Fungal Genetics and Biology 82 (2015) 193–200

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Selective transport between heterogeneous hyphal compartments via the plasma membrane lining septal walls of *Aspergillus niger*

Robert-Jan Bleichrodt [a,b], Arman Vinck [a], Nick D. Read [b], Han A.B. Wösten [a,*]

[a] Microbiology and Kluyver Center for Genomics of Industrial Fermentation, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands
[b] Manchester Fungal Infection Group, Institute of Inflammation and Repair, University of Manchester, CTF Building, 46 Grafton Street, Manchester M13 9NT, United Kingdom

ARTICLE INFO

Article history:
Received 9 September 2014
Revised 19 May 2015
Accepted 21 June 2015
Available online 23 July 2015

Keywords:
Fungus
Glucose
Transport
Septa
Heterogeneity
Mycelium

ABSTRACT

Hyphae of ascomycetes are compartmentalized by septa. The central pore in these septa allows for cytoplasmic streaming. However, many of these pores are closed by Woronin bodies in *Aspergillus*, which prevents cytoplasmic mixing and thus maintains hyphal heterogeneity. Here, glucose uptake and transport was studied in *Aspergillus niger*. Glucose uptake was higher in the hyphal population with high transcriptional activity when compared to the population with low transcriptional activity. Glucose was transported from the colony center to the periphery, but not vice versa. This unidirectional flow was similar in the wild-type and the Δ*hexA* strain that does not form Woronin bodies. This indicated that septal plugging by Woronin bodies does not impact long distance glucose transport. Indeed, the glucose analogue 2-NBDG (2-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-2-deoxyglucose) translocated to neighboring hyphal compartments despite Woronin body mediated plugging of the septum that separated these compartments. Notably, 2-NBDG accumulated in septal cross walls, indicating that intercompartmental glucose transport is mediated by transporters that reside in the plasma membrane lining the septal cross-wall. The presence of such transporters would thus enable selective transport between heterogeneous compartments.

1. Introduction

Filamentous fungi form mycelia consisting of interconnected hyphae that grow at their apices and that branch subapically. Hyphae of the Ascomycota and the Basidiomycota are compartmentalized by septa. These septa consist of invaginations of the cell wall that are lined with the plasma membrane and have a central pore of 50–500 nm (Shatkin and Tatum, 1959; Moore and McAlear, 1962; Lew, 2005). These pores allow streaming of cytosol and even organelles thus enabling mixing of cytoplasm throughout the mycelium. However, Woronin bodies plug approximately 40–50% of the apical septa of hyphae at the periphery of colonies of *Aspergillus*, while the incidence of plugging increases up to 100% in the sub-peripheral colony region (Bleichrodt et al., 2012, 2015). Therefore, cytoplasmic continuity is restricted in an *Aspergillus* mycelium (Bleichrodt et al., 2012, 2015).

The restricted cytoplasmic continuity in the *Aspergillus* mycelium explains why heterogeneity can be observed between zones of a colony with respect to gene expression, growth, and secretion (Wösten et al., 1991, 2013; Moukha et al., 1993a,b; Masai et al., 2006; Levin et al., 2007a,b; Kasuga and Glass, 2008; de Bekker et al., 2011a,b; Krijgsheld et al., 2012, 2013). Even hyphae within a zone of a mycelium can be heterogeneous with respect to cytoplasmic composition (Wösten et al., 1991, 2013; Moukha et al., 1993a; Teertstra et al., 2004; Vinck et al., 2005, 2011; Etxebeste et al., 2009; de Bekker et al., 2011a,b; Bleichrodt et al., 2012; van Veluw et al., 2013; Knaus et al., 2013). For instance, two populations of hyphae can be distinguished at the periphery of an *Aspergillus niger* colony: those with a low and those with a high transcriptional and translational activity (Vinck et al., 2011). The existence of distinct hyphal types in *Aspergillus* can be abolished by inactivation of *hexA* (Bleichrodt et al., 2012) that encodes the major component of Woronin bodies (Jedd and Chua, 2000). These organelles are no longer formed in the absence of *hexA* and, as a consequence, septal plugging is rarely observed (Bleichrodt et al., 2012; Bleichrodt, 2012).

Abbreviations: AU, arbitrary units; FRAP, fluorescence recovery after photo-bleaching; MFS, major facilitator super-family; MM, minimal medium; 2-NBDG, 2-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-2-deoxyglucose; 3-OMG, ^{14}C-3-*O*-methyl glucose; PC, polycarbonate; RFP, red fluorescent protein.

* Corresponding author at: Department of Microbiology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands.

E-mail addresses: robert-jan.bleichrodt@manchester.ac.uk (R.-J. Bleichrodt), armanvinck@gmail.com (A. Vinck), nick.read@manchester.ac.uk (N.D. Read), h.a.b.wosten@uu.nl (H.A.B. Wösten).

http://dx.doi.org/10.1016/j.fgb.2015.06.010
1087-1845/

Here, it is shown that glucose uptake in *A. niger* is higher in hyphae with a high transcriptional activity when compared to hyphae with low transcriptional activity. It is also shown that glucose transport is not affected by septal plugging, explained by transport of glucose via the plasma membrane that lines the septal cross-wall.

