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

G.J. van Veluw[1], W.R. Teertstra[1], C. de Bekker[1], A. Vinck[1], N. van Beek[1], W.H. Muller[2], M. Arentshorst[3], H.C. van der Mei[4], A.F.J. Ram[3], J. Dijksterhuis[5], and H.A.B. Wösten[1*]

[1]*Microbiology and Kluyver Centre for Genomics of Industrial Fermentations, Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*; [2]*Biomolecular Imaging, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*; [3]*Molecular Microbiology and Biotechnology, Institute of Biology Leiden, Leiden University, Sylviusweg 72, 2333 BE Leiden, The Netherlands*; [4]*Department of BioMedical Engineering, University Medical Center Groningen and University of Groningen, Hanzeplein 1, 9713 AV Groningen, The Netherlands*; [5]*CBS-KNAW Fungal Diversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands*

**Correspondence*: H.A.B. Wösten, h.a.b.wosten@uu.nl

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-coloured 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 seems to be less robust when compared to macro-colonies grown on solid medium.

Key words: *Aspergillus,* bioreactor, colony, heterogeneity, mycelium.

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INTRODUCTION

Aspergillus niger is abundant in nature and 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 known as pellets. The morphology of the mycelium impacts the production of enzymes and metabolites. For instance, micro-colonies are needed for the production of citric acid by *A. niger* (Gómez *et al.* 1988). It has also been shown that formation of large pellets 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.* 2003). Large micro-colonies give rise to low viscosity, whereas dispersed mycelium results in a high viscosity. At the same time, the center of large pellets 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). Pellet 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-coloured conidia. The Δ*olvA* and Δ*brnA* strains produce olive-

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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)

and brown-coloured 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). 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 pellets 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 heterogenic 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). Hyphae could even be heterogenic 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 characterised 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 characterised by differences in their RNA profile (de Bekker *et al.* 2011a).

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 heterogenic 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), which 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 (MM (see below) with 2 g/L trypton, 1 g/L casamino acids, 1 g/L yeast extract, 0.5 g/L yeast ribonucleic acids and 1 % glucose). Conidia of strains in which reporter constructs had been introduced were isolated from cultures grown on minimal medium (de Vries *et al.* 2004) 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 minimal medium in 250 mL Erlenmeyers with the same carbon source as the preculture.

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 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 using a Plan-Neofluar 16x/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; 4x 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 normalised 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 normalised 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 twice in excess PBS. Micro-colonies were allowed to settle in between the washing steps. Micro-colonies were analysed based on extinction (EXT) and Time of Flight (TOF) using a COPAS PLUS equipped with a 1 mm nozzle (Union Biometrica) 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 x 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 conidia 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 modelled in the probability distribution $\Phi : \Phi(x) = pN(x; \mu_1,\sigma_1)+(1-p)N(x; \mu_2,\sigma_2)$ (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/links/index.htmL.

RESULTS

Surface characterisation 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 (Fig. 1A). In contrast, the HI of conidia of the Δ*olvA* strain was 0.13 showing that these spores were highly hydrophilic.

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) (Fig. 1B).

Scanning electron microscopy revealed that the diameter of conidia of 3 d 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 (Fig. 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 (Fig. 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.

