Heterogeneity in Microbial Micro-colonies

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Heterogeneity in Microbial Micro-colonies

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Voor mijn ouders

Contents

CHAPTER 1

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General Introduction

Vegetative growth and sporulation in Aspergillus

Aspergillus species are among the most abundant fungi worldwide. They are not very selective with respect to abiotic growth conditions (Table 1). For instance, they can grow over a wide range of temperatures (6-55 °C) and at relatively low humidity. Moreover, *Aspergillus* species feed on a large variety of substrates including animal faeces and human tissue. Nonetheless, they are predominantly found on complex plant polymers (Bennett 2010) and are considered to be common food spoilage fungi. The success of *Aspergillus* is also explained by their effective dispersal. Spores of this genus are among the most dominant fungal structures in the air, dispersing themselves both short and long distances (Bennett 2010). Aspergilli are not only known because of their saprobic life style. *Aspergillus niger* has been reported to be a pathogen of *Zingiber officinale* plants (Pawar et al. 2008). Moreover, a wide variety of aspergilli are opportunistic pathogens of animals and humans. They do not infect healthy individuals but do invade individuals with a compromised immune system (Brakhage 2005, Pitt 1994). Aspergilli (i.e. *Aspergillus fumigatus*, and to a lesser extent species such as *A. niger*, *Aspergillus oryzae*, and *Aspergillus nidulans*) cause invasive aspergillosis (involving several organ systems, particularly pulmonary disease), non-invasive pulmonary aspergilloma, and allergic bronchopulmonary aspergillosis (Denning 1998, Stevens et al. 2000).

Aspergillus spp secrete a wide variety of enzymes that degrade polymers within the substrate into molecules that can be taken up to serve as nutrients. For instance, amylases are secreted to degrade starch, xylanases to degrade xylan and pectinases to degrade pectin within plant material. Similarly, elastase is secreted in the human lung to degrade elastin.

Table 1. Conditions for vegetative growth of selected *Aspergilli*

Species	Optimum Temp (°C)	Temp range (°C)	Optimum pH	pH range	Minimal Water activity	Optimum Water activity	Minimum Relative humidity (%)	Optimum Relative humidity (%)	References
A. niger	35 - 37	6 - 47	6.0	1.5 - 9.8	0.77	0.97	88 - 89	96 - 98	Astoreca et al. 2007, Ayerst 1969, Leong et al. 2006, Mehra & Jaitly 1995, Panasenko 1967, Pitt 1981
A. oryzae	30 - 37	7 - 47	6.0 - 7.5	4 - 8		0.99			Chipeta et al. 2008, Gibson et al. 1994, Nasseri et al. 2002, Panasenko 1967
A. fumigatus	37	10 - 55	5.5 - 6.5	3.0 - 8.0	0.82	0.97	85	98 - 99	Al-Doory 1984, Ayerst 1969, Ogundero 1981, Panasenko 1967, Singh & Sandhu 1982
A. clavatus	20 - 25	5 - 42			0.88		88	98	Panasenko 1967
A. terreus	37	15 - 42	5.0		0.78				Al-Doory 1984, Mehra & Jaitly 1995, Singh & Sandhu 1982
N. fischeri	26 - 45	11 - 52				0.98			Beuchat 1986, Nielsen et al. 1988, Samson et al. 2000, Valik & Pieckova 2001
A. nidulans	35 - 37	6 - 51	7.0	2 - 12	0.78		80	95	Agnihotri 1964, Al-Doory 1984, Lacey 1980, Panasenko 1967

The capacity to secrete large amounts of proteins (and other metabolites such as organic acids) in combination with established fermentation technology and molecular biology make aspergilli such as *A. niger, A. oryzae, Aspergillus sojae,* and *Aspergillus terreus* attractive cell factories for the production of homologous and heterologous proteins (Meyer et al. 2011). The potential of these fungi is exemplified by strains of *A. niger* that produce more than 30 grams per liter of glucoamylase (Finkelstein et al. 1989). Of concern, *Aspergillus spp* can form mycotoxins that are toxic for animals and humans. *Aspergillus flavus* produces aflatoxin, which is one of the most carcinogenic natural molecules. In addition, different aspergilli, including *Aspergillus westerdijkiae*, can form ochratoxin on food products such as coffee and grapes (Leong et al. 2007).

After aspergilli have colonized their substrate they can form asexual or sexual reproductive structures. *A. nidulans* is known to reproduce both sexually and asexually. In contrast, *A. niger* and *A. oryzae* form asexual spores known as conidia but sexual reproduction has not yet been shown to occur in these fungi. Analysis of genome sequences of aspergilli, however, suggests that they should be able to reproduce sexually (Dyer & Paoletti 2005). In the next sections I will describe the process of germination of conidia, formation of a vegetative mycelium, formation of asexual reproductive structures known as conidiophores, and formation of conidia. I will focus on regulatory processes and will describe in some detail the role of hydrophobins and melanin. At the end of this chapter, I will briefly discuss growth and development of filamentous bacteria called streptomycetes. These processes are remarkably similar when compared to those that occur in filamentous fungi such as *Aspergillus*.

Germination of conidia

Three stages can be distinguished during germination of conidia. In the first phase of germination, dormancy is broken by environmental cues such as the presence of water and air either or not in combination with inorganic salts, amino acids or fermentable sugars (Osherov & May 2001). Spores grow isotropically in the second phase of germination. This process that is also known as swelling is observed between 2 and 6 h after inoculation of *A. niger* at 25 °C (van Leeuwen et al. 2013ab). During this stage, the diameter of the spore increases twofold or more due to water uptake. This is accompanied by a decrease in the microviscosity of the cytoplasm (Dijksterhuis et al. 2007). Moreover, molecules are directed to the cell cortex to enable addition of new plasma membrane and cell wall (Momany 2002). In the third phase of germination, a germ tube is formed by polarized growth. To this end, the morphogenetic machinery is redirected to the site of polarization (d'Enfert 1997, Harris & Momany 2004, Harris 2006, Momany 2002). Polarized growth of *A. niger* can be observed 6 h after inoculation at 25 °C (van Leeuwen et al. 2013ab). At a later stage, the growth speed of the germ tube increases.

Transcripts of about one third of the genes can be detected by micro-arrays in dormant conidia of *A. niger* (van Leeuwen et al. 2013ab). Transcripts representing the functional gene classes protein synthesis and protein fate are enriched in the RNA pool. A strong drop in the amount of RNA is observed in the first two hours of germination (van Leeuwen et al. 2013ab). Notably, transcripts belonging to the functional gene classes protein synthesis and its subcategories translation and initiation are over-represented in the up-regulated genes at 2 h. Moreover, the categories transcription (including rRNA synthesis and rRNA processing), energy (respiration), cell cycle & DNA processing are overrepresented in the up-regulated genes at this time point. Up-regulation of genes involved in protein synthesis has also been shown in germinating conidia of *A. fumigatus* (Lamarre et al. 2008). The importance of protein synthesis in early stages of germination is also indicated by the fact that the protein synthesis inhibitor cycloheximide prevents isotropic growth, while inhibitors of the cytoskeleton and DNA- and RNA synthesis do not affect this process (Osherov & May 2000). The total number of genes that are expressed in germinating conidia of *A. niger* gradually increases between 2 and 8 h when cultured at 25 °C (van Leeuwen et al. 2013a). After 4 h of germination, the functional categories metabolism and cell cycle and DNA processing are over-represented in the up-regulated genes. The latter suggests that the conidium prepares itself for mitosis, which occurs a few hours later. No functional gene classes are over- or under-represented in the differentially expressed genes at 6 h and 8 h (van Leeuwen et al. 2013a).

Vegetative growth

A colony can result from a single asexual spore but it may also arise after conidia and / or germlings that are in close vicinity to each other have fused. It has been described that fusion in *A. oryzae, A. sojae* and *Aspergillus tamarii* most often occurs between conidia (>80 %), while fusions between conidia and germlings and fusion of germlings are much less frequent (Ishitani & Sakaguchi 1956). Fusion is mediated by fusion bridges that are formed by conidia or germ tubes. They may be similar to the conidial anastomosis tubes that are formed by *Colletotrichum* and *Neurospora* (Roca et al. 2003, Roca et al. 2005ab). These anastomosis tubes are morphologically and physiologically distinct from germ tubes. They are typically short, thin, and unbranched. Fusion of conidia and germlings have been described to occur within *Aspergillus* strains, between *Aspergillus* strains, between different aspergilli and even between *Aspergillus* and *Penicillium* species (Ishitani & Sakaguchi 1956). However, fusion between strains and between species often results in heterokaryon incompatibility. For instance, heterokaryon incompatibility is a widespread phenomenon among *A. niger* strains. The underlying mechanism is, however, not known (van Diepingen et al. 2009). Fusion of hyphae was reported to be rare when germlings of *A. oryzae, A. sojae* and *A. tamarii* had formed hyphae (Ishitani & Sakaguchi 1956). Whether this also holds for other aspergilli is not known. At least, fusion of hyphae has been shown to occur in other ascomycetes (for references see Ishitani & Sakaguchi 1956).

Colonies can reach a diameter in the (sub-)millimeter (micro-colonies) to centimeter (macro-colonies) scale depending on the size and the composition of the substrate. For instance, micro-colonies are formed on a wheat kernel, whereas macro-colonies can be formed within the lobes of a lung. In the laboratory, aspergilli are routinely grown on agar media or in liquid media. On agar medium, aspergilli form radial symmetrical macro-colonies. The mycelium of *A. nidulans* (Lee & Adams 1994a) and *A. niger* extend their diameter with approximately 0.25 mm per h in excess of nutrients and at a temperature of 37 °C and 30 °C, respectively. Colonies can also be grown between porous polycarbonate membranes on an agar medium (Levin et al. 2007ab, Masai et al. 2006, Wösten et al. 1991). Scanning electron microscopy shows that the periphery of a 7-day-old sandwiched colony of *A. niger* consists of a single layer of hyphae (Figure 1AD). A few millimeters behind the periphery this layer becomes thicker and comprises of up to six layers of hyphae growing on top of each other.

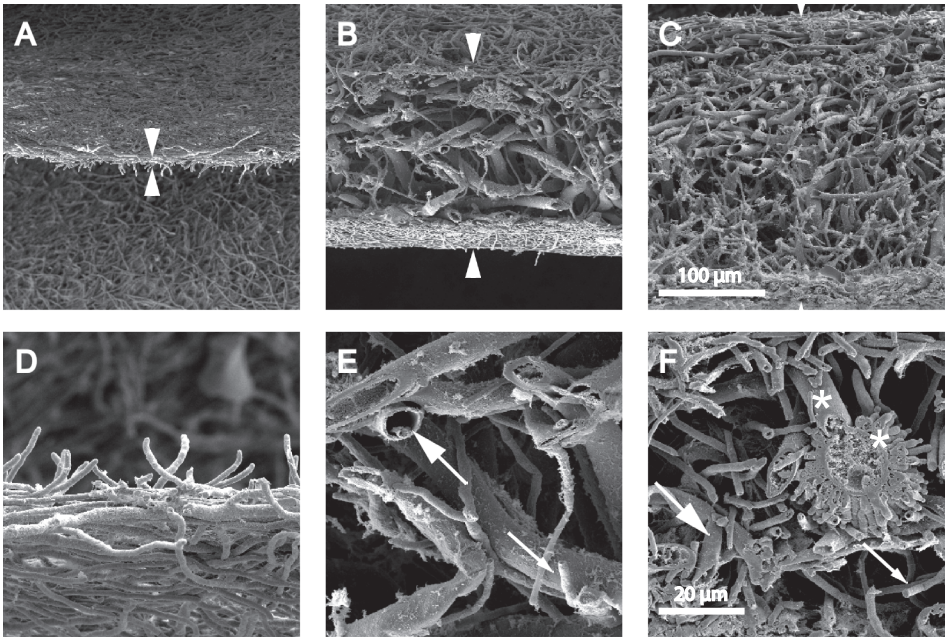

Figure 1. Scanning electron microscopy of cross sections of a 7-day-old sandwiched *A. niger* colony. Cross sections were made at the periphery (A, D), four millimeter behind the periphery (B, E) and at the innermost center (C, F). The thickness of the colony is indicated by the distance between the white triangles. Panels D-F represent higher magnifications of A-C, respectively. Thin and thick arrows point at thin and thick hyphae, respectively. In F asterisks mark a non-sporulating conidiophore. Bars in panel C, for A-C, and F, for D-F, represent 100 and 20 µm, respectively.

Notably, three distinct layers are observed another two millimeters towards the center (Figure 1BE). The upper and lower layer consist of up to five hyphae on top of each other, while

the intermediate layer comprises a loose network of thin and thick hyphae, and some non-sporulating conidiophores. Three distinct layers are also observed in the innermost center of the colony (Figure 1CF). In this case, the upper and lower layers consist of up to twenty and six layers of hyphae, respectively. The intermediate layer comprises a dense network of both thin and thick hyphae, and a relatively high number of non-sporulating conidiophores. An *A. niger* colony grows in a similar way when a 0.45 mm thin agarose layer is present in between the polycarbonate membranes.

Mycelium can grow dispersed, as clumps or as micro-colonies, also known as pellets, during submerged growth in liquid medium. Clumps are aggregated hyphae that are considered to be an intermediate state between pelleted and dispersed growth. The morphology of the mycelium has an enormous impact on the production of enzymes and primary or secondary metabolites. For instance, micro-colonies are required for the production of citric acid by *A. niger* (Vecht-Lifshitz et al. 1990). It is not clear how morphology exactly affects productivity. It has been proposed that this is due to the effect of the fungal morphology on the viscosity of the medium (Bhargava et al. 2003a). Viscosity correlates with the extent of dispersed growth; large micro-colonies thus result in a low viscosity. The center of large micro-colonies may experience oxygen starvation and other nutrients may also become limiting in this part of the mycelium. These conditions may also impact productivity of the micro-colonies.

Micro-colony formation is caused by coagulation of the conidia in the culture. Parameters that affect coagulation of *A. niger* and *A. orzyae* conidia are initial pH, agitation, and medium composition (Metz & Kossen 1977, Carlsen et al. 1996). For instance, the chelating agents EDTA and ferrocyanide lead to small and compact micro-colonies, whereas anionic polymers like carboxypolymethylene and polyacrylate give rise to small but loose micro-colonies. Micro-colony formation can also be manipulated by changing the surface composition of spores. Formation of micro-colonies was affected in strains of *A. nidulans* in which either or both *dewA* and *rodA* were inactivated (Dynesen & Nielsen 2003). The effect was strongest when both hydrophobin genes were deleted, which was accompanied by a huge drop in surface hydrophobicity of the conidia (see below).

Research in the last two decades has shown that macro-colonies of *Aspergillus* are heterogeneous with respect to gene expression, growth, and secretion. Genome wide expression analysis has shown that the RNA composition of central and peripheral zones of macro-colonies of *A. niger* (Levin et al. 2007a) and *A. oryzae* (Masai et al. 2006) is different. In the case of 7-day-old macro-colonies of *A. niger*, 25 % of the active genes show a twofold or more difference in RNA accumulation between the innermost and outermost zone of the mycelium (Levin et al. 2007a). For instance, RNA levels of the glucoamylase gene *glaA* are 3-fold higher at the periphery of maltose grown colonies when compared to the center. Similarly, accumulation of transcripts of the ferulic acid esterase gene *faeA* is 5-fold higher at the periphery of xylose grown colonies. Notably, 9 % of the genes that are active in a 7-day-

old colony are expressed in only one of five concentric zones. For instance, genes related to nitrate metabolism are specifically expressed in the outer zone of the colony, whereas mRNA of the hydrophobin *hfbD* is almost exclusively found in a central zone. Half the variation in RNA profiles is explained by differences in the composition of the medium underlying each zone of the colony, whereas the other half of the variation is caused by medium independent mechanisms (Levin et al. 2007a). These findings imply that differentiation occurs within the vegetative mycelium of *Aspergillus.*

The heterogeneity within macro-colonies of *A. niger* is also indicated by the fact that distinct zones of the mycelium grow and secrete proteins (Levin et al. 2007ab, Masai et al. 2006, Wösten et al. 1991). Proteins are formed throughout the *A. niger* mycelium (Levin et al. 2007ab, Wösten et al. 1991) (Figure 2) but they are mainly secreted at the periphery. Growth is observed in this outer zone but also in the innermost center (Figure 2). Spatial growth and protein production is not affected when 6-day-old macro-colonies are transferred to fresh medium for 16 h. However, after transfer protein secretion is not only observed at the periphery of the macro-colony but also in central parts of the mycelium (Figure 2). These data show that non-growing zones of the mycelium abundantly secrete proteins upon transfer to fresh medium (Levin et al. 2007ab). This is a remarkable finding considering the fact that protein secretion is generally assumed to take place in growing hyphae only (Wessels 1989, Wessels 1990, Wösten et al. 1991).

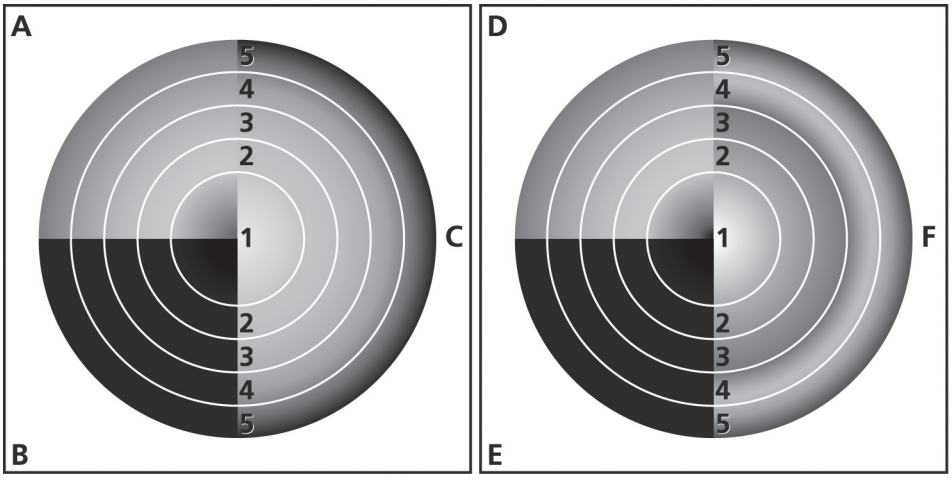

Figure 2. Growth (A, D), protein synthesis (B, E) and protein secretion (C, F) in a 7-day-old xylose grown sandwiched colony of *A. niger* before (A-C) and after transfer (D-F) to fresh medium (Adapted from Levin et al. 2007a).

Heterogeneous gene expression is not only observed between zones of macro-colonies of *Aspergillus*; it is also observed between hyphae in a particular zone. It has been described that

only part of the hyphae at the periphery of macro-colonies of *A. niger* secrete glucoamylase (Wösten et al. 1991). This observation is explained by heterogeneous expression of the glucoamylase gene *glaA* within this zone (Vinck et al. 2005). In fact, two populations of hyphae can be distinguished at the outer zone of the colony; those highly and those lowly expressing *glaA*. The hyphae highly expressing *glaA* also highly express other genes encoding secreted proteins (Vinck et al. 2011). Moreover, these hyphae highly express the glyceraldehyde-3-phosphate dehydrogenase gene *gpdA* and show a high 18S rRNA content. Thus, two populations of hyphae are present at the periphery of a macro-colony; those that are lowly and those that are highly metabolically active. The fact that the lowly active hyphae have a growth rate similar to that of highly active hyphae indicates that a "low" activity of hyphae is sufficient to support hyphal growth. However, a "high" metabolism would be needed to support secretion of large amounts of proteins (Vinck et al. 2011). Single hypha transcriptome analysis indicates that heterogeneity between neighboring hyphae goes beyond two types of hyphae. Individual hyphae each have their own composition of RNA (de Bekker et al. 2011a). So far, we can only guess why hyphae are heterogeneous at the colony periphery. The leading hyphae explore the substrate and they may be exposed to rapid changes in the environment. A heterogeneous hyphal population may contribute to the survival under such conditions.

Conidiophore and conidia formation

After a period of vegetative growth, air-exposed colonies of *A. nidulans* and *A. niger* form two types of aerial hyphae (Figure 3). One type is quite similar to vegetative hyphae of these aspergilli and has a diameter of about 2-3 µm. The second type of aerial hyphae has a diameter of about 4-5 and 6-7 µm in the case of *A. nidulans* and *A. niger,* respectively. These so-called stalks can differentiate into conidiophores (Figure 3). The conidiophore stalk of *A. nidulans* extends about 100 µm into the air and is formed from a specialized foot-cell within the substrate mycelium (Adams et al. 1998). When the stalk has reached its maximum height, the tip swells and forms a vesicle with a diameter of 10 µm. In biserate species like *A. nidulans* and *A. niger*, the vesicle surface buds resulting in a layer of primary sterigmata termed metulae. The metulae in turn bud twice. This results in a second layer of sterigmata called phialides. The phialides give rise to chains of mainly uninucleate conidia. As a result, more than 10.000 conidia can be produced per conidiophore. *A. oryzae* can be both uniserate and biserate. In the case of uniserate species, spore producing phialides are positioned directly at the surface of the conidiophore vesicles.

The 2-3 µm wide aerial hyphae of *A. nidulans* and *A. niger* are formed about 8 h after inoculation of spores on complete medium. Although timing of this type of aerial hyphae seems to be medium-independent, the density of aerial hyphae is lower in the case of minimal medium.

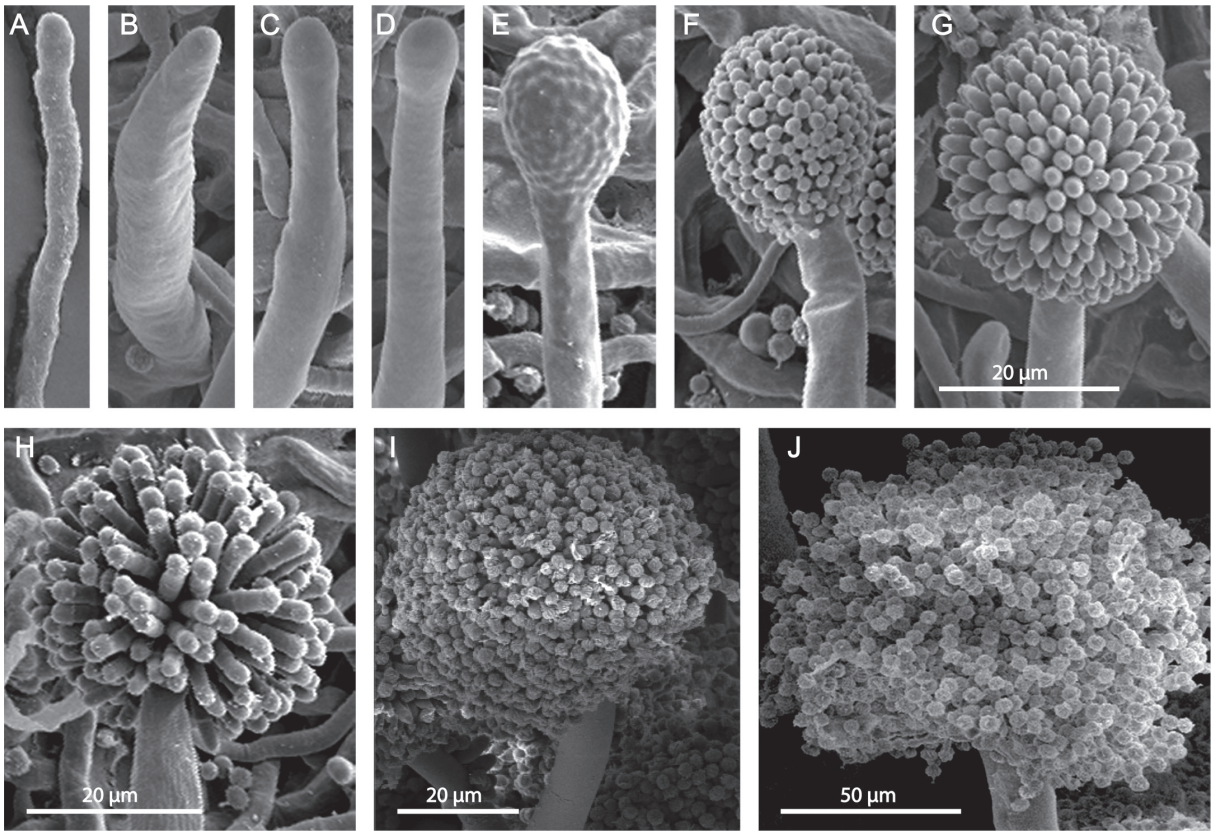

Figure 3. Development of *A. niger* monitored by scanning electron microscopy. The vegetative mycelium forms two types of aerial hyphae. One type is similar to vegetative hyphae (A), while the other type is 2-3 times thicker (B). The tips of the latter aerial hyphae may swell to form a vesicle (C,D). Buds are formed on the vesicle (E) that develop into metulae (F, G). Phialides are formed on top of the metulae (H), which give rise to chains of conidia (I, J). The bar in G also holds for A-F.

The first stalks of *A. nidulans* and *A. niger* are formed 10 h after spore inoculation on complete medium and growth at 37 °C and 30 °C, respectively. In both cases, conidiophores are formed 20 h post-inoculation. Formation of aerial hyphae in both aspergilli starts in the center of the colony and moves outwards ending a few millimeters from the edge of the mycelium. This observation implies that the competence of hyphae to form aerial hyphae is acquired faster when a colony gets older (Adams et al. 1998). The process of aerial growth has been proposed to involve signaling of the cell density of the vegetative mycelium (Lee & Adams 1994b, Wösten et al. 1999a, Wösten & Willey 2000). The signaling molecule would induce hydrophobin genes. These genes encode proteins that lower the water surface tension to enable hyphae to breach the interface to grow into the air (Wösten et al. 1999b, Wösten 2001). Which hydrophobin is secreted into the aqueous environment in *Aspergillus* cultures with the aim to lower the water surface tension is not yet known.

A. nidulans can also form conidia in submerged cultures (Adams et al. 1998). In this case, conidiation is induced when the culture gets stressed or when nutrients are limited (e.g. limitation of the carbon and the nitrogen source). On the other hand, formation of conidiophores in air-exposed colonies is assumed to be induced by an internal signal that activates a genetic program of sporulation (Adams et al. 1998, Krijgsheld et al. 2013). In both cases, competence to sporulate is acquired in a time-dependent way (Skromne et al. 1995). Like *A. nidulans, A. niger* can also form conidiophores in submerged conditions. However, these conidiophores do not form spore chains (Figure 1F).

Regulation of conidiophore and conidia formation

In this chapter I describe the role of FluG, BrlA, AbaA, WetA, VosA, StuA and MedA in formation of conidiophores and conidia. For more details of the regulatory pathway of asexual reproduction I refer to Krijgsheld et al. (2013). *A. nidulans* strains in which the *fluG* (*fluffy*) gene is inactivated (i.e. a Δ*fluG* strain) form aerial hyphae but not conidiophores in excess of nutrients (Lee & Adams 1994b). During nutrient deprivation, however, some conidiophores are being formed on a solid medium. Similarly, submerged cultures of the Δ*fluG* strain start to sporulate in the absence of a carbon source (Lee & Adams 1996). These data indicate that FluG is involved in a developmental program of sporulation but not in the stress-related sporulation pathway. Formation of conidiophores in the Δ*fluG* strain can be rescued by growing the mutant next to a wild-type strain. Complementation is also observed when the strains are physically separated by a dialysis membrane with a size exclusion of 6-8 kDa. This indicates that FluG is involved in the production of a low-molecular weight extracellular signaling molecule that is involved in the formation of conidiophores.

FluG activates the *brlA* (*bristle*) gene. A Δ*brlA* strain of *A. nidulans* forms stalks that do not stop their growth after they have reached a length of 100 μm. These stalks can reach a length 20-30 times longer than those of the wild-type, which results in the characteristic

bristle phenotype (Adams et al. 1988). Moreover, isotropic growth is not initiated at the apex of the stalks of the Δ*brlA* strain. As a result, conidiophore vesicles are not being formed. Conidiophore development becomes independent from *fluG* by placing *brlA* under control of an inducible promoter (Adams et al. 1988). Similar results have been obtained in *A. oryzae* (Ogawa et al. 2010, Yamada et al. 1999). Inactivation of *brlA* in *A. oryzae* results in the inability to form conidiophores. In contrast, fully developed conidiophores are formed in submerged cultures when the *brlA* gene is expressed under the control of the *amyB* promoter. BrlA is also essential for conidiophore formation in *A. fumigatus* (Mah & Yu 2006). However, in contrast to *A. nidulans* (Adams et al. 1988) and *A. oryzae* (Ogawa et al. 2010), the *A. fumigatus* gene seems to function earlier in conidiophore development. This is based on the fact that conidiophore development is completely abolished in a Δ*brlA* strain of *A. fumigatus*. The appearance of the colonies of this strain is more similar to that of the fluffy mutants of *A. nidulans* (see below) (Mah & Yu 2006). In addition, the *A. fumigatus* gene seems to function independent from *fluG.* At least, a Δ*fluG* strain of *A. fumigatus* still sporulates in air-exposed cultures. Possibly, *A. fumigatus* has more than one *brlA* activating mechanism (Mah & Yu 2006). The *brlA* gene of *A. fumigatus* has also been shown to be involved in suppressing ribosomal protein genes during nitrogen stress (Twumasi-Boateng et al. 2009). This finding conforms to the general starvation response in fungi, which involves both down-regulation of ribosomal protein biogenesis and induction of sporulation (Bahn et al. 2007, de la Serna et al. 1999, Gasch et al. 2000, Li et al. 1999, Mogensen et al. 2006, Warner 1999). However, down-regulation of ribosomal protein encoding genes is not impaired during carbon stress in *A. fumigatus* (Twumasi-Boateng et al. 2009). Nevertheless, these findings suggest that *brlA* of *A. fumigatus* is not only a regulator of formation of conidiophores but also influences the vegetative mycelium by affecting its protein synthesizing capacity.

Transcription of *brlA* in *A. nidulans* results in two transcripts that are called *brlAα* and *brlAβ*. Both transcripts are essential for proper conidiophore development (Prade & Timberlake 1993) and are controlled at the transcriptional (*brlAα* and *brlAβ*) and translation level (*brlAβ*) (Han & Adams 2001). Transcript *brlAβ* contains a short upstream ORF (μORF) and a downstream ORF that encodes the same polypeptide as BrlAα but with an N-terminal extension of 23 aa (Prade & Timberlake 1993). Both polypeptides contain two C_2H_2 zinc finger DNA binding motifs. The *brlAα* and *brlAβ* transcripts have different functions during asexual development. As mentioned above, inactivation of *brlA* results in indefinitely elongating stalks. In contrast, aberrant primary conidiophores develop in the Δ*brlAβ* strain that can form secondary conidiophores (*i.e.* a conidiophore that develops from another conidiophore). Asexual development proceeds further in the Δ*blrAα* strain but conidia are not produced (Fischer & Kües 2006). So far, it is not known whether transcription of *brlA* of *A. oryzae* and *A. fumigatus* also results in two transcripts.

BrlA activates a central regulatory pathway controlling temporal and spatial expression of conidiation specific genes (Boylan et al. 1987, Mirabito et al. 1989). This cascade is complex and involves, amongst others, the regulatory genes *abaA*, *wetA, stuA, medA,* and *vosA* (Figure 4). Gene *abaA* (*abacus*) is a regulatory gene that is activated in *A. nidulans* by BrlA during sterigmata differentiation (Boylan et al. 1987, Breakspear & Momany 2007). A Δ*abaA* strain forms metulae that bud apically resulting in chains of cells with metula-like, rather than phialide-like, properties. In other words, phialides are not produced and therefore conidia are not formed (Boylan et al. 1987, Clutterbuck 1969, Sewall et al. 1990). The interaction of AbaA with *brlA* is complex (Figure 4A). Gene *abaA* is activated by BrlA and, in turn, AbaA stimulates formation of *brlAα* transcripts but represses *brlAβ* accumulation (Adams et al. 1998, Andrianopoulos & Timberlake 1994, Han & Adams 2001, Sewall et al. 1990). This is caused by AbaA binding to a responsive element in the *brlAβ* locus (Han & Adams 2001). The net result of *abaA* inactivation is that *brlA* is over-activated (Aguirre 1993). The positive feedback loop of *brlA* itself is likely to be independent of AbaA because the over-expression of *brlAβ* activates expression of *brlAα* in an *abaA* mutant (Han & Adams 2001) (Figure 4A). Taken together, both BrlA and AbaA control transcript levels of *brlAα* and *brlAβ*. AbaA regulates several other genes including *abaA* itself, *medA, wetA* (Figure 4A), *vosA* (Figure 4B), and the structural genes *yA* and *rodA* (for their functions see below) (Andrianopoulos & Timberlake 1994). Recently *abaA* was identified in *A. oryzae* (Ogawa et al. 2010) and *A. fumigatus* (Tao & Yu 2011). The role of *abaA* in *A. oryzae* is similar to that in *A. nidulans.* In the case of *A. fumigatus abaA* also delays autolysis and cell death.

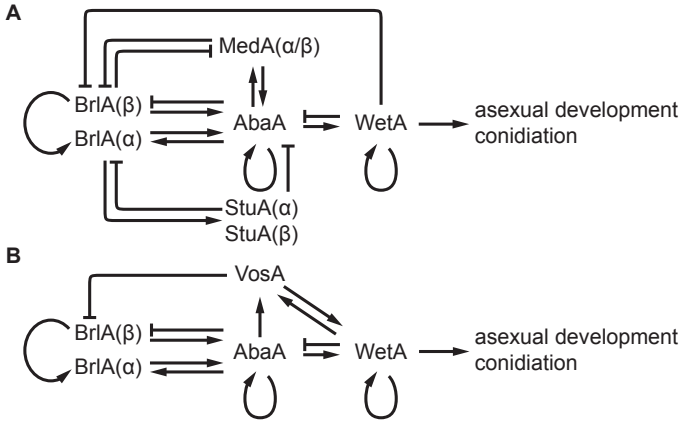

Figure 4. The central regulatory network consisting of BrlA, AbaA and WetA initiates asexual development in *A. nidulans*. StuA and MedA (**A**) and VosA (**B**) are regulators of *brlA, abaA,* and *wetA*.

During the late phase of conidiation, *wetA (wet white)* is activated by *abaA* (Figure 4A). Normal conidiophores are formed by *wetA* mutants. However, the conidia do not form

pigments, are not water repellent, and go in autolysis (Marshall & Timberlake 1991, Sewall et al. 1990). Gene *wetA* activates a set of genes in phialides and spores (e.g. *wA*), which are involved in making the conidial wall impermeable and mature (Marshall & Timberlake 1991). In addition, WetA seems to activate itself (Adams et al. 1998, Boylan et al. 1987, Marshall & Timberlake 1991, Ni & Yu 2007) and represses *abaA* and *brlA* (Tao & Yu 2011) (Figure 4A). Gene *wetA* of *A. oryzae* (Ogawa et al. 2010) has a role similar to that in *A. nidulans.* In the case of *A. fumigatus wetA* seems to have an additional role (Tao & Yu 2011). It would also function in germ tube formation and reduced hyphal branching.

VosA (*viability of spore*) is a putative transcription factor of the velvet family (Tao & Yu 2011). Inactivation of *vosA* results in uncontrolled activation of asexual development, whereas its over-expression blocks sporulation. This may be the result of the observed inhibition of *brlA* by VosA (Tao & Yu 2011) (Figure 4B). It should be noted that *vosA* is lowly expressed in the vegetative mycelium. Yet, these expression levels may be sufficient to control *brlA.* Gene *vosA* is particularly expressed during the formation of conidia and sexual ascospores, where it plays a role in resistance to stress conditions.

The *stuA* and *medA* genes are classified as developmental modifiers. Their encoded proteins affect *brlA* and *abaA* expression (Figure 4A). Mutations in *stuA* (*stunted*) of *A. nidulans* results in shortened aerial hyphae, shortened conidiophores and the absence of metulae and phialides. Conidiophores that are formed have reduced vesicles with abnormal numbers of nuclei. Only a few conidia can directly bud from the conidiophore vesicle. Thus, the morphology of the conidiophores is aberrant in *stuA* mutants, but neither temporal development nor conidiophore density is affected (Wu & Miller 1997). Gene *stuA* has a similar role in asexual development in *A. fumigatus* (Sheppard et al. 2005). StuA is a helix-loop-helix transcription factor with two transcription start sites. This leads to *stuAα* and *stuAβ* transcripts, of which the former is most important for correct development (Aguirre 1993, Miller et al. 1991, Miller et al. 1992). Expression of *stuA* depends on *brlA.* As a result, transcript levels of s*tuA* are increased 20-fold in conidiating cultures (Breakspear & Momany 2007, Busby et al. 1996, Miller et al. 1992). In turn, StuA directly or indirectly represses and spatially restricts *brlA* and *abaA* expression (Figure 4A). With this ability *stuA* is involved in proper spatial distribution of AbaA and BrlA (Miller et al. 1992, Wu & Miller 1997). The StuA protein also stimulates *stuA* expression. This seems to be an indirect effect because its responsive elements are absent in its promoter (Wu & Miller 1997).

