones gevonden worden. Het eiwit glucoamylase wordt namelijk slechts door een subset van hyfen aan de periferie van een *A. niger* kolonie uitgescheiden. Deze heterogeniteit wordt verklaard door verschillen in genexpressie van het glucoamlyase gen. Naburige hyfen zijn dus heterogeen ondanks het feit dat zij dezelfde omgevingscondities ervaren. Het doel van dit proefschrift was om de mate van heterogeniteit in het mycelium van *A. niger* te bepalen en de onderliggende mechanisme(n) te onderzoeken.

De mate van hyfe heterogeniteit in het mycelium van *A. niger*

Voorheen is heterogeniteit in *A. niger* ondermeer bestudeerd aan de hand van expressiepatronen van concentrische zones van kolonies die gegroeid waren op vast medium. Deze macrokolonies hadden een diameter van enkele centimeters. Het bleek dat 9% van de actieve genen slechts in één van de vijf zones tot expressie kwamen. Daarnaast had meer dan 25% van de actieve genen tenminste een tweevoudig verschil

in genexpressie tussen de buitenste en de binnenste zone van de kolonie. De expressie van *glaA* was bijvoorbeeld drie keer hoger aan de periferie dan in het centrum van een kolonie gegroeid op maltose. Deze zonale verschillen in genexpressie konden worden verklaard door zowel medium afhankelijke als medium onafhankelijke mechanismen.

In de industrie wordt *A. niger* gegroeid in vloeibaar medium. Onder deze condities worden microkolonies gevormd met een diameter op (sub)millimeterschaal. In **Hoofdstuk 3** werd onderzocht of heterogeniteit ook bestaat in kolonies die gegroeid zijn in vloeibaar medium. In eerste instantie werd gekeken of er heterogeniteit was tussen microkolonies in een vloeistofculture. Hiertoe werden stammen gebruikt die *GFP* tot expressie brengen vanaf de *glaA* of de *faeA* promoter. De grootte en de GFP fluorescentie van de microkolonies werden bepaald middels een COPAS sorteerder. Statistische analyse en mathematische modellering wezen uit dat zowel de grootte als de fluorescentie van de microkolonies van een 16-uur oude culture geen normale verdeling volgen maar verklaard kunnen worden door twee normaal verdeelde populaties. De populatie van kleine kolonies bevatte ongeveer 25% van de gehele culture, terwijl de populatie van grote kolonies 75% van de culture omvatte. De populatiegrootte van kolonies die GFP laag en hoog tot expressie brachten gaven nooit de 25% : 75% ratio zoals gevonden werd voor de grootte van de kolonies. Dit impliceert dat de heterogeniteit van *glaA* en *faeA* expressie slechts gedeeltelijk wordt verklaard door de grootte van de microkolonies. Microkolonies binnen een vloeistofculture zijn dus heterogeen met betrekking tot zowel koloniegrootte als genexpressie. Deze bevindingen impliceren dat men slechts een beperkt inzicht zal verkrijgen in de biologie binnen een vloeistofculture indien men RNA, eiwit en metaboliet analyses doet aan gehele cultures, zoals op dit moment in het onderzoek gebruikelijk is.

In **Hoofdstuk 3** werd ook de heterogeniteit bestudeerd tussen zones in individuele microkolonies. Daartoe werd laser dissectie (LMPC) gebruikt om het RNA van het centrum en de periferie van microkolonies apart te isoleren. De RNA accumulatie in deze zones werd vervolgens geanalyseerd met behulp van kwantitatieve PCR (QPCR). Voor beide zones werden gelijke hoeveelheden *18S* rRNA, *actine*, *glaA* en *faeA* mRNA gevonden. Echter, het RNA van het centrum was afkomstig van 45 keer meer hyfen dan van de periferie. Uit deze resultaten en uit acridine oranje kleuringen kan geconcludeerd worden dat de hyfen aan de periferie meer RNA bevatten dan de hyfen in het centrum van de microkolonies. Dergelijke heterogene RNA niveaus werden niet gevonden wanneer het centrum en de periferie van macrokolonies werden vergeleken. Echter, RNA hoeveelheden van de buitenste hyfen van macrokolonies waren wel hoger dan in de rest van de periferie. RNA was zelfs het meest abundant in de toppen van de buitenste hyfen. Een hoge RNA concentratie in dit deel van de hyfen zou groei in dit deel van de hyfe mogelijk kunnen maken, een proces wat niet plaatsvindt achter de top van deze filamenten.