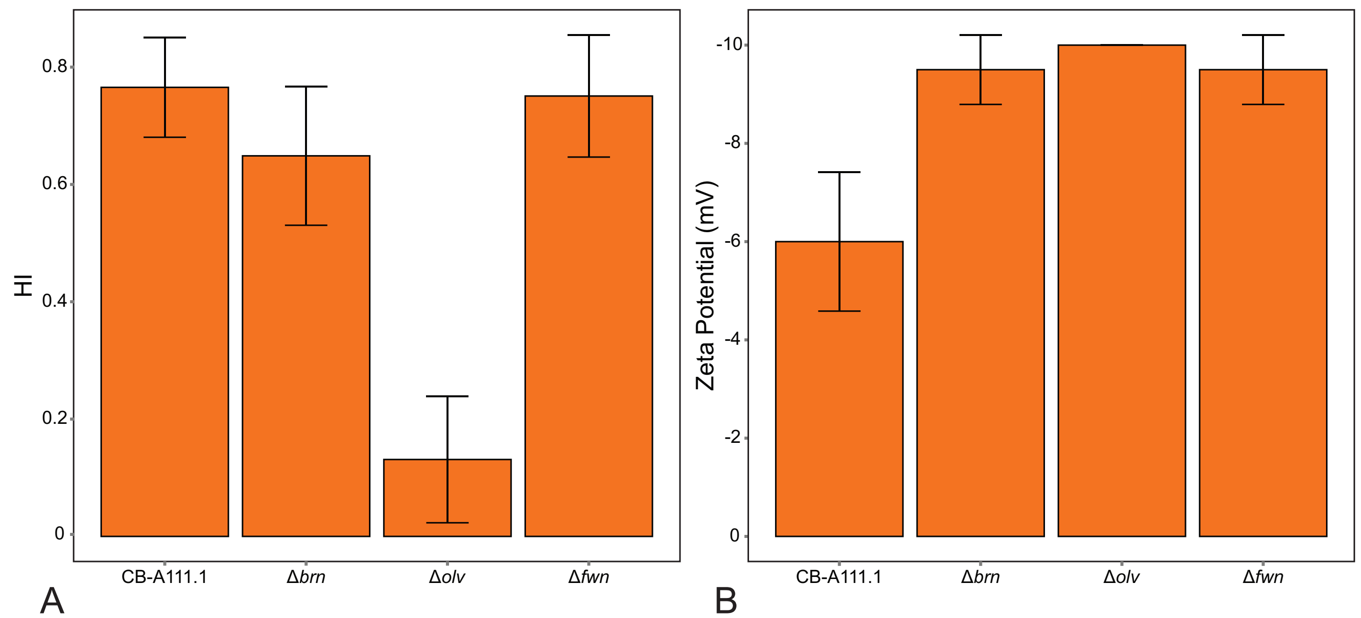

Fig. 1. Hydrophobicity Index (HI) (A) and zeta potential (B) of *A. niger* conidia. The zeta potential was determined in TM medium. Error bars represent standard deviation.

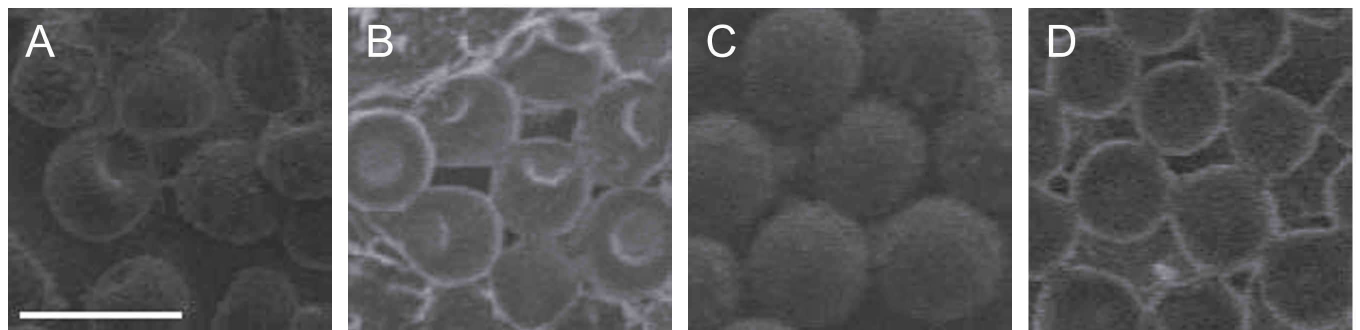

Fig. 2. Scanning electron microscopy of conidia from 3 d old colonies of strains CB-A111.1 (A), Δ*brnA* (B), Δ*olvA* (C), and Δ*fwnA* (D). Bar represents 5 μm.