The *medA* (*medusa*) gene is conserved in filamentous fungi. Like other *A. nidulans* regulatory genes, *medA* is transcribed at two initiation sites. While *stuA* of *A. nidulans* is required for proper spatial expression of *abaA* and *brlA, medA* is involved in proper temporal expression of these genes (Adams et al. 1998, Busby et al. 1996). Accumulation of both *brlA* transcripts is observed earlier in development in a Δ*medA* strain. Moreover, the mutant strain shows higher levels of *brlAβ,* but not *brlAα,* transcripts. As a result, the ratio of *brlA*α

and *brlAβ* transcripts is lowered. Gene *medA* thus acts as a repressor of *brlA* expression. In contrast, it is an activator of *abaA* expression. This is concluded from the observation that *abaA* transcription levels are reduced or even absent in the *medA* mutant (Busby et al. 1996, Miller et al. 1992). The molecular basis of MedA function is still unclear. A Δ*medA* strain forms repeated layers of sterigmata and frequent reinitiated secondary conidiophores (Clutterbuck 1969, Sewall et al. 1990). This phenotype resembles that of a strain of *A. nidulans* in which the chitin synthase genes *chsA* and *chsC* genes have been inactivated (Ichinomiya et al. 2005). In the latter strain, *abaA* expression is reduced. This indicates that *chsA* and *chsC* regulate expression of *abaA*, most probably in an indirect way. The Δ*chsA*Δ*chsC* mutant shows a defective septum formation (Ichinomiya et al. 2005). Therefore, it was proposed that MedA is involved in septum formation on conidiophore structures. Taken together, conidiophore morphogenesis requires a finely tuned balance of at least BrlA, AbaA, MedA, and StuA (Busby et al. 1996), and possibly VosA (Boylan et al. 1987, Ni & Yu 2007).

The role of hydrophobins in asexual development

Regulators activate target genes that fulfill a structural or enzymatic role in the formation of asexual structures. Genes have been identified that are upregulated in conidiophores and / or conidia (Bleichrodt et al. 2013, van Leeuwen et al. 2013ab). Hydrophobin genes are examples of such target genes. Hydrophobins mediate the escape of hyphae into the air and make aerial structures such as conidiophores and conidia hydrophobic (Wösten 2001). This hydrophobicity ensures that reproductive structures do not fall back in the substrate under humid conditions and serves dispersal of conidia by wind or vectors. Hydrophobins may also affect the cell wall architecture (van Wetter et al. 2000) and mediate attachment to hydrophobic substrates (Wösten 2001). In the case of *A. fumigatus* it has been shown that hydrophobins prevent immune recognition by the host (Aimanianda et al. 2009, Aimanianda & Latgé 2010, Bruns et al. 2010, Dagenais et al. 2010, Paris et al. 2003). Moreover, the hydrophobin RolA of *A. oryzae* recruits cutinase by adsorbing to the substrate of the enzyme. As a result, the substrate is efficiently degraded (Takahashi et al. 2005).

The *A. nidulans*, *A. fumigatus*, *A. oryzae*, and *A. niger* genomes contain 6, 4-5, 2, and 8 hydrophobin genes, respectively (Jensen et al. 2010). One or more of the hydrophobins in each species enable hyphae to grow into the air by lowering the surface tension of the aqueous environment (see above). The aerial structures are then coated with hydrophobins to make them hydrophobic. So far, it has not been established which hydrophobins line aerial hyphae and the conidiophore stalk and vesicle of aspergilli. However, hydrophobins have been identified that coat sterigmata and conidia. The hydrophobin gene *rodA* (*rodlet)* of *A. nidulans* is expressed during the final stages of conidiophore formation. It is not expressed by vegetative hyphae and conidia (Boylan et al. 1987, Stringer et al. 1991). Expression of *rodA* is mediated by BrlA but not by AbaA or WetA. This is based on the finding that a Δ*brlA*

strain does not express *rodA* but expression of the hydrophobin gene is not affected in the Δ*abaA* and Δ*wetA* strains. A Δ*rodA* strain forms wettable conidiophores and conidia. This is accompanied by the absence of the rodlet layer (Stringer et al. 1991). As a consequence, Δ*rodA* conidia adhere to each other in water. This affects their dispersal by air flow. The rodlet layer is also absent at the surface of metulae and phialides of the Δ*rodA* strain (Stringer et al. 1991). Experimental data imply that the RodA protein is produced by the sterigmata and diffuses to the outer surface of these structures as well as to that of the conidia to form the rodlet layer. *A. fumigatus* contains an ortholog of *rodA*. Inactivation of this gene results in a phenotype similar to that in *A. nidulans* (Paris et al. 2003, Thau et al. 1994). Moreover, *rodA* of *A. fumigatus* is involved in attachment of spores to particular substrates. The adhesion of the Δ*rodA* conidia is reduced in the case of collagen and bovine serum albumin but is not affected in the case of pneumocytes, fibrinogen, and laminin.

The *dewA* (*detergent wettable*) hydrophobin gene is expressed in sporulating cultures but not in cultures that grow vegetatively (Boylan et al. 1987, Stringer & Timberlake 1995). Unlike RNA of *rodA*, transcripts of *dewA* are present in conidia (Breakspear & Momany 2007). In agreement, immuno-detection showed that DewA hydrophobin is specifically present in cell walls of conidia, especially in mature spores. Expression of *dewA* is not only abolished in the Δ*brlA* strain but also in the Δ*abaA* and Δ*wetA* strains. Forced expression of *brlA* or *abaA* has only resulted in *dewA* expression in strains with an intact copy of *wetA.* Thus, *dewA* is regulated by *brlA* and *abaA* via *wetA* (Stringer & Timberlake 1995). Conidia of Δ*dewA* are still covered with the rodlet layer. Thus, DewA is not essential for the rodlet layer on spores. Yet, when present, it may be part of it. The conidia of the Δ*dewA* strain do not wet in water. However, they wet more easily compared to wild-type spores when detergent is added to the water. A role of DewA in surface hydrophobicity is also indicated from the fact that wettability of the Δ*dewA*Δ*rodA* strain is much more pronounced when compared to the Δ*dewA* and the Δ*rodA* strains. *A. fumigatus* also contains a hydrophobin that is present at the surface of conidia but whose presence is not essential for the rodlet layer of conidia (Paris et al. 2003). This hydrophobin, RodB, is different in sequence when compared to DewA. As mentioned above, the surface of Δ*rodA* conidia of *A. fumigatus* lacks the rodlet layer. Instead, the surface is granular. In contrast, the surface of Δ*rodA*Δ*rodB* conidia is amorphous. Taken together, RodB of *A. fumigatus* may be part of the rodlet layer of conidia when RodA is present. In the absence of RodA, RodB forms a granular structure and does not form rodlets.

The role of melanin and its synthesis

Conidia of the genus *Aspergillus* survive UV due to the presence of melanin or melanin-like pigments in their cell wall. The melanin(-like) pigment is also a virulence factor (Tsai et al. 1999). Melanin contained in the cell wall of *Aspergillus* conidia is synthesized via the 1,8-dihydroxynaphtalene (1,8-DHN) pathway (Figure 5), which is conserved in the Aspergilli

(Baker 2008, Tsai et al. 1999). The DHN pathway results in brown or black melanin. Absence of enzymatic steps or modification of melanin precursors results in pigments with different colors.

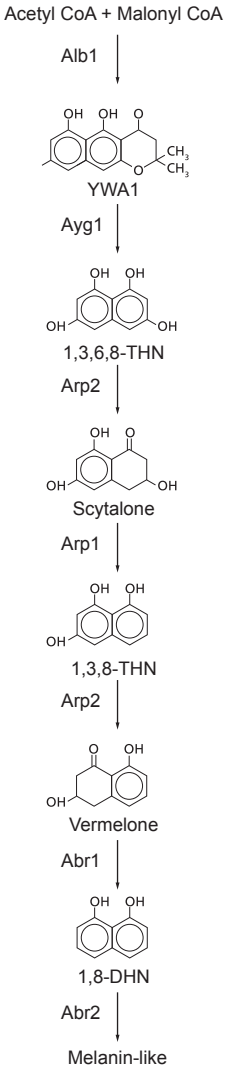

Figure 5. Synthesis of melanin-like pigments by means of the DHN pathway. Proteins of *A. fumigatus* responsible for each of the steps are indicated. Note that absence of particular enzymes and/or modification of melanin precursors will result in melanin-like pigments with colors other than brown or black. Adapted from (Fujii et al. 2004, Tsai et al. 1999, Pihet et al. 2009).

A cluster of six genes has been identified that is involved in the production of the bluish-green pigment in the conidia of *A. fumigatus* (Tsai et al. 1999). Inactivation of one of each genes in the cluster results in spores with different colors. Gene *alb1* (*albino*) encodes the polyketide synthase of the DHN pathway (Figure 5). This polyketide synthase produces the heptaketide naphtopyrone YWA1 (Watanabe et al. 2000), which is the precursor for the green spore pigment of *A. nidulans* (Watanabe et al. 1999). In the case of *A. fumigatus*, *ayg1* (*Aspergillus yellowish green*) converts YWA1 into 1,3,6,8 tetrahydroxynaphtalene (1,3,6,8-THN), which is further modified in the DHN pathway (Fujii et al. 2004). Gene *arp2* (*Aspergillus reddish-pink*) encodes the hydroxynaphthalene (HN) reductase that forms both scytalone and vermelone (Tsai et al. 1999), whereas *arp1* encodes the dehydratase that converts scytalone in 1,3,8-THN, which is the precursor for Arp2 (Tsai et al. 1999). The combination of *alb1*, *ayg1*, *arp1*, and *arp2* are predicted to produce a brown-black melanin. The two other genes in the cluster are assumed to be required for the production of the bluish-green pigment of wild-type *A. fumigatus* spores. Gene *abr1* (*Aspergillus brown*) is a putative multicopper oxidase that converts vermelone to 1,8-DHN (Pihet et al. 2009). Subsequently, 1,8-DHN is polymerized by the laccase encoded by *abr2* (Sugareva et al. 2006).

In contrast to other Aspergilli such as *A. nidulans* and *A. fumigatus*, there is little evidence that supports the involvement of the DHN pathway in *A. niger* (Jørgensen et al. 2011). The *A. niger* genome lacks clear orthologs for *arp1* and *arp2.* Moreover, spore pigmentation of *A. niger* is insensitive to the HN reductase (Arp2) inhibitor tricyclazole A. The *fwnA* (*fawn*), *pptA* (*phosphopantetheinyl transferase*), *olvA* (*olive*), and *brnA* (*brown*) genes have been shown to be involved in the production of the characteristic black spore pigment of *A. niger*. Gene *fwnA* encodes a polyketide synthase. Inactivation of this gene results in fawn-colored conidia. Conidia of the Δ*pptA* strain are white due to the absence of phosphopantetheinyl transferase activity. This activity is required for activation of polyketide synthases (PKSs) and non-ribosomal peptide synthases. The proteins encoded by *olvA* and *brnA* are homologous to the *A. fumigatus* Ayg1 and Abr1 proteins, respectively.

Vegetative growth and sporulation in Streptomyces

Streptomycetes are Gram-positive bacteria. Although they belong to a different domain of life, they adopted a similar life style as filamentous fungi such as aspergilli.

Streptomycetes are abundant in soil representing approximately 10 % of the total microbial community (Janssen 2006). They are saprotrophic, degrading organic polymers such as cellulose, mannan, chitin, xylan, starch, lignin and agar. To this end, a variety of enzymes is secreted by these microbes. Many of these enzymes are of industrial interest for the conversion of renewable energy sources (Vrancken & Anné 2009). Streptomycetes also release antibiotics into their environment, which has been proposed to be instrumental in defending their nutrients (Davelos et al. 2004). Streptomycetes can live on their own or can colonize

fungal hyphae, animals or plants (Fahal 2004, Schrey and Tarkka 2008, Haeder et al. 2009). In the case of plants, *Streptomyces* species can either establish an endophytic relationship or induce a pathogenic interaction (Schrey and Tarkka 2008). Similarly, *Streptomyces* establish a mutual benificial or a pathogenic interaction with animals (Fahal 2004, Haeder et al. 2009).

Streptomycetes such as *Streptomyces coelicolor* are multicellular organisms that undergo a complex program of morphological development (for a review see Claessen et al. 2006). Spore germination results in a mycelium consisting of hyphae that are divided by cross-walls. After a period of vegetative growth, part of the compartments in the hyphae disintegrate, while others proliferate to give rise to a second vegetative mycelium (Manteca et al. 2005, 2006, 2007). At this stage aerial hyphae are also formed that give the colony a typical white and fluffy appearance. These hyphae develop into a chain of pigmented spores that are dispersed to start a new colony.

Regulation of sporulation

Regulation of sporulation is complex. In this section I will describe the role of the *bld* pathway and the *sky* pathway that control production of structural proteins involved in the formation of aerial hyphae. I refer to Chater (2001) for a detailed description of the *bld* pathway and for regulation of later stages of development by the *whi* genes.

In the past, mutants of *S. coelicolor* were isolated that could not produce aerial hyphae and were thus unable to sporulate when grown on rich solid medium. (see Chater 2001). Formation of aerial structures could be complemented by growing these so called bald (*bld*) mutants near a wild-type strain or next to certain other *bld* mutants (Willey et al. 1991, 1993). Based on these observations, it was proposed that most but not all *bld* genes are part of a hierarchical signaling cascade. The hierarchy would be bldJ<bldK/bldL<bldA/bldH<bldG<bldC<bldD/bldM. A strain to the right initially rescues morphogenesis of a strain to the left (Willey et al. 1993, Nodwell et al. 1996, Claessen, 2004), while ultimately also the strain to the right is complemented. Thus, bldJ can be rescued by all other bld mutants, while bldD can only be rescued by the wild-type strain. The *bld* genes in the hierarchy would be involved in the synthesis of, perception of, or response to different extracellular signaling molecules. Each of these signalling molecules would trigger formation of the next signalling compound, ultimately leading to the onset of aerial growth. The *bld* cascade has been proposed to integrate intra- and extracellular conditions (Claessen et al. 2006). The *bldD* gene encodes a protein with DNA binding activity. Experimental evidence suggests that BldD prevents premature expression of *bldN* (Elliott et al. 2001). This gene encodes a sigma factor of the family of extracytoplasmic function (ECF) sigma factors. Its expression depends on BldH and BldG, while it activates the *bldM* gene (Bibb et al. 2000). This shows that the *bld* pathway is much more complex than a linear hierarchical cascade.

The *bld* cascade operates in the vegetative mycelium and was proposed to activate genes encoding structural proteins (i.e. some *chp* genes and the genes encoding SapB; see below) as

well as genes of the *sky* pathway (Claessen et al. 2006). The *sky* pathway would function in the aerial hyphae regulating expression of genes encoding structural proteins including the *chp* and *rdl* genes as well as the genes involved in the synthesis of SapB (see below). To date, components of the sky pathway have not yet been identified.

The role of chaplins, rodlins and SapB in development

The *bld* pathway activates the genes encoding ChpE and ChpH. These genes are part of the chaplin family that contains eight members in *S. coelicolor* (Claessen et al. 2003, Elliot et al. 2003). ChpE and ChpH are secreted into the medium. When confronted with the air-water interface, they assemble into an amphipathic film of amyloids (Figure 6). As a consequence, the water surface tension is reduced from 72 to as low as 32 mJ m^{-2} (Sawyer et al. 2011), which enables hyphae to escape the medium to grow into the air (Wösten et al. 1999). However, in a medium with a high osmolarity hyphae were shown not to be able to escape into the air when only ChpE and ChpH are present as surface active proteins (de Jong et al. 2012). This was explained by the fact that the turgor pressure of hyphae is reduced in such an environment and therefore the hyphae would not have sufficient force to escape the water. To overcome this, wild-type *S. coelicolor* forms SapB (de Jong et al. 2012). SapB is a lantibiotic-like peptide (Kodani et al. 2004) that lowers the surface tension, like ChpE and ChpH, to 32 mJ m^{-2} (Tillotson et al. 1998). SapB formation is the result of a gene cluster consisting of *ramCSAB* with its regulator *ramR* (Keijser et al. 2002, Kodani et al. 2004). It is not clear how SapB facilitates escape of hyphae into the air under high osmolarity. In the absence of the chaplins ChpE and ChpH, it does not mediate aerial growth. Based on these data it has been proposed that SapB intercalates into the chaplin amyloid layer (Figure 6), thereby weakening the mechanical strength of this protein film (de Jong et al. 2012).

Once escaped into the air, the *sky* pathway induces expression of all eight chaplin genes, as well as the genes involved in SapB formation, and the rodlin genes *rdlA* and *rdlB* (Claessen et al. 2006, de Jong et al. 2012). Thus, these proteins are formed by aerial hyphae irrespective of medium composition. The eight chaplins form a protein film consisting of amyloid fibrils at the surface of the aerial hyphae. The chaplin fibrils provide surface hydrophobicity, thereby preventing aerial hyphae to fall back into the aqueous environment. The presence of SapB would affect the properties of assembled chaplins at the surface of the aerial hypha as they are proposed to do at the water-air interface. However, this effect is counteracted by the rodlin proteins RdlA and RdlB (de Jong et al. 2012). These proteins align the chaplin amyloid fibrils into a rigid film consisting of rodlets (Figure 6; Claessen et al. 2004). These rodlets consist of paired rods that are 8-12 nm in width and up to 450 nm in length. In the absence of the rodlins, rodlets are not formed (Claessen et al. 2002). Instead, the surface of aerial hyphae is covered with fine amyloid fibrils of chaplins with a diameter of 4-6 nm. Hyphae are stable in the absence of the rodlins when exposed to low osmolarity media. However, when hyphae grow in medium with high osmolarity they are essential to form aerial hyphae.

Taken together, it was proposed that the rodlets, and not the fine chaplin fibrils, provides mechanical stability to the aerial hyphae when the internal turgor pressure is low.

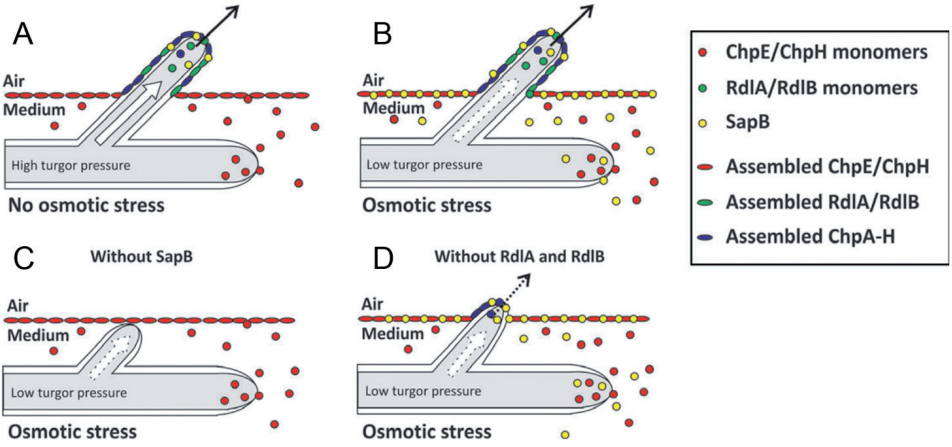

Figure 6. Model of differentiation in *S. coelicolor*. (A) The chaplins ChpE and ChpH are released into the aqueous medium. They assemble into a surface active amphipathic film at the air-medium interface. As a result, the water surface tension is strongly reduced, allowing hyphae to grow into the air. (B) Turgor pressure within hyphae is low when *S. coelicolor* is grown in medium with high osmolarity. This hampers hyphae to breach the chaplin film at the medium-air interface. Therefore, the *bld* pathway activates the ram cluster, resulting in the formation of SapB next to ChpE and ChpH. Co-assembly of these surfactants affects rigidity of the chaplin film. Consequently, hyphae now can escape the aqueous environment. (C) Mutants that do not produce SapB will not form aerial hyphae under osmotic stress but do so when exposed to medium with low osmolarity. (D) Aerial hyphae form eight chaplins (ChpA-H), SapB and the rodlins RdlA and RdlB, irrespective of medium composition. The latter proteins align the chaplin amyloid fibrils into rodlets. The resulting rigid coat that provides stability to the aerial hyphae is essential for aerial hyphae formation in medium with high osmolarity but not under low osmolarity (Taken from de Jong et al. 2012).

Outline of the Thesis

The finding that macro-colonies of *A. niger* are heterogeneous with respect to RNA accumulation, growth and protein secretion raised the question whether heterogeneity is also observed between and within micro-colonies. In this Thesis heterogeneity of micro-colonies was studied in *Aspergillus.* In addition, it was assessed whether this phenomenon can also be observed in micro-colonies of *Streptomyces,* which displays striking similarities in mechanisms involved in growth and development.

In **Chapter 2** it is shown that micro-colonies of *A. niger* that are formed in liquid shaken cultures are heterogeneous with respect to size and gene expression. A population of small and a population of large micro-colonies can be distinguished. Similarly, two populations of

micro-colonies were distinguished when expression of the glucoamylase gene *glaA* and the ferulic acid esterase gene *faeA* were monitored. Notably, the population of lowly expressing micro-colonies is larger than the population of small micro-colonies. This indicates that size of micro-colonies is not the only determinant for expression of genes encoding secreted proteins. It is not yet clear how heterogeneous gene expression is established between zones of micro-colonies. At least, the total amount of RNA per hypha is about 50 times higher at the periphery of 1-mm-wide micro-colonies when compared to the center.

Heterogeneity of micro-colonies of *A. niger* strains in which either the *fwnA*, *olvA* or *brnA* gene was inactivated was studied in **Chapter 3**. These strains are affected in the pigmentation pathway and form fawn-, olive-, and brown-colored conidia respectively, instead of black conidia as observed in the wild-type. The average size of the micro-colonies of the control strain was smaller (628 μm) than that of the deletion strains (790-858 μm). The size distribution of the micro-colonies of the Δ*fwnA* strain was normally distributed, while that of the other strains could be explained by assuming a population of small and a population of large micro-colonies. **Chapter 3** also shows that relative expression levels of *gpdA*, and AmyR and XlnR regulated genes correlate in individual hyphae at the periphery of micro-colonies. This indicates the existence of transcriptionally and translationally highly active and lowly active hyphae as was previously shown in macro-colonies.

Heterogeneity within the mycelium is surprising considering the fact that the cytoplasm of a fungal mycelium is assumed to be continuous. This is based on the fact that the septa within and between hyphae are porous allowing streaming of cytosol and even organelles. In **Chapter 4** it is demonstrated that about half of the apical septa of growing hyphae of *A. oryzae* are closed. Closure of septa correlated with the presence of Woronin body organelles near the septal pore. The location of Woronin bodies in the hyphae was dynamic and, as a result, plugging of the septal pore was reversible. Septal plugging was abolished in a Δ*Aohex1* strain that cannot form Woronin bodies. Notably, hyphal heterogeneity in gene expression was also affected in the Δ*Aohex1* strain. Taken together, it is concluded that Woronin bodies maintain hyphal heterogeneity in a fungal mycelium by impeding cytoplasmic continuity.

Like aspergilli, streptomycetes form micro-colonies in liquid shaken cultures. It is shown that cultures of *Streptomyces* species also consist of two populations of micro-colonies (**Chapter 5**). The population of small micro-colonies had a constant diameter (i.e. approximately 260 μm), whereas the diameter of the population of large colonies varied between strains and medium composition. Size of the population of large *S. coelicolor* micro-colonies, but not that of small micro-colonies, was also influenced by the cellulose synthase-like protein CslA, the cell surface proteins rodlins and chaplins, and the sigma factor BldN.

The results are summarized and discussed in **Chapter 6**.

CHAPTER 2

Abstract

The fungus *Aspergillus niger* forms (sub-) millimeter micro-colonies within a liquid shaken culture. We here show that such micro-colonies are heterogeneous 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 differed 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 culture, respectively. We also show that mRNA accumulation is heterogeneous 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 center 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.

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Heterogeneity in micro-colonies of *Aspergillus niger* in liquid shaken cultures

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 intercompartmental and 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). 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. 1993a). 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. 2007a). 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 center 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. 2007a). 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, we addressed whether micro-colonies within a liquid shaken culture of *A. niger* are heterogeneous with respect to size and gene expression. Moreover, it was assessed whether zones within individual micro-colonies are heterogeneous with respect to gene expression. To this end, 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 microbiology.

Materials and Methods

Strains

A. niger strain N593 (*pyrA6 cspA1*) (Goosen et al. 1987), AR9#2 (Siedenberg et al. 1999), and UU-A005.4 (Vinck et al. 2011) were used in this study. These strains are derivatives of *A. niger* N402 (Bos et al. 1988). Strains AR9#2 and UU-A005.4 express GFP from the *glaA* and *faeA* promoter, respectively. Strain AR9#2 contains 7 copies of the reporter construct, which have integrated at 4 different sites in the genome. Strain UU-A005.4 contains about 20 copies of the reporter construct, which have integrated at 13 different positions in the genome.

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. Micro-colonies were sorted based on size (time of flight, TOF) and fluorescence using a COPAS PLUS profiler equipped with a 1 mm nozzle (Union Biometrica, Holliston, MA). 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 was 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, Munich, Germany) equipped with an Axiovert 200M Zeiss inverted microscope (Carl Zeiss AG, Oberkochen, Germany) and a 3CCD color camera (HV-D30, Hitachi Kokusai Electric Inc., Tokyo, Japan). The 10× objective was routinely used. The 40× 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, Munich, Germany) and subsequently air dried. Micro-colonies of approximately 750 μm were selected and cut using LMPC. Larger colonies cannot 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) (Carl Zeiss MicroImaging, Munich, Germany), after which the laser dissected and catapulted these zones from the center (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 placed above the section. The cap contained 50 μl RNA*later* (Qiagen, Hilden, Germany) to enable RNA extraction.

RNA isolation

Individual micro-colonies or parts thereof were soaked in 50 µl RNA*later* (Qiagen, Hilden, Germany), 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., Melsungen, Germany) in a chilled container at 1500 rpm for 60 s. The frozen material was taken up in 250 µl Trizol Reagent (Invitrogen, Carlsbad, CA) 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, Hilden, Germany) 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 purified total RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, germany). QPCR was performed using the ABI Prism 7900HT SDS and SYBR Green chemistry (Applied Biosystems, Carlsbad, CA). Cycle threshold (Ct) levels were measured for *18S* rDNA, the *actin* gene, and for *glaA* and *faeA*. Primers were designed according to the recommendations of the PCR master-mix manufacturer (Applied Biosystems, Carlsbad, CA). 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 1). 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 1). The *glaA* cDNA was amplified with an efficiency of 1.98 and *faeA* cDNA with an efficiency of 1.92.

Table 1. 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, Franklin Lakes, NJ) 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 (Carl Zeiss AG, Oberkochen, Germany) equipped with a HBO 100 W mercury lamp and a Leica LFC 420C camera (2592×1944 pixels) using standard FITC and TRITC filters.

Statistical analysis

Statistical significance of differences in QPCR data was 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): φ: $\varphi(x) = pN(x;\mu_1,\sigma_1) + (1\text{-}p)N(x;\mu_2,\sigma_2)$, where $0 < p < 1$ and $x|\rightarrow N(x;\mu,\sigma)$ is the probability density of the normal distribution with parameters μ and σ. 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 fraction 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_1) 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://web.science.uu.nl/microbiology/images/fung/fittools.zip; http://web.science.uu.nl/microbiology/images/fung/manual%20fittools.pdf

Results

Fluorescence and volume of micro-colonies are not normally distributed

A. niger strains AR9#2 and UU-A005.4 express GFP from the promoter of the glucoamlyase gene *glaA* and the feruoyl esterase gene *faeA*, 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* (Boel et al. 1984, 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 volume

of the micro-colonies within the liquid shaken cultures of strains AR9#2 and UU-A005.4 can be explained by two normally distributed populations (Figure 1, Table 2 & 3).

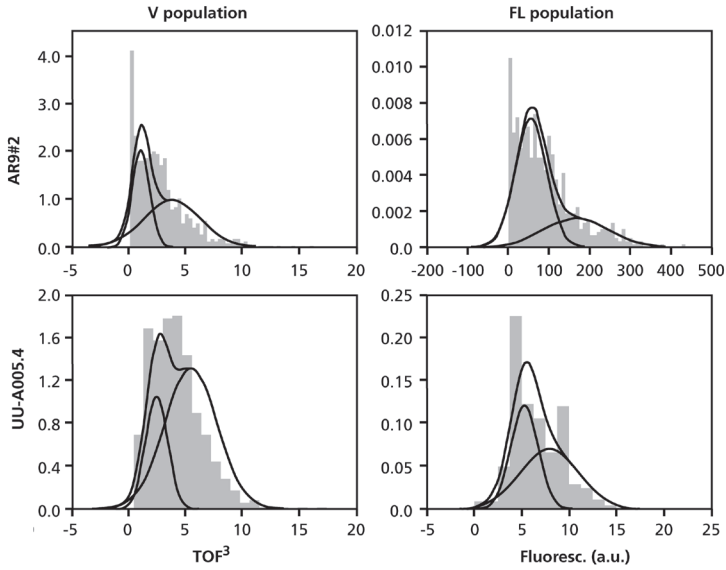

Figure 1. Heterogeneity of micro-colonies of *A. niger* in liquid shaken cultures with respect to size and gene expression. The dark grey areas represent the distribution of volume (V population) and *glaA* and *faeA* expression (FL population) of micro-colonies within liquid cultures. These distributions 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 2. Volume and fluorescence distribution of micro-colonies in liquid shaken cultures of *A. niger* can be explained by the existence of two populations of micro-colonies.

Strain	*Reporter construct*	*N*	*Participation level micro-colonies with small V*	*Participation level micro-colonies with high V*	*Participation level micro-colonies with low FL*	*Participation level micro-colonies with high FL*
AR9#2	*PglaA::GFP*	662	0.39	0.61	0.68	0.32
UU-A005.4	*PfaeA::GFP*	522	0.25	0.75	0.44	0.56

N = number of analyzed micro-colonies, FL = fluorescence, V = micro-colony volume (TOF^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, Table 2 & 4).

Table 3. Statistical analysis of the volume distribution of micro-colonies of *A. niger* in liquid shaken cultures. GFP was expressed from the *glaA* and *faeA* promoters.

Strain	Expression construct	N	μ_1	μ_2	σ_1	σ_2	p	CI
AR9#2	*PglaA::GFP*	662	1.0	3.8	0.8	2.5	0.39	0.25 - 0.43
UU-A005_4	*PfaeA::GFP*	522	2.3	5.4	0.9	2.3	0.25	0.25 - 0.25

N = number of analyzed micro-colonies; $\mu_{1,2}$ = mean of component 1 and 2, respectively; $\sigma_{1,2}$ = standard deviation of component 1 and 2, respectively; p = participation fraction of component 1; CI = 95% confidence interval of the participation fraction of component 1.

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. 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, Table 2 & 4). Volume and GFP expression of micro-colonies was also heterogeneous in other strains tested (Table 5 & 6). These data show that heterogeneity is not the result of the copy number and the site of integration.

Table 4. Statistical analysis of the fluorescence distribution of micro-colonies of *A. niger* in liquid shaken cultures. GFP was expressed from the *glaA* and *faeA* promoters.

Strain	Expression construct	N	μ_1	μ_2	σ_1	σ_2	p	CI
AR9#2	*PglaA::GFP*	662	55.2	167.5	38.0	79.1	0.68	0.31 - 0.78
UU-A005_4	*PfaeA::GFP*	522	5.3	8.0	1.5	3.2	0.44	0.30 - 0.99

N = number of analyzed micro-colonies; $\mu_{1,2}$ = mean of component 1 and 2, respectively; $\sigma_{1,2}$ = standard deviation of component 1 and 2, respectively; p = participation fraction of component 1; CI = 95% confidence interval of the participation fraction of component 1.

Table 5. Statistical analysis of the volume distribution of micro-colonies of *A. niger* in liquid shaken cultures. GFP was expressed from the *glaA* or *faeA* promoter, whereas dTomato was expressed from the *glaA* or *aamA* promoter. N593 is not transformed with a reporter construct.

Strain	Expression construct	N	μ_1	μ_2	σ_1	σ_2	p	CI
N593	-	264	1.4	2.5	0.3	1.0	0.25	0.25 - 0.25
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* & *PaamA::dTomato*	247	6.5	9.3	3.9	5.7	0.25	0.25 - 0.43
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* & *PglaA::dTomato*	361	4.4	8.0	1.4	2.1	0.25	0.25 - 0.25

N = number of analyzed micro-colonies; $\mu_{1,2}$ = mean of component 1 and 2, respectively $\sigma_{1,2}$ = standard deviation of component 1 and 2, respectively; p = participation fraction of component 1; CI = 95% confidence interval of the participation fraction of component 1.

Table 6. Statistical analysis of the fluorescence distribution of micro-colonies of *A. niger* in liquid shaken cultures. GFP was expressed from the *glaA* or *faeA* promoter, whereas dTomato was expressed from the *glaA* or *aamA* promoter.

Strain	Expression construct	N	μ_1	μ_2	σ_1	σ_2	p	CI
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* & *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 - 0.49 R: 0.34 - 0.50
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* & *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 - 0.81 R: 0.37 - 0.69

G = GFP; R = dTomato. N = number of analyzed micro-colonies; $\mu_{1,2}$ = mean of component 1 and 2, respectively; $\sigma_{1,2}$ = standard deviation of component 1 and 2, respectively; p = participation fraction of component 1; CI = 95% confidence interval of the participation fraction of component 1.

Heterogeneous 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 (Boel et al. 1984, de Vries & Visser 1999) 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 center 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).

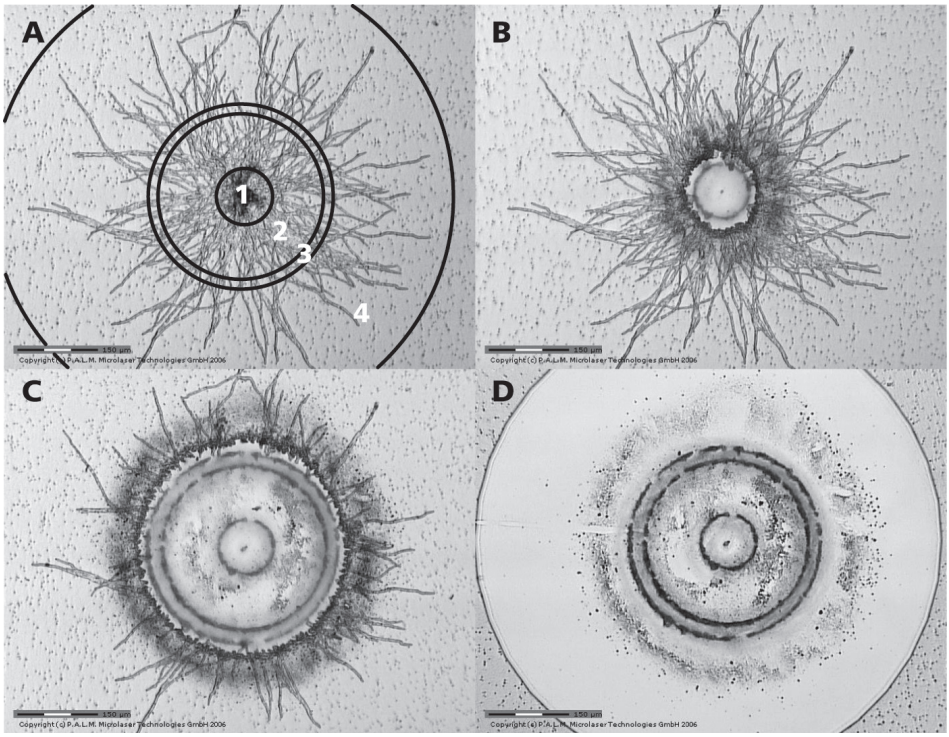

Figure 2. Laser dissection and pressure catapulting of micro-colonies of *A. niger* grown in a liquid shaken culture. Four zones are distinguished in a micro-colony (A). First, the central zone 1 was cut (B). This was followed by cutting zone 2 (C). After cutting zone 3, the outer zone 4 was dissected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

Levels of *18S* rRNA, *actin*, *glaA* and *faeA* mRNA were determined in zones 2 and 4 of individual micro-colonies by QPCR. Similar Ct values were obtained in the case of the central zone 2 and the peripheral zone 4 ($p \geq 0.95$; Table 7).

Table 7. 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.