Om RNA profielen van geselecteerde hyfen of delen daarvan te kunnen analyseren werd een RNA extractie en amplificatie protocol ontwikkeld (**Hoofdstuk 5**). Hiermee werd aangetoond dat de eerste 100 µm van een buitenste hyfe van een macrokolonie van *A. niger* 1 pg RNA bevat. Deze 100 µm is slechts een deel van het eerste hyfe compartiment aangezien het eerste septum pas gevonden werd op ongeveer 400 µm van de top. Het RNA extractie en amplificatie protocol werd gebruikt om voor het

eerst in een microbe een subcellulaire RNA samenstelling te analyseren. Hiertoe werd de RNA samenstelling van de toppen van de buitenste hyfen vergeleken met die van de periferie in zijn geheel. RNA werd geisoleerd, omgezet in cDNA, geamplificeerd en gehybridiseerd op DNA arrays van *A. niger.* Profielen van de hyfentoppen en de periferie werden apart van elkaar genormaliseerd en onderworpen aan statistische analyses. Hierna werden de profielen indirect met elkaar vergeleken. RNA profielen van functionele gencategorieën werden vergeleken middels over-representatie analyse. Slechts 8 en 3 categorieën werden gevonden die significant bijdroegen aan de top 10% van genen met de hoogste RNA abundantie in respectievelijk de periferie en de hyfe toppen. De categorieën die specifiek waren voor de periferie waren voornamelijk gerelateerd aan "eiwitbestemming" wat impliceert dat deze gencategorie minder belangrijk is in de toppen van de buitenste hyfen. Het schalen van functionele gencategorieën aan de hand van de p-waarden van de over-representatie analyse binnen de top 10% van genen met de hoogste expressiesignalen onthulde dat de gengroepen die gerelateerd zijn aan interacties met de omgeving belangrijker in de hyfe toppen van de buitenste hyfen zijn. Dit zou verklaard kunnen worden door het feit dat deze toppen in een onontgonnen onbekend substraat komen, terwijl de milieuomstandigheden binnen de kolonie reeds door de hyfen in kaart zijn gebracht en hierop is geanticipeerd. De toppen van de buitenste hyfen bleken ook verrijkt te zijn voor RNAs betrokken bij transcriptie en transport. Deze laatstgenoemde categorie werd ook gevonden in de over-representatie analyse op individuele genen. Verhoogde expressie van transportgenen zou de opname van moleculen uit verse substraten kunnen faciliteren. De analyses lieten ook duidelijk zien dat de hyfe toppen verrijkt zijn in rRNAs en in RNAs van andere genen die betrokken zijn bij translatie. Deze data impliceren dat de RNA samenstelling van de toppen van de buitenste hyfen van een kolonie actieve groei en anticipatie op een nieuwe omgeving mogelijk maken..

In **Hoofdstuk 4** heb ik onderzocht of er naast *glaA* ook nog andere genen die coderen voor gesecreteerde eiwitten heterogeen tot expressie komen aan de periferie van macrokolonies. Hiertoe werd GFP gebruikt als reporter om de expressie van het acid amylase gen *aamA*, het α-glucuronidase gen *aguA*, en het feruline zuur esterase gen *faeA* te bestuderen. Transformanten werden gemaakt met behulp van protoplasten die waren verkregen met een nieuwe enzymcocktail die beschreven is in **Hoofdstuk 2**. Voor alle genen kon de verdeling van de GFP fluorescentie verklaard worden door aan te nemen dat er sprake was van een gewogen gemiddelde van twee normaal verdeelde componenten. Deze componenten representeren een populatie hyfen die *GFP* laag en een populatie hyfen die *GFP* hoog tot expressie brachten. In alle gevallen was de populatie van hyfen met een lage expressie hoger dan die van de populatie met een hoge expressie. Dit impliceert dat het aantal hyfen dat bijdraagt aan de uitscheiding van enzymen laag is, waarschijnlijk veel lager dan 1% van de totale macrokolonie. In vervolgexperimenten werden co-lokalisatie studies gedaan, waarbij gebruik werd gemaakt van stammen die *GFP* en *dTomato* tot expressie brengen vanaf de *amyR*, *glaA*, *aamA*, *faeA* of *aguA* promoteren. Deze studies lieten zien dat hyfen die één van deze genen hoog tot expressie brengen de andere genen ook hoog tot expressie brengen, zelfs indien het genen betrof die door andere transcriptiefactoren worden geïnduceerd (bijvoorbeeld *glaA* en *faeA*). Deze resultaten impliceren dus