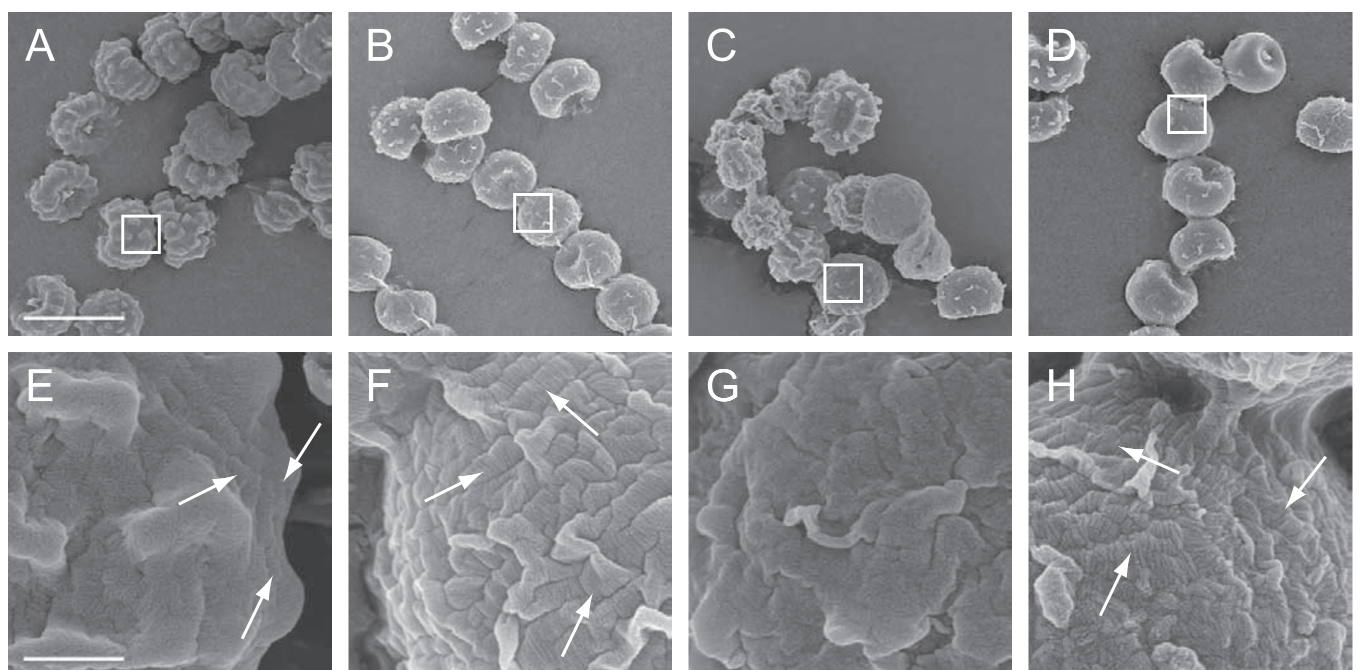

Fig. 3. Scanning electron microscopy of conidia of 3 d old colonies of strains CB-A111.1 (A, E), Δ*brnA* (B, F), Δ*olvA* (C, G) and Δ*fwnA* (D, H). Bar represents 5 μm (A–D) and 500 nm (E–H). Rodlets are visible on the conidia of the control, Δ*brnA* and Δ*fwnA* strains and are indicated by white arrows.