	18S rRNA	*Act*	*glaA*	*faeA*
Center (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)

Act encodes actin, *glaA* glucoamylase, and *faeA* ferulic acid esterase.

However, the RNA from the central zone 2 was derived from 45 times more hyphal material than from the peripheral zone 4, which contained approximately 100 hyphae only. This indicates that RNA levels of these genes per unit mass of hypha are much higher in zone 4 when compared to zone 2. In agreement, acridine orange staining showed that the periphery of micro-colonies of *A. niger* is rich in RNA, while the center is not (El-Enshasy et al. 2006). Acridine orange binds single- and double-stranded nucleic acids, resulting in red and green fluorescence, respectively. We repeated this experiment in our experimental set up. As expected, red fluorescence (indicative for RNA) was more pronounced in the peripheral zone 4 when compared to green fluorescence (indicative for DNA), while the reverse was observed in the central zone 2 (Figure 3).

Figure 3. Heterogeneous 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 center of a micro-colony (A), whereas single stranded mRNA is predominantly observed at the periphery (B). (C) represents an overlay of (A) and (B). Zones within the colony are indicated by vertical lines.

Discussion

Previously, it has been shown that zones of cm-scale macro-colonies of *A. niger* are heterogeneous with respect to gene expression and protein secretion (Wösten et al. 1991, Levin et al. 2007a, Vinck et al. 2005, 2011). In liquid cultures such as industrial bioreactors, however, (sub)-millimeter scale micro-colonies are formed. These micro-colonies are exposed to a more homogenous medium than macro-colonies that are grown on a static solid medium. This fact and the enormous size difference between micro- and macro-colonies raised the question whether zones in micro-colonies of *A. niger* are also heterogeneous in RNA abundance and composition. Moreover, it was addressed for the first time whether a liquid shaken culture of *A. niger* consists of a homogenous population of micro-colonies or that distinct populations of micro-colonies can be distinguished that differ in size and gene expression.

Micro-colonies of 800 µm were subjected to laser micro-dissection and laser pressure catapulting, which enabled isolation of RNA from the central zone 2 and the peripheral zone 4. QPCR could not show differences in the levels of *18S* rRNA, and of RNA of the *actin*

gene and of *glaA* and *faeA* between the zones. However, the RNA from the central zone 2 originated from 45 times more hyphae when compared to the peripheral zone 4. Acridine orange staining confirmed that the hyphae in zone 2 contained less RNA, while staining with propidium iodide showed that ≥ 99 % of the hyphae were alive (unpublished results). The huge difference in RNA abundance between the periphery and the center was not observed in macro-colonies grown on a solid medium (unpublished results). We have no explanation for this. The size and open structure of the micro-colonies suggest that the center 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 heterogeneous with respect to volume and expression of *glaA* and *faeA.* Statistical analyses showed that 16 h-old cultures consist of two populations of micro-colonies that can be distinguished on basis of their volume. The small micro-colonies 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 (Table 6).

The fact that micro-colonies within liquid cultures are heterogeneous 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, an 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 in a liquid culture. COPAS enables rapid sorting of populations of living or fixed micro-colonies. We envision that protein production in liquid cultures can be increased by reducing heterogeneity of the micro-colonies.

Acknowledgements

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CHAPTER 3

Abstract

Black pigmented conidia of *Aspergillus niger* give rise to micro-colonies when incubated in liquid shaken medium. These micro-colonies are heterogeneous with respect to gene expression and size. We here studied the biophysical properties of the conidia of a control strain and of strains in which the *fwnA*, *olvA* or *brnA* gene is inactivated. These strains form fawn, olive-, and brown-colored conidia, respectively. The Δ*olvA* strain produced larger conidia (3.8 μm) when compared to the other strains (3.2-3.3 μm). Moreover, the conidia of the Δ*olvA* strain were highly hydrophilic, whereas those of the other strains were hydrophobic. The zeta potential of the Δ*olvA* conidia in medium was also more negative when compared to the control strain. This was accompanied by the near absence of a rodlet layer of hydrophobins. Using the Complex Object Parametric Analyzer and Sorter it was shown that the ratio of individual hyphae and micro-colonies in liquid shaken cultures of the deletion strains was lower when compared to the control strain. The average size of the micro-colonies of the control strain was also smaller (628 μm) than that of the deletion strains (790-858 μm). The size distribution of the micro-colonies of the Δ*fwnA* strain was normally distributed, while that of the other strains could be explained by assuming a population of small and a population of large micro-colonies. In the last set of experiments it was shown that relative expression levels of *gpdA,* and AmyR and XlnR regulated genes correlate in individual hyphae at the periphery of micro-colonies. This indicates the existence of transcriptionally and translationally highly active and lowly active hyphae as was previously shown in macro-colonies. However, the existence of distinct populations of hyphae with high and low transcriptional and translational activity in micro-colonies seems to be less robust when compared to macro-colonies grown on solid medium.

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Heterogeneity in liquid shaken cultures of *Aspergillus niger* inoculated with melanised conidia or conidia of pigmentation mutants

Introduction

Aspergillus niger is abundant in nature and is an important industrial microorganism because of its ability to secrete large amounts of proteins and metabolites such as citric acid (Finkelstein et al. 1989, Conesa et al. 2001, Punt et al. 2002, Papagianni 2007, Andersen et al. 2011). Submerged growth of *A. niger* in liquid medium results in dispersed mycelium, in clumps or in micro-colonies (also known as pellets). The morphology of the mycelium impacts the production of enzymes and metabolites. For instance, micro-colonies highly produce citric acid by *A. niger* (Gómez et al. 1988). It has also been shown that formation of large micro-colonies coincides with increased extracellular glucoamylase activity and reduced extracellular protease activity (Papagianni & Moo-Young, 2002). The mechanisms underlying the impact of morphology on productivity is not yet clear. Possibly, the effect of the fungal morphology on the viscosity of the medium plays a role (Bhargava et al. 2003b). Large micro-colonies give rise to low viscosity, whereas dispersed mycelium results in a high viscosity. At the same time, the center of large micro-colonies may experience oxygen starvation and other nutrients may also become limiting in this part of the mycelium (Gómez et al. 1988). These gradients are expected to be less pronounced during dispersed growth.

Conidia are used to inoculate liquid cultures of *A. niger*. Micro-colony formation is the result of a two-step aggregation process. First, conidia aggregate. This is followed by aggregation of germ tubes (Lin et al. 2008). Initial pH, agitation, and medium composition influence the degree of coagulation of conidia (Metz & Kossen 1977). Micro-colony formation can also be manipulated by changing the surface properties of the conidia. Formation of micro-colonies was affected in strains of *Aspergillus nidulans,* in which either or both *dewA* and *rodA* were

inactivated (Dynesen & Nielsen 2003). The effect was strongest when both hydrophobin genes were inactivated and this correlated with increased wettability of the mutant conidia. The pigment aspergillin that is contained in the cell wall of *A. niger* conidia may also directly or indirectly influence surface properties of the spore. Part of aspergillin is melanin. The *pptA*, *fwnA*, *olvA* and *brnA* genes have been shown to be involved in melanin synthesis in *A. niger*. Conidia of the Δ*pptA* strain are white due to the absence of phosphopantetheinyl transferase activity. This activity is required for activation of polyketide synthases (PKSs). In fact, inactivation of *pptA* abolishes synthesis of all polyketides and non-ribosomal peptides (Jørgensen et al. 2011). The phenotype of this gene can therefore be considered pleiotropic. Gene *fwnA* encodes a polyketide synthase. Inactivation of this gene results in fawn-colored conidia. The Δ*olvA* and Δ*brnA* strains produce olive- and brown-colored conidia, respectively. The function of BrnA is not yet known. The protein encoded by *olvA* is highly homologous to the *A. fumigatus* Ayg1 protein. Ayg1 converts the heptaketide naphtopyrone YWA1 into 1,3,6,8 tetrahydroxynaphtalene, which is further modified in the DHN pathway to produce melanin (Fujii et al. 2004).

Micro-colonies formed by a wild-type strain of *A. niger* are not homogenous in size and gene expression (de Bekker et al. 2011b, Chapter 2). Flow cytometry showed that a population of small and a population of large micro-colonies can be distinguished in a liquid culture. Similarly, populations of micro-colonies were detected that either highly or lowly express the glucoamylase *glaA* gene. The population of micro-colonies lowly expressing *glaA* was over-represented and did only partly overlap with the population of small micro-colonies. It was also shown that zones within a micro-colony are heterogeneous with respect to RNA content. The hyphae at the periphery of the colony would contain 50 times more RNA than those in the center of 1-mm wide micro-colonies (de Bekker et al. 2011b, Chapter 2). Hyphae could even be heterogeneous within a zone of a micro-colony. At least, this is the case at the periphery of macro-colonies of *A. niger.* It was shown that in this zone two populations of hyphae could be distinguished. One population has a high rRNA content and highly expresses the glyceraldehyde-3-phosphate dehydrogenase gene *gpdA* and genes encoding secreted proteins. The other population is characterized by lower rRNA content and lower expression of *gpdA* and genes encoding secreted proteins (Vinck et al. 2005, 2011). Recently it was shown by whole genome expression analysis that neighboring hyphae at the periphery of the colony are characterized by differences in their RNA profile (de Bekker et al. 2011a). These data suggest that the periphery of macro-colonies could consist of more than two types of hyphae.

Here, the role of the conidial pigment in heterogeneity between micro-colonies was studied. Moreover, it was assessed whether hyphae at the periphery of micro-colonies are heterogeneous with respect to expression of *gpdA* and genes encoding secreted proteins.

Materials and Methods

Strains and culture conditions

All strains used in this study are derivatives of *A. niger* N402 (Table 1). CB-A111.1 is a derivative of *A. niger* N593 (Goosen et al. 1987) that contains pGW635 (Goosen et al. 1989) resulting in uridine prototrophy. Cultures were grown at 30 °C. Conidia were isolated with saline Tween (0.5 % NaCl and 0.005 % Tween-80) from 3-day-old cultures. The conidia of CB-A111.1 and the pigmentation mutant strains were isolated from cultures that had been grown on solid complete medium (minimal medium (MM, de Vries et al. 2004) with 2 g l^{-1} trypton, 1 g l^{-1} casamino acids, 1 g l^{-1} yeast extract, 0.5 g l^{-1} yeast ribonucleic acids and 1 % glucose). Conidia of strains in which reporter constructs had been introduced were isolated from cultures grown on minimal medium containing 50 mM glucose (CB-A114.2, CB-A114.22, CB-A115.3, CB-A115.9, CB-A116.2 and CB-A116.11) or 200 mM xylose (CB-A117.1, CB-A117.5, CB-A118.24, CB-A118.28, CB-A121.4 and CB-A121.7) to prevent the conidia to become fluorescent. Liquid cultures used to assess heterogeneity had been inoculated with 1.5 10^8 conidia and had been grown for 16 h at 250 rpm in 1 L Erlenmeyers in 250 ml transformation medium (TM) (Kusters-van Someren et al. 1991) with 25 mM of carbon source. Glucose was used to assess heterogeneity in micro-colony size and maltose (CB-A121.4 and CB-A121.7), xylose (CB-A114.2, CB-A114.22, CB-A115.3, CB-A115.9, CB-A116.2 and CB-A116.11) or a combination of xylose and maltose (CB-A117.1, CB-A117.5, CB-A118.24 and CB-A118.28) was used to assess heterogeneity at the hyphal level. To induce fluorescence of reporter proteins for heterogeneity studies, 5 ml of culture was transferred for 6 h to 50 ml MM in 250 ml Erlenmeyers with the same carbon source as the preculture.

Table 1. Strains used in this study.

Strains	*Parent*	*Genotype*	*Reference*
CB-A111.1	N593 (pyrG$^-$)	pGW635 (pyrG)	This study
AW6.1	MA169.4	*brnA::AopyrG*	Jørgensen et al. 2011
AW8.4	MA169.4	*olvA::AopyrG*	Jørgensen et al. 2011
MA93.1	N402	*fwnA::hygB*	Jørgensen et al. 2011
CB-A114.2 & CB-A114.22	UU-A005.4	*faeA::GFP, faeA::dTomato*	Vinck et al. 2011
CB-A115.3 & CB-A115.9	UU-A005.4	*faeA::GFP, aguA::dTomato*	Vinck et al. 2011
CB-A116.2 & CB-A116.11	UU-A005.4	*faeA::GFP, gpdA::dTomato*	Vinck et al. 2011
CB-A117.1 & CB-A117.5	UU-A005.4	*faeA::GFP, aamA::dTomato*	Vinck et al. 2011
CB-A118.24 & CB-A118.28	UU-A005.4	*faeA::GFP, glaA::dTomato*	Vinck et al. 2011
CB-A121.4 & CB-A121.7	CB-A112.11	*glaA::GFP, aamA::dTomato*	Vinck et al. 2011

Microbial adhesion to hydrocarbons (MATH) assay

Conidia were tested for hydrophobicity with the MATH assay as described (Smith et al. 1998). In short, the optical density (OD) was determined at 470 nm before and after extraction with hexadecane. The hydrophobicity index (HI) was calculated using the formula $(OD_{before} – OD_{after}) / OD_{before}$

Zeta-potential

The zeta potential of conidia was obtained by particulate micro-electrophoresis with a Lazer Zee meter 501 (PenKem, Bedford Hills, N.Y.). The micro-electrophoresis chamber was filled with 30 ml spore solution (10^6 - 10^7 conidia ml^{-1} TM or 100-times diluted TM) and a voltage difference of 150 V was applied. Conidia were detected by scattering of incident laser light. Image analysis revealed the velocity of conidia and zeta potentials were derived using the Smoluchowski equation (Hiemenz 1977).

Microscopy

GFP and dTomato expression was studied by confocal laser scanning microscopy (CLSM). Micro-colonies were imaged with an inverted Zeiss LSM 5 system (Zeiss, www.zeiss.com) using a Plan-Neofluar 16×/0.5 oil immersion lens. GFP and dTomato were excited using a 488 nm and a 543 nm laser, respectively. GFP fluorescence was detected with a 505-530 nm band pass filter, while a 560 nm long pass filter was used in the case of dTomato. Under- and over-exposure was prevented by adjusting gain and amplifier offset settings. Images were captured as a z-stack of optical slices using the multi-track scanning mode (optimal interval 2.02 mm; 4× line average; 8 bit scan depth). Subsequently, the z-stack was projected with maximum intensity (1024 × 1024 pixels) using Zeiss software.

Image analysis

Hyphal fluorescence was quantified as described (Vinck et al. 2011). In short, the intensity of GFP was calculated with KS400 software by selecting hyphae in the green channel. The mask was copied to the red channel to determine the corresponding dTomato fluorescence. A custom Python script was used to correlate intensity of GFP and dTomato. Areas less than 100 mm^2 were discarded. For each channel the signal was normalized by dividing the hyphal fluorescence by the total green or red fluorescence for that picture. The Pearson correlation coefficient between GFP and dTomato was calculated for the normalized data.

Flow cytometry using the COPAS PLUS

Samples of 5 ml were taken from 16-h-old liquid shaken cultures and fixed for 20 min in 70 % ethanol (EtOH) in a final volume of 50 ml. The EtOH was removed by washing 2 times

in excess PBS. Micro-colonies were allowed to settle in between the washing steps. Micro-colonies were analyzed based on extinction (EXT) and time of flight (TOF) using a COPAS PLUS equipped with a 1 mm nozzle (Union Biometrica, Holliston, MA) and a 488 nm solid state laser. The TOF depends on the Feret diameter.

Electron microscopy

Cryoscanning electron microscopy was performed to determine the size and ornamentation of condia. To this end, a 1 µl spore solution was dried on 4 % water agar. Small cubes (3 × 3 mm) of agar were excised and transferred to a copper cup for snap-freezing in nitrogen slush. Agar blocks were glued to the copper surface with frozen tissue medium (KP-Cryoblock, Klinipath, Duiven, Netherlands). Samples were examined in a JEOL 5600LV scanning electron microscope (JEOL, Tokyo, Japan) equipped with an Oxford CT1500 Cryostation for cryo-electron microscopy (cryoSEM). Ice was removed from the sample surface by sublimation at -85 ºC. Electron micrographs were acquired from uncoated frozen samples using an acceleration voltage of 3 kV and 30 averaged fast scans (SCAN 2 mode). Rodlets were viewed with a field emission scanning electron microscope equipped with a through lens detector at 5 kV and a working distance of 3.5 mm (FEI, www.fei.com). To this end, fresh condia were attached on a carbon adhesive stub and sputter coated with a 9 nm Pt/Pd layer.

Statistical analysis

A two-way ANOVA with Tukey post-hoc test ($p < 0.05$) was used to assess statistical significance of differences in hydrophobicity, zeta potential and diameter of conidia as well as differences in micro-colony heterogeneity. To assess whether distributions in size or fluorescence can be explained by a mixture of two normal distributions the data was modeled in the probability distribution φ: $\varphi(x) = pN(x;\mu_1,\sigma_1) + (1\text{-}p)N(x;\mu_2,\sigma_2)$ (Vinck et al. 2005). In this model, μ_1 and μ_2 represent the means of the populations, σ_1 and σ_2 their standard deviations and p the participation fraction. The five parameters in the model (p; μ_1; σ_1;μ_2; σ_2;) were fit to empirical data by means of the maximum likelihood principle. 95 % confidence interval estimates were obtained by means of bootstrapping (1000 replicates) and refitting with the model using the open source Scilab language. The scripts of the Scilab functions are available at http://web.science.uu.nl/microbiology/images/fung/fittools.zip ; http://web.science.uu.nl/microbiology/images/fung/manual%20fittools.pdf

Results

Surface characterization of conidia of the wild-type strain and melanin mutants

Hydrophobicity of the conidia of the control strain CB-A111.1 and the pigmentation mutant strains Δ*fwnA*, Δ*olvA*, and Δ*brnA* was determined by the MATH assay. To this end, aqueous

suspensions of conidia were extracted with hexadecane. The ratio of conidia in the aqueous solution before and after hexadecane extraction was determined by the OD_{470} resulting in a hydrophobicity index (HI) between 0-1. Values ≤ 0.7 are considered hydrophilic (Holder et al. 2007). Conidia of CB-A111.1 and the Δ*fwnA* and Δ*brnA* strains had a HI between 0.65 and 0.77. Their values were not significantly different (Figure 1A). In contrast, the HI of conidia of the Δ*olvA* strain was 0.13 showing that these spores were highly hydrophilic.

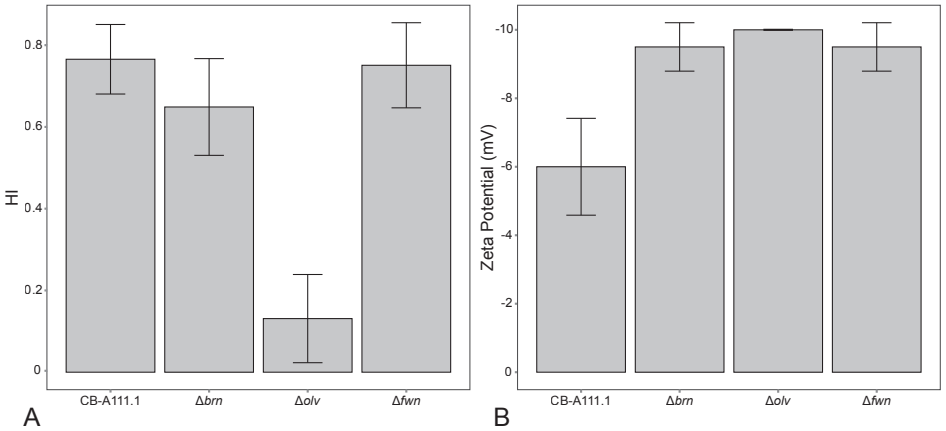

Figure 1. Hydrophobicity Index (HI) (A) and zeta potential (B) of *A. niger* conidia. The zeta potential was determined in TM medium. Error bars represent standard deviation.

Surface charge, as assessed by the zeta potential, of the conidia of CB-A111.1, Δ*olvA*, and Δ*brnA* ranged between -31 and -41 mV in 100-fold diluted TM medium. Their zeta potential was not significantly different. The conidia of the Δ*fwnA* mutant had a zeta potential of -47 ± 0.7 mV. This value was significantly different from that of the conidia of the control strain but not from that of the other pigmentation mutant strains. Zeta potential of all conidia was at least 4-fold lower in TM medium (used to grow the strains). The zeta potential of the conidia of the control strain (-6 ± 1.4 mV) was significantly lower when compared to that of the Δ*olvA* spores (-10 ± 0 mV) and showed a trend towards significance for the Δ*brnA* and Δ*fwnA* conidia (-9.5 ± 0.7 mV; p = 0.051) (Figure 1B).

Scanning electron microscopy revealed that the diameter of conidia of 3-day-old cultures of the control strain and the pigmentation mutant strains ranged between 3.2 and 3.8 µm. The conidia of CB-A111.1, Δ*brnA*, and Δ*fwnA* (3.2-3.3 µm) were significantly smaller than those of Δ*olvA* (3.8 µm). In all cases, the majority of the conidia lacked ornamentations with a width > 200 nm (Figure 2). High resolution scanning electron microscopy revealed large areas of 13-16 nm wide rodlets on conidia of all strains except for the Δ*olvA* strain (Figure 3). In the latter case some thin bundles of rodlets could be distinguished.

Taken together, these data show that the biophysical and structural properties of the conidia of the Δ*olvA* strain are most distinct when compared to the control strain and the Δ*brnA* and Δ*fwnA* strains.

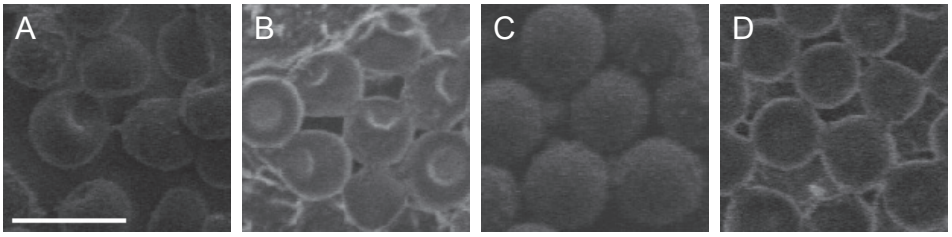

Figure 2. Scanning electron microscopy of conidia from 3-day-old colonies of strains CB-A111.1 (A), Δ*brnA* (B), Δ*olvA* (C), and Δ*fwnA* (D). Bar represents 5 µm.

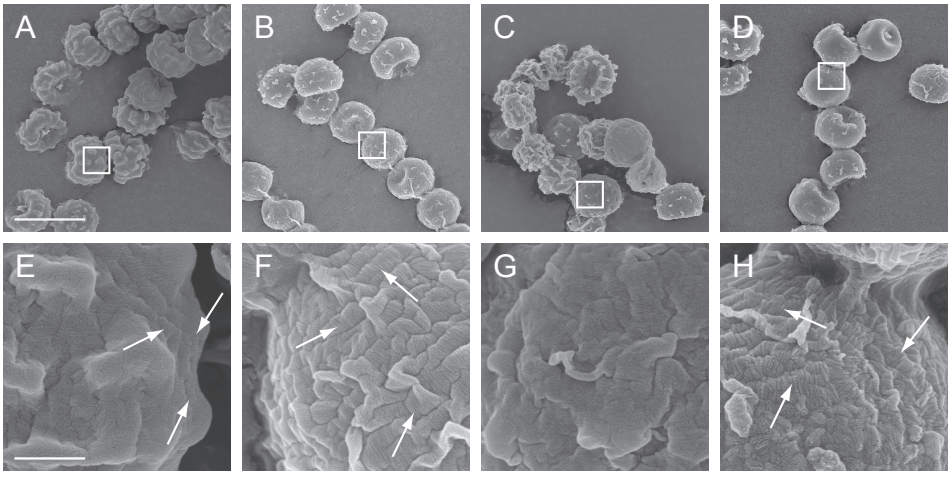

Figure 3. Scanning electron microscopy of conidia of 3-day-old colonies of strains CB-A111.1 (A,E), Δ*brnA* (B,F), Δ*olvA* (C,G) and Δ*fwnA* (D,H). Bar represents 5 µm (A-D) and 500 nm (E-H). Inset in A-D represents the area shown in E-H. Rodlets are visible on the conidia of the control, Δ*brnA* and Δ*fwnA* strains and are indicated by white arrows.

Culture profiling by flow cytometry

The control strain CB-A111.1 and the pigmentation mutant strains Δ*fwnA*, Δ*olvA*, and Δ*brnA* were grown for 16 h in TM as liquid shaken cultures. The resulting micro-colonies were analyzed on basis of their diameter as expressed as the time of flight (TOF) in milliseconds (Figure 4). Individual hyphae were detected in the extinction range between 0-150, whereas micro-colonies were detected above 200. A mixture of hyphae and micro-colonies was observed in the range between 150-200 (Data not shown). The percentage of events representing individual hyphae was not significantly different in the case of the CB-

A111.1 and Δ*fwnA* strains. They were found to be 64 % and 39 %, respectively (Table 2). The number of individual hyphae was very low in the case of the Δ*brnA* and Δ*olvA* strains (i.e. 6 % and 9 %, respectively). The percentage of events with an extinction between 150 and 200 was relatively low in all cases with a maximum of 6 % for the control strain. The percentage of events representing micro-colonies was 90-94 % in the case of the Δ*brnA* and Δ*olvA* strains. This was statistically different from the values obtained with CB-A111.1 (30 %) and Δ*fwnA* (60 %) (Table 2). Also, the number of events of the Δ*fwnA* strain with an extinction > 200 was significantly different from that of the control strain. The average TOF of the micro-colonies (i.e. with an EXT > 200) produced by the control (821) was significantly different from that of the pigmentation mutant strains (1173-1321) (Table 2). Using the formula defined by de Bekker et al. (2011b, Chapter 2), the average TOF value of the micro-colonies of the control strain corresponds to a diameter of 628, whereas that of the pigmentation mutants corresponds to 790-858 µm.

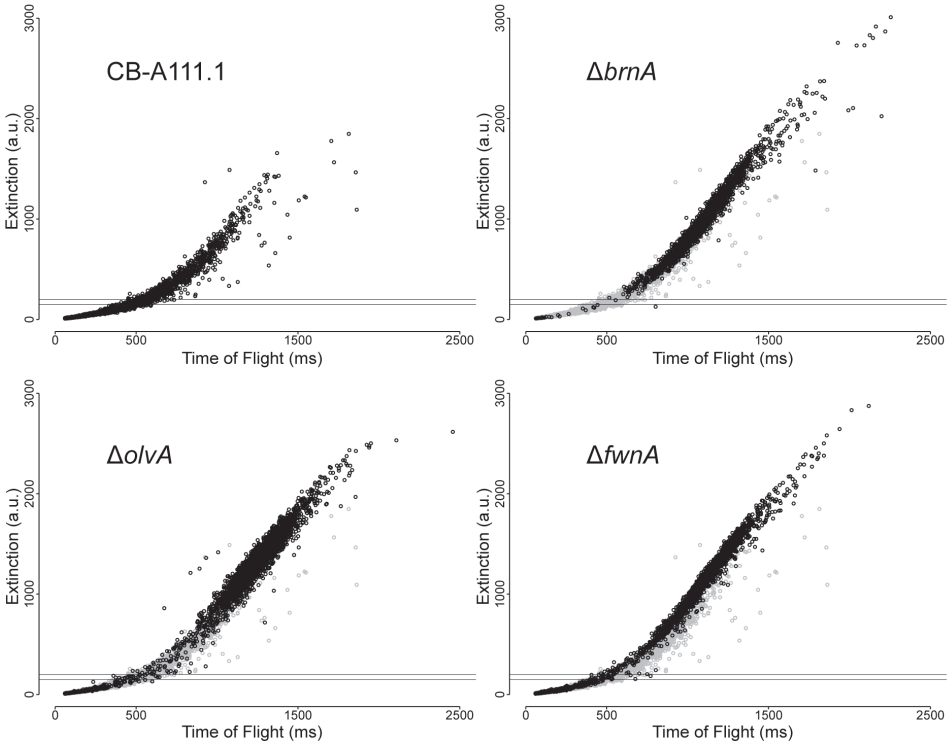

Figure 4. Distribution of diameter (time of flight in milliseconds × 0.4) and optical density (extinction in arbitrary units) of hyphae and micro-colonies of liquid cultures of pigmentation mutants of *A. niger* and the control strain CB-A111.1. The horizontal lines represent an extinction of 150 and 200. The grey dots in the graphs of the pigmentation mutants represent the events of the control strain.

Table 2. Fraction of individual hyphae and micro-colonies in *A. niger* cultures. Individual hyphae with occasional branches were observed at an extinction ≤ 150. A mixture of hyphae and micro-colonies was observed at an extinction between 150 and 200. Micro-colonies were observed at an extinction > 200. The average size of the micro-colonies (fraction with an extinction > 200) is indicated by the mean TOF. In all cases, standard deviation is indicated.

Strains	*Events (%) ≤ 150*	*Events (%) > 150 - ≤ 200*	*Events (%) > 200*	*mean TOF micro-colony fraction*
CB-A111.1	64 ± 16	6 ± 1	30 ± 16	821 ± 95
Δ*brnA*	6 ± 6	0 ± 0	94 ± 6	1173 ± 50
Δ*olvA*	9 ± 3	1 ± 0	90 ± 4	1321 ± 189
Δ*fwnA*	39 ± 15	1 ± 0	60 ± 15	1243 ± 125

In the next step, we assessed whether the size distribution of the micro-colonies was normally distributed. To this end, the TOF of the events > 200 was divided by the mean TOF of the population to normalize the data. Mathematical modeling of the pooled data showed that the size distribution of the micro-colonies within liquid shaken cultures of the CB-A111.1, Δ*brnA*, and Δ*olvA* strains can be explained by two normally distributed populations (Figure 5 and Table 3). The population of large micro-colonies was underrepresented in the CB-A111.1 and Δ*brnA* strains, whereas in the Δ*olvA* strain this population was over-represented. The Δ*fwnA* strain did not show a distribution of 2 populations. It should be noted that the average diameter of the two populations of strain CB-A111.1 differed almost 150 µm, whereas this was only 62 and 43 µm in the case of the Δ*brnA* and Δ*olvA* strains. These data show that the ratio of individual hyphae and micro-colonies in liquid shaken cultures are different between the pigmentation mutants and the control strain. Moreover, the size distribution of micro-colonies of the pigmentation mutants is different when compared to the control strain.

Table 3. Heterogeneity in size between micro-colonies of liquid shaken cultures of the control strain and the pigmentation mutants of *A. niger*. Heterogeneity is defined as non-overlapping confidence intervals (CI) of the mean of both populations (μ_1 and μ_2) and a CI of the participation fraction (p) between 0.025 - 0.975.

Strains	*CI* μ_1		*CI* μ_2		*CI p*		*Diameter (µm) fraction 1*	*Diameter (µm) fraction 2*	*hetero-geneity*
CB-A111.1	9.148	9.657	12.237	14.394	0.744	0.918	608	755	Yes
Δ*brnA*	9.765	9.854	10.624	11.375	0.794	0.875	780	842	Yes
Δ*olvA*	9.306	9.620	10.116	10.216	0.181	0.290	825	868	Yes
Δ*fwnA*	9.689	10.160	9.641	10.137	0.335	0.764	818	-	No

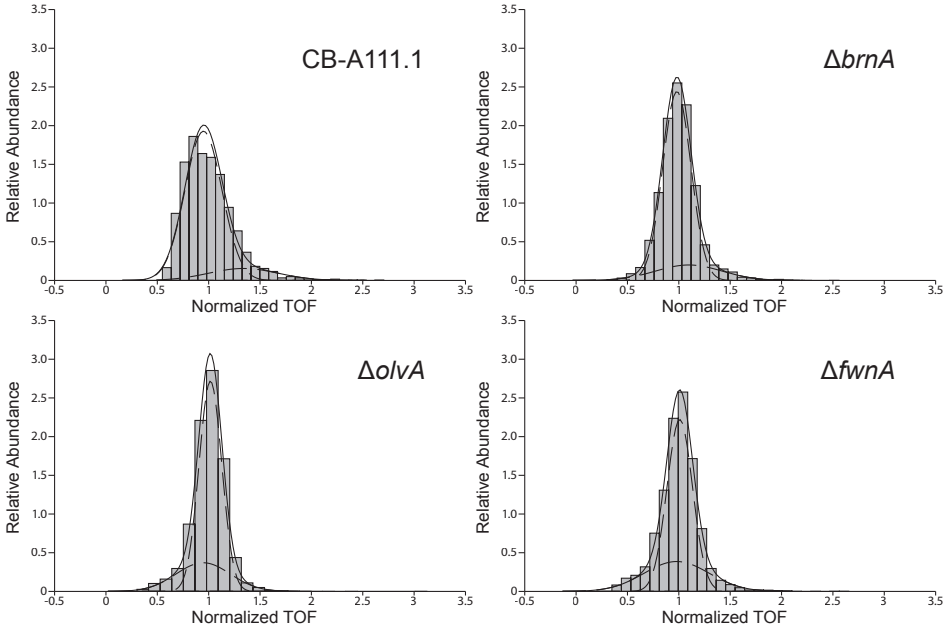

Figure 5. Size distributions of micro-colonies of liquid shaken cultures of the control strain CB-A111.1 and the Δ*brnA*, Δ*olvA* and Δ*fwnA* strains. The time of flight (TOF) of each micro-colony was divided by the average TOF of the micro-colonies in the culture to compile the data of a biological triplicate in one histogram. The scale of the Y-axis is set such that the surface area of the histogram equals 1.

Correlation of gene expression in A. niger strains

Relative expression levels of genes encoding secreted proteins correlate in hyphae at the periphery of macro-colonies (Vinck et al. 2011). This was shown by using strains expressing dTomato and GFP from promoters of genes that are regulated by the amylolytic regulator AmyR (*glaA* and *aamA*) and the xylanolytic regulator XlnR (*faeA* and *aguA*). In addition, it was shown that expression of the glyceraldehyde-3-dehydrogenase gene *gpdA* correlated with *faeA*. Here, it was assessed whether expression levels of *gpdA* and genes encoding secreted proteins also correlate in hyphae at the periphery of micro-colonies. To this end, fluorescence of GFP and dTomato was quantified in individual hyphae of strains expressing the reporter genes from the *gpdA* promoter and / or promoters of genes encoding secreted proteins. Expression of genes was not high enough in micro-colonies grown in TM, probably due to the presence of yeast extract. Therefore, fluorescence was induced for 6 h in MM. In general, GFP and dTomato expression resulting from the different promoters correlated in the individual hyphae at the periphery of micro-colonies (Table 4). The highest correlation was found between *faeA* driven GFP expression and *faeA* driven dTomato expression (correlation coefficient 0.8). The lowest correlation (but still highly significant) was found between *faeA* driven GFP expression and *glaA* or *aamA* driven dTomato expression (correlation coefficient

of 0.64-0.65). Taken together, expression of *gpdA,* and AmyR and XlnR regulated genes correlates in individual hyphae of micro-colonies.

Table 4. Correlation coefficients of expression of genes of *A. niger* using GFP and dTomato as a reporter. Strains were grown as macro-colonies (Vinck et al. 2011) or micro-colonies.