2. Materials and methods

2.1. Strains and growth conditions

Strains of *A. niger* and *A. oryzae* (Table 1) were grown at 30 °C in boxes with constant light (~700 lux). Colonies were grown as sandwiched cultures using MM (0.6% $NaNO_3$, 0.15% KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4{\cdot}7H_2O$, 0.2 ml l^{-1} 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 and Santer, 1957], pH 6.0) containing 1.5% agar and 200 mM xylose or 25 mM maltose as carbon source. 2 µl spore suspension (a total of 1000 spores) was added on top of a PC membrane (76 mm; Profiltra, Almere, the Netherlands) overlaying 25 ml agar medium in a 9-cm Petri dish. After 24 h a Lumox membrane (20 × 20 mm, manually cut; Greiner Bio-One, Alphen a/d Rijn, the Netherlands) was placed on top of the culture with the hydrophobic site facing the colony (de Bekker et al., 2011a). When cultures were grown for more than 3 days a 76 mm PC membrane was used instead of a Lumox membrane. Alternatively, 5000 spores were seeded in poly-d-lysine coated glass bottom dishes (P35GC-1.5-14-C; MatTek, Ashland, USA) in 2 ml MM + 25 mM maltose liquid medium and grown for 24 h.

Spores were harvested from 3-day-old cultures that had been grown on complete medium (MM with 0.2% tryptone, 0.1% casamino acids, 0.1% yeast extract, 0.05% yeast ribonucleic acids, and 1% glucose) using 0.9% NaCl (w/v), 0.05% (v/v) Tween-20. Spores were routinely diluted to a final concentration of 5×10^5 spores ml^{-1}. Spores were diluted to 1×10^8 spores ml^{-1} in the case of radioactive labeling experiments.

2.2. Expression of RFP

Gene *TagRFP-T* was amplified by PCR using Phusion® High-Fidelity DNA polymerase (Fisher Scientific, Landsmeer, the Netherlands), TagRFP-158T-C1 plasmid DNA (Merzlyak et al., 2007) and primers 5′-GTTCCATGGGTGCTGGTGCTGGTGCTGGTGCT GGTGCTATGGTGTCTAAGGGCG-3′ and 5′-AAGCTTTCAATTAAGTTT GTGCCCCAG-3′. The blunt end PCR product of *TagRFP-T* was inserted in the SmaI site of pUC19. The cloned fragment was cut from the resulting vector pRB068 with NcoI/HindIII and ligated in pGPDGFP (Lagopodi et al., 2002) that had been digested with the same enzymes. The resulting plasmid pRB069 encompasses *TagRFP-T* under control of the *Aspergillus nidulans gpdA* promoter and the *trpC* terminator.

2.3. Transformation

Protoplasts of *A. niger* were obtained according to de Bekker et al. (2009) and transformed using polyethylene glycol (Punt and van den Hondel, 1992). N593 was co-transformed with pRB069 and pGW635 that contains *pyrG* as a selection marker (Goosen et al., 1989). Transformants were selected on MMS medium (MM with 0.95 M sucrose and 1.5% agar) based on *pyrG* prototrophy. A representative transformant RB#69.1 was selected based on RFP fluorescence.

2.4. Short distance inter-compartmental glucose transport

The wild-type *A. niger* strain N402, and strain RB#69.1 that expresses TagRFP-T (henceforth called RFP) controlled by the *gpdA* promoter, were grown as sandwiched colonies (see above; Wösten et al., 1991) on MM with 25 mM maltose as carbon source. After 1–2 days, the colonies were transferred for 1 or 24 h to MM without a carbon source or with 25 mM maltose containing 200 µM of the inert fluorescent glucose analogue 2-NBDG (Oliveira et al., 2002) (Life Technologies, Bleiswijk, the Netherlands). Alternatively, strain N402 was grown for 24 h as a liquid culture in a poly-d-lysine coated glass bottom dish (see above) using MM with 25 mM maltose, after which the medium was replaced with MM containing 200 µM 2-NBDG and incubated for 1–2 h.

2-NBDG fluorescence at the colony level was monitored using a Leica MZFLIII binocular (Leica Microsystems CMS, Mannheim, Germany) equipped with a HBO 100 W mercury lamp and a Photometrics Cool SNAP camera (1392 × 1024 pixels) connected to a computer. Uptake of 2-NBDG in individual hyphae was visualized by CLSM with an inverted Zeiss LSM 5 system using a Plan-Neofluar 25×/0.8 Imm corr objective (Carl Zeiss Micro-Imaging, Oberkochen, Germany) with oil immersion, a 488 nm laser (5 mW power), a pinhole of 2.9 airy units, a pixel dwelling time of 1.60 µs, and a LP530 filter. Images were captured as 512 × 512 or 1024 × 1024 pixels.