dat heterogeniteit gereguleerd wordt op een hoger niveau dan het niveau van de transcriptionele regulatoren die genen die coderen voor secretie eiwitten activeren. Deze conclusie werd versterkt door de significante, hoge correlatie die werd gevonden tussen de expressie van *faeA* en het gen *gpdA.* Het laatstgenoemde gen codeert voor een enzym uit het primaire metabolisme (glyceraldehyde-3-phosphate dehydrogenase). Daarnaast werd een sterke correlatie gevonden tussen de expressie van *glaA* en de abundantie van *18S* rRNA. Samengevat impliceren de resultaten dat er tenminste twee populaties van hyfen zijn aan de periferie van een macrokolonie van *A. Niger*. Deze populaties verschillen van elkaar in transcriptionele en translationele activiteit. Opvallend genoeg zijn er geen indicaties dat hyfen met een hoge transcriptionele activiteit ook harder groeien. Mogelijk is de transcriptionele en translationele activiteit in hyfen met een lage expressie voldoende om groei te ondersteunen. Een hoge transcriptionele en translationele activiteit zou hoge secretie van eiwitten mogelijk maken.

Het mechanisme van heterogeniteit

Macrokolonies worden normaalgesproken beent met meerdere sporen of met een stukje mycelium wat vele hyfen bevat. Heterogene genexpressie zou dus het resultaat kunnen zijn van heterogeniteit in de hyfen of sporen binnen de gebruikte ent. Om deze hypothese te onderzoeken werden in **Hoofdstuk 4** kolonies geanalyseerd die het resultaat zijn van een enkele spore of hyfe. Deze cultures lieten ook heterogene *glaA* expressie zien. De variatie in *glaA* expressie komt dus tot stand tijdens de vorming van het mycelium en is dus niet het resultaat van een programmering in de sporen of hyfen van waaruit het mycelium tot stand is gekomen. Om het mechanisme achter heterogeniteit verder te onderzoeken werden twee strategieën gevolgd. In de eerste strategie werd het effect van butyraat en 5'-azacytidine bestudeerd. Deze chemicaliën veranderen de chromatine structuur door respectievelijk hyperacetylatie van histonen te veroorzaken en door het remmen van methylatie van histonen en het DNA. Beide chemicaliën leiden tot een meer open DNA structuur en zouden daarmee heterogene genexpressie kunnen beïnvloeden. De bimodale expressie van *glaA* werd inderdaad beïnvloed in macrokolonies die gegroeid waren in de aanwezigheid van butyraat of 5'-azacytidine. Dit impliceert dat de chromatine structuur van het DNA betrokken is bij het tot stand komen van hyfe heterogeniteit.

De tweede strategie om het mechanisme achter hyfe heterogeniteit te onderzoeken in macrokolonies was via de analyse van het complete transcriptoom van vijf naburig gelegen hyfen aan de rand van een kolonie (**Hoofdstuk 6**). Hiertoe werd het protocol dat beschreven is in **Hoofdstuk 5** aangepast. Het RNA van vijf naburige enkele hyfen (welke elk 1 pg totaal RNA bevatten) werd geamplificeerd tot cDNA, geamplificeerd en gehybridiseerd op DNA micro-aarays van *A. niger*. Dit resulteerde in een present call voor 4-7% van de probe sets op deze chips. Genen met een absent call kwamen over het algemeen laag tot expressie aan de periferie van de kolonie of binnen een pool van hyfe-toppen. Hieruit werd geconcludeerd dat laag tot expressie komende genen niet goed geamplificeerd werden. De 2608 gevonden probe sets met een present call in tenminste een van de vijf individuele hyfen omvatten alle functionele gencategorieën. Tenminste 10-30% van de probe sets van elke categorie kreeg een present call. rRNAs en tRNAs waren in alle vijf de hyfen over-gerepresenteerd, terwijl in 4 van de 5 hyfen

een over-representatie van genen betrokken bij ribosoom biogenese werd gevonden. Negen andere ongerelateerde categorieën werden gevonden in 1 of 2 van de 5 hyfen. Uit deze analyse kan dus geconcludeerd worden dat zeer verschillende genklasses heterogeen tot expressie komen in de buitenste hyfen van de kolonie. Dit werd ook geconcludeerd uit het feit dat bijna alle genklassen waren vertegenwoordigd in de 12% van de detecteerbare genen die een relatief hoge standaarddeviatie hadden wanneer de genexpressie tussen de 5 enkele hyfen werd vergeleken. Hiërarchische clustering liet zien dat elke hyfe een cluster van genen had die hogere signalen hadden vergeleken met de andere vier hyfen. In de meerderheid van deze clusters waren genen betrokken bij ribosoom biogenese en tRNAs over-gerepresenteerd. Één van de hyfen bevatte helemaal geen over-gerepresenteerde categorieën terwijl in een andere hyfe 7 van de 10 gevonden categorieën over-gerepresenteerd waren. Twee van deze categorieën waren betrokken bij energiehuishouding. Dit impliceert dat deze hyfe metabolisch meer actief zou kunnen zijn geweest vergeleken bij de andere vier hyfen. Tenslotte werd de top 100 van genen met de hoogste hybridisatie signalen in elke hyfe geanalyseerd als een maat voor heterogeniteit. Deze top 100s omvatten 207 verschillende genen waarvan er 43 in de top 100 van alle 5 hyfen teruggevonden werden. Bij deze 43 genen bevonden zich alle 4 de rRNAs, 4 tRNAs en 14 genen betrokken bij metabolisme. Daarentegen waren er 119 genen die exclusief bij één van de hyfen werden gevonden. Deze set genen omvatte onder andere 13 tRNAs, 7 genen coderend voor cytoplasmatische ribosomale eiwitten en *glaA*. Heterogeniteit van *18S rRNA* en *glaA* werd bevestigd middels QPCR.