Strain	*Promoters*	*Macro-colony*	*Micro-colony*	*Carbon source*
CB-A114.2	*faeA//faeA*	0.70	0.86	xylose
CB-A114.22	*faeA//faeA*	0.72	0.74	xylose
CB-A115.3	*faeA//aguA*	0.73	0.79	xylose
CB-A115.9	*faeA//aguA*	0.77	0.62	xylose
CB-A116.2	*faeA//gpdA*	0.87	0.75	xylose
CB-A116.11	*faeA//gpdA*	0.80	0.71	xylose
CB-A117.1	*faeA//aamA*	0.46	0.73	xylose/maltose
CB-A117.5	*faeA//aamA*	0.52	0.56	xylose/maltose
CB-A118.24	*faeA//glaA*	0.35	0.80	xylose/maltose
CB-A118.28	*faeA//glaA*	0.46	0.49	xylose/maltose
CB-A121.4	*glaA//aamA*	0.80	0.74	maltose
CB-A121.7	*glaA//aamA*	0.78	0.80	maltose

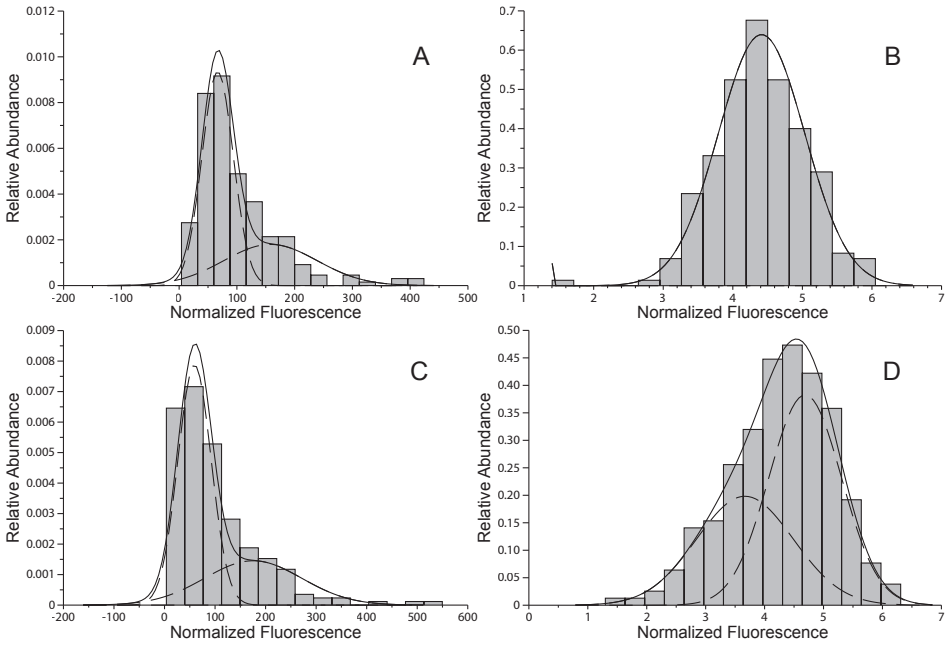

Figure 6. Representative fluorescence distributions before (A,C) and after (B,D) log transformation of hyphae at the periphery of micro-colonies of strain CB-A118.28 expressing GFP from the *faeA* promoter (A,B) and dTomato from the *glaA* promoter (C,D). A, C and D show non-overlapping CI's of the means. The scale of the Y-axis is set such that the surface area of the histogram equals 1.

Table 5. Heterogeneous gene expression in hyphae of micro-colonies without log transformation of the fluorescence intensities of the individual hyphae. Heterogeneity is defined as non-overlapping confidence intervals (CI) of the mean of both populations (μ_1 and μ_2) and a CI of the participation fraction (p) between 0.025-0.975.

Strain	*Promoter*	*CI* μ_1		*CI* μ_2		*CI p*		*Heterogeneity*
CB-A 114.2	*faeA*	57.75	78.33	144.69	240.85	0.57	0.85	Yes
	faeA	48.94	83.76	136.00	276.46	0.47	0.90	Yes
CB-A 114.22	*faeA*	47.40	69.04	121.65	172.68	0.32	0.74	Yes
	faeA	50.19	84.12	130.18	300.53	0.48	0.92	Yes
CB-A 115.3	*faeA*	65.32	77.00	170.06	223.84	0.68	0.85	Yes
	aguA	69.75	89.50	151.63	280.78	0.67	0.94	Yes
CB-A 115.9	*faeA*	56.57	89.17	141.41	350.60	0.57	0.95	Yes
	aguA	63.66	80.97	160.52	234.61	0.64	0.88	Yes
CB-A 116.2	*faeA*	44.99	84.77	125.88	298.54	0.36	0.92	Yes
	gpdA	40.56	90.70	123.91	392.00	0.33	0.96	Yes
CB-A 116.11	*faeA*	53.70	89.80	129.79	356.56	0.47	0.95	Yes
	gpdA	55.93	89.22	155.57	465.86	0.63	0.96	Yes
CB-A 117.1	*faeA*	61.28	76.68	165.70	235.15	0.65	0.84	Yes
	aamA	50.52	65.18	151.82	201.41	0.53	0.73	Yes
CB-A 117.5	*faeA*	60.05	82.29	152.78	308.23	0.63	0.90	Yes
	aamA	46.45	73.00	148.21	246.00	0.51	0.83	Yes
CB-A 118.24	*faeA*	59.79	82.08	145.66	245.57	0.58	0.87	Yes
	glaA	57.96	83.08	147.24	256.03	0.57	0.89	Yes
CB-A 118.28	*faeA*	55.56	87.70	133.41	259.09	0.47	0.92	Yes
	glaA	45.19	71.54	142.39	223.02	0.47	0.79	Yes
CB-A 121.4	*glaA*	50.70	77.69	128.64	188.70	0.40	0.79	Yes
	aamA	29.81	92.14	111.78	302.03	0.20	0.94	Yes
CB-A 121.7	*glaA*	49.69	85.73	113.19	190.52	0.23	0.85	Yes
	aamA	49.14	65.64	140.56	187.48	0.46	0.71	Yes

In the next analysis it was assessed whether the differences in fluorescence intensity of the reporters between the hyphae can be explained by assuming the existence of two distinct populations of hyphae; one highly and one lowly expressing the selected genes. The parameters obtained are the mean, standard deviation and participation fraction of the population lowly expressing GFP or dTomato (μ_1, σ_1 and p) and the mean and standard deviation of the population high expressing hyphae (μ_2, σ_2). The participation fraction of the second population is given by 1 - p. Confidence intervals of the means were obtained by bootstrapping and refitting with the model. The data can be explained with a normal

distribution if the confidence intervals of the means overlap. In the case of absence of overlap and when the CI of p is between 0.025 and 0.975, data can be explained by the presence of a bimodal distribution. In all cases, bimodal distributions were obtained (Table 5). However, fluorescence intensity distributions were skewed to the right (Figure 6). Therefore, fluorescence data were log transformed followed by modeling the data assuming a bimodal distribution (see above). In this case, only 2 out of the 24 cases reporter expression was heterogeneous (Table 6).

Table 6. Heterogeneous gene expression in hyphae of micro-colonies using log transformed fluorescence intensities of the individual hyphae. Heterogeneity is defined as non-overlapping confidence intervals (CI) of the mean of both populations (μ_1 and μ_2) and a CI of the participation fraction (p) between 0.025-0.975.

Strain	*Promoter*	*CI* μ_1		*CI* μ_2		*CI p*		*Heterogeneity*
CB-A 114.2	*faeA*	2.86	4.44	4.32	6.65	0.02	1.00	No
	faeA	2.84	4.37	4.34	6.60	0.02	0.99	No
CB-A 114.22	*faeA*	3.72	4.39	4.38	6.84	0.05	0.99	No
	faeA	1.21	4.52	4.24	6.49	0.00	0.99	No
CB-A 115.3	*faeA*	3.55	4.48	4.36	5.67	0.05	0.96	No
	aguA	2.90	4.57	4.37	5.82	0.02	0.98	No
CB-A 115.9	*faeA*	3.18	4.42	4.38	6.55	0.04	0.99	No
	aguA	1.91	4.48	1.91	5.52	0.00	1.00	No
CB-A 116.2	*faeA*	3.25	4.39	4.40	6.38	0.05	0.99	No
	gpdA	2.65	4.34	4.37	6.32	0.03	0.98	No
CB-A 116.11	*faeA*	2.16	4.44	4.30	6.19	0.01	0.98	No
	gpdA	-0.73	4.45	4.21	6.17	0.01	0.98	No
CB-A 117.1	*faeA*	2.80	4.37	4.35	6.15	0.03	0.99	No
	aamA	1.93	4.33	4.24	5.90	0.01	0.97	No
CB-A 117.5	*faeA*	2.40	4.43	4.25	6.79	0.01	0.99	No
	aamA	1.63	4.33	3.96	5.78	0.01	0.96	No
CB-A 118.24	*faeA*	2.79	4.44	4.35	6.35	0.02	0.99	No
	glaA	1.65	4.43	1.65	5.33	0.00	0.99	No
CB-A 118.28	*faeA*	1.40	4.44	1.40	5.98	0.00	1.00	No
	glaA	2.34	4.16	4.33	5.30	0.03	0.91	Yes
CB-A 121.4	*glaA*	2.63	4.39	4.42	5.44	0.02	0.96	No
	aamA	3.13	4.36	4.49	5.02	0.11	0.92	Yes
CB-A 121.7	*glaA*	3.69	4.62	3.88	5.51	0.06	0.94	No
	aamA	2.33	4.32	4.37	5.43	0.01	0.93	No

Discussion

Macro-colonies of fungi with a diameter > 5 cm have been shown to be heterogeneous with respect to gene expression, growth and secretion (Krijgsheld et al. 2013). Heterogeneity was not only observed between zones (Wösten et al. 1991, Moukha et al. 1993ab, Masai et al. 2006, Levin et al. 2007ab) but even between neighboring hyphae (Moukha et al. 1993b, Teertstra et al. 2004, Vinck et al. 2005, 2011, de Bekker et al. 2011a, Etxebeste et al. 2009). Heterogeneity within a liquid shaken culture has been studied less. Recently, it was described that micro-colonies within a liquid culture of *A. niger* are heterogeneous with respect to size and gene expression (de Bekker et al. 2011b, Chapter 2). Moreover, it was shown that hyphae in the outer zone contains more RNA than hyphae in the central zone of the micro-colony (de Bekker et al. 2011b, Chapter 2). Here we studied whether pigmentation of conidia, used to inoculate the cultures, impacts heterogeneity in a liquid shaken culture. Moreover, it was assessed whether neighboring hyphae within a liquid shaken culture are heterogeneous with respect to expression of genes encoding GpdA and secreted proteins.

Liquid shaken cultures of *A. niger* that have been inoculated with conidia consist of individual hyphae (either or not with occasional branches) and micro-colonies. In the case of TM medium, these micro-colonies are smaller than 1 mm (de Bekker et al. 2011b, Chapter 2). The incidence of individual hyphae and micro-colonies was different in the control strain when compared to the pigmentation mutant strains Δ*fwnA*, Δ*brnA*, and Δ*olvA.* The percentage of events representing micro-colonies was 90-94 % in the case of the Δ*brnA* and Δ*olvA* strains, which was higher than that of Δ*fwnA* (60 %). All pigmentation mutant strains had a higher incidence of micro-colonies when compared to the control strain CB-A111.1 (30 %). These data show that the control strain forms relatively more single hyphae. This may be due to shearing or to conidia that have germinated later in the cultivation process. It should be noted that the biomass of an individual hypha is less than 0.1 % of that of a micro-colony (de Bekker et al. 2011b, Chapter 2). Therefore, the biomass of the individual hyphae represents maximally a few percent of the culture. This agrees with a study performed with *A. nidulans* (Dynesen & Nielsen 2003). At pH 5.8 only 0.1 % of the biomass of the culture consisted of free hyphal elements. The percentage increased by lowering the pH of the culture medium. For instance, more than 50 % of the biomass of *A. nidulans* was contained in free hyphae at pH 3.4. The percentage of biomass present in individual hyphae could also be increased by inactivating the hydrophobin genes *rodA* and *dewA*. These mutant strains produce more wettable conidia. However, our results show that wettability of these asexual spores is not correlated per se with an increased incidence of hyphal elements in the liquid shaken culture. Strains Δ*brnA* and Δ*olvA* formed a similar number of hyphal elements. Yet, the conidia of the Δ*brnA* strain were hydrophobic, while those of the Δ*olvA* strain were highly hydrophilic.

The average size of the micro-colonies of the control strain was 628 µm, while that of the pigmentation mutants was between 790-858 µm. This is of interest for biotechnological applications because of the fact that mycelial morphology determines productivity of the bioreactor (Gomez et al. 1988, Papagianni & Moo-Young 2002, Bhargava et al. 2003b). To optimally control productivity one would like to have a homogenous morphology of the mycelium. This is not the case in liquid shaken cultures of *A. niger*. Liquid shaken cultures of *A. niger* strains AR9#2 and UU-A005.4 consisted of two populations of micro-colonies (de Bekker et al. 2011b, Chapter 2). The population of large and small micro-colonies had an average diameter of 595 and 505 µm, respectively. Here, it was shown that cultures of the control strain CB-A111.1 are characterized by two populations with an average diameter of 608 and 755 µm, respectively. The Δ*brnA* and Δ*olvA* strains also formed heterogeneous cultures. However, the average diameter of the population of large and small micro-colonies were less distinct (i.e. 780 and 842 µm and 825 and 868 µm). The micro-colonies of the Δ*fwnA* strain were even normally distributed with an average diameter of 818 µm. Taken together, the pigmentation mutants form larger and more homogenous micro-colonies than CB-A111.1 and the AR9#2 and UU-A005.4 strains.

The size of micro-colonies is influenced by aggregation of conidia and of germlings (Lin et al. 2008). This implies that the size of micro-colonies depends on the surface properties of both conidia and hyphae. Whole genome expression analysis indicates that *brnA*, *olvA*, and *fwnA* are more than 8 times down-regulated in vegetative hyphae when compared to aerial structures (Bleichrodt et al. 2013). This indicates that the pigmentation genes can only affect the size of micro-colonies via their impact on surface properties of conidia. Conidia of the Δ*brnA* and the Δ*fwnA* strains were similar in size and displayed a similar hydrophobicity as the control strain. They did show a trend towards higher negative surface charge as indicated by the zeta potential. The rodlets were still present at the spore surface of these pigmentation mutants. The properties of the conidia of the Δ*olvA* strain were distinct from that of the control strain. In contrast to conidia of CB-A111.1, conidia of 3-day-old cultures of the Δ*olvA* strain were larger, more negatively charged, and highly hydrophilic. Moreover, rodlets formed by hydrophobins were almost completely absent. The latter is a remarkable finding. It may be that the pigment in the cell wall of the conidia affects assembly of hydrophobins. Assembly of the SC3 hydrophobin of *Schizophyllum commune* is promoted by glucan polymers in the cell wall (Scholtmeijer et al. 2009). Spore pigments may do the same but the effect may also be indirect for instance by promoting the interaction between glucan and hydrophobin. The differences in biophysical and structural properties of the Δ*olvA* strain do not result in differences in incidence and size distribution of micro-colonies in the liquid shaken cultures when compared to the other pigmentation mutants. Previously, it has been shown that hydrophilicity of conidia and absence of the rodlet layer contributes to smaller micro-colonies in *A. nidulans* (Dynesen & Nielsen, 2003). This was not the case in *A. niger.*

Possibly, different mechanisms underlie micro-colony formation in *A. nidulans* and *A. niger.* However, the differences may also be due to different growth conditions. It has been shown that the type and concentration of the carbon source, the levels of nitrogen and phosphate, trace elements, dissolved oxygen and carbon dioxide, as well as pH and temperature affect the morphology of the culture. Moreover, the geometry of the flask or bioreactor, the agitation system, the rheology and the type of culture (batch, fed-batch or continuous) impact the morphology of the mycelium (Papagianni 2004).

The periphery of macro-colonies consists of a population of hyphae that show a high transcriptional and translational activity and a population of hyphae that show a lower transcriptional and translational activity (Vinck et al. 2011). Similar results were obtained with micro-colonies formed within liquid cultures. By quantifying fluorescence of the reporters GFP and dTomato it was shown that relative expression levels of *gpdA* and genes encoding secreted proteins correlated in individual hyphae at the periphery of micro-colonies. As expected, the highest correlation was found when GFP and dTomato were expressed in the same strain from the same promoter (correlation coefficient 0.8). The correlation of expression of the XlnR regulated genes *faeA* and *aguA* and the AmyR regulated genes *glaA* and *aamA* were also highly significant and ranged between 0.56 and 0.8. Thus, relative expression of *gpdA,* and AmyR and XlnR regulated genes correlate. The distribution of expression of genes encoding secreted proteins can be explained by the existence of two distinct populations of hyphae at the periphery of macro-colonies. The presence of such populations was also observed in micro-colonies. However, the distributions were skewed to the right (i.e. a relatively low number of highly fluorescent hyphae were observed). Log-transformation of the fluorescence intensities resulted in normal distributions of expression of the reporters in most of the cases. In contrast, bimodal distributions were still obtained after log-transformation of fluorescence intensities of individual hyphae at the periphery of macro-colonies of *Aspergillus oryzae* expressing GFP from the *A. niger glaA* or *aguA* promoter (Bleichrodt et al. 2012, Chapter 4). This indicates that heterogeneity of expression of genes at the periphery of the micro-colonies is less robust as observed in macro-colonies. Possibly, signaling between hyphae is involved in promoting heterogeneity. In contrast to solid media, gradients of signaling molecules cannot be formed between hyphae that are grown in liquid shaken cultures. Growth of individual hyphae in micro-channels may give proof for a role of signaling molecules in heterogeneity of gene expression in aspergilli.

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CHAPTER 4

Abstract

Hyphae of higher fungi are compartmentalized by septa. These septa contain a central pore that allows for inter-compartmental and inter-hyphal cytoplasmic streaming. The cytoplasm within the mycelium is therefore considered to be a continuous system. In this study, however, it was demonstrated by laser dissection that 40 % of the apical septa of exploring hyphae of *Aspergillus oryzae* are closed. Closure of septa correlated with the presence of a peroxisome-derived organelle, known as Woronin body, near the septal pore. The location of Woronin bodies in the hyphae was dynamic and, as a result, plugging of the septal pore was reversible. Septal plugging was abolished in a Δ*Aohex1* strain that cannot form Woronin bodies. Notably, hyphal heterogeneity was also affected in the Δ*Aohex1* strain. Wild-type strains of *A. oryzae* showed heterogeneous distribution of GFP between neighboring hyphae at the outer part of the colony when the reporter was expressed from the promoter of the glucoamylase gene *glaA* or the α-glucuronidase gene *aguA*. In contrast, GFP fluorescence showed a normal distribution in the case of the Δ*Aohex1* strain. Taken together, it is concluded that Woronin bodies maintain hyphal heterogeneity in a fungal mycelium by impeding cytoplasmic continuity.

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Hyphal heterogeneity in *Aspergillus oryzae* is maintained by dynamic closure of septa by Woronin bodies

Introduction

Cellular heterogeneity within an isogenic cell population is common in prokaryotic and eukaryotic organisms. Heterogeneity of cells can be beneficial for organisms in many ways. For instance, it is important for cell specialization (Kaech & Wherry 2007) and it may function in the survival of (members of) the population under adverse conditions (Nobile & Mitchell 2007, Veening et al. 2008). It has been shown that hyphae within the mycelium of fungi are also heterogeneous. Heterogeneity has been observed in the case of gene expression, growth and secretion (Wösten et al. 1991, Moukha et al. 1993ab, Teertstra et al. 2004, Vinck et al. 2005, 2011, Masai et al. 2006, Levin et al. 2007ab, Kasuga & Glass 2008, Etxebeste et al. 2009, de Bekker et al. 2011ab, Chapter 2 and 3). For instance, only part of the hyphae at the periphery of a vegetative mycelium of *Aspergillus niger* secretes glucoamylase (Wösten et al. 1991). This is due to heterogeneous expression of *glaA* in this part of the colony. In fact, two populations of hyphae can be distinguished; one that highly and one that lowly expresses this gene (Vinck et al. 2005). Hyphae that show high *glaA* expression also highly express other genes encoding secreted proteins. Moreover, they have a high rRNA content and highly express the glyceraldehyde-3-phosphate dehydrogenase gene *gpdA* (Vinck et al. 2011). From these studies it was concluded that two populations of hyphae can be distinguished at the outer part of the vegetative mycelium; one with a "high" and one with a "low" transcriptional and translational activity. The low activity would be sufficient to support growth but a high activity would be needed to support secretion of high amounts of protein.

Hyphae of Ascomycota and Basidiomycota are compartmentalized by porous septa. The diameter of the septal pore varies between 50 and 500 nm, which allows for passage of

cytosol and even organelles (Shatkin & Tatum 1959, Moore & McAlear 1962, Gull 1978, Lew 2005). Upon mechanical injury, septa of ascomycetes are plugged by Woronin bodies to prevent excessive cytoplasmic bleeding (Trinci & Collinge 1974, Collinge & Markham 1985, Jedd & Chua 2000, Tenney et al. 2000, Soundararajan et al. 2004, Maruyama et al. 2005, Dhavale & Jedd 2007). The Woronin bodies of *Aspergillus nidulans* are usually located at the septal pore or in apical regions (Momany et al. 2002). In the case of *Neurospora crassa,* they are tethered via WSC and Leashin to the cell cortex (Ng et al. 2009, Jedd 2011). Woronin bodies originate from peroxisomes by Pex11- and WSC-mediated budding (Jedd & Chua 2000, Liu et al. 2008, Escaño et al. 2009). Pex14 functions in biogenesis of Woronin bodies in *N. crassa* by playing a role in import of HEX1 in the organelle (Managadze et al. 2007). The lumen of Woronin bodies is filled with hexagonal rods of this HEX1 protein (Jedd & Chua 2000, Hoch & Maxwell 1974, Managadze et al. 2010). Deletion of *HEX1* in *N. crassa*, *Magnaporthe grisea* and *A. oryzae* results in the absence of Woronin bodies (Jedd & Chua 2000, Tenney et al. 2000, Soundararajan et al. 2004, Maruyama et al. 2005).
Here, it is shown that Woronin bodies of *A. oryzae* not only plug septa in damaged hyphae but also in intact growing hyphae. By doing so, they maintain hyphal heterogeneity in a fungal mycelium by impeding cytoplasmic continuity.

Materials and methods

Strains and growth conditions

Strains used in this study are listed in Table 1. RIB40 was used as wild-type strain (Machida et al. 2005). Strain NSRKu70-1-1A (Escaño et al. 2009) is a derivative of NSRKu70-1-1 (Δ*ku70 niaD*$^{-}$ *sC*$^{-}$ *adeA*$^{-}$; Escaño et al. 2009) expressing the *adeA* selection marker gene. NSRKu70-1-1AS (Tanabe et al. 2011) is a derivative of NSRKu70-1-1A, which has been transformed with the *sC* selection marker gene. NS4 (*niaD*$^{-}$ *sC*$^{+}$) (Yamada et al. 1997) is the parental strain of NSR13 (*niaD*$^{-}$ *sC*$^{-}$ *adeA*$^{-}$) (Jin et al. 2004). Inactivation of *Aohex1* in strain NSRKu70-1-1A, or in NSR13, resulted in strains NSRK-ΔHx5 and NSR-ΔHx11, respectively. Strain 11NSR-NAGHs expresses EGFP-AoHex1 under control of the *amyB* promoter in strain NSR-ΔHx11. Strains RB#140.1, RB#140.2, RB#154.3 and RB#154.4 are derivatives of *A. oryzae* NSRKu70-1-1AS (Δ*ku70*). The former two express sGFP under the control of the *A. niger glaA* promoter, while the latter two express sGFP under the control of the *A. niger aguA* promoter. Similarly, RB#141.3 and RB#141.4, express sGFP under the control of the *A. niger glaA* promoter, while RB#156.2 and RB#156.4 express sGFP under the control of the *A. niger aguA* promoter, but these strains are derivatives of *A. oryzae* NSRK-ΔHx5 (Δ*ku70* Δ*Aohex1*).

Table 1. Strains used in this study.

Strain	*Parental Strain*	*Genotype*	*Auxotrophy*	*Reporter plasmid*	*Reference*
RIB40		Wild-type			Machida et al. 2005
NSR13	NS4		*niaD*$^-$ *sC*$^-$ *adeA*$^-$		Jin et al. 2004
NSRKu70-1-1A	NSRKu70-1-1	Δ*ku70*	*niaD*$^-$ *sC*$^-$		Escaño et al. 2009
NSRKu70-1-1AS	NSRKu70-1-1A	Δ*ku70*	*niaD*$^-$		Tanabe et al. 2011
NSRK-ΔHx5	NSRKu70-1-1A	Δ*ku70* Δ*Aohex1*	*niaD*$^-$		This study
NSR-ΔHx11	NSR13	Δ*Aohex1*	*niaD*$^-$ *sC*$^-$		This study
11NSR-NAGHs	NSR-ΔHx11	Δ*Aohex1*	*sC*$^-$	PUNAGHs	This study
RB#140.1	NSRKu70-1-1AS	Δ*ku70*		pAN52-10S65TGFPn/s	This study
RB#140.2	NSRKu70-1-1AS	Δ*ku70*		pAN52-10S65TGFPn/s	This study
RB#141.3	NSRK-ΔHx5	Δ*ku70* Δ*Aohex1*		pAN52-10S65TGFPn/s	This study
RB#141.4	NSRK-ΔHx5	Δ*ku70* Δ*Aohex1*		pAN52-10S65TGFPn/s	This study
RB#154.3	NSRKu70-1-1AS	Δ*ku70*		P*aguA*_sGFP+	This study
RB#154.4	NSRKu70-1-1AS	Δ*ku70*		P*aguA*_sGFP+	This study
RB#156.2	NSRK-ΔHx5	Δ*ku70* Δ*Aohex1*		P*aguA*_sGFP+	This study
RB#156.4	NSRK-ΔHx5	Δ*ku70* Δ*Aohex1*		P*aguA*_sGFP+	This study

To obtain spores for inoculation, *A. oryzae* was grown on 3.7 % PDA (Potato Dextrose Agar, Sigma Aldrich, www.sigmaaldrich.com). Spores were harvested in 0.9 % NaCl (w/v) containing 0.05 % (v/v) Tween-20 and diluted to a final concentration of 5 10^5 spores ml^{-1}. For microscopy, *A. oryzae* was grown in glass bottom dishes (MatTek, www.glass-bottom-dishes.com, P35G-1.5-20-C) essentially as described by van Peer et al. (2009). Strains with nitrate prototrophy were grown on CD + Met medium (0.3 % $NaNO_3$, 0.2 % KCl, 0.1 % KH_2PO_4, 0.05 % $MgSO_4.7H_2O$, 0.002 %, $FeSO_4.7H_2O$, 1 % glucose, 0.0015 % methionine, pH 5.5; Maruyama et al. 2010), while strains with nitrate auxotrophy (*niaD*$^-$) were grown on M medium (0.2 % NH_4Cl, 0.1 % $(NH_4)_2SO_4$, 0.05 % KCl, 0.05 % NaCl, 0.1 % KH_2PO_4, 0.05 % $MgSO_4.7H_2O$, 0.002 % $FeSO_4.7H_2O$, 1 % glucose, pH 5.5; Ohneda et al. 2005). The glass bottom dishes were filled with 30 µl CD + Met medium or M medium containing 1 % agarose. To this end, the glass bottom dishes and the agar medium were pre-warmed at 50 °C. Spores (250 spores in 0.5 µl) were placed in the middle of an 18 mm cover glass and placed upside down on the non-solidified agarose medium. This resulted in a 118 µm thin agarose layer. After solidifying the agarose medium, 2 ml liquid medium was added on top of the culture.

For septal plugging experiments, cultures were grown for 2 days at 30 °C under water saturating conditions, after which they were either or not exposed to stress conditions. Temperature stress was imposed by incubation at 4 °C or 45 °C for 30 min. For pH stress, the

liquid medium was replaced by CD + Met medium with a pH of 2.0 or 11.0 and subsequent incubation for 1.5 h. To this end, the pH was adjusted with HCl or NaOH, respectively. Starvation stress was imposed from t = 0 onwards during 2 days of growth. For carbon-starvation, solid medium contained 0.2 % glucose and liquid medium contained no glucose. For nitrogen-starvation solid medium contained nitrate/ammonium (see above), but liquid medium did not contain a nitrogen source.

Inactivation of the Aohex1 gene

The 1.9 kb upstream flanking region of the *Aohex1* open reading frame was amplified with primers aB4F and aB1R (Table 2) and using RIB40 genomic DNA as a template. The fragment was inserted into pDONR™ P4-P1R (Invitrogen, www.invitrogen.com) by BP recombination reaction generating the 5' entry clone plasmid, pg5'Ahx1. The 1.6 kb downstream flanking region of *Aohex1* was amplified with primers aB2F and aB3R (Table 2) and inserted into pDONR™ P2R-P3 (Invitrogen) by BP recombination reaction generating the 3' entry clone plasmid, pg3'Ahx1. The 5' and 3' entry clones together with the center entry clone pgEsC (containing the *sC* marker gene) were subjected with LR clonase in the presence of pDEST R4-R3 (destination vector) to obtain the final plasmid pgΔdAoHex1. The *Aohex1* gene deletion fragment (~6.5 kb) was amplified by PCR using primers aB4F and aB3R and pgΔdAoHex1 as template. Strain NSRKu70-1-1A (Escaño et al. 2009) was transformed with the deletion fragment as described (Ohneda et al. 2005). Transformants with the *sC*$^+$ phenotype were selected on M medium containing 1.5 % agar and 2 % glucose. Disruption of the *Aohex1* gene in strain NSRK-ΔHx5 was confirmed by Southern blotting using restriction enzymes *Bam*HI and *Eco*T221. Plasmid pgEsC containing the *sC* selection marker gene was introduced into *A. oryzae* strain NSRKu70-1-1A generating the control strain NSRKu70-1-1AS.

Table 2. Primers used in this study.

Primer name	*Sequence*
aB4F	GGGGACAACTTTGTATAGAAAAGTTGGGACCAATGCGACCATGAAG
aB1R	GGGGACTGCTTTTTTGTACAAACTTGCAGTAGTAGTGCTAAGAACCTTGAC
aB2F	GGGGACAGCTTTCTTGTACAAAGTGGATCATTTTCCCGCTTTGATCTGGTC
aB3R	GGGGACAACTTTGTATAATAAAGTTGCAAGATTCTAGGTGCTTGGTTTGAG
5-hex1-F	GGGGACAACTTTGTATAGAAAAGTTGGGACCAATGCGACCATGAAG
5-hex1-R	GGGGACTGCTTTTTTGTACAAACTTGCAGTAGTAGTGCTAAGAACCTTGAC
3-hex1-F	GGGGACAGCTTTCTTGTACAAAGTGGATCATTTTCCCGCTTTGATTCGGTC
3-hex1-R	GGGGACAACTTTGTATAATAAAGTTGCAAGATTCTAGGTGCTTGGTTTGAG

The *Aohex1* deletion vector pgDAHx1 was also constructed using the Multisite Gateway cloning system (Invitrogen) (Mabashi et al. 2006). The upstream (2.0 kb) and downstream (2.0 kb) regions of the *Aohex1* gene were amplified by PCR using the primer combinations 5-hex1-F and 5-hex1-R, and 3-hex1-F and 3-hex1-R, respectively (Table 2). The amplified upstream and downstream regions of *Aohex1* were introduced into pDNORP4-P1R and pDONRP2R-P3, respectively, with the Gateway BP clonase reaction. The resulting plasmids were subjected to Gateway LR clonase reaction together with the center entry clone plasmid containing the *A. oryzae adeA* gene as a selection marker (Jin et al. 2007) and the destination vector pDESTR4-R3 (Invitrogen). The resulting plasmid pgDAHx1 was used as a template to amplify the deletion cassette by PCR with the primers 5-hex1-F and 3-hex1-R. The amplified deletion fragment was introduced into *A. oryzae* NSR13 according to Maruyama and Kitamoto (2011). A representative transformant was selected in M medium for *adeA* prototrophy and named NSR-ΔHx11. Disruption of the *Aohex1* gene was confirmed by Southern analysis. For this, genomic DNA was digested with *Bam*HI and *Nco*I and the 2.0 kb fragment of the *Aohex1* upstream region was used as a probe.

Expression of EGFP-AoHex1

The expression vector pUNAGHs (Juvvadi et al. 2007) encompassing the fusion of *EGFP* and *Aohex1* under the control of the *amyB* promoter was introduced into NSR-ΔHx11 using *niaD* as a selection marker according to (Maruyama & Kitamoto, 2011). Strain 11NSR-NAGHs (Table 1) showed fluorescence representative for the transformants obtained.

Expression of sGFP under control of the glaA and aguA promoters

A. oryzae strains NSRKu70-1-1AS and NSRK-ΔHx5 were co-transformed with pAN52-10S65TGFPn/s (Siedenberg et al. 1999) and pNR10 (Yoon et al. 2010) or with P*aguA*_sGFP+ (Vinck et al. 2011) and pNR10 as described (de Bekker et al. 2009, Punt & van den Hondel 1992). These plasmids contain *sGFP*(S65T) under the regulation of the *glaA* (pAN52-10S65TGFPn/s) or *aguA* (P*aguA*_sGFP+) promoter of *A. niger* and *niaD* (pNR10) under the control of the *amyB* promoter of *A. oryzae*. Nitrate prototrophic (*niaD*$^{+}$) strains were selected on MMS medium (minimal medium pH 6.0, 0.95 M sucrose and 1.5 % agar; de Bekker et al. 2009).

Fluorescence microscopy

The reporter protein EGFP-AoHex1 was monitored over periods of up to 16 h on a Zeiss confocal laser scanning microscope (CSLM; Zeiss LSM 5 PASCAL; Zeiss, www.zeiss.com) using a 488 nm laser and a LP505 filter. For live cell imaging, images were taken in the Z-plane in three slices (slice thickness 0.4 μm) every 15 min using a Plan-Neofluor 25×/0.8 Imm corr objective. The pixel time was 2.51 μs, the laser power 0.125 mW, and the pinhole

136 µm. Images were taken with a resolution of 512 × 512 pixels and exported as tiff files using the Zeiss LSM Image Browser v4.2 (www.zeiss.co.jp). Composition and layout of exported images was made with GIMP v2.6 (http://www.gimp.org/).

Heterogeneous expression of sGFP under the control of the glaA and aguA promoters

A. oryzae was grown as a sandwiched culture (de Bekker et al. 2011a) on minimal medium (0.6 % $NaNO_3$, 0.15 % KH_2PO_4, 0.05 % KCl, 0.05 % $MgSO_4.7H_2O$, 0.2 ml/l Vishniac [per liter: 10 g EDTA, 4.4 g $ZnSO_4$, 1.01 g $MnCl_2$, 0.32 g $CoCl_2$, 0.315 g $CuSO_4$, 0.22 g ammonium heptamolybdate, 1.47 g $CaCl_2$ and 1.0 g $FeSO_4$; Vishniac & Santer 1957], pH 6.0; de Vries & Visser 1999) containing 3 % agar and 200 mM xylose (*glaA* repressing) or 50 mM glucose (*aguA* repressing). To this end, 2 µl containing 1000 spores was spotted at the center of a polycarbonate membrane (76 mm; Profiltra, www.profiltra.nl) that was placed on top of the solidified medium. After 24 h a Lumox membrane (20 × 20 mm, manually cut; Greiner Bio-One, www.greinerbioone.com) was placed on top of the culture with the hydrophobic side facing the colony. After 42 h of growth, the sandwiched colony was transferred for 6 h to minimal medium plates containing 25 mM maltose (inducing *glaA*) for strains RB#140.1, RB#140.2, RB#141.3 and RB#141.4. For strains RB#154.3, RB#154.4, RB#156.2 and RB#156.4 the sandwiched colony was transferred for 6 h to minimal medium plates containing 25 mM xylose (inducing *aguA*). The Lumox membrane was removed and a piece of the polycarbonate membrane (approximately 10 × 10 mm) carrying the colony was cut and placed up-side-down in a glass bottom microscopy dish (MatTek, P35G-1.5-20-C) on a 20 µl drop of minimal medium. GFP fluorescence was monitored on the Zeiss LSM 5 system equipped with a Plan-Neofluar 16×/0.5 oil immersion objective. GFP was excited with a 488 nm laser and images were captured as a Z-stack of optical slices (pinhole 1-2 airy units; optimal interval 2.02 mm; 4× line average; 8 bit scan depth). Maximum intensity projections of the Z-sections (1024 × 1024 pixels) were used for further analysis. The fluorescence intensity was quantified by measuring the mean pixel value of hyphae using a macro in the KS400 software (Version 3.0; Carl Zeiss Vision, www.zeiss.de). Sections of 50-100 µm of leading hyphae were selected by hand and fluorescence was automatically quantified as the sum grey value per hypha with the background value from an equivalent area subtracted (Vinck et al. 2005). Signals were normalized with a custom Python script by dividing single hyphal fluorescence by the total fluorescence of all selected hyphae per image. To examine whether hyphal fluorescence followed a bimodal distribution, the normalized data were log transformed and subsequently modeled using 5 parameters (p, μ_1, σ_1, μ_2 and σ_2) as described (Vinck et al. 2005). 95 % Confidence Intervals (CI) of the parameters were estimated by bootstrapping (1000 replicates). Customs scripts in the Scilab programming language were used to fit the data. (http://web.science.uu.nl/microbiology/images/fung/fittools.zip; http://web.science.uu.nl/microbiology/images/fung/manual%20fittools.pdf).

Analysis of plugging

Compartments were ruptured by laser dissection using the laser pressure catapulting function (LPC) of the PALM laser dissection microscope (Zeiss, www.zeiss.com). To this end, 60-70 % of the power of the pulsed UV-laser was used. Each experiment was done *in duplo* using 20 hyphae in each experiment. ANOVA analysis was used with Bonferroni post-hoc correction when multiple comparisons were made between treatments. ANOVA analysis was used with Dunnet's post-hoc correction when differences in septal plugging in a strain were assessed between a control condition and stress conditions. In all cases, a difference was assumed significant when $p < 0.05$.