To study intercellular 2-NBDG flow between hyphal compartments a Leica SP8 confocal system (Leica Microsystems CMS, Mannheim, Germany) was used with the internal FRAP mode with the Argon laser at 69.8% power. Photo-bleaching was obtained by 10 s exposure (continuous scanning) to 50% 488 nm light (Argon laser) using a pinhole of 3.0 airy units. Time-lapse movies of 1 min were produced with an image captured every 1.3 s (HC PL APO UVIS CS2 63×/1.20 W CORR objective, pinhole 3.0 airy units, 488 nm Argon laser at 5%, continuous emission filter 505–600 nm, PMT detector). 2-NBDG content was measured in 20 wide and thin hyphae as arbitrary fluorescence units in ~100 μm^2 ROIs using imageJ 1.48 v. A *T*-test was performed to compare the levels of 2-NBDG in wide and thin hyphae. Differences were assumed significant when $p < 0.05$.

FRAP was performed using a Zeiss LSM 700 system with a Plan-Neofluar 40×/1.3 objective (Carl Zeiss Micro-Imaging,

Table 1
Strains used in this study.

Strain	Parental strain	Genotype	Auxotrophy	Reporter plasmid	References
N402	NRRL 3	Δ*cspA1*			Bos et al. (1988)
N593	N402	Δ*cspA1*	*pyrG*$^-$		Goosen et al. (1987)
RB#69.1	N593	Δ*cspA1*		pRB069 (P*gpdA*::*TagRFP-T*)	This study
N402Δ*AnhexA*	N402	Δ*cspA1* Δ*AnhexA*			Bleichrodt (2012)
NSRKu70-1-1AS	NSRku70-1-1A	Δ*ku70*	*niaD*$^-$		Tanabe et al. (2011)
NSRK-ΔH×5	NSRku70-1-1A	Δ*ku70* Δ*AohexA*	*niaD*$^-$		Bleichrodt et al. (2012)
CB-A109.1	N593	Δ*cspA1*		pCB021 (P*glaA*::*dTomato*)	Vinck et al. (2011)

Oberkochen, Germany) with oil immersion to study intercellular 2-NBDG and RFP flow in strain RB#69.1. Time-lapse movies of 2–5 min were obtained by CLSM with an image taken every 1.97 s. Photo-bleaching of 2-NBDG was obtained by a 5 s exposure to 12.5 mW 488 nm laser light. RFP was monitored using a LP 560 filter and CLSM settings of 0.05 mW 543 nm laser power, pinhole 3.03 airy units, pixel time 1.60 μs, and 1.97 s scan time. Photobleaching of RFP was performed for 10 s with 0.5 mW 543 nm laser light.

2.5. Monitoring long distance glucose transport

Five-day-old xylose-grown sandwiched colonies of *A. niger* strains N402 and N402Δ*AnhexA* and *A. oryzae* strains NSRKu70-1-1AS and NSRK-ΔH×5 were transferred to fresh solid medium containing 25 mM maltose or to a medium without a carbon source. Colonies were labeled with 1 μCi [^{14}C]D-maltose (specific activity, 2.04 gBq mmol^{-1}; MP Biomedicals, Amsterdam, the Netherlands) or [^{14}C]3-O-methyl-D-glucose (specific activity, 2.04 gBq mmol^{-1}; MP Biomedicals) for 5 or 24 h. Label was absorbed to a rice paper disc (diameter 20 mm) and placed on top of the sandwiched culture either at its center or periphery. After labeling, colonies were either or not fixed with 4% formaldehyde for 1 h at RT. Colonies were washed 3 times for 60 min with 100 mM D-maltose or 100 mM D-glucose at RT. After air-drying, colonies were exposed to Kodak X-OMAT films (Sigma–Aldrich, Zwijndrecht, the Netherlands).

2.6. Heterogeneity within Aspergillus colonies

To monitor heterogeneity in glucose uptake, a 1-day-old xylose grown sandwiched *A. niger* culture was transferred to MM with 200 μM 2-NBDG immediately after placing the Lumox membrane (see growth conditions). After 42 h of growth, the sandwiched colony was transferred for 6 h to MM plates containing 25 mM maltose. 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, Ashland, USA; P35G-1.5-20-C) on a 20 μl drop of MM. 2-NBDG and dTomato fluorescence was monitored on a Zeiss LSM 700 AxioObserver system equipped with a Plan-Apochromat 20×/0.8 M27 objective (Carl Zeiss Micro-Imaging, Oberkochen, Germany). 2-NBDG was excited with a 10% 488 nm laser BP 490-555, and dTomato with a 10% 555 nm laser LP560 in multi-track mode. Images were captured as Z-stacks of optical slices; pinhole 98 μm (2-NBDG), 104 μm (dTomato); interval 1.00 μm; 8 bit scan depth, pixel time 1.58 μs, line average 2). 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, Sliedrecht, the Netherlands). Hyphal regions of interest of ~50 μm were selected manually and fluorescence was automatically quantified as the sum gray 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 en 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).