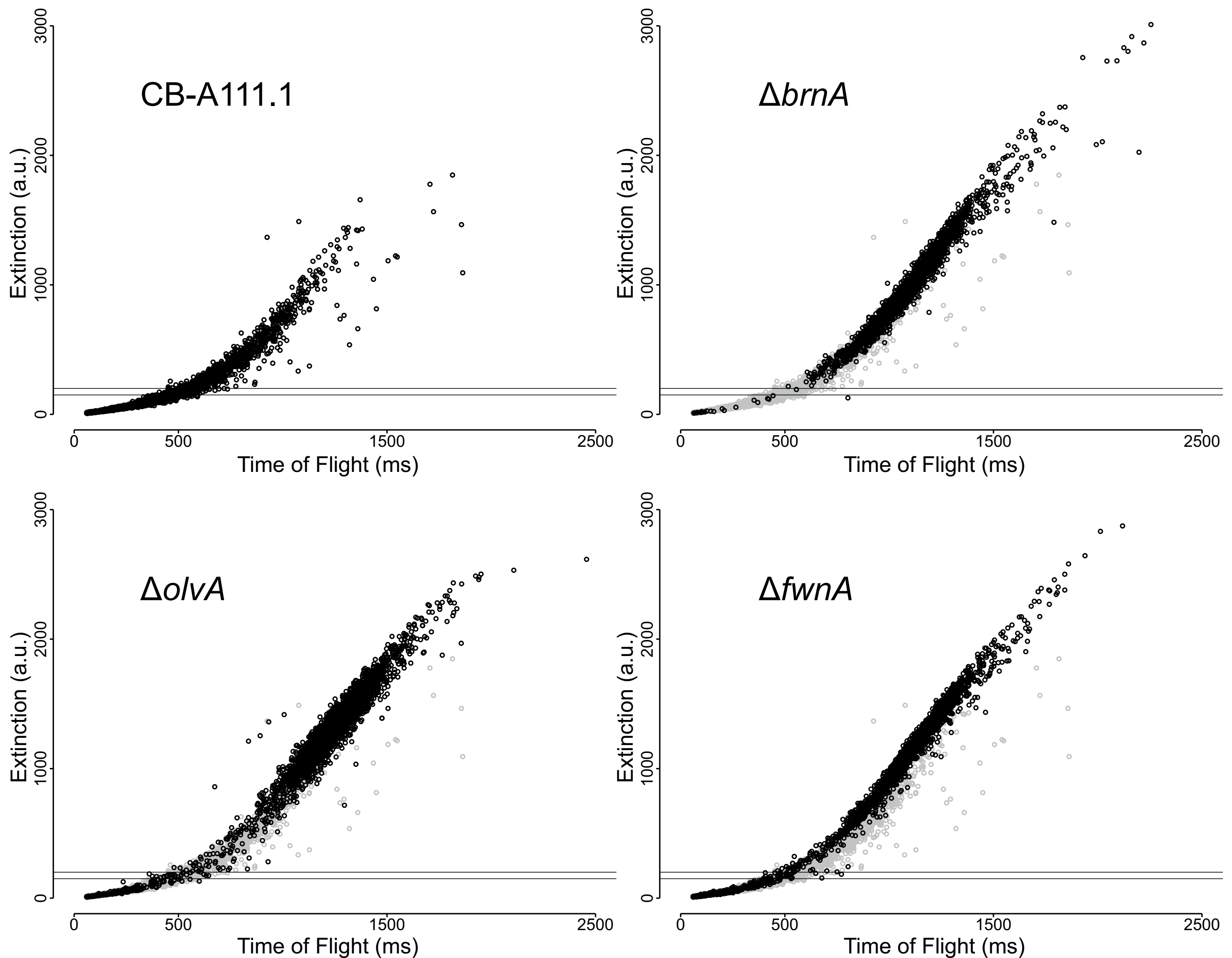

Fig. 4. Distribution of diameter (time of flight in milliseconds x 0.4) and optical density (extinction in arbitrary units) of hyphae and micro-colonies of liquid cultures of pigmentation mutants of *A. niger* and the control strain CB-A111.1. The horizontal lines represent an extinction of 150 and 200.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

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 analysed on basis of their diameter as expressed as the time of flight (TOF) in milliseconds (Fig. 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). Using the formula defined by de Bekker *et al*. (2011b), 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.

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 normalise the data. Mathematical modelling 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 (Fig. 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 CBB-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.