Results

Septal closure during vegetative growth is not dependent on environmental conditions

It was assessed whether septa in intact growing hyphae are open or closed. To this end, the wild-type *A. oryzae* RIB40 strain was grown for 2 days at 30 ºC in a glass bottom microscopy dish in CD + Met medium. The apical compartments of hyphae at the most outer part of RIB40 colonies were dissected using a UV laser and simultaneously it was monitored whether cytoplasm from the sub-apical, adjacent compartment was streaming through the septum of the damaged compartment. The septum was scored as 'open' when cytoplasm was streaming through the septum towards the ruptured compartment (see Supplemental Movie 1). When cytoplasmic movement quickly ceased within seconds, the septum was scored 'quickly closed' (Supplemental Movie 2). In the case cytoplasmic streaming was not observed, the septum was scored as 'closed' (Supplemental Movie 3). Using these criteria it was found that 57.5 % ± 10.6 of the apical septa of the wild-type *A. oryzae* strain were open (this included the septa that were scored quickly closed) (Figure 1). In more detail, 5 % ± 7.1, 52.5 % ± 17.7 and 42.5 % ± 10.6 of the septa were scored as 'open', 'quickly closed' and 'closed', respectively. Similar percentages of open septa were found for the second and third septa (67.5 % ± 3.5 and 50 % ± 7.1, respectively; Figure 1).

To assess whether the septal plugging state of neighboring septa correlates, the first three compartments of hyphae were sequentially dissected. It was observed that neighboring septa of closed compartments can be either open or closed (Table 3). Taken together, these data show that there is no difference in septal plugging incidence between the first three septa of intact growing hyphae at the periphery of an *A. oryzae* RIB40 colony and that closure of neighboring septa does not correlate.

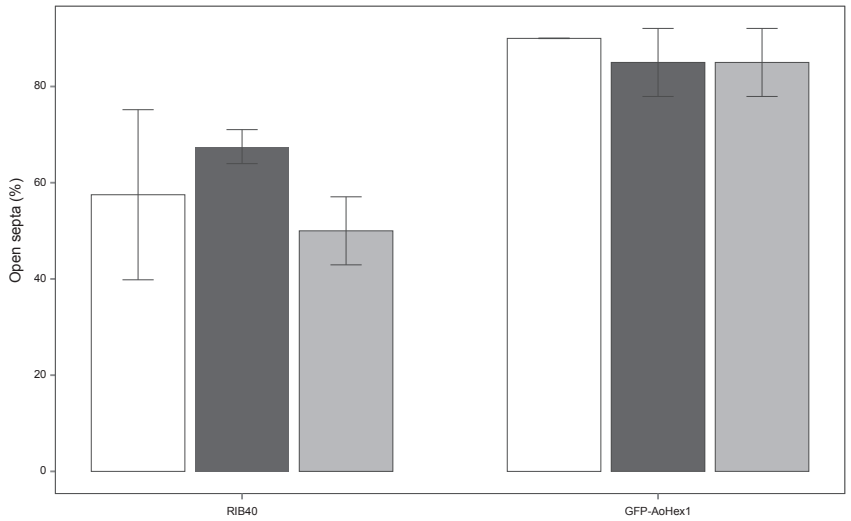

Figure 1. Percentage of open septa of leading hyphae of the wild-type *A. oryzae* strain RIB40 and the Δ*Aohex1* strain that had been complemented with EGFP-AoHex1. White, black and grey bars show percentages of the first, second and third septa, respectively. Experiments were done with 20 hyphae in duplicate. Bars represent standard deviations.

Table 3. Septal plugging of the three most apical septa is independent. 1 = septum open, 0 = septum closed.

Hypha number	*First septum*	*Second septum*	*Third septum*
1	1	1	1
2	0	0	0
3	1	1	1
4	0	1	0
5	0	0	0
6	0	0	1
7	0	1	0
8	1	0	0
9	1	0	1
10	0	0	0

A. oryzae RIB40 was grown on CD + Met medium for 2 days at 30 ºC, after which it was subjected to 4 °C or 45 °C, hypo- or hypertonic conditions, or pH 2.0 or 11.0. Alternatively, RIB40 was grown for 2 days with C- or N-limitation. Laser dissection showed that none of the conditions significantly influenced the septal plugging incidence of the first (*i.e.* the apical) septum (Figure 2). After incubating the mycelium of RIB40 for 30 min at 4 ºC or

45 ºC, 72.5 % ± 3.5 and 65 % ± 0 of the septa were open, respectively (Figure 2). When it was subjected to 1 M $MgSO_4$ or to H_2O for 1.5 h, 57.5 % ± 3.5 and 77.5 % ± 3.5 of the septa were open, respectively. Similarly, 62.5 % ± 10.6 and 67.5 % ± 10.6 of the septa were open after incubation of the mycelium at pH 2.0 or pH 11.0 for 1.5 h, respectively. These numbers were 57.5 % ± 10.6 and 70 % ± 7.1, when the mycelium was exposed to carbon- and nitrogen starvation for 2 days (Figure 2).

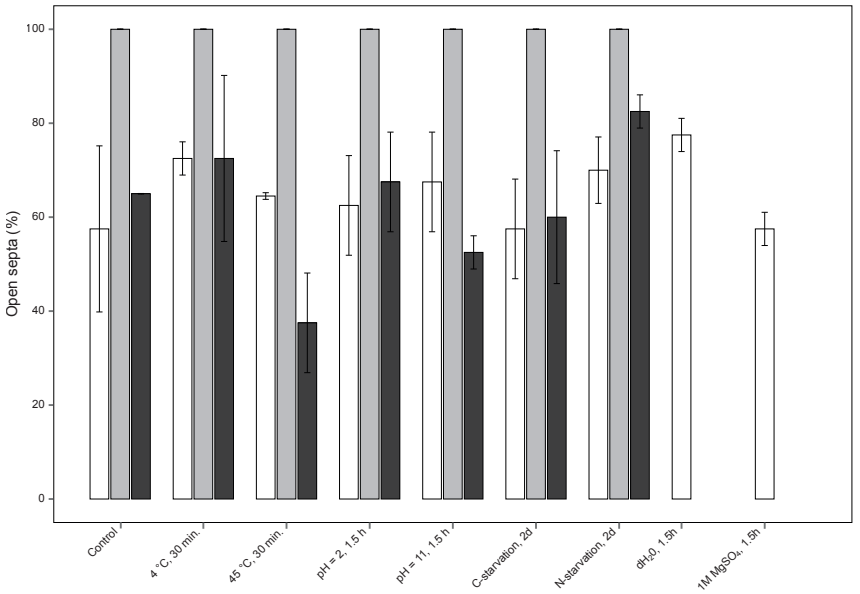

Figure 2. Percentage of open apical septa of leading hyphae of the wild-type *A. oryzae* strain RIB40 (white bars), control strain NSRKu70-1-1AS (black bars) and the Δ*Aohex1* strain NSRK-ΔHx5 (grey bars) under various environmental conditions. Strains were grown for 2 days on CD + Met medium (RIB40) or M medium (NSRKu70-1-1AS and NSRK-ΔHx5), after which they were exposed to 4 °C or 45 °C, pH 2.0 or 11.0, C- or N-limitation, and in the case of RIB40, to hypo- or hypertonic conditions. Experiments were done with 20 hyphae in duplicate. Bars represent standard deviations.

Woronin bodies are responsible for septal closure during vegetative growth

The *A. oryzae* Δ*Aohex1* deletion strain NSRK-ΔHx5 was grown for 2 days at 30 ºC on M medium and was exposed to standard environmental conditions and to 4 °C, 45 °C, pH 2.0, pH 11.0, and to carbon- and nitrogen starvation. In all cases, 100 % ± 0 of the apical septa were open (Figure 2). In contrast, on average 62 % ± 3.5 of the septa of the control strain NSRKu70-1-1AS were open during these conditions. Notably, cytoplasmic movement was observed throughout at least seven compartments after damaging the apical compartment of leading hyphae of NSRK-ΔHx5. In contrast, cytoplasmic movement was only observed in the second and sometimes the third compartment of strain NSRKu70-1-1AS.

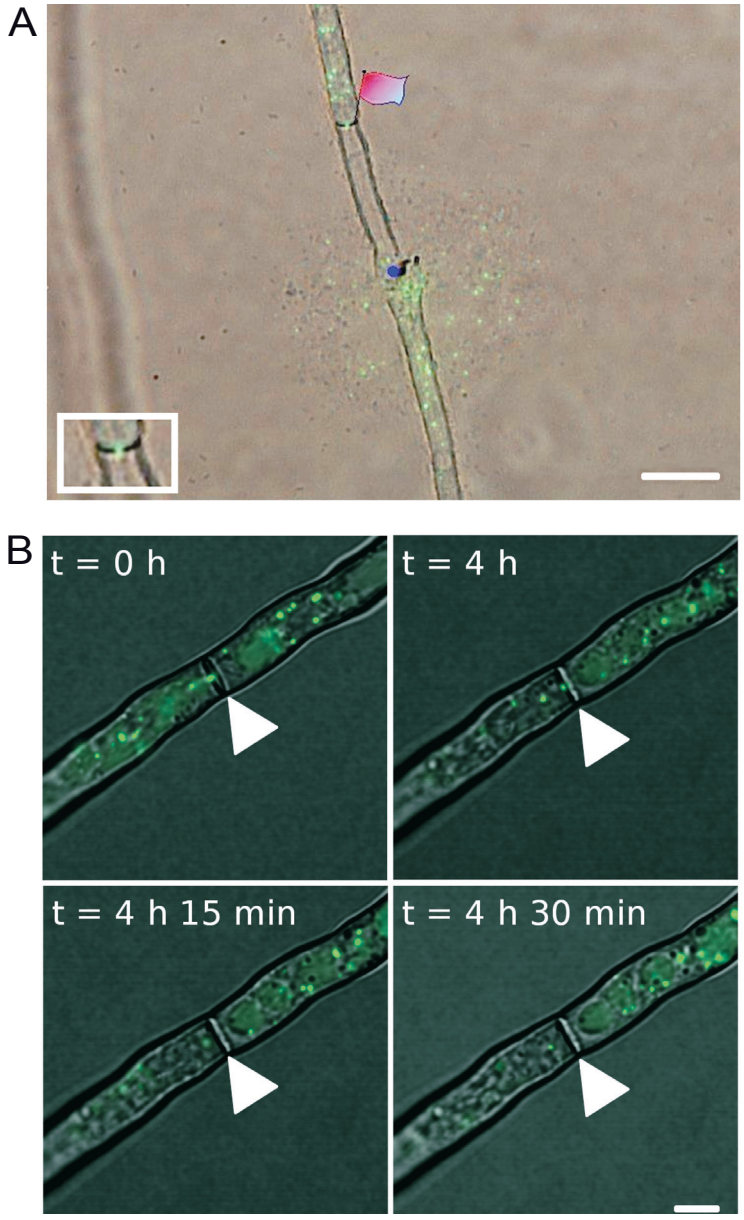

Figure 3. Woronin bodies close septa reversibly. Strain 11NSR-NAGHs was grown for 2 days on CD + Met medium and a septum with a Woronin body (tagged with EGFP-AoHex1) was selected. (A) No cytoplasmic streaming was observed through the septum (indicated by the flag) after dissection of the hypha at the spot indicated by the blue dot. Inset shows a magnification of the Woronin body plugging the septum. (B) The location of a Woronin body at the septum (arrowhead) was monitored in time using CLSM. After 4 hours the Woronin body was still localized at the septum but 15 minutes later the organelle had lost its position. Bar represents 25 (A) and 5 (B) µm, respectively.

Septal closure in strain NSRK-ΔHx5 was partially rescued after introduction of a vector encompassing a gene encoding EGFP-AoHex1 under control of the *amyB* promoter. In the resulting strain 11NSR-NAGHs 90 % ± 0, 85 % ± 7.1 and 85 % ± 7.1 of the first, second and third septa of the leading hyphae, respectively, were open under standard growth conditions (Figure 1). The partial complementation of AoHex1 by EGFP-AoHex1 may be caused by a lower binding of the fusion protein to WSC when compared to the native AoHex1 protein. This binding is needed for proper localization of the Woronin body at the septal pore. Septa of the 11NSR-NAGHs strain with a Woronin body localized at the septal pore (visualized by the reporter protein EGFP-AoHex1) were always closed (Figure 3A). In contrast, in the absence of a Woronin body, the septum was open in 100 % of the cases. These results show that Woronin bodies of *A. oryzae* close septa during vegetative growth under various environmental conditions. CLSM live cell imaging of three hyphae showed that Woronin body localization at the septum is dynamic. Woronin bodies moved away from the septum between 15 minutes to several hours of growth (Figure 3B).

Heterogeneous distribution of GFP resulting from glaA- and aguA-driven expression is abolished in a ΔAohex1 strain

Constructs pAN52-10S65TGFPn/s (Siedenberg et al. 1999) and P*aguA*sGFP+ (Vinck et al. 2011) encompassing the *sGFP* gene under control of the *glaA* and *aguA* promoter of *A. niger,* respectively, were introduced in strains NSRKu70-1-1AS and NSRK-ΔHx5 of *A. oryzae.* Transformants were screened by fluorescence microscopy and two representative strains of each transformation were selected. These strains were called RB#140.1 and RB#140.2 (*glaA,* Δ*ku70* background); RB#154.3 and RB#154.4 (*aguA,* Δ*ku70* background); RB#141.3 and RB#141.4 (*glaA,* Δ*ku70*Δ*Aohex1* background); and RB#156.2 and RB#156.4 (*aguA,* Δ*ku70*Δ*Aohex1* background). Hyphal heterogeneity of GFP accumulation at the outer part of colonies of these strains was assessed by modeling log transformed fluorescence intensity distributions assuming the existence of two populations of hyphae (*i.e.* one with high and one with low GFP fluorescence) (Figure 4). For this, 50-100 µm sections of apical compartments of hyphae were measured. The confidence intervals of the mean in populations with low (μ_1) and high (μ_2) GFP fluorescence were not overlapping in *A. oryzae* strains RB#140.1, RB#140.2; RB#154.3 and RB#154.4 that all contain Woronin bodies (Table 4). This showed that *glaA* and *aguA* driven expression of GFP is heterogeneous in these strains. In contrast, the confidence intervals of the means in strains RB#141.3, RB#141.4; RB#156.2 and RB#156.4, all having the *Aohex1* deletion, did overlap. Thus, the assumption that GFP distribution is heterogeneous in these strains was falsified (Figure 4, Table 4). Taken together, these data show that Woronin bodies maintain hyphal heterogeneity in the *A. oryzae* mycelium.

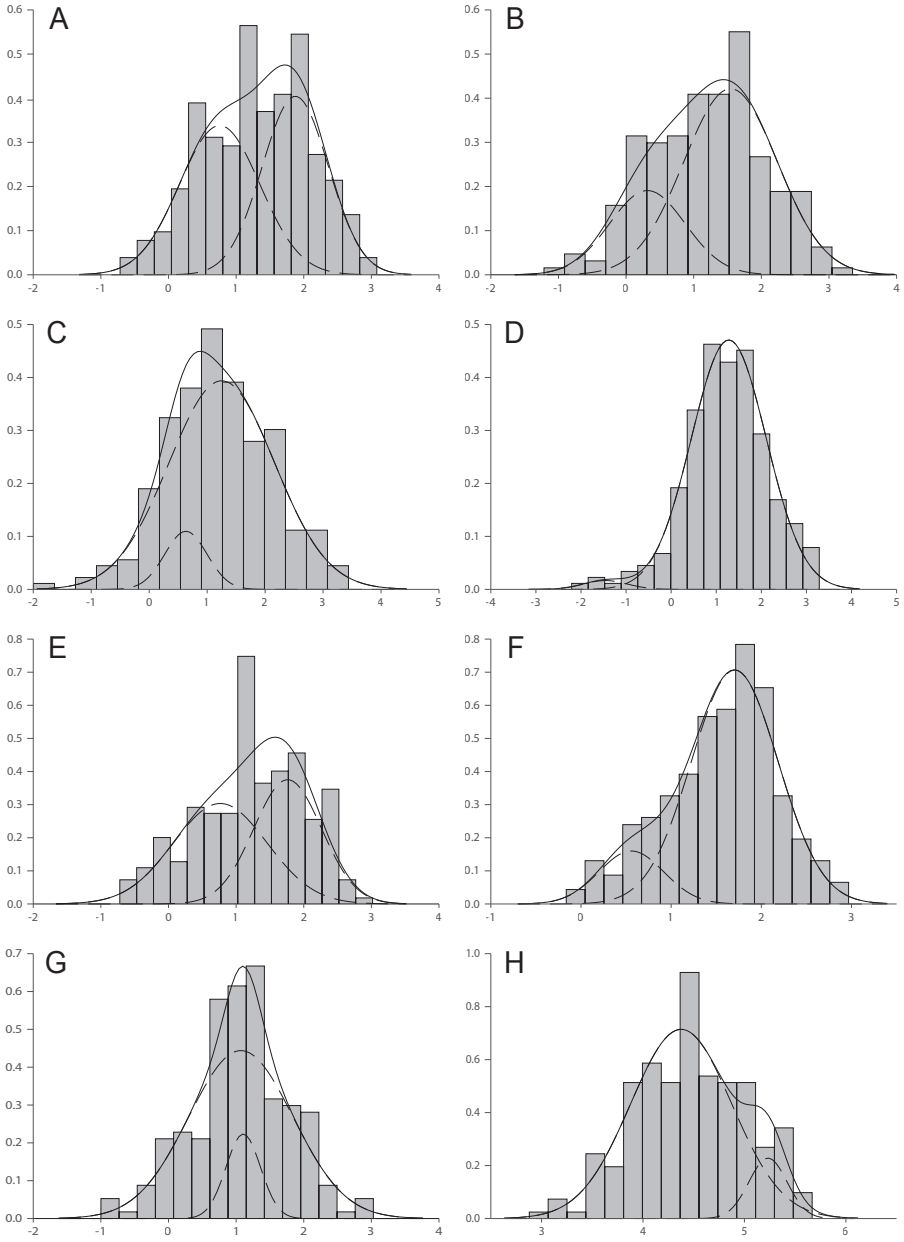

Figure 4. Fluorescence intensity distributions resulting from *glaA* driven GFP expression in *A. oryzae* strains with (A, B) and without Woronin bodies (Δ*Aohex1*) (C, D) and resulting from *aguA* driven GFP expression in *A. oryzae* strains with (E, F) and without Woronin bodies (Δ*Aohex1*) (G, H). Data (± 200 hyphae for each strain) were fitted assuming the existence of bimodal fluorescence distribution between the hyphae at the periphery of the mycelium after log transformation.

Table 4. 95 % confidence intervals (CI) of the mean assuming a bimodal fluorescence distribution of *glaA* or *aguA* driven GFP expression after log transformation in hyphae of strains with (RB#140.1 and RB#140.2 (*glaA*); RB#154.3 and RB#154.4 (*aguA*)) and without (RB#141.3 and RB#141.4 (*glaA*); RB#156.2 and RB#156.4 (*aguA*))

Strains	Background	N	CI (μ_1)	CI (μ_2)	CI (p)
RB#140.1	Δ*ku70*	203	-0.10 - 1.18	1.43 - 2.30	0.078 - 0.892
RB#140.2	Δ*ku70*	209	-0.18 - 1.19	1.23 - 2.71	0.041 - 0.955
RB#141.3	Δ*ku70* Δ*Aohex1*	247	-2.00 - 1.27	0.65 - 3.04	0.005 - 0.972
RB#141.4	Δ*ku70* Δ*Aohex1*	242	-2.21 - 1.31	1.01 - 2.73	0.007 - 0.942
RB#154.3	Δ*ku70*	220	-0.50 - 1.21	1.28 - 2.35	0.051 - 0.940
RB#154.4	Δ*ku70*	220	0.10 - 1.49	1.58 - 2.00	0.034 - 0.819
RB#156.2	Δ*ku70* Δ*Aohex1*	212	-0.86 - 1.18	0.97 - 2.81	0.017 - 0.974
RB#156.4	Δ*ku70* Δ*Aohex1*	220	-0.02 - 1.64	1.61 - 2.98	0.024 - 0.976

Woronin bodies. N = sample size, CI (μ_1) and CI (μ_2) represent the confidence intervals of the lower and upper limits of population 1 and 2, respectively. CI (*p*) represents the confidence interval of the lower- and upper limits of the participation fraction of population 1.

Discussion

Intercellular cytoplasmic connections have been identified in multicellular eukaryotic organisms belonging to the four classical kingdoms. In the case of animals, gap junctions provide the cytoplasmic contact. They mediate interchange of molecules < 1000 daltons. Plasmodesmata in plants provide intercellular transport of for instance photo-assimilates, mRNA and proteins. The pore sizes in septa of fungi are even larger. They allow inter-compartmental streaming of macromolecules and even organelles. Gap junctions and plasmodesmata can regulate their pore size and in this way function in developmental processes (Heinlein 2002, Norris et al. 2008). Little is known about closure of fungal septa. So far, it is generally believed that septa are open in intact fungal hyphae. In this view, the mycelium would consist of a continuous cytoplasm. Recently, it was shown that septa of *Schizophyllum commune* can already be plugged in intact growing hyphae (van Peer et al. 2009). It was demonstrated that apical septa are open in intact vegetative hyphae of this basidiomycete. In contrast, only 50 % and 10 % of the second and third septa are open, respectively. Septal plugging was shown to be reversible and to depend on environmental conditions (van Peer et al. 2009). A strain in which the *spc33* gene was inactivated did not form septal pore caps and, as a result, septal plugging was abolished (van Peer et al. 2010). In this study it was shown that septa of intact hyphae of the ascomycete *A. oryzae* can also be reversibly plugged. In this case, Woronin bodies are essential for closure of septa. These results refute the general view that a fungal mycelium consists of a continuous cytoplasm. It should be noted that it is not yet clear how general these results apply to other species of ascomycetes and basidiomycetes.

We do demonstrate that septal closure results in heterogeneity of cytoplasmic composition of neighboring hyphae within a mycelium of *A. oryzae*.

Using laser dissection it was shown that about 60 % of the first three septa of hyphae at the periphery of an *A. oryzae* colony are open. The plugging state of the first septum did not correlate with that of the second or the third septum. The plugging state also did not depend on the environmental conditions. Septa were always closed when a GFP tagged Woronin body had been localized at the septal pore. Conversely, septa were always open when a Woronin body was absent. This strongly indicated a role for Woronin bodies in plugging septa of intact vegetative hyphae. Indeed, all septa were open, irrespective of the growth conditions, in a Δ*Aohex1* strain that cannot form these organelles. Live cell imaging showed that Woronin body localization near septa was dynamic in intact vegetative hyphae. Taken together, these results imply that closure of septa in *A. oryzae* is a reversible process. In contrast to *S. commune,* there seems not to be a mechanism directing septal closure in intact vegetative hyphae. It seems to be a stochastic driven process.

The general accepted view that the cytoplasm is continuous in a fungal mycelium due to the porosity of septa (Jennings et al. 1974, 1987) is in conflict with hyphal heterogeneity as has been observed in *A. niger* (Wösten et al. 1991, Vinck et al. 2005, 2011, Levin et al. 2007ab, Etxebeste et al. 2009, de Bekker et al. 2011b, Chapter 2 and 3) and *A. oryzae* (Maruyama et al. 2006). Septal plugging in intact hyphae, however, explains why hyphae can be heterogeneous with respect to RNA and protein composition. The fact that each septum has a chance of 40 % to be closed implies that only in about 5 % of the cases the cytoplasm of two hyphae is in physical contact when they are separated by 6 septa. Absence of Woronin bodies would result in a continuous cytoplasm and consequently hyphal heterogeneity would be abolished. This hypothesis was tested. It was shown that GFP distribution was heterogeneous between neighboring hyphae of a wild-type *A. oryzae* strain when the reporter was expressed from the *A. niger glaA* and *aguA* promoters (see Figure 5A). Two populations of hyphae were distinguished: those with a high and those with a low GFP fluorescence. In contrast, populations of hyphae with low and high GFP content could not been shown to exist in the Δ*Aohex1* mutant (see Figure 5B). From this it is concluded that indeed hyphal heterogeneity is abolished in strains that do not form Woronin bodies. We propose that heterogeneous gene expression still occurs in the Δ*Aohex1* mutant. However, since all septa are open, cytoplasmic streaming evenly distributes gene products over the entire cytoplasm.

So far, it is not clear why colonies send out exploring hyphae that are heterogeneous with respect to transcriptional and translational activity. Possibly, this increases the chance that (some of the) hyphae survive when a colony is exposed to stress conditions like the presence of antibiotics, reactive oxygen species or high temperature. Plugging of septa by Woronin bodies would maintain diversity of RNA and protein composition between the hyphae, and thereby

would promote survival of the mycelium. In agreement with this, the *A. niger* Δ*Anhex1* strain died when exposed for three days to 45 °C, whereas the strain with Woronin bodies survived (Bleichrodt 2012). This effect could not be explained by more excessive bleeding (Data not shown).

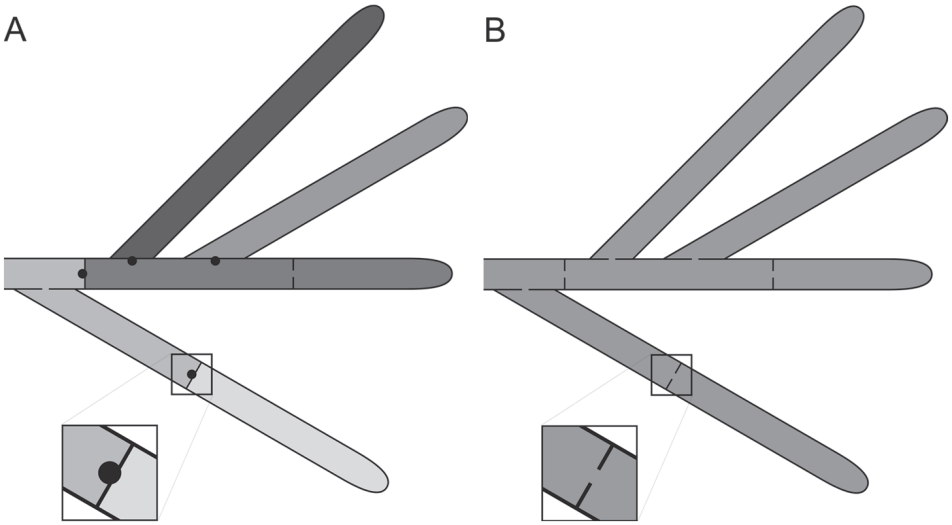

Figure 5. Diagram showing the effect of septal plugging on hyphal heterogeneity. Wild type (A) shows normal septal plugging levels that maintain the differences in RNA and / or protein levels between neighbouring hyphae**.** Hyphae are shown with different gene expression levels (indicated by differences in grayscale). Black dots indicate Woronin bodies that close septa (magnified in the inlay). The *hex1* mutant (B) shows homogeneous levels of gene products. Although there is heterogeneous gene transcription between neighbouring hyphae, these levels cannot be maintained. Since all septa are open (magnified in the inlay) cytoplasmic streaming distributes gene products evenly over the entire cytoplasm.

Acknowledgements

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CHAPTER 5

Abstract

Streptomycetes are proficient producers of enzymes and antibiotics. When grown in bioreactors, these filamentous microorganisms form micro-colonies that consist of interconnected hyphae. We here employed a flow cytometry approach designed for large particles (COPAS) and demonstrate that liquid-grown *Streptomyces* cultures consist of two distinct populations of micro-colonies. One population contains small micro-colonies with a constant diameter of around 260 μm, whereas the other population contains larger micro-colonies whose diameter varies between strains and medium composition. It is also shown that the cellulose synthase-like protein CslA, the cell surface proteins rodlins and chaplins, and the sigma factor BldN influence the size of the larger micro-colonies but not that of small micro-colonies. Considering the fact that production of enzymes and metabolites depends on micro-colony size, these results provide new leads towards rational strain design of *Streptomyces* strains tailored for industrial fermentations.

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Size fractionation of micro-colonies reveals two populations in liquid-grown *Streptomyces* cultures

Introduction

Streptomycetes are Gram-positive mycelial soil bacteria that are commercially very attractive for the production of a wide range of natural products such as antibiotics, anticancer agents and immunosuppressants (Hopwood 2007). Moreover, streptomycetes produce a plethora of enzymes that allow them to utilize almost any naturally occurring polymer, such as cellulose, mannan, chitin, xylan, starch, glycan and agar. Many of these enzymes are industrially important for the conversion of renewable energy sources (Vrancken & Anné 2009). Streptomycetes grow by means of hyphae that grow at their tips and that branch subapically. This results in the formation of millimeter scale macro-colonies or sub-millimeter micro-colonies depending on the size and the nature of the environment. The reproductive phase is initiated by the formation of aerial hyphae that differentiate into structures that form chains of spores. Aerial hyphae are formed on mycelium grown on solid medium (Flärdh & Buttner 2009, Kelemen & Buttner 1998). The transition from vegetative to aerial growth is coordinated by regulatory genes known as *bld* genes. They ensure that the formation of aerial hyphae only starts when environmental conditions are suitable to support their growth (Claessen et al. 2006, Flärdh & Buttner 2009, Nodwell et al. 1999). The extracytoplasmic function sigma factor BldN of *Streptomyces venezualea* binds to the promoters of the *chp* and *rdl* genes for chaplins and rodlins, respectively, thus suggesting that this activates their expression (Bibb et al. 2012). The chaplin proteins assemble into amyloid fibrils that are aligned by the rodlins into so called rodlets at the surface of aerial hyphae and spores. The amyloid fibrils of the chaplins provide surface hydrophobicity to the aerial structures (Claessen et al. 2002, 2003, 2004, Elliot et al. 2003).

Industrial-scale production with streptomycetes occurs in large bioreactors. Under this condition, micro-colonies are formed (Celler et al. 2012). Such micro-colonies can have different sizes. Elongation and branching of hyphae, as well as fragmentation and lysis are regarded as factors controlling the size of these multicellular structures (Nielsen et al. 1995, Nielsen 1996). Moreover, the cell wall plays an important role in micro-colony architecture. For instance, the absence of the cellulose synthase-like protein CslA in *Streptomyces coelicolor* (de Jong et al. 2009b, Xu et al. 2008) leads to a significant decrease in micro-colony size. CslA produces an extracellular polysaccharide at the hyphal tip, which has been suggested to be cellulose (de Jong et al. 2009b, Xu et al. 2008). Gene *cslA* is particularly expressed when hyphae are in contact with a hydrophobic substrate or during aerial growth but expression is also observed in liquid shaken cultures (de Jong et al. 2009b). The polysaccharide resulting from CslA activity is thought to maintain the integrity of the hyphal tip that is subject to constant remodeling due to ongoing cell wall synthesis (Flärdh 2003, Xu et al. 2008).

Flow cytometry has been used to study populations of single-celled organisms (Hutter & Eipel 1979, Phillips & Martin 1983). This method cannot be used for micro-colonies of *Streptomyces* because they are too large to pass the nozzle. Recently, the COPAS (Complex Object Parametric Analyzer and Sorter) platform has been used to analyze micro-colonies of the fungus *Aspergillus niger* with sizes ranging from 30-1500 microns (de Bekker et al. 2011b, van Veluw et al. 2013, Chapters 2 and 3). Here, COPAS was used for the first time to analyze bacterial multicellular structures. It is shown that *Streptomyces* liquid cultures consist of two populations of micro-colonies that differ in size. The cellulose synthase-like protein CslA, the cell surface proteins rodlins and chaplins, and the sigma factor BldN are shown to play a role in establishing the size of the larger micro-colonies. Moreover, the size of these larger micro-colonies, but not that of the small micro-colonies, is species- and medium-dependent.

Materials and Methods

Strains and culture conditions

The strains used in this study are shown in Table 1. Media were prepared as described (Kieser et al. 2000). *Streptomyces* species were grown at 30 °C on solid R5 or MS agar plates, or as shaken cultures (180 rpm) in YEME, TSBS, or R5 with glucose or in NMMP defined medium containing glucose. Liquid media were inoculated with 10^6 spores ml^{-1}. In case of the non-sporulating *S. coelicolor* Δ*bldN* strain, a 2-day-old colony grown on R5 agar medium was used as the inoculum, a wild-type colony serving as a control.

Flow cytometry using the COPAS PLUS

Micro-colonies were harvested and fixed with 4 % formaldehyde for 30 min on ice. They were washed twice with phosphate-buffered saline (PBS) and stored at -20 °C until further

use. Micro-colonies were analyzed based on size (time of flight [TOF]) using a COPAS Plus profiler equipped with a 1 mm nozzle (Union Biometrica, Holliston, MA). All data points with an extinction [EXT] ≥ 25 (i.e. excluding single hyphae and debris) were used for subsequent analysis.

Table 1. Strains used in this study.

Organism	*Strain*	*Description*	*Reference or source*
S. coelicolor	M145	Wild-type SCP1$^-$ SCP2$^-$	Kieser et al. 2000
	cslA (Tn5062)	*sco2836::Tn5062*	de Jong et al. 2009b
	rdlAB	*rdlAB::aac(3)IV*	Claessen et al. 2004
	chpABCDEFGH	*chpAD::scar chpB::vph chpCH::aadA chpE::scar chpF::scar chpG::aac(3)IV*	Claessen et al. 2004
	nepA	*nepA::aac(3)IV*	de Jong et al. 2009a
	J2177	*bldN::hyg*	Bibb et al. 2000
S. griseus	ATCC 13273	Wild-type	ATCC
S. lividans	1326	Wild-type	Kieser et al. 2000
S. scabies	ISP5078	Wild-type	Gift from prof. R Loria

Statistical analysis

The statistical analyses were performed as described (van Veluw et al. 2013, Chapter 3). Briefly, the log-transformed datasets were fit by a probability distribution assuming two normal distributions (Vinck et al. 2005). This model determines five parameters: the participation fraction (p) two means (μ_1; μ_2) and two standard deviations (σ_1; σ_2). For each parameter the 95 % confidence interval (CI) estimate was obtained by refitting with the model after bootstrapping (1000 replicates) using the open source Scilab language. Datasets with non-overlapping CI's of the means and a $0.025 < p < 0.975$ were considered to be derived from a culture with two populations of micro-colonies.

Results

Use of large particle flow cytometry with streptomycetes

S. coelicolor forms micro-colonies of variable size in liquid-grown cultures (Figure 1A). To analyze the size distribution, large particle flow cytometry was deployed using COPAS. *S. coelicolor* micro-colonies were analyzed on the basis of their time of flight (TOF) in milliseconds (Figure 1B). To relate the micro-colony diameter to TOF, 27 micro-colonies were measured by microscopy (Figure 1C). This revealed a relationship between TOF and micro-colony diameter whereby the diameter equals 0.57 × TOF + 159 µm.

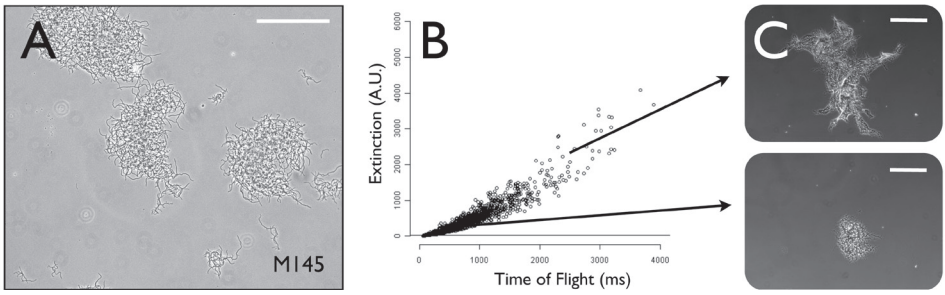

Figure 1. Heterogeneity in micro-colony size in liquid cultures of *S. coelicolor*. Size distribution of micro-colonies (A) was analyzed by COPAS (B). The COPAS technology allows separation of colonies according to size (C). Bars represent 400 (A) and 300 (C) µm, respectively.

Distinct populations of Streptomyces micro-colonies

To analyze the size distribution of the *S. coelicolor* micro-colonies, the TOF of the events with an extinction ≥ 25 were divided by the mean TOF of the population. Mathematical modeling of the pooled data of biological triplicates showed that the micro-colony size in liquid-grown cultures was not normally distributed when YEME, NMMP, R5, and TSBS were used as growth medium. Instead, the size distribution could be fitted assuming the existence of two normally distributed populations (Figure 2, Table 2).