Hoofdstuk 6 is het eerste onderzoek in microben waarbij het transcriptoom is geanalyseerd van een enkele cel. Hiermee werd aangetoond dat naburig gelegen hyfen heterogeen zijn wat betreft hun genexpressie ondanks het feit dat zij groeiden onder identieke mileuomstandigheden. Deze heterogeniteit wordt teruggevonden in alle functionele gencategorieën. Dit impliceert dat heterogeniteit veroorzaakt zou kunnen worden door stochastische genexpressie en / of epigenetische processen. Toekomstige studies zullen worden gewijd aan het verbeteren van de gevoeligheid van RNA profiling. Dit zou uiteindelijk moeten resulteren in modellen die de transcriptie in enkele cellen beschrijft en hun relatie tot de gemiddelde expressie binnen een zone in het mycelium.

CURRICULUM VITAE

Charissa de Bekker was born on January 19, 1983 in Veghel, the Netherlands. She followed her secondary education at the Zwijsen College in Veghel, the Netherlands and graduaded in 2001 with a Gymnasium diploma. In September of the same year she started to study Biology at Utrecht University. In 2004 she received her bachelor degree in Biology, after which she enrolled the Prestige Master "Biomolecular Sciences" of Utrecht University. As part of this master she did a 9-months internship in the Molecular Microbiology Group of the Department of Biology at Utrecht University under supervision of dr. R.P. de Vries and dr. A.M. Levin. In 2005 Charissa received the Biomolecular Sciences Travel Grant to perform her second internship for 6 months in the Microbiology Unit of the Organic Chemistry Department at Oxford University. After obtaining her MSc diploma in 2006 Charissa started her PhD at the Molecular Microbiology Group of the Department of Biology at Utrecht University under supervision of prof. dr. H.A.B. Wösten. The research during this period on heterogeneity of gene expression in *Aspergillus niger* was financially supported by STW (project number 07453) and is described in this Thesis. In April 2011 Charissa will start as a postdoctoral researcher at Pennsylvania State University where she will study metabolite and gene expression heterogeneity in colonies of ant infecting fungi.

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ABSTRACTS & PRESENTATIONS

de Bekker, C. Ruhr-Universität Bochum, Fakultät für Biologie & Biotechnologie, November 22, 2010. Bochum, Germany (Oral Presentation)

de Bekker, C., Vinck, A., Wösten, H.A.B. IMC9, August 1-6, 2010. Edinburgh, United Kingdom (Oral presentation)

de Bekker, C., van Veluw, G.J., Vinck, A., Wösten, H.A.B. IMC9, August 1-6, 2010. Edinburgh, United Kingdom (Oral presentation)

de Bekker, C., Vinck, A., Wösten, H.A.B. Single Cell Analysis Conference, May 25-26, 2010. Dublin, Ireland (Poster presentation)

de Bekker, C., Vinck, A., Wösten, H.A.B. ECFG10, March 29 – April 1, 2010. Noordwijkerhout, the Netherlands (Poster presentation)

de Bekker, C., Vinck, A., Wösten, H.A.B. 7th Symposium Kluyver Centre for Genomics of Industrial Fermentation, January 26-28, 2010. Noordwijkerhout, the Netherlands (Poster presentation)

de Bekker, C., Vinck, A., Wösten, H.A.B. 3rd International Conference on Environmental, Industrial and Applied Microbiology, December 2-4, 2009. Lisbon, Portugal (Oral Presentation)

de Bekker, C., van Veluw, G.J., Vinck, A., Wiebenga, L.A., Wösten, H.A.B. 3rd International Conference on Environmental, Industrial and Applied Microbiology, December 2-4, 2009. Lisbon, Portugal (Oral Presentation)

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de Bekker, C. 6th Symposium Kluyver Centre for Genomics of Industrial Fermentation, January 27-29, 2009. Noordwijkerhout, the Netherlands (Oral presentation)

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