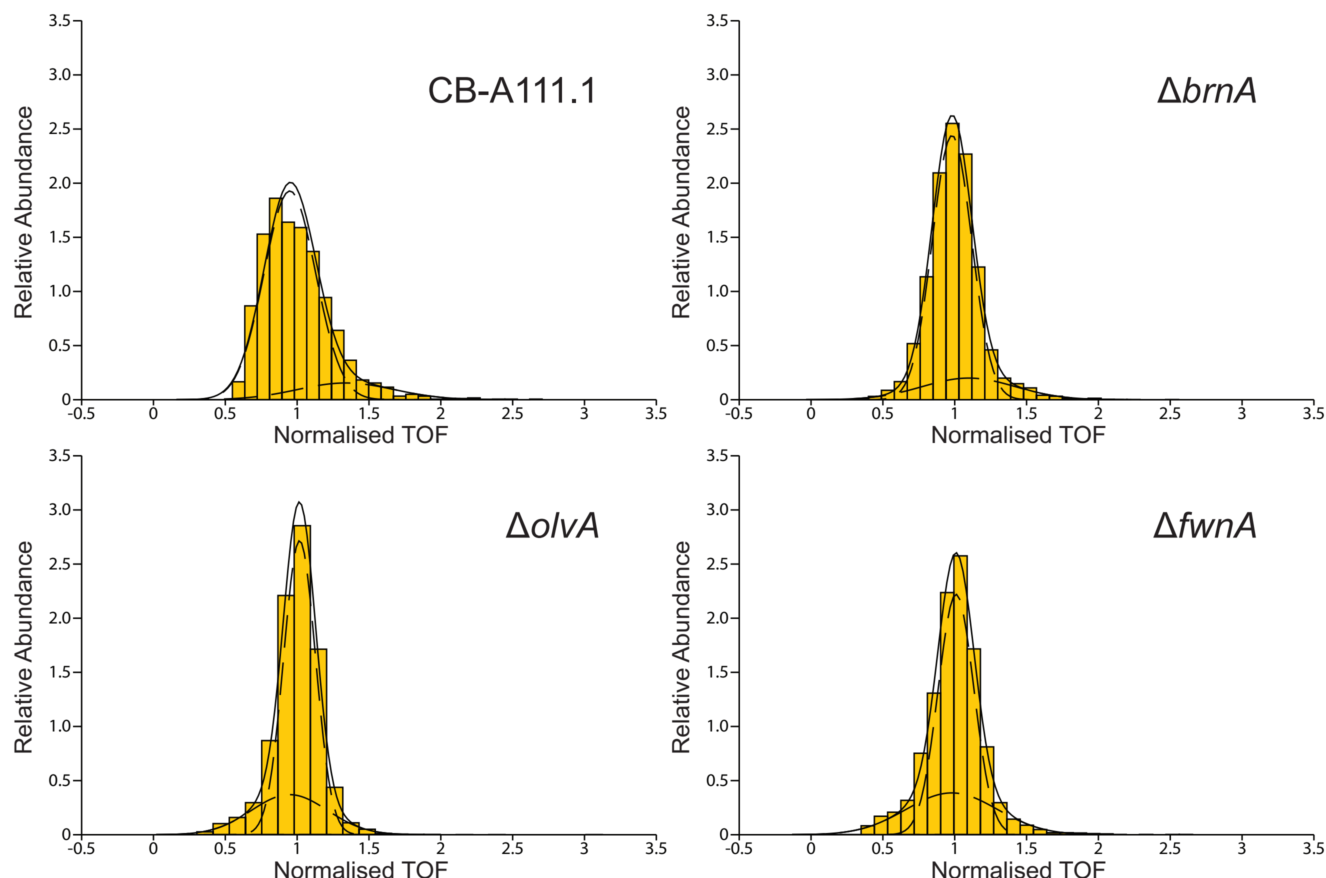

Fig. 5. Size distributions of micro-colonies of liquid shaken cultures of the control strain CB-A111.1 and the Δ*brnA*, Δ*olvA* and Δ*fwnA* strains. The TOF of each micro-colony was divided by the average TOF of the micro-colonies in the culture to compile the data of a biological triplicate in one histogram. 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 correlate in individual hyphae of micro-colonies.