3. Results

3.1. Long distance streaming of glucose

Sandwiched colonies of *A. niger* strain N402 were grown for 5 days on 200 mM xylose, after which they were transferred to medium containing 25 mM maltose or to medium without a carbon source. After transfer, the center of colonies was exposed for 5 or 24 h with 1 μCi (*i.e.* 7.2 10^{-4} mM) ^{14}C-labeled maltose applied on a 2 cm disc of rice paper. D-maltose is extra-cellularly converted into glucose, which is taken up by the fungus and metabolized (vanKuyk et al., 2012). Autoradiography showed that part of the ^{14}C-glucose that had been taken up had been transported to the periphery of the colony (Fig. 1A). Labeling was more intense when colonies had been labeled for 24 h instead of 5 h and when the medium did not contain a carbon source. However, distribution patterns were similar (data not shown). Labeling of N402Δ*hexA* (Fig. 1E) was similar to strain N402 (Fig. 1A). Thus, septal pore plugging by Woronin bodies does not impact the transport of glucose through the colony.

By fixing colonies with formaldehyde and washing with non-labeled maltose (as in Fig. 1A and E) only label that has been incorporated in macro-molecules (*e.g.* polysaccharides, nucleic acids and proteins) will be retained in the colony. In contrast, labeled glucose or other small organic molecules also remain in the mycelium by washing non-fixed colonies (Levin et al., 2007a). No difference in the spatial distribution of label was observed between fixed and non-fixed colonies (Compare Fig. 1A and E with Fig. 1B and F). This shows that ^{14}C-glucose is readily incorporated into macro-molecules. The rapid metabolism of the sugar may impact its distribution through the mycelium. Therefore, colonies were labeled with the inert glucose analogue ^{14}C-3-OMG. Distribution of 3-OMG in fixed wild-type and Δ*hexA* colonies was different from that of ^{14}C-glucose (compare Fig. 1A and E with Fig. 1C and G). In the case of 3-OMG, label was present at the periphery, but was absent where it was taken up (*i.e.* in the colony center). Apparently, 3-OMG quickly moves outwards after uptake. Non-fixed colonies that had been labeled with ^{14}C-3-OMG contained more label than fixed colonies (compare Fig. 1C and G and Fig. 1D and H). Apparently, most of the ^{14}C-3-OMG label is washed out in the fixed samples illustrating that most of the label is not incorporated into macro-molecules. The fact that fixed colonies do contain ^{14}C-label suggests that *A. niger* can metabolize this inert sugar analogue, albeit at a low level.

Glucose and 3-OMG did not translocate to the center of the colony when ^{14}C-maltose or ^{14}C-3-OMG was applied at the periphery of the N402 or N402Δ*hexA* mycelium (Fig. 2). In contrast, they did move laterally within the colony periphery (Fig. 2). All labeling experiments described above were also performed with the *A. oryzae* control strain NSRKu70-1-1AS and the Δ*hexA* strain NSRK-ΔH×5. Similar results were obtained when compared to the *A. niger* strains N402 and N402Δ*AnhexA* (data not shown). Taken together, the results show that glucose and 3-OMG stream from the center to the periphery of the *Aspergillus* colony, but not vice versa, and that the presence of Woronin bodies does not impact long distance glucose transport.

3.2. Intercellular streaming of glucose within the periphery of the colony

Colonies of the wild-type *A. niger* strain N402 and strain RB#69.1 that expresses cytosolic RFP were incubated with the fluorescent glucose analog 2-NBDG. After 1 h, the entire N402 colony had taken up 2-NBDG (Fig. 3A). Much less 2-NBDG was taken up when the medium also contained 25 mM maltose (data not