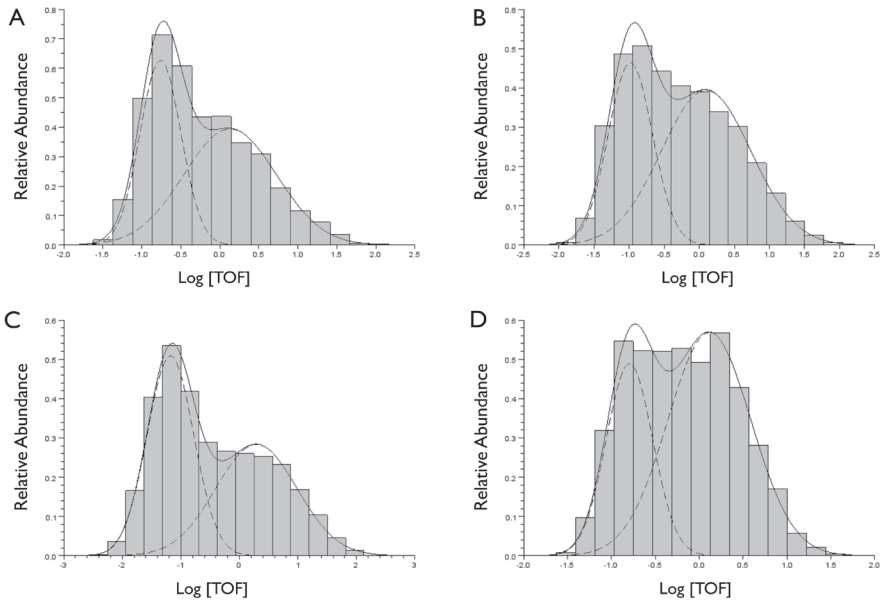

Figure 2. Relation of medium and size distribution of micro-colonies in liquid-grown cultures of *S. coelicolor*. *S. coelicolor* was grown for 24 h in NMMP with glucose (A), R5 (B), TSBS (C) or YEME (D). Two populations of micro-colonies that differ in size were detected in all media (see also Table 2).

Table 2. Heterogeneity between micro-colonies in liquid cultures of *S. coelicolor*. Heterogeneity is defined as non-overlapping confidence intervals (CI) of the means of both populations (μ_1 and μ_2) and a confidence interval (CI) of the participation fraction (p) between 0.025 - 0.975.

Medium	*Time (h)*	μ_1	*95% CI* (μ_1)	μ_2	*95% CI* (μ_2)	*p*	*95% CI (p)*
NMMP	24	260	258 - 262	405	399 - 413	0.413	0.391 - 0.437
	48	285	284 - 287	347	331 - 365	0.791	0.711 - 0.850
	72	281	278 -284	364	358 - 373	0.551	0.507 - 0.608
	96	285	282 - 289	347	333 - 363	0.630	0.520 - 0.725
	168	278	275 - 281	457	443 - 477	0.585	0.550 - 0.626
R5	24	261	257 - 264	458	446 - 475	0.368	0.334 - 0.409
	48	256	254 - 259	521	507 - 537	0.363	0.340 - 0.391
	72	259	256 - 261	450	438 - 465	0.550	0.522 - 0.579
	96	256	253 - 260	398	387 - 410	0.511	0.469 - 0.553
	168	251	249 - 254	346	338 - 354	0.483	0.437 - 0.531
TSBS	24	290	287 - 294	728	693 - 761	0.507	0.478 - 0.536
	48	285	281 - 290	629	609 - 650	0.457	0.427 - 0.486
	72	281	276 - 285	581	559 - 603	0.489	0.449 - 0.523
	96	274	270 - 279	477	463 - 493	0.456	0.416 - 0.496
	168	271	268 - 275	454	440 - 469	0.506	0.467 - 0.546
YEME	24	246	243 - 250	376	364 - 388	0.324	0.272 - 0.377
	48	257	253 - 261	474	458 - 494	0.316	0.276 - 0.362
	72	254	250 - 259	408	395 - 425	0.360	0.306 - 0.423
	96	248	246 - 251	366	357 - 376	0.383	0.344 - 0.427
	168	242	238 - 245	304	289 - 324	0.703	0.592 - 0.805

These two populations were observed between day 1 and day 7 (Figure 2, Table 2). The average size (μ_1) of the population of small micro-colonies was 266 µm (± 10 %) regardless of the medium that was used or the age of the cultures. In contrast, the average size (μ_2) of the population of large micro-colonies was highly variable in time (e.g. varying between 454 and 728 µm in TSBS). A difference in the size in the population of large micro-colonies was also observed between different media (varying from 457 µm in case of NMMP to 728 µm in case of TSBS medium; Table 2). The populations of large micro-colonies formed in R5- and YEME-grown cultures reached their maximal size (521 µm in case of R5 and 474 µm in case of YEME medium; Table 2) after 48 h of growth, after which their size decreased to 346 and 304 µm, respectively. In contrast, in TSBS medium the micro-colonies decreased in size after 24 h of growth. Unlike in other media, in minimal media (NMMP) the diameter of the large micro-colonies increased after an initial decrease (Table 2). The largest micro-colonies in NMMP-grown cultures (457 µm) were detected after 7 days of growth. Taken together, these data show that the size of the large micro-colonies formed by *S. coelicolor* is dynamic in time and depends on the medium composition.

Two populations of micro-colonies could also be distinguished in liquid-grown cultures of *Streptomyces lividans*, and the more phylogenetically distant species *Streptomyces scabies* and *Streptomyces griseus* (Figure 3, Table 3).

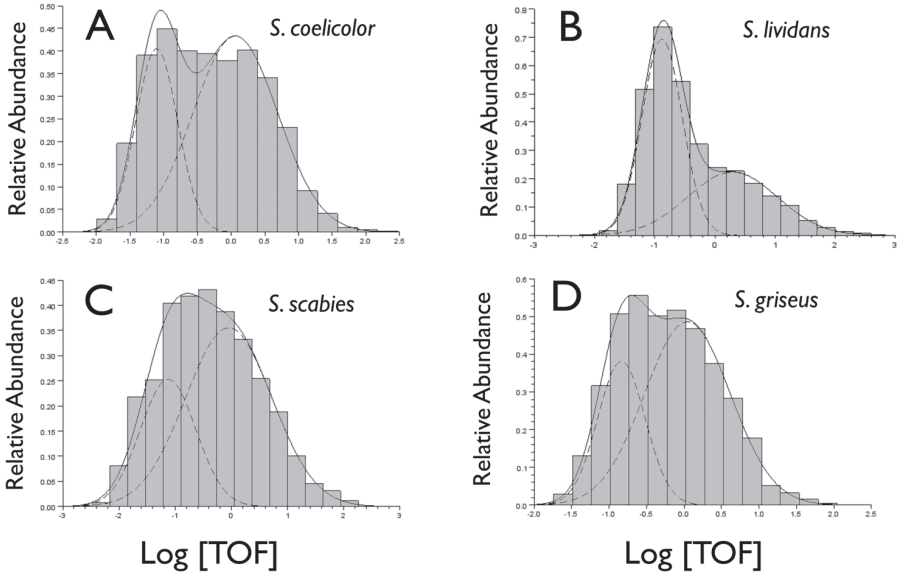

Figure 3. Size distributions of micro-colonies of liquid-grown cultures of *S. coelicolor* (A), *S. lividans* (B), *S. scabies* (C), and *S. griseus* (D) grown for 48 h in YEME medium. Two populations of micro-colonies that differ in size are detected in all strains (see also Table 3).

Table 3. Heterogeneity between micro-colonies in liquid-grown cultures of different *Streptomyces* species and strains. Heterogeneity is defined as non-overlapping confidence intervals (CI) of the means of both populations (μ_1 and μ_2) and a confidence interval (CI) of the participation fraction (p) between 0.025 - 0.975.

Strain	*Time (h)*	μ_1	*95% CI* (μ_1)	μ_2	*95% CI* (μ_2)	*p*	*95% CI (p)*
S. coelicolor	24	246	243 - 250	376	364 - 388	0.324	0.272 - 0.377
	48	257	253 - 261	474	458 - 494	0.316	0.276 - 0.362
	72	254	250 - 259	408	395 - 425	0.360	0.306 - 0.423
	96	248	246 - 251	366	357 - 376	0.383	0.344 - 0.427
	168	242	238 - 245	304	289 - 324	0.703	0.592 - 0.805
S. lividans	24	244	233 - 266	302	285 - 399	0.457	0.238 - 0.916
	48	262	260 - 264	492	475 - 511	0.582	0.558 - 0.609
	72	276	269 - 284	412	396 - 446	0.446	0.369 - 0.553
	96	267	263 - 272	374	365 - 385	0.520	0.465 - 0.577
	168	264	258 - 272	376	365 - 390	0.431	0.360 - 0.518

Strain	Time (h)	μ_1	95% CI (μ_1)	μ_2	95% CI (μ_2)	p	95% CI (p)
S. scabies	24	251	247 - 258	332	320 - 356	0.564	0.479 - 0.694
	48	271	258 - 286	489	462 - 525	0.313	0.235 - 0.410
	72	279	267 - 298	557	499 - 715	0.526	0.418 - 0.692
	96	252	247 - 258	426	410 - 446	0.349	0.298 - 0.408
	168	259	253 - 265	400	383 - 423	0.491	0.422 - 0.567
S. griseus	24	247	215 - 281	385	293 - 1971	0.629	0.163 - 0.967
	48	252	248 - 256	384	374 - 398	0.302	0.256 - 0.363
	72	249	247 - 252	370	363 - 378	0.424	0.387 - 0.461
	96	253	248 - 259	374	360 - 389	0.397	0.324 - 0.471
	168	239	236 - 243	288	270 - 316	0.749	0.588 - 0.871
Δ*cslA*	24	238	235 - 244	271	263 - 300	0.603	0.455 - 0.871
	48	244	242 - 248	312	307 - 318	0.328	0.278 - 0.396
	72	255	251 - 258	378	369 - 388	0.391	0.349 - 0.436
	96	255	250 - 259	328	320 - 338	0.466	0.388 - 0.542
	168	245	243 - 248	310	296 - 329	0.760	0.677 - 0.827
Δ*rdlAB*	24	267	263 - 270	383	354 - 423	0.815	0.741 - 0.875
	48	280	272 - 291	424	409 - 445	0.435	0.365 - 0.522
	72	260	256 - 266	356	345 - 371	0.448	0.370 - 0.542
	96	257	252 - 264	340	331 - 357	0.445	0.365 - 0.570
	168	260	256 - 263	351	339 - 367	0.559	0.492 - 0.631
Δ*chpABCDEFGH*	24	259	247 - 269	344	306 - 462	0.743	0.490 - 0.921
	48	268	261 - 276	404	384 - 429	0.531	0.450 - 0.618
	72	266	261 - 388	388	372 - 409	0.406	0.329 - 0.500
	96	260	256 - 355	355	343 - 368	0.484	0.411 - 0.558
	168	270	262 - 281	379	360 - 417	0.553	0.439 - 0.720
J2177 (*bldN*::hyg)	24	260	253 - 267	497	406 - 932	0.833	0.735 - 0.929
	48	280	271 - 289	755	708 - 814	0.305	0.256 - 0.355
	72	279	271 - 289	653	608 - 730	0.389	0.333 - 0.465
	96	264	257 - 272	519	497 - 546	0.265	0.214 - 0.319
	168	267	263 - 272	526	500 - 556	0.506	0.465 - 0.550

The average diameter of small micro-colonies (μ_1) remained constant in time and was similar for all streptomycetes (averaging 256 μm ± 10%). In contrast, the average size of the large micro-colonies (μ_2) fluctuated in case of *S. lividans* and *S. scabies* (Figure 4, Table 3). Less variation was observed for *S. griseus*, which is known to sporulate in submerged cultures (Kendrick & Ensign 1983). This again shows that streptomycetes form two different populations of micro-colonies in liquid-grown cultures, with a dynamic size distribution for large micro-colonies, while that of the small micro-colonies is constant.

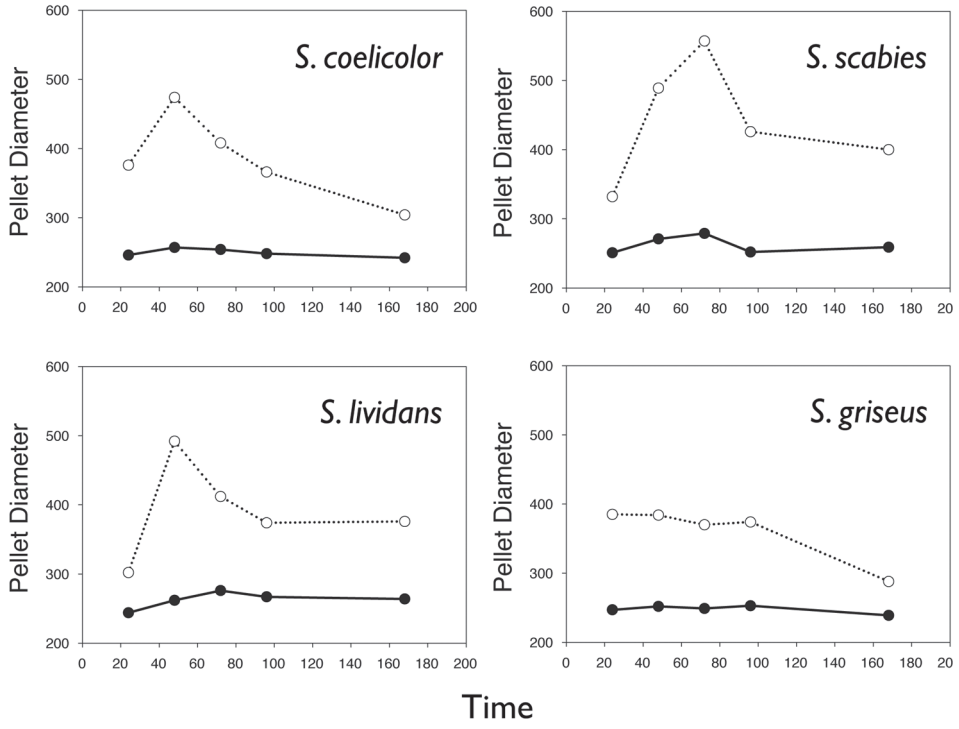

Figure 4. Dynamics of mean micro-colony size of the populations of small and large micro-colonies in liquid-grown cultures of *S. coelicolor* (A), *S. lividans* (B), *S. scabies* (C), and *S. griseus* (D).

CslA, chaplins and rodlins affect the micro-colony diameter in the population of large micro-colonies

The *cslA* mutant strain was previously shown to form smaller micro-colonies (Xu et al. 2008). Yet, again two populations were detected in this strain throughout growth (Table 3). The average size of the small micro-colonies was about 250 μm, similar to that observed for the parental wild-type strain. In contrast, the size of the large micro-colonies was different from the parental strain. After 24 h, the average size of the large *cslA* micro-colonies was 271 μm compared to 376 μm for the parental strain (Table 3). A comparable difference was detected after 48 h with 312 μm for the *cslA* mutant and 474 μm for the parental strain. At later time points the differences decreased until the sizes were almost identical after 168 h (310 μm for the *cslA* mutant and 304 μm for the parental strain).

Micro-colonies of the Δ*rdlAB* and Δ*chpABCDEFGH* strains were analyzed to investigate the role of rodlins and chaplins, respectively, in the morphology of micro-colonies in liquid-grown cultures (Table 3). Like the parental *S. coelicolor* strain M145, two distinct

populations of micro-colonies could be distinguished. The small micro-colonies had a diameter of approximately 265 µm, irrespective of culture age and this was similar to that of parental strain (Table 3). In contrast, the large micro-colonies formed by the Δ*rdlAB* and Δ*chpABCDEFGH* mutants were 424 µm and 404 µm in diameter, respectively, which is significantly smaller than the 474 µm observed for the parental strain after 48 h (Table 3). However, the size difference was less pronounced than observed for the *cslA* mutant.

Previous work demonstrated that expression of the *chp* and *rdl* genes depends on the developmental sigma factor BldN (Bibb et al. 2012, Elliot et al. 2003). Micro-colonies from liquid cultures of the *bldN* null mutant showed a bimodal distribution like the parental strain (Table 3). The average size of small micro-colonies formed by the *bldN* mutant was comparable to that formed by the parental strain (i.e. approximately 270 µm). However, the average micro-colony diameter in the population of large micro-colonies was approximately 60 % larger in the *bldN* mutant after 48 and 72 h of growth, namely 474 µm for the parental strain versus 755 µm for the *bldN* mutant at 48 h; and 408 µm for the parental strain versus 653 µm for the *bldN* mutant at 72 h (Table 3).

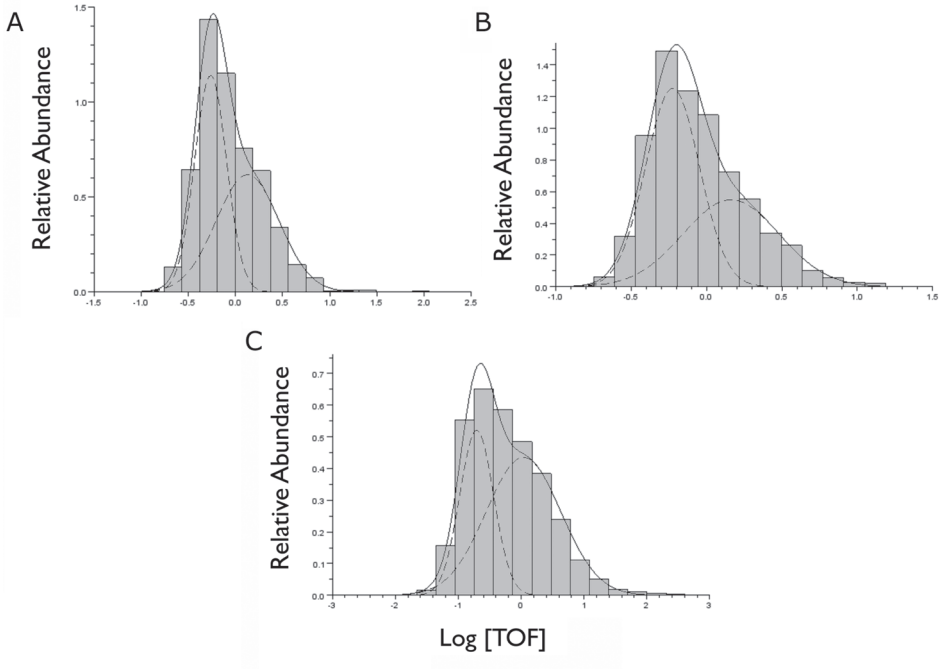

Figure 5. The effect of synchronous spore germination on the size distribution of micro-colonies. Two populations of micro-colonies were detected after 24 h using untreated (A) or heat-shocked (B) spores. Two populations that differ in size are also detected in the *nepA* mutant whose spores germinate more synchronously (C).

Synchronous spore germination does not affect micro-colony heterogeneity

To exclude that differences in spore germination could contribute to culture heterogeneity, we inoculated cultures with spores that had been pregerminated by 10 min heat shock at 50 °C followed by preincubation in rich 2×YT media (Kieser et al. 2000) and analyzed the size distribution of micro-colonies after 24 h of growth (Figure 5). Phase contrast microscopy showed that indeed the pre-germination procedure resulted in synchronous spore germination (not shown). Notably, again two distinct populations of micro-colonies were observed (Figure 5), strongly suggesting that germination efficiency did not play a role in culture heterogeneity. Consistent with this idea, a bimodal distribution was also observed in the Δ*nepA* mutant strain (Figure 5C) that germinates synchronously (de Jong et al. 2009a).

Discussion

A major complicating factor in the use of streptomycetes for industrial fermentation is that, like filamentous fungi, these microorganisms grow as branched hyphae, producing characteristic mycelia. This results in slow growth and high viscosity of the fermentation broth. The concomitant low yield per unit of time is a major bottleneck for their industrial fermentation. Understanding how morphology correlates to the production of natural products and enzymes is of great importance for industry-scale production. Antibiotics are typically produced by large micro-colonies (Wardell et al. 2002), while enzymes are optimally produced by small fragments (van Wezel et al. 2006). These results imply that a reduction in micro-colony size heterogeneity can improve product formation. In this Chapter it was shown that two populations of micro-colonies of *Streptomyces* can be discriminated in liquid shaken cultures based on size.

The presence of two populations of micro-colonies in liquid cultures of streptomycetes was observed irrespective of media composition and culture age. The micro-colony diameter in the population of small micro-colonies was similar between the tested streptomycetes and was also not affected by culture age (*i.e.* between day 1 and day 7) and medium composition. In contrast, the size of the large micro-colonies did vary. Micro-colony size is affected by parameters such as the geometry of the flask or bioreactor, the composition of the growth medium, pH, temperature, and the stirring speed (Celler et al. 2012, Cui et al. 1998, Tough & Prosser 1996). Our data suggest that these parameters would impact in particular the population consisting of large micro-colonies and not that of the small ones. Similarly, the CslA protein, as well as the chaplins, rodlins, and BldN were shown to affect only the size of the large micro-colonies. How these proteins influence micro-colony size is unclear. The observed decrease in size in case of the Δ*cslA*, Δ*rdlAB* and Δ*chpABCDEFGH* mutants imply that the cell surface has a critical role in establishing or maintaining micro-colony architecture. The increased micro-colony size observed in the *bldN* null mutant strain indicates that this effect

is not mediated by the regulatory role of BldN on the expression of rodlins and chaplins. This means that other members of the BldN regulon are responsible for the large micro-colony phenotype (Elliot et al. 2003). Taken together, these data suggest that the population of small micro-colonies is an intrinsic property of growth of streptomycetes not influenced by environmental conditions and cell wall components. In contrast, the diameter of large micro-colonies is determined by such factors and differs between *Streptomyces* species.

So far, it is not clear whether the population of large micro-colonies develops from the population of small ones or that they develop in an independent way. The size of fungal micro-colonies is influenced by aggregation of spores (primary aggregation) and of germlings (secondary aggregation) (Lin et al. 2008). This implies that the micro-colony size depends on the surface properties of both spores and hyphae (van Veluw et al. 2013, Chapter 3). Significantly, in *Streptomyces* two populations of micro-colonies were observed irrespective whether spores or mycelium were used as an inoculum. This implies that heterogeneity is not the result of spore aggregation. However, the large micro-colonies might result from the aggregation of small micro-colonies. Heterogeneity in surface properties of the small micro-colonies may result in distinct populations. This is consistent with the important role that cell surface proteins have on micro-colony size.

In terms of application, the COPAS technology employed here should enable -omics technologies on different cell types (*i.e.* small and large micro-colonies). This may reveal targets for the design of producer strains with improved productivity.

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CHAPTER 6

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Summary and General Discussion

Filamentous microbes play an important part in nature. They are essential in nutrient recycling by degrading dead organic material. Moreover, they can establish mutual beneficial or pathogenic interactions with other organisms including bacteria, fungi, plants and animals. Filamentous microbes are also important for mankind. For instance, they are used as cell factories for the production of pharmaceuticals and enzymes and metabolites (e.g. organic acids) that are used in the industry. In this Thesis I studied the filamentous fungi *Aspergillus niger* and *Aspergillus oryzae* as well as filamentous bacteria belonging to *Streptomyces*. Although these microbes belong to different domains of life, their life style and mode of growth are remarkably similar. In the next paragraphs I will briefly introduce the model organisms that were used in this study, followed by the aim of this Thesis.

A colony of *A. niger* or *A. oryzae* can result from a single asexual spore, known as conidium, or may result from conidia and / or germlings that have aggregated. Colonies can reach a diameter in the (sub-)millimeter (micro-colonies) to centimeter (macro-colonies) scale depending on the size and the composition of the substrate. For instance, micro-colonies are formed on a wheat kernel, whereas macro-colonies can be formed within the lobes of a lung. In the laboratory, aspergilli are routinely grown on agar media or in liquid media. On agar medium, aspergilli form radial symmetrical macro-colonies. In liquid cultures, mycelium can grow dispersed or as micro-colonies. The morphology of the mycelium has an enormous impact on the production of enzymes and primary and secondary metabolites. The underlying mechanisms however, are not yet clear. The size of micro-colonies is influenced by aggregation of conidia and / or germlings (Lin et al. 2008). Parameters that affect coagulation of *A. niger* and *A. orzyae* conidia are initial pH, agitation, and medium composition (Metz

& Kossen 1977, Carlsen et al. 1996). Micro-colony formation can also be manipulated by changing the surface composition of spores. Formation of micro-colonies was affected in strains of *A. nidulans* in which either or both *dewA* and *rodA* were inactivated (Dynesen & Nielsen 2003). The effect was strongest when both hydrophobin genes were deleted, which was accompanied by a huge drop in surface hydrophobicity of the conidia.

Research in the last two decades has shown that macro-colonies of *Aspergillus* are heterogeneous with respect to gene expression, growth, and secretion. For instance, enzymes like glucoamylase and ferulic acid esterase are secreted at the periphery of the colony. Their RNA levels are 3-fold and 5-fold higher, respectively, at the periphery of the colony when compared to the colony center (Wösten et al. 1991, Levin et al. 2007a). Half of the variation in RNA profiles is caused by medium-independent mechanisms (Levin et al. 2007a), which implies that differentiation occurs within the vegetative mycelium of *Aspergillus.* Heterogeneity in gene expression and secretion is not only observed between zones of macro-colonies of *Aspergillus*, it is also observed between hyphae in a particular zone. This is illustrated by the fact that only part of the hyphae at the periphery of macro-colonies of *A. niger* secrete glucoamylase (Wösten et al. 1991), which is explained by heterogeneous expression of the glucoamylase gene *glaA* within this zone (Vinck et al. 2005). The lowly expressing *glaA* hyphae have a low transcriptional and translational activity, whereas these activities are high in the high expressing *glaA* hyphae (Vinck et al. 2011). Single hypha transcriptome analysis indicates that heterogeneity between neighboring hyphae goes beyond two types of hyphae. Individual hyphae each have their own RNA composition (de Bekker et al. 2011a).

Streptomycetes such as *Streptomyces coelicolor* also have the ability to form micro- and macro-colonies. Macro-colonies, which are a few millimeters in diameter, are formed on an agar medium, while sub-millimeter size micro-colonies are formed in liquid shaken cultures. Heterogeneity in macro-colonies of streptomycetes has not been studied in the same detail as in fungi. However, it is known that the vegetative mycelium of these colonies undergoes differentiation too. Part of the compartments in established macro-colonies disintegrate at a certain moment, while others proliferate to give rise to a second vegetative mycelium (Manteca et al. 2005, 2006, 2007). At this stage aerial hyphae are also formed. These hyphae develop into a chain of pigmented spores that are dispersed to start a new colony.

The finding that macro-colonies of *Aspergillus* are heterogeneous with respect to RNA accumulation, growth and protein secretion raised the question whether heterogeneity is also observed between and within micro-colonies of this fungal genus. This was the subject of this Thesis. In addition, it was assessed whether heterogeneity can be observed between micro-colonies of streptomycetes.

Heterogeneity between microbial micro-colonies in liquid shaken cultures

The complex object parametric analyzer and sorter (COPAS) device was originally designed to analyze and sort stages and mutants of the nematode *C. elegans.* Later on, COPAS was also applied to other organisms like zebrafish, mosquitos as well as pollen of plants (www.unionbio.com). In **Chapter 2**, **3**, and **5**, COPAS was used for the first time to analyze and sort microbial micro-colonies. Diameter and gene expression of micro-colonies of liquid cultures of the *A. niger* strains AR9#2 and UU-A005.4 (**Chapter 2**) and CB-A111.1 (**Chapter 3**) were analyzed by COPAS. AR9#2 and UU-A005.4 express GFP from the glucoamylase promoter *glaA* and the ferulic acid esterase promoter *faeA*, respectively, while CB-A111.1 is a control strain that is complemented for the auxotrophic marker uridine. Statistical analyses showed that the liquid cultures of all three strains consisted of two populations of micro-colonies that differed in size. The population of small micro-colonies of strains AR9#2 and UU-A005.4 had an average diameter of 505 µm, while the population of large micro-colonies measured on average 595 µm. These values were 605 and 755 µm, respectively, for strain CB-A111.1. In the next set of experiments it was assessed whether expression of *glaA* and *faeA* is heterogeneous between micro-colonies within liquid cultures. Indeed, two populations of micro-colonies could be distinguished in the case of *glaA* and *faeA* expression. The population of micro-colonies highly expressing *glaA* or *faeA* was smaller than the population of large micro-colonies (**Chapter 3**). This indicates that heterogeneity in *glaA* and *faeA* expression in a liquid shaken culture of *A. niger* is not only caused by the heterogeneity in the size of the micro-colonies. This is strengthened by the finding that heterogeneity in gene expression is also observed between micro-colonies with one particular diameter (Recter & Wösten, unpublished results).

In **Chapter 5** it was assessed whether colonies of *Streptomyces* are also heterogeneous in size. Indeed, two populations of micro-colonies could be shown in liquid cultures of *S. coelicolor, Streptomyces lividans, Streptomyces scabies,* and *Streptomyces griseus.* The diameter of the population of small micro-colonies was similar in all streptomycetes. The diameter of the population of small micro-colonies of *S. coelicolor* was not affected by culture age and medium composition. In contrast, the size of the large micro-colonies did vary. Micro-colony size in *Streptomyces* is determined by parameters such as the geometry of the flask or bioreactor, the composition of the growth medium, pH, temperature, and the stirring speed (Celler et al. 2012, Cui et al. 1998, Tough & Prosser 1996). The data presented in **Chapter 5** suggest that these parameters impact in particular the population of large micro-colonies and not that of the small ones. It is not clear whether the population of large micro-colonies develops from the population of small ones or that they are formed independently.

Fungal micro-colonies are the result of aggregation of spores (primary aggregation) and / or of germlings (secondary aggregation) (Lin et al. 2008). This implies that micro-colony size depends on the surface properties of both spores and hyphae (van Veluw et al. 2013, **Chapter**

3). In *Streptomyces* two populations of micro-colonies were observed irrespective whether spores or mycelium were used as an inoculum (**Chapter 5**), implying that heterogeneity of these filamentous bacteria is not the result of spore aggregation. However, spore surface properties do seem to determine micro-colony size. Micro-colonies of the Δ*rdlAB* and Δ*chpABCDEFGH* strains were still heterogeneous with respect to size. The population of small micro-colonies of these mutant strains that do not form the spore cell wall proteins rodlins and chaplins, respectively, were similar to that of the wild-type strain measuring about 250 μm. Notably, the diameter of the population of large micro-colonies was affected. The large micro-colonies formed by the Δ*rdlAB* and Δ*chpABCDEFGH* mutants were 424 μm and 404 μm in diameter, respectively, while those of the wild-type had a diameter of 474 μm after 48 h of culturing. Similar results as obtained with the Δ*rdlAB* and Δ*chpABCDEFGH* mutants were found for the Δ*cslA* strain of *S. coelicolor.* The protein encoded by *cslA* forms an extracellular cellulose-like polymer (de Jong et al. 2009b). It has been proposed to contribute to chaplin assembly at the surface of aerial hyphae and spores and to anchor fimbriae of chaplins to the cell wall during attachment of hyphae to a hydrophobic surface. Assembly of chaplins at the spore surface would be important in the light of the results of **Chapter 5.** It should be noted that *cslA* is also lowly expressed in liquid shaken cultures (de Jong et al. 2009b). Therefore, this gene may also affect size of the large micro-colonies by affecting surface properties of vegetative hyphae in liquid cultures. Taken together, the results of **Chapter 5** imply that the surface of spores and vegetative hyphae has a critical role in establishing or maintaining architecture of large micro-colonies. In contrast, the diameter of the population of small micro-colonies would be an intrinsic property of streptomycetes that would not be influenced by environmental conditions and cell wall components.

In the case of *Aspergillus* it was assessed whether the cell wall component melanin has an influence on colony heterogeneity. To this end, deletion strains were analyzed that are affected in the biosynthesis of this spore pigment. The *fwnA*, *olvA* and *brnA* genes have been shown to be involved in melanin synthesis in *A. niger*. Gene *fwnA* encodes a polyketide synthase. Absence of this gene results in fawn-colored conidia. The Δ*olvA* and Δ*brnA* strains produce olive- and brown-colored conidia, respectively. The function of BrnA is not yet known. Based on homology to the *A. fumigatus* Ayg1 protein, OlvA is expected to convert the heptaketide naphtopyrone YWA1 into 1,3,6,8 tetrahydroxynaphtalene, which is further modified in melanin (Fujii et al. 2004). The Δ*fwnA,* Δ*olvA* and Δ*brnA* strains formed on average larger micro-colonies than the control strain CB-A111.1 (*i.e.* 628 versus 790-858 μm). This indicates that melanin impacts colony size. The underlying mechanism is not yet known. It may be that the average number of aggregated spores leading to a micro-colony is larger in the case of the pigmentation mutants. This hypothesis is based on the finding that spore clusters leading to micro-colonies are larger in large micro-colonies when compared to small micro-colonies (unpublished results). The Δ*brnA* and Δ*olvA* strains formed heterogeneous

cultures like the control strain CB-A111.1. However, the differences in diameter between the populations of large and small micro-colonies were smaller (i.e. 780 and 842 μm and 825 and 868 μm). The micro-colonies of the Δ*fwnA* strain were even normally distributed with an average diameter of 818 μm. Taken together, the pigmentation mutants form larger and more homogenous micro-colonies than CB-A111.1 and the AR9#2 and UU-A005.4 strains. Conidia of the pigmentation mutants were characterized to assess whether surface properties had been changed compared to spores of a control strain. Conidia of the Δ*brnA* and the Δ*fwnA* strains were similar in size and displayed a similar hydrophobicity as the control strain. The latter can be explained by the fact that the rodlets were still present at the spore surface of these pigmentation mutants. The conidia of the Δ*olvA* strain had distinct properties when compared to the control strain. Conidia of the Δ*olvA* strain were larger, more negatively charged, highly hydrophilic, and rodlets were almost completely absent. The latter suggests that the pigment in the cell wall of conidia affects assembly of hydrophobins into rodlets. It has been shown that assembly of hydrophobins is promoted by glucan polymers (Scholtmeijer et al. 2009). The same could hold for melanin. As mentioned above, the differences in biophysical and structural properties of the Δ*olvA* strain do not result in differences in incidence and size distribution of micro-colonies in the liquid shaken cultures when compared to the other pigmentation mutants. Previously, it has been shown that hydrophilicity of conidia and absence of the rodlet layer contributes to smaller micro-colonies in *A. nidulans* (Dynesen & Nielsen, 2003). This was not the case in *A. niger.* Possibly, different mechanisms underlie micro-colony formation in *A. nidulans* and *A. niger.* However, the differences may also be due to different growth conditions.

The results of **Chapter 3** and **5** are of interest for biotechnological applications because mycelial morphology impacts productivity of the bioreactor (Gomez et al. 1988, Papagianni & Moo-Young, 2002, Bhargava et al. 2003). For instance, formation of large micro-colonies coincides with increased release of glucoamylase in *A. niger* (Papagianni & Moo-Young, 2002) and erythromycin in *Saccharopolyspora erythraea* (Wardell et al. 2002). To optimally control productivity one would like to have a homogenous morphology (*i.e.* a particular diameter) of the micro-colonies. Omics approaches may identify mechanisms underlying heterogeneity between micro-colonies in liquid cultures. Recently, it was shown that 37 out of 1100 detected proteins were differentially produced when large and small micro-colonies of *S. coelicolor* were compared (van Veluw et al. 2012). Interestingly, proteins involved in the production of the antibiotic CDA were higher in abundance in large micro-colonies. This is in line with the finding that large micro-colonies produce more erythromycin in *S. erythraea* (Wardell et al. 2002).

Heterogeneity within microbial micro-colonies in liquid shaken cultures

RNA was isolated from a central and peripheral zone by subjecting micro-colonies of *A. niger* to laser micro-dissection and laser pressure catapulting (**Chapter 2**). QPCR could not show zonal differences in the relative levels of *18S* rRNA, and RNA of *actin*, *glaA*, and *faeA*. However, the amount of RNA per individual hypha was 45 times higher at the periphery when compared to the center of the micro-colony. These results show that there are fundamental differences between macro- and micro-colonies. On the one hand, there are zonal differences in gene expression in macro-colonies. On the other hand, there are no indications for zonal differences in the amount of RNA per hypha. At the moment, there is no explanation for these differences between micro- and macro-colonies.

In **Chapter 3** it was assessed whether expression of genes is heterogeneous between neighboring hyphae within the outer zone of micro-colonies grown in liquid shaken cultures, like it was observed in macro-colonies (Vinck et al. 2005, 2011, de Bekker et al. 2011a). Indeed, the presence of distinct populations of hyphae was observed in micro-colonies but the heterogeneity in gene expression seems to be less robust. This is indicated by the fact that heterogeneity was no longer observed after log-transformation of the fluorescence data. Possibly, signaling between hyphae is involved in promoting heterogeneity. In contrast to solid media, gradients of signaling molecules cannot be formed between hyphae that are grown in liquid shaken cultures.