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 (Fig. 6). Therefore, fluorescence data were log transformed followed by modelling the data assuming a bimodal distribution (see above). In this case, only 2 out of the 24 cases reporter expression was heterogeneous (Table 6).

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.* 1993a,b, Masai *et al.* 2006, Levin *et al.* 2007a,b) but even between neighboring hyphae (Moukha *et al.* 1993a, Teertstra *et al.* 2004, Vinck *et al.* 2005, 2011, Etxebeste *et*

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

Table 3. Heterogeneity between micro-colonies in 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 (μ1 and μ2) and a CI of the participation fraction (pf) between 0.025–0.975.

Strains	CI μ1		CI μ2		CI pf		Diameter (μm) fraction 1	Diameter (μm) fraction 2	heterogeneity
CB-A111.1	0.9148	0.9657	1.2237	1.4394	0.744	0.918	608	755	Yes
Δ*brnA*	0.9765	0.9854	1.0624	1.1375	0.794	0.875	780	842	Yes
Δ*olvA*	0.9306	0.9620	1.0116	1.0216	0.181	0.290	825	868	Yes
Δ*fwnA*	0.9689	1.0160	0.9641	1.0137	0.335	0.764	818	-	No

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

Strain	Promoters	Colony	Micro-colony	Carbon source
CB-A114.2	*faeA//faeA*	0.7	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*	0.8	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	0.8	xylose/maltose
CB-A118.28	*faeA//glaA*	0.46	0.49	xylose/maltose
CB-A121.4	*glaA//aamA*	0.8	0.74	maltose
CB-A121.7	*glaA//aamA*	0.78	0.8	maltose

al. 2009, de Bekker *et al.* 2011a). 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 heterogenic with respect to size and gene expression (de Bekker *et al.* 2011b). Moreover, it was shown that hyphae in the outer zone contain more RNA than hyphae in the central zone of the micro-colony (de Bekker *et al.* 2011b). 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). 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). Therefore, the biomass of the individual hyphae represents maximally a few percent of the culture. This agrees with a study performed with *Aspergillus 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*

Table 5. Heterogenic 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 (μ1 and μ2) and a CI of the participation fraction (pf) between 0.025–0.975.

Strain	Promoter	CI μ1		CI μ2		CI pf		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	276.46	0.47	0.9	Yes
CB-A 114.22	*faeA*	47.4	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	170.06	223.84	0.68	0.85	Yes
	aguA	69.75	89.5	151.63	280.78	0.67	0.94	Yes
CB-A 115.9	*faeA*	56.57	89.17	141.41	350.6	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	90.7	123.91	392	0.33	0.96	Yes
CB-A 116.11	*faeA*	53.7	89.8	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	165.7	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	0.9	Yes
	aamA	46.45	73	148.21	246	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	87.7	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*	50.7	77.69	128.64	188.7	0.4	0.79	Yes
	aamA	29.81	92.14	111.78	302.03	0.2	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

Table 6. Heterogenic 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 (μ1 and μ2) and a CI of the participation fraction between 0.025–0.975.

Strain	Promoter	CI μ1		CI μ2		CI pf		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

Table 6. (Continued).

Strain	Promoter	CI µ1		CI µ2		CI pf		Heterogeneity
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