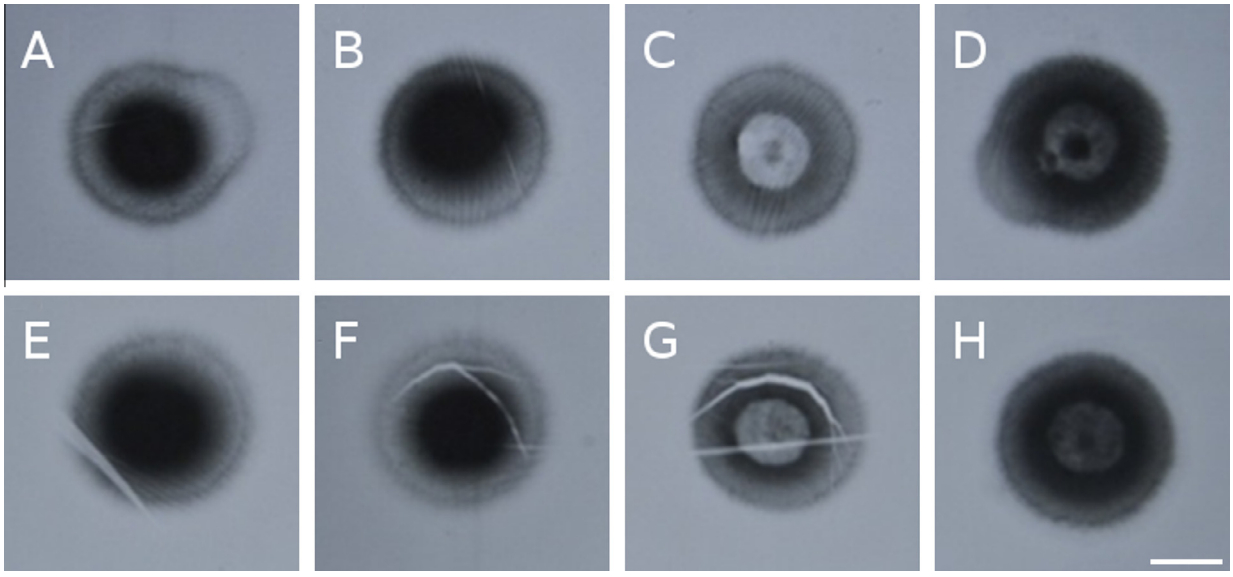

Fig. 1. Long-distance glucose transport from the center to the periphery of colonies. Fixed (A, C, E, G) and non-fixed (B, D, F, H) colonies of *A. niger* strains N402 (A–D) and N402Δ*AnhexA* (E–H) that had been labeled for 24 h at the center of the mycelium with ^{14}C maltose (A, B, E, F) or ^{14}C 3-OMG (C, D, G, H). Scale bar represents 2 cm.

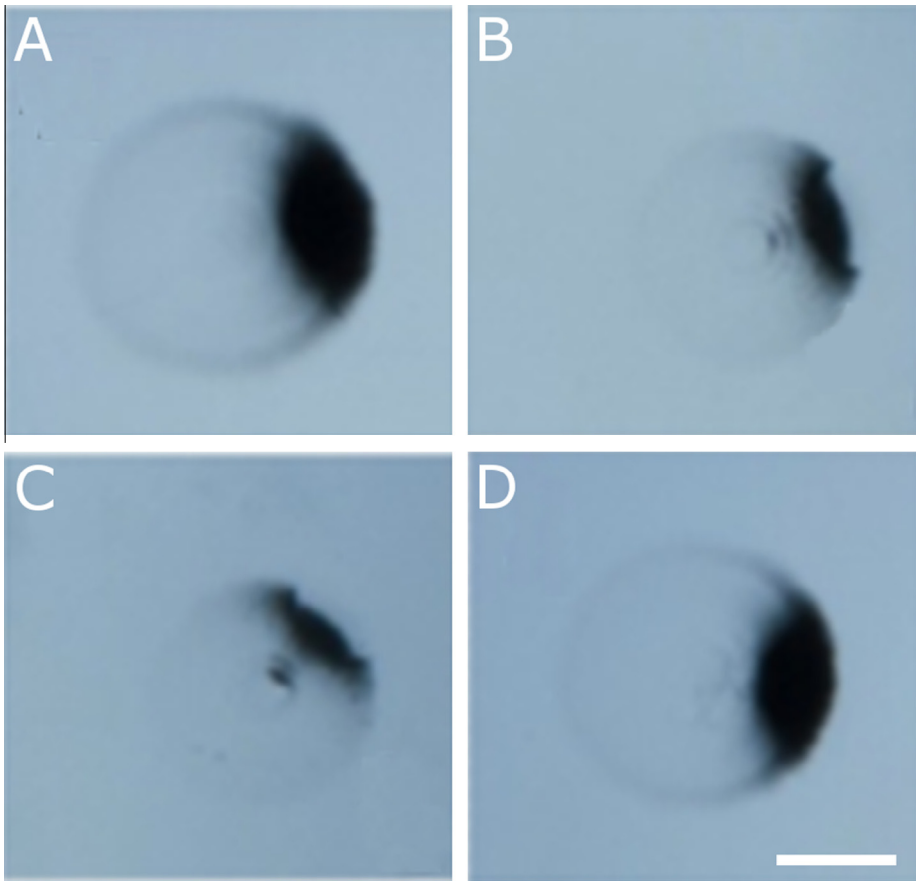

Fig. 2. Long-distance glucose transport within the periphery of colonies. Wild-type N402 (A and C) and N402Δ*AnhexA* (B and D) colonies of *A. niger* that had been labeled for 24 h at the periphery of the mycelium with ^{14}C maltose (A and B) or ^{14}C 3-O-methyl-D-glucose (C and D). Scale bar represents 2 cm.

shown), which can be explained by competition for uptake between glucose and 2-NBDG. The fluorescent analogue of glucose localized to the cytosol and the septa (Fig. 3D) and accumulated in vacuoles after 4–24 h (Fig. 3E). FRAP of cytosolic 2-NBDG was observed in the wild-type strain N402 within 1 min in 20 out of 20 hyphae after photo-bleaching a compartment (Fig. 4). This implies that 2-NBDG had been streaming from the left or right neighboring compartment to the bleached compartment. Strain RB#69.1 that expresses RFP under control of the promoter of the glucoamylase gene *glaA* was incubated with 2-NBDG to assess