Heterogeneity in gene expression between hyphae at the outer zone of micro-and macro-colonies was a surprising finding considering the fact that it is generally believed that a fungal mycelium shares a common cytoplasm due to the presence of porous septa that allow cytoplasmic streaming. However, it was shown in the basidiomycete *Schizophyllum commune* that septa can be plugged in intact growing hyphae (van Peer et al. 2009), thus abolishing cytoplasmic continuity. In **Chapter 4** it was shown that this is also the case in *A. oryzae.* Using laser dissection it was shown that about 40 % of the first three septa of hyphae at the periphery of an *A. oryzae* colony are closed. The plugging state of these apical septa did neither depend on the plugging state of neighboring septa, nor on environmental conditions. Notably, culturing temperature and osmolarity of the medium did affect the plugging state in *A. niger* (Bleichrodt 2012). Septa were always closed when a peroxisome-like organelle, known as Woronin body, had been localized at the septal pore. Conversely, septa were open when a Woronin body was absent. The role of Woronin bodies in plugging septa of intact vegetative hyphae was confirmed in a Δ*Aohex1* strain that cannot form these organelles. Live cell imaging showed that the presence of Woronin bodies near septa was dynamic, implying that septal closure is reversible. The dynamics of septal closure seems to be a stochastic process in *A. oryzae* but it may be controlled in *A. niger*.

Septal plugging in intact hyphae explains why hyphae can be heterogeneous with respect to RNA and protein composition. The fact that each septum of *A. oryzae* hyphae has a chance

of 60 % to be open implies that only in about 1 % of the cases the cytoplasm of two hyphae is in physical contact when they are separated by 9 septa. Absence of Woronin bodies would result in a continuous cytoplasm and, consequently, hyphal heterogeneity would be abolished. Indeed, expression of *glaA* and *aguA* was normally distributed in neighboring hyphae of the Δ*Aohex1* strain of *A. oryzae*, while it was heterogeneous in the wild-type background. We propose that heterogeneous gene expression still occurs in the Δ*Aohex1* mutant. However, since all septa are open, cytoplasmic streaming evenly distributes gene products within the mycelium.

Preliminary data indicates that heterogeneity of exploring hyphae increases viability when the colony is exposed to stress conditions like the presence of antibiotics or high temperature (Bleichrodt 2012 and unpublished results). This would also be an attractive mechanism for streptomycetes. This has not yet been studied. However, as mentioned above differentiation does occur in a vegetative mycelium showing that different hyphae within a vegetative mycelium of *S. coelicolor* can have differences in RNA and protein content.

The fact that microbial micro-colonies are heterogeneous 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, an 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 or an individual colony. Therefore, individual populations should be studied to understand mechanisms underlying biological processes in a liquid culture. COPAS sorting and analysis and laser dissection can be used to separate such populations.

REFERENCES

Adams TH, Boylan MT, Timberlake WE (1988). *brlA* is necessary and sufficient to direct conidiophore development in *Aspergillus nidulans*. *Cell* **54**: 353-362.

Adams TH, Wieser JK, Yu JH (1998). Asexual sporulation in *Aspergillus nidulans*. *Microbiology and Molecular Biology Reviews* **62**: 35-54.

Agnihotri VP (1964). Studies on Aspergilli. XVI. Effect of pH, temperature, and carbon and nitrogen interaction. *Mycopathologia et Mycologia Applicata* **24**: 305-314.

Aguirre J (1993). Spatial and temporal controls of the *Aspergillus brlA* developmental regulatory gene. *Molecular Microbiology* **8**: 211-218.

Aimanianda V, Bayry J, Bozza S, Kniemeyer O, Perruccio K, Elluru SR, Clavaud C, Paris S, Brakhage AA, Kaveri SV, Romani L, Latgé JP (2009). Surface hydrophobin prevents immune recognition of airborne fungal spores. *Nature* **460**: 1117-1121.

Aimanianda V, Latgé JP (2010). Fungal hydrophobins form a sheath preventing immune recognition of airborne conidia. *Virulence* **1**: 185-187.

Al-Doory Y (1984). Chapter 3. In: *Mould Allergy* (Al-Doory Y, Domsom JF, eds.) Lee & Febiger: 27-40.

Andersen MR, Salazar MP, Schaap PJ, van de Vondervoort PJ, Culley D, Thykaer J, Frisvad JC, Nielsen KF, Albang R, Albermann K, Berka RM, Braus GH, Braus-Stromeyer SA, Corrochano LM, Dai Z, van Dijck PW, Hofmann G, Lasure LL, Magnuson JK, Menke H, Meijer M, Meijer SL, Nielsen JB, Nielsen ML, van Ooyen AJ, Pel HJ, Poulsen L, Samson RA, Stam H, Tsang A, van den Brink JM, Atkins A, Aerts A, Shapiro H, Pangilinan J, Salamov A, Lou Y, Lindquist E, Lucas S, Grimwood J, Grigoriev IV, Kubicek CP, Martinez D, van Peij NN, Roubos JA, Nielsen J, Baker SE (2011). Comparative genomics of citric-acid-producing *Aspergillus niger* ATCC 1015 versus enzyme-producing CBS 513.88. *Genome Research* **21**: 885-897.

Andrianopoulos A, Timberlake WE (1994). The *Aspergillus nidulans abaA* gene encodes a transcriptional activator that acts as a genetic switch to control development. *Molecular and Cellular Biology* **14**: 2503-2515.

Astoreca A, Magnoli C, Ramirez ML, Combina M, Dalcero A (2007). Water activity and temperature effects on growth of *Aspergillus niger*, *A. awamori* and *A. carbonarius* isolated from different substrates in Argentina. *International Journal of Food Microbiology* **119**: 314-318.

Ayerst G (1969). The effects of moisture and temperature on growth and spore germination in some fungi. *Journal of Stored Products Research* **5**: 127-141.

Bahn YS, Xue C, Idnurm A, Rutherford JC, Heitman J, Cardenas ME (2007). Sensing the environment: lessons from fungi. *Nature Reviews. Microbiology* **5**: 57-69.

Baker SE (2008). *Aspergillus* genomics and DHN-melanin conidial pigmentation. In: *Aspergillus in the genomics era* (Varga J, Samson RA, eds.) Wageningen Acedemic Publishers: 73-85.

Bennett JW (2010). An overview of the genus *Aspergillus*. In: *Aspergillus: Molecular Biology and Genomics* (Machida M, Gomi K, eds.) Horizon Scientific Press: 1-17.

Beuchat LR (1986). Extraordinary heat resistance of *Talaromyces flavus* and *Neosartorya fischeri* ascospores in fruit products. *Journal of Food Science* **51**: 1506-1510.

Bhargava S, Wenger KS, Marten MR (2003a). Pulsed feeding during fed-batch *Aspergillus oryzae* fermentation leads to improved oxygen mass transfer. *Biotechnology Progress* **19**: 1091-1094.

Bhargava S, Wenger KS, Marten MR (2003b). Pulsed addition of limiting-carbon during *Aspergillus oryzae* fermentation leads to improved productivity of a recombinant enzyme. *Biotechnology and Bioengineering* **82**: 111-117.

Bibb MJ, Molle V, Buttner MJ (2000). σ^{BldN}, an extracytoplasmic function RNA polymerase sigma factor required for aerial mycelium formation in *Streptomyces coelicolor* A3(2). *Journal of Bacteriology* **182**: 4606-4616.

Bibb MJ, Domonkos A, Chandra G, Buttner MJ (2012). Expression of the chaplin and rodlin hydrophobic sheath proteins in *Streptomyces venezuelae* is controlled by σ(BldN) and a cognate anti-sigma factor, RsbN. *Molecular Microbiology* **84**: 1033-1049.

Bleichrodt R (2012). Cytoplasmic Streaming in *Aspergillus.* Thesis, University of Utrecht.

Bleichrodt R, van Veluw GJ, Recter B, Maruyama J, Kitamoto K, Wösten HAB (2012). Hyphal heterogeneity in *Aspergillus oryzae* is the result of dynamic closure of septa by Woronin bodies. *Molecular Microbiology* **86**: 1334-1344.

Bleichrodt R, Vinck A, Krijgsheld P, van Leeuwen MR, Dijksterhuis J, Wösten HAB (2013). Cytoplasmic streaming in vegetative mycelium and aerial structures of *Aspergillus niger. Studies in Mycology* **74**: 31-46.

Boel E, Hjort I, Svensson B, Norris F, Norris KE, Fiil NP (1984). Glucoamylases G1 and G2 from Aspergillus niger are synthesized from two different but closely related mRNAs. *The EMBO Journal* **3**: 1097-1102.

Bos CJ, Debets AJ, Swart K, Huybers A, Kobus G, Slakhorst SM (1988). Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*. *Current Genetics* **14**: 437-443.

Boylan MT, Mirabito PM, Willett CE, Zimmerman CR, Timberlake WE (1987). Isolation and physical characterization of three essential conidiation genes from *Aspergillus nidulans*. *Molecular and Cellular Biology* **7**: 3113-3118.

Brakhage AA (2005). Systemic fungal infections caused by *Aspergillus* species: epidemiology, infection process and virulence determinants. *Current Drug Targets* **6**: 875-886.

Breakspear A, Momany M (2007). *Aspergillus nidulans* conidiation genes *dewA*, *fluG*, and *stuA* are differentially regulated in early vegetative growth. *Eukaryotic Cell* **6**: 1697-1700.

Bruns S, Kniemeyer O, Hasenberg M, Aimanianda V, Nietzsche S, Thywissen A, Jeron A, Latgé JP, Brakhage AA, Gunzer M (2010). Production of extracellular traps against *Aspergillus fumigatus* in vitro and in infected lung tissue is dependent on invading neutrophils and influenced by hydrophobin RodA. *PLoS Pathogens* **6**: e1000873.

Busby TM, Miller KY, Miller BL (1996). Suppression and enhancement of the *Aspergillus nidulans* medusa mutation by altered dosage of the bristle and stunted genes. *Genetics* **143**: 155-163.

Carlsen M, Spohr AB, Nielsen J, Villadsen J (1996). Morphology and physiology of an alpha-amylase producing strain of *Aspergillus oryzae* during batch cultivations. *Biotechnology and Bioengineering* **49**: 266-276.

Celler K, Picioreanu C, van Loosdrecht MC, van Wezel GP (2012). Structured morphological modeling as a framework for rational strain design of *Streptomyces species*. *Antonie Van Leeuwenhoek* **102**: 409-423.

Chater KF (2001). Regulation of sporulation in *Streptomyces coelicolor* A3(2): a checkpoint multiplex? *Current Opinion in Microbiology* **4**: 667-673.

Chipeta ZA, du Preez JC, Christopher L (2008). Effect of cultivation pH and agitation rate on growth and xylanase production by *Aspergillus oryzae* in spent sulphite liquor. *Journal of Industrial Microbiology & Biotechnology* **35**: 587-594.

Claessen D, Wösten HAB, van Keulen G, Faber OG, Alves AM, Meijer WG, Dijkhuizen L (2002). Two novel homologous proteins of *Streptomyces coelicolor* and *Streptomyces lividans* are involved in the formation of the rodlet layer and mediate attachment to a hydrophobic surface. Molecular Microbiology **44**: 1483-1492.

Claessen D, Rink R, de Jong W, Siebring J, de Vreugd P, Boersma FG, Dijkhuizen L, Wösten HAB (2003). A novel class of secreted hydrophobic proteins is involved in aerial hyphae formation in *Streptomyces coelicolor* by forming amyloid-like fibrils. *Genes & Development* **17**: 1714-1726.

Claessen D (2004). Structural proteins involved in morphological differentiation of streptomycetes. Thesis, University of Groningen.

Claessen D, Stokroos I, Deelstra HJ, Penninga NA, Bormann C, Salas JA, Dijkhuizen L, Wösten HAB (2004). The formation of the rodlet layer of streptomycetes is the result of the interplay between rodlins and chaplins. *Molecular Microbiology* **53**: 433-443.

Claessen D, de Jong W, Dijkhuizen L, Wösten HAB (2006). Regulation of *Streptomyces* development: reach for the sky! *Trends in Microbiology* **14**: 313-319.

Clutterbuck AJ (1969). A mutational analysis of conidial development in *Aspergillus nidulans*. *Genetics* **63**: 317-327.

Collinge AJ, Markham P (1985). Woronin bodies rapidly plug septal pores of severed *Penicillium chrysogenum* hyphae. *Experimental Mycology* **9**: 80-85.

Conesa A, Punt PJ, van Luijk N, van den Hondel CAMJJ (2001). The secretion pathway in filamentous fungi: a biotechnological view. *Fungal Genetics and Biology* **33**: 155-171.

Cui YQ, Okkerse WJ, van der Lans RG, Luyben KC (1998). Modeling and measurements of fungal growth and morphology in submerged fermentations. *Biotechnology and Bioengineering* **60**: 216-229.

Dagenais TR, Giles SS, Aimanianda V, Latgé JP, Hull CM, Keller NP (2010). *Aspergillus fumigatus* LaeA-mediated phagocytosis is associated with a decreased hydrophobin layer. *Infection and Immunity* **78**: 823-829.

Davelos AL, Xiao K, Flor JM, Kinkel LL (2004). Genetic and phenotypic traits of streptomycetes used to characterize antibiotic activities of field-collected microbes. *Canadian Journal of Microbiology* **50**: 79-89.

de Bekker C, Wiebenga LA, Aguilar G, Wösten HAB (2009). An enzyme cocktail for efficient protoplast formation in *Aspergillus niger*. *Journal of Microbiological Methods* **76**: 305-306.

de Bekker C, Bruning O, Jonker MJ, Breit TM, Wösten HAB (2011a). Single cell transcriptomics of neighboring hyphae of *Aspergillus niger*. *Genome Biology* **12**: R71.

de Bekker C, van Veluw GJ, Vinck A, Wiebenga LA, Wösten HAB (2011b). Heterogeneity of *Aspergillus niger* microcolonies in liquid shaken cultures. *Applied and Environmental Microbiology* **77**: 1263-1267.

de Jong W, Manteca A, Sanchez J, Bucca G, Smith CP, Dijkhuizen L, Claessen D, Wösten HAB (2009a). NepA is a structural cell wall protein involved in maintenance of spore dormancy in *Streptomyces coelicolor*. *Molecular Microbiology* **71**: 1591-1603.

de Jong W, Wösten HAB, Dijkhuizen L, Claessen D (2009b). Attachment of *Streptomyces coelicolor* is mediated by amyloidal fimbriae that are anchored to the cell surface via cellulose. *Molecular Microbiology* **73**: 1128-1140.

de Jong W, Vijgenboom E, Dijkhuizen L, Wösten HAB, Claessen D (2012). SapB and the rodlins are required for development of *Streptomyces coelicolor* in high osmolarity media. *FEMS Microbiology Letters* **329**:154-159.

de la Serna I, Ng D, Tyler BM (1999). Carbon regulation of ribosomal genes in *Neurospora crassa* occurs by a mechanism which does not require Cre-1, the homologue of the *Aspergillus* carbon catabolite repressor, CreA. *Fungal Genetics and Biology* **26**: 253-269.

de Vries RP, Visser J (1999). Regulation of the feruloyl esterase (*faeA*) gene from *Aspergillus niger*. *Applied and Environmental Microbiology* **65**: 5500-5503.

de Vries RP, Burgers K, van de Vondervoort PJ, Frisvad JC, Samson RA, Visser J (2004). A new black *Aspergillus* species, *A. vadensis*, is a promising host for homologous and heterologous protein production. *Applied and Environmental Microbiology* **70**: 3954-3959.

d'Enfert C (1997). Fungal spore germination: Insights from the molecular genetics of *Aspergillus nidulans* and *Neurospora crassa*. *Fungal Genetics and Biology* **21**: 163-172.

Denning DW (1998). Invasive aspergillosis. *Clinical Infectious Diseases* **26**: 781-803.

Dhavale TJ, Jedd G (2007). The fungal Woronin body. In: *The Mycota VIII - Biology of the fungal cell* (Howard RJ, Gow NAR, eds.) Springer: 87-96.

Dijksterhuis J, Nijsse J, Hoekstra FA, Golovina EA (2007). High viscosity and anisotropy characterize the cytoplasm of fungal dormant stress-resistant spores. *Eukaryotic Cell* **6**:157-170.

Dyer PS, Paoletti M (2005). Reproduction in *Aspergillus fumigatus*: sexuality in a supposedly asexual species? *Medical Mycology* **43**: S7-S14.

Dynesen J, Nielsen J (2003). Surface hydrophobicity of *Aspergillus nidulans* conidiospores and its role in pellet formation. *Biotechnology Progress* **19**: 1049-1052.

El-Enshasy H, Kleine J, Rinas U (2006). Agitation effects on morphology and protein productive fractions of filamentous and pelleted growth forms of recombinant *Aspergillus niger*. *Process Biochemistry* **41**: 2103-2112.

Elliot MA, Bibb MJ, Buttner MJ, Leskiw BK (2001). BldD is a direct regulator of key developmental genes in *Streptomyces coelicolor* A3(2). *Molecular Microbiology* **40**: 257-269.

Elliot MA, Karoonuthaisiri N, Huang J, Bibb MJ, Cohen SN, Kao CM, Buttner MJ (2003). The chaplins: a family of hydrophobic cell-surface proteins involved in aerial mycelium formation in *Streptomyces coelicolor*. *Genes & Development* **17**: 1727-1740.

Escaño CS, Juvvadi PR, Jin FJ, Takahashi T, Koyama Y, Yamashita S, Maruyama J, Kitamoto K (2009). Disruption of the *Aopex11-1* gene involved in peroxisome proliferation leads to impaired Woronin body formation in *Aspergillus oryzae*. *Eukaryotic Cell* **8**: 296-305.

Etxebeste O, Herrero-García E, Araújo-Bazán L, Rodríguez-Urra AB, Garzia A, Ugalde U, Espeso EA (2009). The bZIP-type transcription factor FlbB regulates distinct morphogenetic stages of colony formation in *Aspergillus nidulans*. *Molecular Microbiology* **73**: 775-789.

Fahal AH (2004). Mycetoma: a thorn in the flesh. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **98**: 3-11.

Finkelstein DB, Rambosek J, Crawford MS, Soliday CL, McAda PC, Leach J (1989). Protein secretion in *Aspergillus niger*. In: *Genetics and Molecular Biology of Industrial Microorganisms* (Hershberger CL, Queener SW, Hegeman G, eds). American Society for Microbiology: 295-300.

Fischer R, Kües U (2006). Asexual Sporulation in Mycelial Fungi. In: *The Mycota I -Growth, Differentiation and Sexuality* (Kües U, Fischer R, eds.) Springer: 263-292.

Flärdh K (2003). Essential role of DivIVA in polar growth and morphogenesis in *Streptomyces coelicolor* A3(2). *Molecular Microbiology* **49**: 1523-1536.

Flärdh K, Buttner MJ (2009). *Streptomyces* morphogenetics: dissecting differentiation in a filamentous bacterium. *Nature Reviews. Microbiology* **7**: 36-49.

Freudenberg S, Fasold K, Müller SR, Siedenberg D, Kretzmer G, Schügerl K, Giuseppin M (1996). Fluorescence microscopic investigation of *Aspergillus awamori* growing on synthetic and complex media and producing xylanase. *Journal of Biotechnology* **46**: 265-273.

Fujii I, Yasuoka Y, Tsai HF, Chang YC, Kwon-Chung KJ, Ebizuka Y (2004). Hydrolytic polyketide shortening by ayg1p, a novel enzyme involved in fungal melanin biosynthesis. *Journal of Biological Chemistry* **279**: 44613-44620.

Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO (2000). Genomic expression programs in the response of yeast cells to environmental changes. *Molecular Biology of the Cell* **11**: 4241-4257.

Gibson AM, Baranyi J, Pitt JI, Eyles MJ, Roberts TA (1994). Predicting fungal growth: the effect of water activity on *Aspergillus flavus* and related species. *International Journal of Food Microbiology* **23**: 419-431.

Gómez R, Schnabel I, Garrido J. Pellet growth and citric acid yield of *Aspergillus niger* 110 (1988). *Enzyme and Microbial Technology* **10**:188-191.

Goosen T, Bloemheuvel G, Gysler C, de Bie DA, van den Broek HWJ, Swart K (1987). Transformation of *Aspergillus niger* using the homologous orotidine-5′-phosphate-decarboxylase gene. *Current Genetics* **11**: 499-503.

Goosen T, van Engelenburg F, Debets F, Swart K, Bos K, van den Broek H (1989). Tryptophan auxotrophic mutants in *Aspergillus niger*: Inactivation of the *trpC* gene by cotransformation mutagenesis. *Molecular and General Genetics* **219**: 282-288.

Gull K (1978). Form and function of septa in filamentous fungiIn: *The Filamentous Fungi III - Developmental Mycology* (Smith JE, Berry DR, eds.) Edward Arnold: 78-93.

Haeder S, Wirth R, Herz H, Spiteller D (2009). Candicidin-producing *Streptomyces* support leaf-cutting ants to protect their fungus garden against the pathogenic fungus *Escovopsis*. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 4742-4746.

Han S, Adams TH (2001). Complex control of the developmental regulatory locus *brlA* in *Aspergillus nidulans*. *Molecular Genetetics and Genomics* **266**: 260-270.

Harris SD, Momany M (2004). Polarity in filamentous fungi: moving beyond the yeast paradigm. *Fungal Genetics and Biology* **41**: 391-400.

Harris SD (2006). Cell polarity in filamentous fungi: shaping the mold. *International Review of Cytology* **251**: 41-77.

Heinlein M (2002). Plasmodesmata: dynamic regulation and role in macromolecular cell-to-cell signalling. *Current Opinion in Plant Biology* **5**: 543-552.

Hiemenz PC (1977). Electrophoresis and other electrokinetic phenomena. In: *Principles of colloid and surface chemistry* (Lagowski JJ, ed.) Marcel Dekker: 452-487.

Hoch HC, Maxwell DP (1974). Proteinaceous hexagonal inclusions in hyphae of *Whetzelinia sclerotiorum* and *Neurospora crassa*. *Canadian Journal of Microbiology* **20**: 1029-1035.

Holder DJ, Kirkland BH, Lewis MW, Keyhani NO (2007). Surface characteristics of the entomopathogenic fungus *Beauveria (Cordyceps) bassiana*. *Microbiology* **153**: 3448-3457.

Hopwood DA (2007). *Streptomyces in nature and medicine: the antibiotic makers*, Oxford University Press.

Hutter KJ, Eipel HE (1979). Microbial determinations by flow cytometry. *Journal of General Microbiology* **113**: 369-375.

Ichinomiya M, Ohta A, Horiuchi H (2005). Expression of asexual developmental regulator gene *abaA* is affected in the double mutants of classes I and II chitin synthase genes, *chsC* and *chsA*, of *Aspergillus nidulans*. *Current Genetics* **48**: 171-183.

Ishitani C, Sakaguchi KI (1956). Hereditary variation and recombination in Koji molds (*Aspergilus oryzae* and *A. sojae*) v. heterocaryosis. *Journal of General and Applied Microbiology* **2**: 345-399.

Janssen PH (2006) Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Applied and Environmental Microbiology* **72**: 1719-1728.

Jedd G, Chua NH (2000). A new self-assembled peroxisomal vesicle required for efficient resealing of the plasma membrane. *Nature Cell Biology* **2**: 226-231.

Jedd G (2011). Fungal evo-devo: organelles and multicellular complexity. *Trends in Cell Biology* **21**: 12-19.

Jennings DH, Thornton JD, Galpin MF, Coggins CR (1974). Translocation in fungi. *Symposia of the Society for Experimental Biology* **28**: 139-156.

Jennings DH (1987). Translocation of solutes in fungi. *Biological Reviews* **62**: 215-243.

Jensen BG, Andersen MR, Pedersen MH, Frisvad JC, Sondergaard I (2010). Hydrophobins from *Aspergillus* species cannot be clearly divided into two classes. *BMC Research Notes* **3**: 344.

Jin FJ, Maruyama J, Juvvadi PR, Arioka M, Kitamoto K (2004). Adenine auxotrophic mutants of *Aspergillus oryzae*: development of a novel transformation system with triple auxotrophic hosts. *Bioscience, Biotechnology, and Biochemistry* **68**: 656-662.

Jin FJ, Watanabe T, Juvvadi PR, Maruyama J, Arioka M, Kitamoto K (2007). Double disruption of the proteinase genes, *tppA* and *pepE*, increases the production level of human lysozyme by *Aspergillus oryzae*. *Applied Microbiology and Biotechnology* **76**: 1059-1068.

Jørgensen TR, Park J, Arentshorst M, van Welzen AM, Lamers G, Vankuyk PA, Damveld RA, van den Hondel CAMJJ, Nielsen KF, Frisvad JC, Ram AFJ (2011). The molecular and genetic basis of conidial pigmentation in *Aspergillus niger*. *Fungal Genetics and Biology* **48**: 544-553.

Juvvadi PR, Maruyama J, Kitamoto K (2007). Phosphorylation of the *Aspergillus oryzae* Woronin body protein, AoHex1, by protein kinase C: evidence for its role in the multimerization and proper localization of the Woronin body protein. *The Biochemical Journal* **405**: 533-540.

Kaech SM, Wherry EJ (2007). Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. *Immunity* **27**: 393-405.

Kasuga T, Glass NL (2008). Dissecting colony development of *Neurospora crassa* using mRNA profiling and comparative genomics approaches. *Eukaryotic Cell* **7**: 1549-1564.

Keijser BJ, van Wezel GP, Canters GW, Vijgenboom E (2002). Developmental regulation of the *Streptomyces lividans ram* genes: involvement of RamR in regulation of the *ramCSAB* operon. *Journal of Bacteriology* **184**: 4420-4429.

Kelemen GH, Buttner MJ (1998). Initiation of aerial mycelium formation in *Streptomyces*. *Current Opinion in Microbiology* **1**: 656-662.

Kendrick KE, Ensign JC (1983). Sporulation of *Streptomyces griseus* in submerged culture. *Journal of Bacteriology* **155**: 357-366.

Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000). *Practical Streptomyces genetics*, The John Innes Foundation.

Kodani S, Hudson ME, Durrant MC, Buttner MJ, Nodwell JR, Willey JM (2004). The SapB morphogen is a lantibiotic-like peptide derived from the product of the developmental gene *ramS* in *Streptomyces coelicolor*. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 11448-11453.

Krijgsheld P, Bleichrodt R, van Veluw GJ, Wang F, Müller WH, Dijksterhuis J, Wösten HAB (2013). Development in *Aspergillus*. *Studies in Mycology* **74**: 1-29.

Kusters-van Someren MA, Harmsen JAM, Kester HCM, Visser J (1991). Structure of the *Aspergillus niger pelA* gene and its expression in *Aspergillus niger* and *Aspergillus nidulans*. *Current Genetics* **20**: 293-299.

Lacey J (1980). The microflora of graindust. In: *Occupational pulmonary disease: focus on graindust and health* (Dosman JA, Cotton DJ, eds.) Academic Press: 417-440.

Lamarre C, Sokol S, Debeaupuis JP, Henry C, Lacroix C, Glaser P, Coppee JY, Francois JM, Latgé JP (2008). Transcriptomic analysis of the exit from dormancy of *Aspergillus fumigatus* conidia. *BMC Genomics* **9**: 417.

Lee BN, Adams TH (1994a). Overexpression of *flbA*, an early regulator of *Aspergillus* asexual sporulation, leads to activation of *brlA* and premature initiation of development. *Molecular Microbiology* **14**: 323-334.

Lee BN, Adams TH (1994b). The *Aspergillus nidulans fluG* gene is required for production of an extracellular developmental signal and is related to prokaryotic glutamine synthetase I. *Genes & Development* **8**: 641-651.

Lee BN, Adams TH (1996). *fluG* and *flbA* function interdependently to initiate conidiophore development in *Aspergillus nidulans* through *brlA* beta activation. *The EMBO Journal* **15**: 299-309.

Leong SL, Hocking AD, Scott ES (2006). Effect of temperature and water activity on growth and ochratoxin A production by Australian *Aspergillus carbonarius* and *A. niger* isolates on a simulated grape juice medium. *International Journal of Food Microbiology* **110**: 209-216.

Leong SL, Hien LT, An TV, Trang NT, Hocking AD, Scott ES (2007). Ochratoxin A-producing Aspergilli in Vietnamese green coffee beans. *Letters in Applied Microbiology* **45**: 301-306.

Levin AM, de Vries RP, Conesa A, de Bekker C, Talon M, Menke HH, van Peij NNME, Wösten HAB (2007a). Spatial differentiation in the vegetative mycelium of *Aspergillus niger*. *Eukaryotic Cell* **6**: 2311-2322.

Levin AM, de Vries RP, Wösten HAB (2007b). Localization of protein secretion in fungal colonies using a novel culturing technique; the ring-plate system. *Journal of Microbiological Methods* **69**: 399-401.

Lew RR (2005). Mass flow and pressure-driven hyphal extension in *Neurospora crassa*. *Microbiology* **151**: 2685-2692.

Li B, Nierras CR, Warner JR (1999). Transcriptional elements involved in the repression of ribosomal protein synthesis. *Molecular and Cellular Biology* **19**: 5393-5404.

Lin PJ, Grimm LH, Wulkow M, Hempel DC, Krull R (2008). Population balance modeling of the conidial aggregation of *Aspergillus niger*. *Biotechnology and Bioengineering* **99**: 341-350.

Liu F, Ng SK, Lu Y, Low W, Lai J, Jedd G (2008). Making two organelles from one: Woronin body biogenesis by peroxisomal protein sorting. *The Journal of Cell Biology* **180**: 325-339.

Mabashi Y, Kikuma T, Maruyama J, Arioka M, Kitamoto K (2006). Development of a versatile expression plasmid construction system for *Aspergillus oryzae* and its application to visualization of mitochondria. *Bioscience, Biotechnology, and Biochemistry* **70**: 1882-1889.

Machida M, Asai K, Sano M, Tanaka T, Kumagai T, Terai G, Kusumoto K, Arima T, Akita O, Kashiwagi Y, Abe K, Gomi K, Horiuchi H, Kitamoto K, Kobayashi T, Takeuchi M, Denning DW, Galagan JE, Nierman WC, Yu J, Archer DB, Bennett JW, Bhatnagar D, Cleveland TE, Fedorova ND, Gotoh O, Horikawa H, Hosoyama A, Ichinomiya M, Igarashi R, Iwashita K, Juvvadi PR, Kato M, Kato Y, Kin T, Kokubun A, Maeda H, Maeyama N, Maruyama J, Nagasaki H, Nakajima T, Oda K, Okada K, Paulsen I, Sakamoto K, Sawano T, Takahashi M, Takase K, Terabayashi Y, Wortman JR, Yamada O, Yamagata Y, Anazawa H, Hata Y, Koide Y, Komori T, Koyama Y, Minetoki T, Suharnan S, Tanaka A, Isono K, Kuhara S, Ogasawara N, Kikuchi H (2005). Genome sequencing and analysis of *Aspergillus oryzae*. *Nature* **438**: 1157-1161.

Mah JH, Yu JH (2006). Upstream and downstream regulation of asexual development in *Aspergillus fumigatus*. *Eukaryotic Cell* **5**: 1585-1595.

Managadze D, Würtz C, Sichting M, Niehaus G, Veenhuis M, Rottensteiner H (2007). The peroxin PEX14 of *Neurospora crassa* is essential for the biogenesis of both glyoxysomes and Woronin bodies. *Traffic* **8**: 687-701.

Managadze D, Würtz C, Wiese S, Meyer HE, Niehaus G, Erdmann R, Warscheid B, Rottensteiner H (2010). A proteomic approach towards the identification of the matrix protein content of the two types of microbodies in *Neurospora crassa*. *Proteomics* **10**: 3222-3234.

Manteca A, Fernandez M, Sanchez J (2005). A death round affecting a young compartmentalized mycelium precedes aerial mycelium dismantling in confluent surface cultures of *Streptomyces antibioticus*. *Microbiology* **151**: 3689-3697.

Manteca A, Mader U, Connolly BA, Sanchez J (2006). A proteomic analysis of *Streptomyces coelicolor* programmed cell death. *Proteomics* **6**: 6008-6022.

Manteca A, Claessen D, Lopez-Iglesias C, Sanchez J (2007). Aerial hyphae in surface cultures of *Streptomyces lividans* and *Streptomyces coelicolor* originate from viable segments surviving an early programmed cell death event. *FEMS Microbiology Letters* **274**: 118-125.

Marshall MA, Timberlake WE (1991). *Aspergillus nidulans wetA* activates spore-specific gene expression. *Molecular and Cellular Biology* **11**: 55-62.

Maruyama J, Juvvadi PR, Ishi K, Kitamoto K (2005). Three-dimensional image analysis of plugging at the septal pore by Woronin body during hypotonic shock inducing hyphal tip bursting in the filamentous fungus *Aspergillus oryzae*. *Biochemical and Biophysical Research Communications* **331**: 1081-1088.

Maruyama J, Kikuchi S, Kitamoto K (2006). Differential distribution of endoplasmic reticulum network as visualized by the BipA-EGFP fusion protein in hyphal compartments across the septum of the filamentous fungus, *Aspergillus oryzae*. *Fungal Genetics and Biology* **43**: 642-654.

Maruyama J, Escaño CS, Kitamoto K (2010). AoSO protein accumulates at the septal pore in response to various stresses in the filamentous fungus *Aspergillus oryzae*. *Biochemical and Biophysical Research Communications* **391**: 868-873.

Maruyama J, Kitamoto K (2011). Targeted gene disruption in Koji mold *Aspergillus oryzae*. *Methods in Molecular Biology* **765**: 447-456.

Masai K, Maruyama J, Sakamoto K, Nakajima H, Akita O, Kitamoto K (2006). Square-plate culture method allows detection of differential gene expression and screening of novel, region-specific genes in *Aspergillus oryzae*. *Applied Microbiology and Biotechnology* **71**: 881-891.

Mehra S, Jaitly A (1995). pH and temperature optima for growth and sporulation in some common fungi from city waste. *Mycoscience* **36**: 243-246.

Metz B, Kossen NWF (1977). The growth of molds in the form of pellets - a literature review. *Biotechnology and Bioengineering* **19**: 781-799.

Meyer V, Wu B, Ram AFJ (2011). *Aspergillus* as a multi-purpose cell factory: current status and perspectives. *Biotechnology Letters* **33**: 469-476.

Miller KY, Toennis TM, Adams TH, Miller BL (1991). Isolation and transcriptional characterization of a morphological modifier: the *Aspergillus nidulans* stunted (*stuA*) gene. *Molecular and General Genetics* **227**: 285-292.

Miller KY, Wu J, Miller BL (1992). StuA is required for cell pattern formation in *Aspergillus*. *Genes & Development* **6**: 1770-1782.

Mirabito PM, Adams TH, Timberlake WE (1989). Interactions of three sequentially expressed genes control temporal and spatial specificity in *Aspergillus* development. *Cell* **57**: 859-868.

Mogensen J, Nielsen HB, Hofmann G, Nielsen J (2006). Transcription analysis using high-density micro-arrays of *Aspergillus nidulans* wild-type and *creA* mutant during growth on glucose or ethanol. *Fungal Genetics and Biology* **43**: 593-603.

Momany M (2002). Polarity in filamentous fungi: establishment, maintenance and new axes. *Current Opinion in Microbiology* **5**: 580-585.

Momany M, Richardson EA, Van Sickle C, Jedd G (2002). Mapping Woronin body position in *Aspergillus nidulans*. *Mycologia* **94**: 260–266.

Moore RT, McAlear JH (1962). Fine structures of mycota. Observations on septa of ascomycetes and basidiomycetes. *American Journal of Botany* **49**: 86-94.

Moukha SM, Wösten HAB, Mylius EJ, Asther M, Wessels JGH (1993a). Spatial and temporal accumulation of mRNAs encoding two common lignin peroxidases in *Phanerochaete chrysosporium*. *Journal of Bacteriology* **175**: 3672-3678.

Moukha SM, Wösten HAB, Asther M, Wessels JGH (1993b). In situ localization of the secretion of lignin peroxidases in colonies of *Phanerochaete chrysosporium* using a sandwiched mode of culture. *Journal of General Microbiology* **139**: 969-978.

Nasseri S, Assadi MM, Sepehr MN, Rostami K, Shariat M, Nadafi K (2002). Chromium removal from tanning effluent using biomass of *Aspergillus oryzae*. *Pakistan Journal of Biological Sciences* **5**: 1056-1059.

Ng SK, Liu F, Lai J, Low W, Jedd G (2009). A tether for Woronin body inheritance is associated with evolutionary variation in organelle positioning. *PLoS Genetics* **5**: e1000521.

Ni M, Yu JH (2007). A novel regulator couples sporogenesis and trehalose biogenesis in *Aspergillus nidulans*. *PloS One* **2**: e970.

Nielsen PV, Beuchat LR, Frisvad JC (1988). Growth of and fumitremorgin production by *Neosartorya fischeri* as affected by temperature, light, and water activity. *Applied and Environmental Microbiology* **54**: 1504-1510.