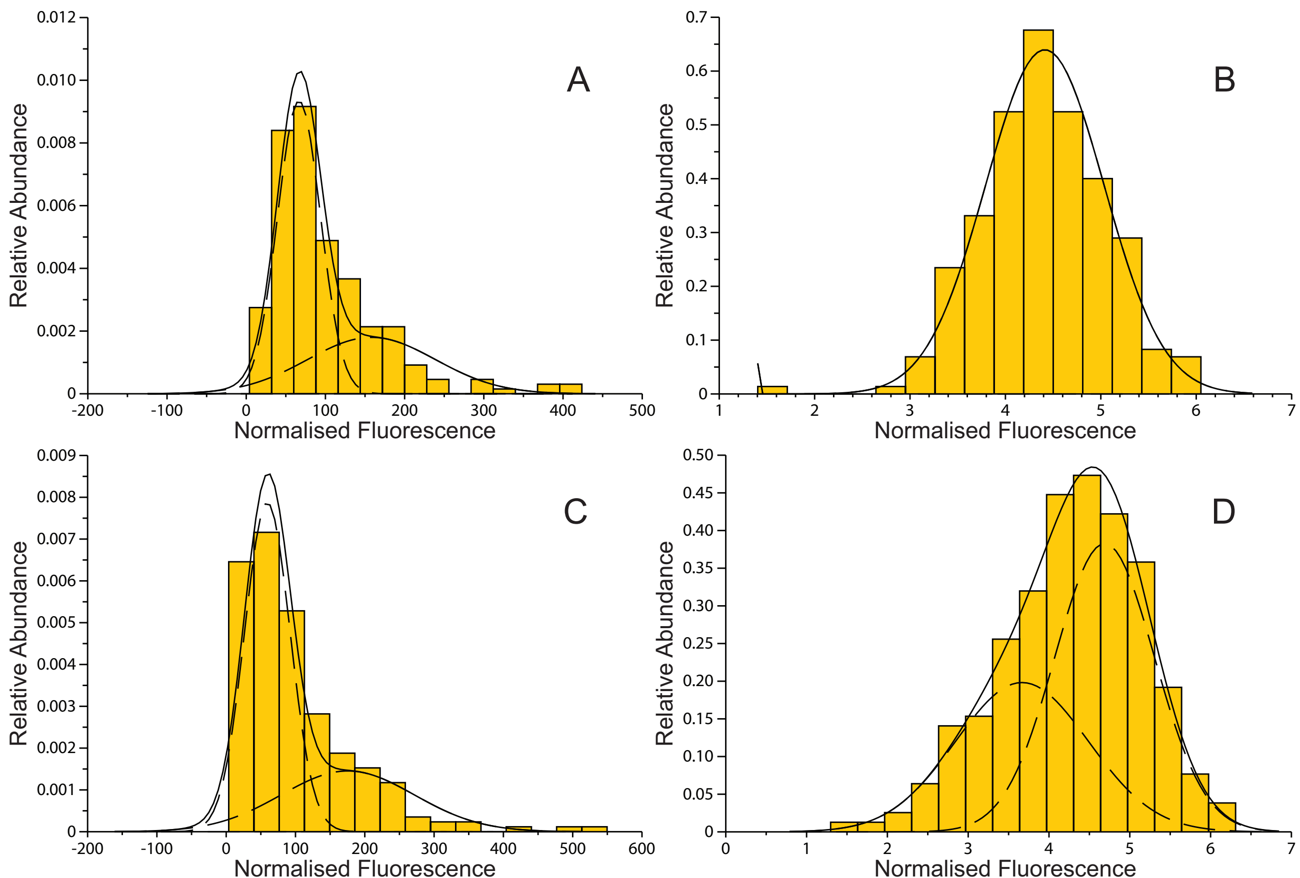

Fig. 6. Representative fluorescence distributions before (A, C) and after (B, D) log transformation of hyphae at the periphery of micro-colonies of strain CB-A118.28 expressing GFP from the *faeA* promoter (A, B) and dTomato from the *glaA* promoter (C, D). A, C and D show non-overlapping CI's of the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

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.* 2003). 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. The population of large and small micro-colonies had an average diameter of 595 and 505 µm, respectively. Here, we showed that cultures of the control strain CB-A111.1 are characterised 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 was 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 d 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.* 2010). 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 pellet 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* promoter (G.J. van Veluw, R. Bleichrodt and H.A.B. Wösten, unpubl. results). This indicates that heterogeneity of expression of genes at the periphery of the micro-colonies is less robust as observed in macro-colonies. Possibly, signalling between hyphae is involved in promoting heterogeneity. In contrast to solid media, gradients of signalling 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 signalling molecules in heterogeneity of gene expression in aspergilli.

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