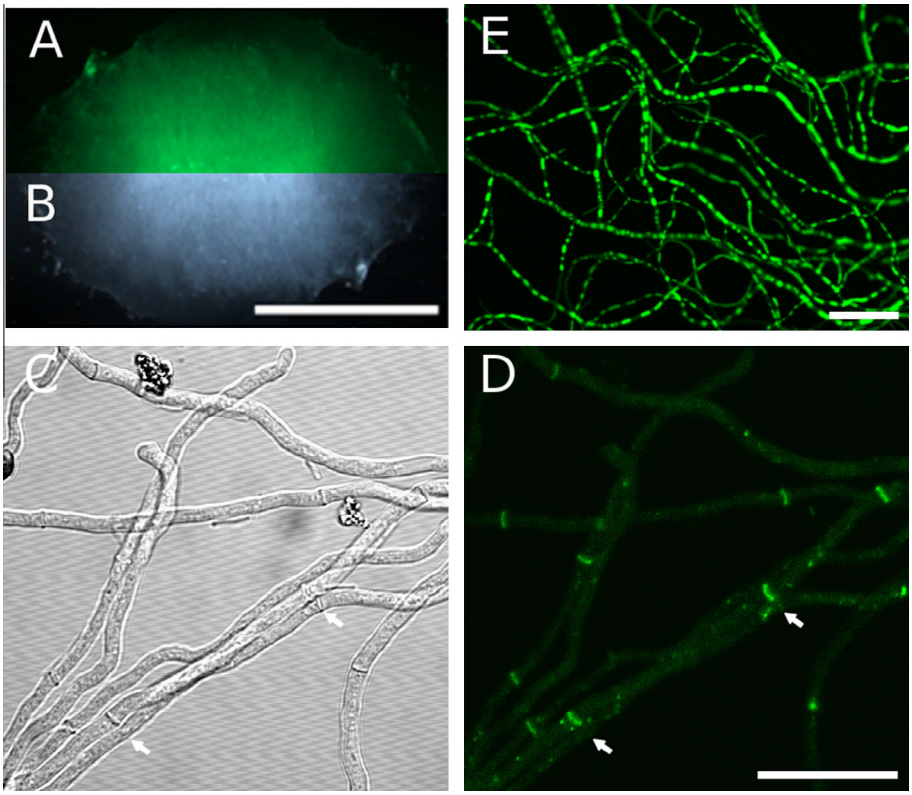

Fig. 3. Uptake of 2-NBDG by a colony of *A. niger*. Strain N402 was grown for 1–2 days as a sandwiched culture or in a glass bottom dish on MM with 25 mM maltose. The colonies were subsequently incubated in MM containing 200 μM 2-NBDG for 1–2 h (A–D) or 24 h (E). Distribution of 2-NBDG fluorescence in a N402 colony (A), bright field image as a control (B). After 1–2 h 2-NBDG is distributed in the cytosol of peripheral hyphae and accumulates in septa (D), bright field (C). 2-NBDG is eventually taken up by vacuoles (E) after it has been taken up from the medium. Bars represent 5 mm (A and B), 50 μm (C–E). Arrows indicate septa (C and D).

whether septal plugging blocks 2-NBDG transport. FRAP of RFP was not observed in compartments in which both septal pores had been plugged (Fig. 5C, F and G). In contrast, FRAP did occur in the case of 2-NBDG (Fig. 5B, E and G). The relatively low recovery observed for 2-NBDG to its initial fluorescence level can be explained by the fact that the vacuoles contained most of the 2-NBDG before bleaching and that recovery of fluorescence was not observed in the vacuoles within this time frame. A recovery rate of ~50% was obtained within parts of hyphae lacking vacuoles (as is seen in Fig. 4D). From these data it is concluded that septal plugging is not blocking intercompartmental streaming of glucose, but blocks intercellular streaming of RFP.

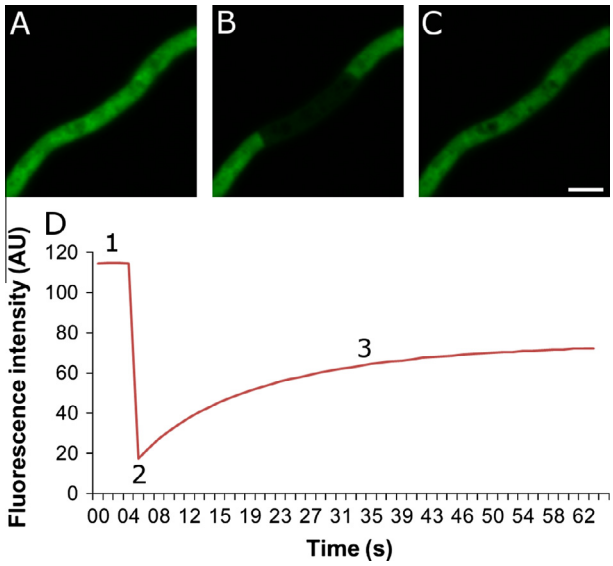

Fig. 4. Glucose transport between neighboring hyphal compartments. FRAP was performed on hyphal compartments of strain N402 that had been grown for 1 d and subsequently incubated with 200 μM 2-NBDG for 24 h. A compartment and its fluorescence intensity (AU) are shown before (A and D1) and after bleaching (B and D2) and after recovery of fluorescence (C and D3). Scale bar represents 20 μm (C).