Nielsen J, Johansen CL, Jacobsen M, Krabben P, Villadsen J (1995). Pellet formation and fragmentation in submerged cultures of *Penicillium chrysogenum* and its relation to penicillin production. *Biotechnology Progress* **11**: 93-98.

Nielsen J (1996). Modelling the morphology of filamentous microorganisms. *Trends in Biotechnology* **14**: 438-443.

Nobile CJ, Mitchell AP (2007). Microbial biofilms: e pluribus unum. *Current Biology* **17**: 349-353.

Nodwell JR, McGovern K, Losick R (1996). An oligopeptide permease responsible for the import of an extracellular signal governing aerial mycelium formation in *Streptomyces coelicolor*. *Molecular Microbiology* **22**: 881-893.

Nodwell JR, Yang M, Kuo D, Losick R (1999). Extracellular complementation and the identification of additional genes involved in aerial mycelium formation in *Streptomyces coelicolor*. *Genetics* **151**: 569-584.

Norris RP, Freudzon M, Mehlmann LM, Cowan AE, Simon AM, Paul DL, Lampe PD, Jaffe LA (2008). Luteinizing hormone causes MAP kinase-dependent phosphorylation and closure of connexin 43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption. *Development* **135**: 3229-3238.

Ogawa M, Tokuoka M, Jin FJ, Takahashi T, Koyama Y (2010). Genetic analysis of conidiation regulatory pathways in koji-mold *Aspergillus oryzae*. *Fungal Genetics and Biology* **47**: 10-18.

Ogundero VW (1981). Cultural and nutritional studies of zoopathogenic fungi associated with livestock feeds in Nigeria. *Zeitschrift für Allgemeine Mikrobiologie* **21**: 255-259.

Ohneda M, Arioka M, Kitamoto K (2005). Isolation and characterization of *Aspergillus oryzae* vacuolar protein sorting mutants. *Applied and Environmental Microbiology* **71**: 4856-4861.

Osherov N, May GS (2000). Conidial germination in *Aspergillus nidulans* requires RAS signaling and protein synthesis. *Genetics* **155**: 647-656.

Osherov N, May GS (2001). The molecular mechanisms of conidial germination. *FEMS Microbiology Letters* **199**: 153-160.

Panasenko V (1967). Ecology of microfungi. *The Botanical Review* **33**: 189-215.

Papagianni M, Moo-Young M (2002). Protease secretion in glucoamylase producer *Aspergillus niger* cultures: fungal morphology and inoculum effects. *Process Biochemistry* **37**:1271-1278.

Papagianni M (2004). Fungal morphology and metabolite production in submerged mycelial processes. *Biotechnology Advances* **22**: 189–259.

Papagianni M (2007). Advances in citric acid fermentation by *Aspergillus niger*: biochemical aspects, membrane transport and modeling. *Biotechnology Advances* **25**: 244-263.

Paris S, Debeaupuis JP, Crameri R, Carey M, Charles F, Prevost MC, Schmitt C, Philippe B, Latgé JP (2003). Conidial hydrophobins of *Aspergillus fumigatus*. *Applied and Environmental Microbiology* **69**: 1581-1588.

Pawar NV, Patil VB, Kamble SS, Dixit GB (2008). First Report of *Aspergillus niger* as a Plant Pathogen on Zingiber officinale from India. *Plant Disease* **92**: 1368-1368.

Petersen KL, Lehmbeck J, Christensen T (1999). A new transcriptional activator for amylase genes in *Aspergillus*. *Molecular & General Genetics* **262**: 668–676.

Phillips AP, Martin KL (1983). Immunofluorescence analysis of *Bacillus* spores and vegetative cells by flow cytometry. *Cytometry* **4**: 123-131.

Pihet M, Vandeputte P, Tronchin G, Renier G, Saulnier P, Georgeault S, Mallet R, Chabasse D, Symoens F, Bouchara JP (2009). Melanin is an essential component for the integrity of the cell wall of *Aspergillus fumigatus* conidia. *BMC Microbiology* **9**: 177.

Pitt JI (1981). Food spoilage and biodeterioration. In: *Biology of Conidial Fungi II* (Cole GT, Kendrick B, eds.) Academic Press: 111-142.

Pitt JI (1994). The current role of *Aspergillus* and *Penicillium* in human and animal health. *Journal of Medical and Veterinary Mycology* **32 S1**: 17-32.

Prade RA, Timberlake WE (1993). The *Aspergillus nidulans brlA* regulatory locus consists of overlapping transcription units that are individually required for conidiophore development. *The EMBO Journal* **12**: 2439-2447.

Punt PJ, van den Hondel CAMJJ (1992). Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. *Methods in Enzymology* **216**: 447-457.

Punt PJ, van Biezen N, Conesa A, Albers A, Mangnus J, van den Hondel CAMJJ (2002). Filamentous fungi as cell factories for heterologous protein production. *Trends in Biotechnology* **20**: 200-206.

Roca MG, Davide LC, Mendes-Costa MC, Wheals A (2003). Conidial anastomosis tubes in *Colletotrichum*. *Fungal Genetics & Biology* **40**: 138-145.

Roca MG, Arlt J, Jeffree CE, Read ND (2005a). Cell biology of conidial anastomosis tubes in *Neurospora crassa*. *Eukaryotic Cell* **4**: 911-919.

Roca MG, Read ND, Wheals AE (2005b). Conidial anastomosis tubes in filamentous fungi. *FEMS Microbiology Letters* **249**: 191-198.

Samson RA, Hoekstra ES, Frisvad JC, Filtenborg O (2000). Identification of the common food- and airborne fungi, *Neosatorya fischeri*. In: *Introduction to food- and airborne fungi* (Samson RA, Hoekstra ES, Frisvad JC, Filtenborg O, eds.) Centraalbureau voor Schimmelcultures: 44-47.

Sawyer EB, Claessen D, Haas M, Hurgobin B, Gras SL (2011). The assembly of individual chaplin peptides from *Streptomyces coelicolor* into functional amyloid fibrils. *PLoS One* **6**: e18839.

Scholtmeijer K, de Vocht ML, Rink R, Robillard GT, Wösten HAB (2009). Assembly of the fungal SC3 hydrophobin into functional amyloid fibrils depends on its concentration and is promoted by cell wall polysaccharides. *Journal of Biological Chemistry* **284**: 26309-26314.

Schrey SD, Tarkka MT (2008). Friends and foes: streptomycetes as modulators of plant disease and symbiosis. *Antonie van Leeuwenhoek* **94**: 11-19.

Sewall TC, Mims CW, Timberlake WE (1990). *abaA* controls phialide differentiation in *Aspergillus nidulans*. *Plant Cell* **2**: 731-739.

Shatkin AJ, Tatum EL (1959). Electron microscopy of *Neurospora crassa* mycelia. *The Journal of Biophysical and Biochemical Cytology* **6**: 423-426.

Sheppard DC, Doedt T, Chiang LY, Kim HS, Chen D, Nierman WC, Filler SG (2005). The *Aspergillus fumigatus* StuA protein governs the up-regulation of a discrete transcriptional program during the acquisition of developmental competence. *Molecular Biology of the Cell* **16**: 5866-5879.

Siedenberg D, Mestric S, Ganzlin M, Schmidt M, Punt PJ, van den Hondel CAMJJ, Rinas U (1999). *glaA* promoter controlled production of a mutant green fluorescent protein (S65T) by recombinant *Aspergillus niger* during growth on defined medium in batch and fed-batch cultures. *Biotechnology Progress* **15**: 43-50.

Singh S, Sandhu D (1982). Growth response of some thermophilous fungi at different incubation temperatures. *Proceedings: Plant Sciences* **91**: 153-158.

Skromne I, Sanchez O, Aguirre J (1995). Starvation stress modulates the expression of the *Aspergillus nidulans brlA* regulatory gene. *Microbiology* **141**: 21-28.

Smith S, Chohan R, Armstrong R, Whipps J (1998). Hydrophobicity and surface electrostatic charge of conidia of the mycoparasite *Coniothyrium minitans*. *Mycological Research* **102**: 243-249.

Soundararajan S, Jedd G, Li X, Ramos-Pamploña M, Chua NH, Naqvi NI (2004). Woronin body function in *Magnaporthe grisea* is essential for efficient pathogenesis and for survival during nitrogen starvation stress. *The Plant Cell* **16**: 1564-1574.

Stevens DA, Kan VL, Judson MA, Morrison VA, Dummer S, Denning DW, Bennett JE, Walsh TJ, Patterson TF, Pankey GA (2000). Practice guidelines for diseases caused by *Aspergillus*. *Clinical Infectious Diseases* **30**: 696-709.

Stringer MA, Dean RA, Sewall TC, Timberlake WE (1991). Rodletless, a new *Aspergillus* developmental mutant induced by directed gene inactivation. *Genes & Development* **5**: 1161-1171.

Stringer MA, Timberlake WE (1995). *dewA* encodes a fungal hydrophobin component of the *Aspergillus* spore wall. *Molecular Microbiology* **16**: 33-44.

Sugareva V, Härtl A, Brock M, Hübner K, Rohde M, Heinekamp T, Brakhage AA (2006). Characterisation of the laccase-encoding gene *abr2* of the dihydroxynaphtalene-like melanin gene cluster of *Aspergillus fumigatus*. *Archives of Microbiology* **186**: 345-355.

Takahashi T, Maeda H, Yoneda S, Ohtaki S, Yamagata Y, Hasegawa F, Gomi K, Nakajima T, Abe K (2005). The fungal hydrophobin RolA recruits polyesterase and laterally moves on hydrophobic surfaces. *Molecular Microbiology* **57**: 1780-1796.

Tanabe Y, Maruyama J, Yamaoka S, Yahagi D, Matsuo I, Tsutsumi N, Kitamoto K (2011). Peroxisomes are involved in biotin biosynthesis in *Aspergillus* and *Arabidopsis*. *The Journal of Biological Chemistry* **286**: 30455-30461.

Tao L, Yu JH (2011). AbaA and WetA govern distinct stages of *Aspergillus fumigatus* development. *Microbiology* **157**: 313-326.

Teertstra WR, Lugones LG, Wösten HAB (2004). In situ hybridisation in filamentous fungi using peptide nucleic acid probes. *Fungal Genetics and Biology* **41**: 1099-1103.

Tenney K, Hunt I, Sweigard J, Pounder JI, McClain C, Bowman EJ, Bowman BJ (2000). *Hex-1*, a gene unique to filamentous fungi, encodes the major protein of the Woronin body and functions as a plug for septal pores. *Fungal Genetics and Biology* **31**: 205-217.

Thau N, Monod M, Crestani B, Rolland C, Tronchin G, Latgé JP, Paris S (1994). Rodletless mutants of *Aspergillus fumigatus*. *Infection and Immunity* **62**: 4380-4388.

Tillotson RD, Wösten HAB, Richter M, Willey JM (1998). A surface active protein involved in aerial hyphae formation in the filamentous fungus *Schizophyllum commune* restores the capacity of a bald mutant of the filamentous bacterium *Streptomyces coelicolor* to erect aerial structures. *Molecular Microbiology* **30**: 595-602.

Tough AJ, Prosser JI (1996). Experimental verification of a mathematical model for pelleted growth of *Streptomyces coelicolor* A3(2) in submerged batch culture. *Microbiology* **142**: 639-648.

Trinci AP, Collinge AJ (1974). Occlusion of the septal pores of damaged hyphae of *Neurospora crassa* by hexagonal crystals. *Protoplasma* **80**: 57-67.

Tsai HF, Wheeler MH, Chang YC, Kwon-Chung KJ (1999). A developmentally regulated gene cluster involved in conidial pigment biosynthesis in *Aspergillus fumigatus*. *Journal of Bacteriology* **181**: 6469-6477.

Twumasi-Boateng K, Yu Y, Chen D, Gravelat FN, Nierman WC, Sheppard DC (2009). Transcriptional profiling identifies a role for BrlA in the response to nitrogen depletion and for StuA in the regulation of secondary metabolite clusters in *Aspergillus fumigatus*. *Eukaryotic Cell* **8**: 104-115.

Valik L, Pieckova E (2001). Growth modelling of heat-resistant fungi: the effect of water activity. *International Journal of Food Microbiology* **63**: 11-17.

van Diepingen AD, Pal K, van der Lee TAJ, Hoekstra RF, Debets AJM (2009). The het-c heterokaryon incompatibility gene in *Aspergillus niger*. *Mycological research* **113**: 222-229.

van Leeuwen MR, Krijgsheld P, Bleichrodt R, Menke H, Stam H, Stark J, Wösten HAB, Dijksterhuis J (2013a). Germination of conidia of *Aspergillus niger* is accompanied by major changes in RNA profiles. *Studies in Mycology* **74**: 59-70.

van Leeuwen MR, Krijgsheld P, Wyatt TT, Golovina EA, Menke H, Dekker A, Stark J, Stam H, Grijpstra J, Bleichrodt R, Wösten HAB, Dijksterhuis J (2013b). The effect of natamycin on the transcriptome of conidia of *Aspergillus niger*. *Studies in Mycology* **74**: 71-85.

van Peer AF, Müller WH, Boekhout T, Lugones LG, Wösten HAB (2009). Cytoplasmic continuity revisited: closure of septa of the filamentous fungus *Schizophyllum commune* in response to environmental conditions. *PLoS One* **4**: e5977.

van Peer AF, Wang F, van Driel KG, de Jong JF, van Donselaar EG, Müller WH, Boekhout T, Lugones LG, Wösten HAB (2010). The septal pore cap is an organelle that functions in vegetative growth and mushroom formation of the wood-rot fungus *Schizophyllum commune*. *Environmental Microbiology* **12**: 833-844.

van Veluw GJ, Petrus ML, Gubbens J, de Graaf R, de Jong IP, van Wezel GP, Wösten HA, Claessen D (2012). Analysis of two distinct mycelial populations in liquid-grown *Streptomyces* cultures using a flow cytometry-based proteomics approach. *Applied Microbiology and Biotechnology* **96**: 1301-1312.

van Veluw GJ, Teertstra WR, de Bekker C, Vinck A, van Beek N, Muller WH, Arentshorst M, van der Mei HC, Ram AFJ, Dijksterhuis J, Wösten HAB (2013). Heterogeneity in liquid shaken cultures of *Aspergillus niger* inoculated with melanised conidia or conidia of pigmented mutants. *Studies in Mycology* **74**: 47-57.

van Wetter MA, Wösten HAB, Sietsma JH, Wessels JG (2000). Hydrophobin gene expression affects hyphal wall composition in *Schizophyllum commune*. *Fungal Genetics and Biology* **31**: 99-104.

van Wezel GP, Krabben P, Traag BA, Keijser BJF, Kerste R, Vijgenboom E, Heijnen JJ, Kraal B (2006). Unlocking *Streptomyces* spp. for use as sustainable industrial production platforms by morphological engineering. *Applied and Environmental Microbiology* **72**: 5283-5288.

Vecht-Lifshitz SE, Magdassi S, Braun S (1990). Pellet formation and cellular aggregation in *Streptomyces tendae*. *Biotechnology and Bioengineering* **35**: 890-896.

Veening JW, Smits WK, Kuipers OP (2008). Bistability, epigenetics, and bet-hedging in bacteria. *Annual Review of Microbiology* **62**: 193-210.

Vinck A, Terlou M, Pestman WR, Martens EP, Ram AFJ, van den Hondel CAMJJ, Wösten HAB (2005). Hyphal differentiation in the exploring mycelium of *Aspergillus niger*. *Molecular Microbiology* **58**: 693-699.

Vinck A, de Bekker C, Ossin A, Ohm RA, de Vries RP, Wösten HAB (2011). Heterogenic expression of genes encoding secreted proteins at the periphery of *Aspergillus niger* colonies. *Environmental Microbiology* **13**: 216-225.

Vishniac W, Santer M (1957). The thiobacilli. *Bacteriological Reviews* **21**: 195–213.

Vrancken K, Anné J (2009). Secretory production of recombinant proteins by *Streptomyces*. *Future Microbiology* **4**: 181-188.

Wardell JN, Stocks SM, Thomas CR, Bushell ME (2002). Decreasing the hyphal branching rate of *Saccharopolyspora erythraea* NRRL 2338 leads to increased resistance to breakage and increased antibiotic production. *Biotechnology and Bioengineering* **78**: 141-146.

Warner JR (1999). The economics of ribosome biosynthesis in yeast. *Trends in Biochemical Sciences* **24**: 437-440.

Watanabe A, Fujii I, Sankawa U, Mayorga ME, Timberlake WE, Ebizuka Y (1999). Re-identification of *Aspergillus nidulans wA* gene to code for a polyketide synthase of naphthopyrone. *Tetrahedron Letters* **40**: 91-94.

Watanabe A, Fujii I, Tsai H, Chang YC, Kwon-Chung KJ, Ebizuka Y (2000). *Aspergillus fumigatus alb1* encodes naphthopyrone synthase when expressed in *Aspergillus oryzae*. *FEMS Microbiology Letters* **192**: 39-44.

Wessels JGH (1989). The cell wall and protein secretion in fungi. In: *Proceedings of the EMBO-ALKO Workshop on Molecular Biology of Filamentous Fungi* (Nevalainen H, Penttilä M, eds.) Foundation for Biotechnical and Industrial Research: 177-186.

Wessels JGH (1990). Role of cell wall architecture in fungal tip growth generation. In: *Tip Growth in Plant and Fungal Walls* (Heath IB, ed.) Academic Press: 1-29.

Willey J, Santamaria R, Guijarro J, Geistlich M, Losick R (1991). Extracellular complementation of a developmental mutation implicates a small sporulation protein in aerial mycelium formation by *Streptomyces coelicolor*. *Cell* **65**: 641-650.

Willey J, Schwedock J, Losick R (1993). Multiple extracellular signals govern the production of a morphogenetic protein involved in aerial mycelium formation by *Streptomyces coelicolor*. *Genes & Development* **7**: 895-903.

Wösten HAB, Moukha SM, Sietsma JH, Wessels JGH (1991). Localization of growth and secretion of proteins in *Aspergillus niger*. *Journal of General Microbiology* **137**: 2017-2023.

Wösten HAB, Richter M, Willey JM (1999a). Structural proteins involved in emergence of microbial aerial hyphae. *Fungal Genetics and Biology* **27**: 153-160.

Wösten HAB, van Wetter MA, Lugones LG, van der Mei HC, Busscher HJ, Wessels JG (1999b). How a fungus escapes the water to grow into the air. *Current Biology* **9**: 85-88.

Wösten HAB, Willey JM (2000). Surface-active proteins enable microbial aerial hyphae to grow into the air. *Microbiology* **146**: 767-773.

Wösten HAB (2001). Hydrophobins: multipurpose proteins. *Annual Review of Microbiology* **55**: 625-646.

Wu J, Miller BL (1997). Aspergillus asexual reproduction and sexual reproduction are differentially affected by transcriptional and translational mechanisms regulating stunted gene expression. *Molecular and Cellular Biology* **17**: 6191-6201.

Xu H, Chater KF, Deng Z, Tao M (2008). A cellulose synthase-like protein involved in hyphal tip growth and morphological differentiation in *Streptomyces*. *Journal of Bacteriology* **190**: 4971-4978.

Yamada O, Lee BR, Gomi K (1997). Transformation system for *Aspergillus oryzae* with double auxotrophic mutations, *niaD* and *sC*. *Bioscience, Biotechnology, and Biochemistry* **61**: 1367-1369.

Yamada O, Lee BR, Gomi K, Iimura Y (1999). Cloning and functional analysis of the *Aspergillus oryzae* conidiation regulator gene *brlA* by its disruption and misscheduled expression. *Journal of Bioscience and Bioengineering* **87**: 424-429.

Yoon J, Aishan T, Maruyama J, Kitamoto K (2010). Enhanced production and secretion of heterologous proteins by the filamentous fungus *Aspergillus oryzae* via disruption of vacuolar protein sorting receptor gene *Aovps10*. *Applied and Environmental Microbiology* **76**: 5718-5727.

SAMENVATTING

Draadvormige (filamenteuze) micro-organismen hebben een belangrijke rol in de natuur. Ze zijn essentieel in het recyclen van organisch materiaal zoals bijvoorbeeld in compost. Verder kunnen deze microben interacties aangaan met andere organismen, waaronder bacteriën, schimmels, planten en dieren. Filamenteuze microben zijn ook belangrijk voor de samenleving. Ze worden bijvoorbeeld gebruikt om medicijnen te produceren of enzymen en metabolieten die gebruikt worden in de industrie. In dit Proefschrift bestudeerde ik de filamenteuze schimmels *Aspergillus niger* en *Aspergillus oryzae* en filamenteuze bacteriën van het geslacht *Streptomyces*. De bestudeerde microben behoren tot verschillende domeinen van het leven, maar hun levensstijl vertoont opvallend veel overeenkomsten.

Kieming van één of meerdere sporen van *A. niger* of *A. oryzae* resulteert in een kolonie die een millimeter of minder (micro-kolonies) tot centimeters (macro-kolonies) groot wordt. De grootte van het mycelium hangt af van het substraat waarop gegroeid wordt. Micro-kolonies worden bijvoorbeeld gevormd op tarwe korrels en macro-kolonies kunnen worden gevormd in de long. In het lab worden aspergilli routinematig gegroeid op voedingsbodems van agar of in vloeibare cultures. Op agar worden macro-kolonies gevormd, terwijl in vloeibaar medium vele miljoenen micro-kolonies ontstaan. Macro-kolonies van *Aspergillus* zijn heterogeen ten aanzien van gen-expressie, groei en uitscheiding. Zo worden enzymen als glucoamylase alleen aan de buitenkant van de kolonie uitgescheiden. De heterogeniteit van gen-expressie en secretie bestaat niet alleen tussen zones van een *Aspergillus* macro-kolonie, maar ook tussen hyfen binnen een bepaalde zone. Zo zijn er aan de buitenkant van een macro-kolonie hyfen die wel en hyfen die geen glucoamylase uitscheiden.
Streptomyceten kunnen ook micro- en macro-kolonies vormen. Macro-kolonies die op agar groeien zijn een paar millimeter in diameter, terwijl sub-millimeter micro-kolonies worden gevormd in vloeistofculturen. Heterogeniteit in macro-kolonies van streptomyceten is niet in dezelfde mate bestudeerd als in schimmels, alhoewel het bekend is dat binnen de kolonie bepaalde hyfen doodgaan terwijl anderen juist ontwikkelen tot sporen vormende luchthyfen. De vinding dat macro-kolonies van *Aspergillus* heterogeen zijn in hun gen-expressie, groei en eiwit uitscheiding vormt de basis voor de vraag of dit ook voorkomt tussen en binnen micro-kolonies van filamenteuze schimmels en bacteriën. Deze vraag vormde de basis voor dit Proefschrift en de resultaten zijn van belang om productie van medicijnen, enzymen en metabolieten in filamenteuze microben verder te optimaliseren.

Heterogeniteit tussen microbiële micro-kolonies in vloeistof culturen

Een flowcytometer voor grote deeltjes (COPAS) werd in **Hoofdstuk 2**, **3**, en **5** voor het eerst gebruikt om microbiële micro-kolonies te analyseren. De diameter en gen-expressie van micro-kolonies van *A. niger* werd geanalyseerd met COPAS in **Hoofdstuk 2** en **3**. Statistische analyse liet zien dat in vloeistofculturen twee populaties van micro-kolonies worden gevormd die verschillen in grootte. Daarnaast werd aangetoond dat expressie van

glaA en *faeA* (beide coderend voor uitgescheiden eiwitten) heterogeen is tussen micro-kolonies in vloeistofculturen. De populatie van micro-kolonies dat een hoge expressie van *glaA* of *faeA* vertoonde was kleiner dan de populatie van grote micro-kolonies (**Hoofdstuk 3**). Dit betekent dat de heterogeniteit van *glaA* en *faeA* expressie in vloeistofcultures van *A. niger* niet alleen veroorzaakt wordt door de grootte van de micro-kolonies.

In **Hoofdstuk 5** werd aangetoond dat ook micro-kolonies van *Streptomyces* heterogeen zijn qua grootte. Twee populaties van micro-kolonies konden onderscheiden worden in vloeistofculturen van *Streptomyces coelicolor*, *Streptomyces lividans*, *Streptomyces scabies* en *Streptomyces griseus*. Opvallend was dat de diameter van de populatie van kleine micro-kolonies niet afhankelijk was van de soort, de leeftijd van de cultuur of de samenstelling van het medium. De diameter van de grote kolonies varieerde echter wel. Het is nog niet duidelijk of de grote micro-kolonies ontstaan uit de kleine kolonies of dat ze onafhankelijk van elkaar worden gevormd.

Micro-kolonies van schimmels ontstaan uit geaggregeerde sporen en / of door samenklontering van ontkiemende sporen. Dit impliceert dat de micro-kolonie grootte afhangt van de eigenschappen van sporen en hyfen. In *Streptomyces* werden twee populaties van micro-kolonies gevonden onafhankelijk van het feit of mycelium of sporen werden gebruik om de culture te starten (**Hoofdstuk 5**), hetgeen impliceert dat heterogeniteit niet het resultaat is van sporenaggregatie. De oppervlakte eigenschappen van de sporen lijken echter wel de micro-kolonie grootte te beïnvloeden. De gemiddelde grootte in de populatie van kleine micro-kolonies van stammen waarin de rodlin genen of de chaplin genen waren uitgeschakeld was onveranderd vergeleken met een natuurlijke stam. Echter, de grootte van micro-kolonies binnen de populatie van grote micro-kolonies verschilde van de ouderstam. Ook de Δ*cslA* mutant vertoonde vergelijkbare resultaten. Men gaat er vanuit dat het cellulose-achtige polymeer dat door *cslA* gemaakt wordt de opbouw van de eiwit coating van chaplins en rodlins op de buitenkant van de luchthyfen en sporen bevordert. Aangezien *cslA* tot expressie komt in vloeistof cultures zou dit gen ook verantwoordelijk kunnen zijn voor de grootte van de micro-kolonies middels de eigenschappen van de vegetatieve hyfen. De resultaten van **Hoofdstuk 5** impliceren dat de oppervlakte eigenschappen van sporen en vegetatieve hyfen een belangrijke rol hebben in het vormen en behouden van de architectuur van de grote micro-kolonies. Dit in tegenstelling tot de diameter van de kleine kolonies, dit zou een intrinsieke eigenschap zijn van streptomyceten en niet onder invloed staan van omgevingsfactoren en celwand componenten.

Ook in het geval van *Aspergillus* is onderzocht of een celwand component, het pigment melanine, een invloed heeft op kolonie heterogeniteit. Hiertoe zijn deletiestammen geanalyseerd die op verschillende plaatsen in de melanine biosynthese route gestoord zijn. De *fwnA*, *olvA* en *brnA* genen zijn nodig bij het opbouwen van melanine in *A. niger*. Afwezigheid van deze genen resulteert in lichtbruine, olijfkleurige en bruin-gekleurde sporen. De Δ*fwnA,*

Δ*olvA* en Δ*brnA* stammen vormden gemiddeld grotere kolonies dan de controle stam (**Hoofdstuk 3**). Dit impliceert dat melanine een invloed heeft op de grootte van de kolonie. Daarnaast was het opvallend dat de micro-kolonies van de Δ*fwnA* stam, en niet die van de Δ*brnA* en Δ*olvA* stammen, normaal verdeeld waren. Om de effecten van de pigmenten in de celwand op grootte en de heterogenitieit van micro-kolonies beter te begrijpen werden de oppervlakte eigenschappen van de sporen van de pigmentatiemutanten bestudeerd. De sporen van de Δ*brnA* en de Δ*fwnA* stammen waren vergelijkbaar in grootte en waterafstotendheid als de controle stam. Dit laatste kan worden verklaard door het feit dat de waterafstotende laag van rodlets van hydrophobines nog steeds aanwezig was op het sporen oppervlak. De sporen van de Δ*olvA* stam weken af van de controle stam. Deze waren groter, meer negatief geladen, waterminnend, en de rodlet laag was bijna helemaal verdwenen. Dit laatste suggereert dat melanine en bepaalde voorloper pigmenten betrokken zijn bij de opbouw van de rodlet laag van hydrofobines. Opmerkelijk genoeg leidde deze verschillen in eigenschappen van de Δ*olvA* stam niet tot verschillen in de diameter verdeling van micro-kolonies in vloeistof culturen wanneer deze vergeleken worden met de andere pigmentatie stammen.

Heterogeniteit in microbiële micro-kolonies in vloeistof culturen

RNA werd geïsoleerd van een centrale zone en de buitenste zone van een *A. niger* micro-kolonie door middel van laser micro-dissectie (**Hoofdstuk 2**). QPCR analyse van *18S* rRNA, en RNA van *actine*, *glaA*, en *faeA* liet geen relatieve verschillen zien tussen deze zones. Echter, de hoeveelheid RNA per individuele hyfe bleek 45 keer hoger aan de buitenkant vergeleken met het centrum van de micro-kolonie. Een dergelijk verschil is niet gevonden in macro-kolonies. Deze resultaten laten dus zien dat er een fundamenteel verschil is tussen macro- en micro-kolonies. Macro-kolonies worden gekenmerkt door verschillen in gen-expressie tussen zones, terwijl zones in micro-kolonies worden gekenmerkt door verschillen in totaal RNA per hyfe. Ondanks het feit dat er in micro-kolonies geen verschillen tussen zones konden worden aangetoond, werden er wel verschillen in gen-expressie tussen hyfen aan de buitenkant van de micro-kolonie gevonden (**Hoofdstuk 3**). De heterogeniteit blijkt echter minder robuust te zijn vergeleken met macro-kolonies. Het is mogelijk dat uitgescheiden signaal moleculen heterogeniteit van het mycelium beïnvloeden. Op vaste media kunnen gradiënten van signaalmoleculen worden opgebouwd, wat niet mogelijk is in een vloeibaar medium dat voortdurend wordt gemengd.

Heterogeniteit in gen-expressie tussen hyfen in de buitenste zone van micro- en macro-kolonies is een verrassende vinding vanwege de algemene aanvaarde aanname dat cytoplasma in het mycelium van schimmels een continu systeem zou zijn vanwege de aanwezigheid van poreuze septa. In **Hoofdstuk 4** is echter beschreven dat septa van *A. oryzae* kunnen worden afgesloten met speciale organellen die Woronin bodies worden genoemd. Met laser dissectie werd aangetoond dat 40 % van de eerste drie septa dicht zijn in hyfen aan de buitenkant van

een *A. oryzae* kolonie, terwijl in een Δ*Aohex1* stam die geen Woronin bodies kan vormen alle septa open waren. De open of dichte staat van septa was onafhankelijk van de staat van naburige septa en onafhankelijk van omgevingsfactoren. Ook bleek dat het afsluiten van septa dynamisch is; Woronin bodies konden uit een pore van een septa migreren, nadat het enkele uren het septum had afgesloten. Zoals het nu lijkt is de dynamiek van de doorlaatbaarheid van septa van *A. oryzae* een stochastisch proces.

Het afsluiten van septa in intacte hyfen kan verklaren waarom hyfen heterogeen zijn in RNA en eiwit verdeling. Het feit dat elk septum in *A. oryzae* een kans van 60 % heeft om open te zijn impliceert dat het cytoplasma van twee compartimenten die door 9 septa worden gescheiden in maar 1 % van de gevallen fysiek met elkaar verbonden is. De afwezigheid van Woronin bodies zou resulteren in een continu cytoplasma. Als gevolg hiervan zou er geen heterogeniteit meer zijn tussen hyfen door het continue mengen van het cytoplasma door cytoplasmatische stroming. Fluorescentie intensiteit van GFP was inderdaad normaal verdeeld tussen naburige hyfen van een *A. oryzae* stam zonder Woronin bodies wanneer de reporter tot expressie was gebracht vanaf de *glaA* of *aguA* promoter, terwijl een heterogene verdeling werd gevonden in de wild-type achtergrond. Wij stellen voor dat heterogeniteit van gen-expressie nog steeds aanwezig is in een stam zonder Woronin bodies, maar doordat alle septa open zijn, wordt door stroming van het cytoplasma alles gelijkmatig verdeeld.

Het feit dat microbiële micro-kolonies heterogeen zijn wat betreft grootte en gen-expressie heeft verregaande implicaties hoe de analyse van RNA, eiwit en metabolieten van hele culturen moet worden geïnterpreteerd. Als men een hele culture gebruikt, wordt een gemiddelde samenstelling of activiteit van de micro-kolonies bepaald. Dit gemiddelde hoeft helemaal niet weer te geven wat er gebeurt in de individuele kolonies en daarmee in de cultuur in het geheel. Hierom is het van belang dat individuele populaties bestudeerd worden om mechanismen van biologische processen te begrijpen in vloeistofculturen. COPAS analyse en laser dissectie kunnen hierbij worden ingezet.

CURRICULUM VITAE

Gerard Jerre van Veluw was born on July 18th, 1981 in Nijkerk, The Netherlands. He followed his secondary education at the Menso Alting College in Hoogeveen, The Netherlands. In September 2000 he began his coursework in Biomedical Sciences at Utrecht University. As part of his studies he did an internship at the Hubrecht Institute of the Royal Netherlands Academy of Arts and Sciences in Utrecht under the supervision of dr. Ellen Nollen in the group of prof. dr. Ronald Plasterk. This was followed by an internship in the group of prof. dr. Ton Schumacher at the Netherlands Cancer Institute in Amsterdam supervised by dr. Sine Reker Hadrup. Jerre obtained his MSc diploma in December 2007. In 2008 he started his PhD project in the Molecular Microbiology group of the Department of Biology at Utrecht University under the supervision of prof. dr. Han Wösten. Research on the heterogeneity of microbial micro-colonies was financially supported during this period by the Dutch Ministry of Economic Affairs through the IOP Genomics program and is described in this thesis. In January 2012 Jerre started working as a post-doctoral researcher within the Molecular Microbiology group of Utrecht University. He studies schizophyllan production in *Schizophyllum commune* for enhanced oil recovery. This is done in collaboration with BASF in Ludwigshafen, Germany.

PUBLICATIONS

Wösten HAB, van Veluw GJ, de Bekker C, Krijgsheld P (2013). Heterogeneity in the mycelium: implications for the use of fungi as cell factories. *Biotechnology Letters* April 17.

Krijgsheld P, Bleichrodt R, van Veluw GJ, Wang F, Müller WH, Dijksterhuis J, Wösten HAB (2013). Development in *Aspergillus*. *Studies in Mycology* **74**: 1-29.

van Veluw GJ, Teertstra WR, de Bekker C, Vinck A, van Beek N, Muller WH, Arentshorst M, van der Mei HC, Ram AFJ, Dijksterhuis J, Wösten HAB (2013). Heterogeneity in liquid shaken cultures of *Aspergillus niger* inoculated with melanised conidia or conidia of pigmented mutants. *Studies in Mycology* **74**: 47-57.

Bleichrodt RJ, van Veluw GJ, Recter B, Maruyama J, Kitamoto K, Wösten HAB (2012). Hyphal heterogeneity in *Aspergillus oryzae* is the result of dynamic closure of septa by Woronin bodies. *Molecular Microbiology* **86**: 1334-1344.

van Veluw GJ, Petrus ML, Gubbens J, de Graaf R, de Jong IP, van Wezel GP, Wösten HAB, Claessen D (2012). Analysis of two distinct mycelial populations in liquid-grown *Streptomyces* cultures using a flow cytometry-based proteomics approach. *Applied Microbiology and Biotechnology* **96**: 1301-1312.

Battaglia E, Visser L, Nijssen A, van Veluw GJ, Wösten HAB, de Vries RP (2011) Analysis of regulation of pentose utilisation in *Aspergillus niger* reveals evolutionary adaptations in *Eurotiales*. *Studies in Mycology* **69**: 31-38.

de Bekker C, van Veluw GJ, Vinck A, Wiebenga LA, Wösten HAB (2011) Heterogeneity of *Aspergillus niger* microcolonies in liquid shaken cultures. *Applied and Environmental Microbiology* **77**: 1263-1267.

van Ham TJ, Holmberg MA, van der Goot AT, Teuling E, Garcia-Arencibia M, Kim HE, Du D, Thijssen KL, Wiersma M, Burggraaff R, van Bergeijk P, van Rheenen J, van Veluw GJ, Hofstra RMW, Rubinsztein DC, Nollen EAA (2010). Identification of MOAG-4/SERF as a regulator of age-related proteotoxicity. *Cell* **142**: 601-612.

Hadrup SR, Bakker AH, Shu CJ, Andersen RS, van Veluw GJ, Hombrink P, Castermans E, thor Straten P, Blank C, Haanen JB, Heemskerk MH, Schumacher TNM (2009) Parallel detection of antigen-specific T-cell responses by multidimensional encoding of MHC multimers. *Nature Methods* **6**: 520-526.

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