3.3. Glucose concentration is higher in wide hyphae than in thin hyphae

A. niger strain N402 was grown as sandwiched colonies on MM with 25 mM maltose as a carbon source. Light microscopy revealed the presence of two types of hyphae within the sub-periphery and more central parts of the colony. Thin hyphae (2.4 μm ± 0.3) that were orientated more-or-less randomly and a second hyphal type that was more than twice as wide (6.4 μm ± 0.7) and traversed from the center toward the colony periphery over long distances (Fig. 6). Glucose uptake of 2-NBDG was significantly higher ($p = 0.008$) in wide hyphae when compared to thin hyphae with mean pixel values (in arbitrary fluorescence units) of 32.6 (28.0–37.2; 95% confidence interval) and 24.5 (21.3–27.8), respectively. This was measured in regions of interest of ~250 μm^2 within 20 leading hyphae of each population. These data indicate that wide hyphae contain a higher glucose concentration than thin hyphae.

3.4. Hyphae that highly express glaA take up more glucose than hyphae that lowly express glaA

2-NBDG uptake was co-localized with dTomato expression from the promoter of the glucoamylase gene *glaA* to assess whether high transcriptional activity correlates with high glucose uptake. To this

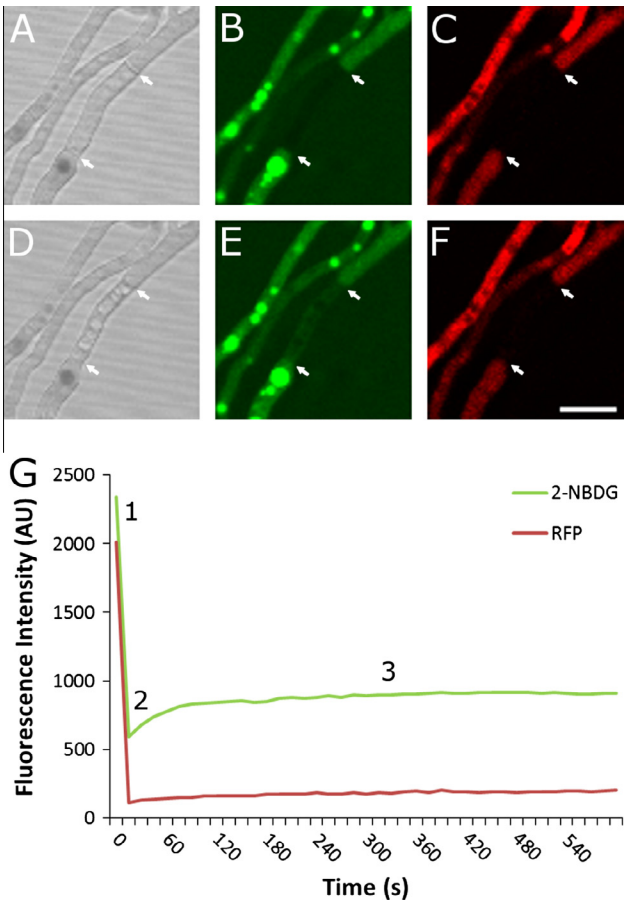

Fig. 5. Septal plugging does not impede intercompartmental glucose streaming but does impede intercellular protein streaming. *A. niger* strain RB#69.1 was grown as a sandwiched culture on MM with 25 mM maltose. After 1 day, the colonies were transferred to MM containing 200 μM 2-NBDG for 24 h. The second compartment of a hypha is shown directly after photo-bleaching of 2-NBDG (B) or RFP (C) and after 2 min, respectively (E and F). (A) and (D) represent bright field images. Arrows indicate septa. Bar represents 10 μm (F). Fluorescence intensity of the second compartment is shown before bleaching (G1), directly after bleaching (G2) and after recovery of fluorescence (G3).

end, 1-day-old xylose-grown colonies of CB-A109.1 were transferred to MM with 200 μM 2-NBDG, followed by a transfer after 18 h to MM agar medium containing 25 mM maltose. The latter induced dTomato expression from the *glaA* promoter. After 6 h, optical Z-stacks were produced of the colony periphery and maximum intensity projections were made (Fig. 7A). Fluorescence intensities resulting from dTomato and 2-NBDG were quantified for more than 200 leading hyphae. Both *glaA* expression (dTomato) and glucose uptake (2-NBDG) showed heterogeneity within the hyphae at the periphery of the colony. Two populations of hyphae could be distinguished: one that highly express *glaA* and shows high 2-NBDG uptake, while the other showed low *glaA* expression and low 2-NBDG uptake (Table 2, Figs. 7 and 8A and B). The Pearson correlation coefficient of dTomato and 2-NBDG fluorescence signals was 0.84.

4. Discussion

Nutrients can be transported over long distances in a fungal mycelium (Jennings, 1994). Hyphal fusion plays an important role in long-distance translocation of nutrients in *Neurospora crassa* (Simonin et al., 2012). This was shown by the severely reduced transport of nutrients in a *soft* mutant (Δ*so*) that lacks hyphal fusion. Even a 50% reduction in hyphal fusion frequency, as occurs in the Δ*prm-1* mutant, results in decreased nutrient translocation rates. Translocation of nutrients and organelles in *N. crassa* is also facilitated by its open septa (Hickey et al., 2002; Simonin et al., 2012). Notably, most septa are closed in *A. niger* (Bleichrodt, 2012) and hyphal fusion has not been observed. Yet, we showed here that glucose was efficiently transported from the center to the periphery of the mycelium. Glucose transport was not increased in a *hexA* deletion strain that does not form Woronin bodies and, as a result, has only open septa. Labeling with 2-NBDG provided an explanation for this paradox. This fluorescent glucose analogue accumulated in the cross walls of the septa, implying that it had been transported across the plasma membrane lining the cross walls. Glucose transport across the plasma membrane is mediated by transporters that belong to the MFS class (Nelissen et al., 1997). These permeases typically consist of 12 transmembrane domains that form a channel in the cell membrane. They allow the passive passage of sugars down a concentration gradient. Interestingly, the hexose transporter *mstE* was found to localize to septa in *A. nidulans* (Forment et al., 2006). This suggests that intercellular glucose transport is mediated by hexose transporters in the septa.

Wide hyphae were shown to traverse from the center to the sub-periphery of the *A. niger* colony. These hyphae are also present in *N. crassa* (Steele and Trinci, 1975; McLean and Prosser, 1987; Bistis et al., 2003; Lew, 2005), where they are called 'leader or trunk' hyphae. Cytoplasmic bulk flow in these hyphae is uni-directional toward the colony periphery and exhibits velocities as high as 60 μm s^{-1} (Lew, 2005). ^{14}C labeling showed that glucose transport was also uni-directional to the colony periphery in the case of *A. niger*. Transport of the sugar from the periphery to the colony center was not observed. Indeed, the wide hyphae showed a higher concentration of the glucose analoque 2-NBDG than thin hyphae. It is likely that wide hyphae mediate the long-distance uni-directional glucose transport observed from the center to the periphery of the colony. We observed trans-lateral movement of glucose in the periphery of the colony (Fig. 2). For this, the leading hyphae would have to transport glucose back to wide hyphae that then transport it to a neighboring sector at the outer part of the

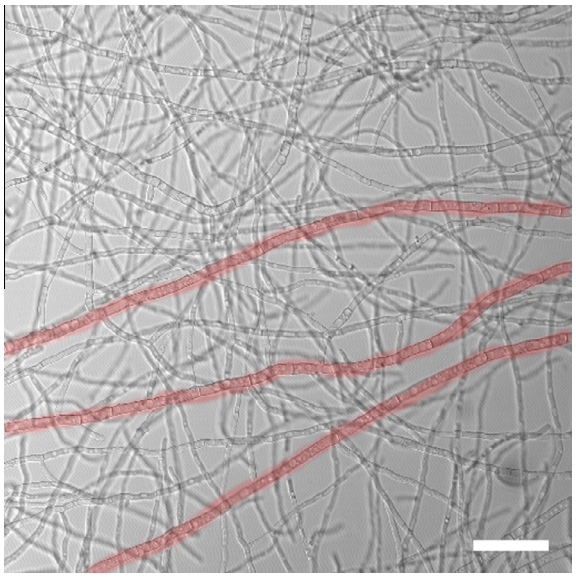

Fig. 6. Wide hyphae (highlighted in red) orientated from left to right within the sub-peripheral part of an *A. niger* N402 wild-type colony surrounded by thin hyphae orientated more-or-less randomly. Scale bar represents 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

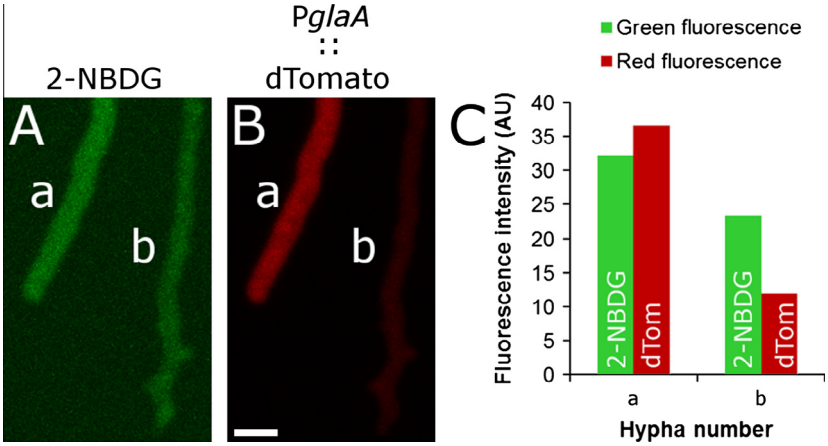

Fig. 7. Glucoamylase (*glaA*) expression correlates with glucose uptake. dTomato (strain CB-A109.1) was expressed under control of the *glaA* promoter. Correlation of fluorescence intensity between 2-NBDG (A) and dTomato (B). Fluorescence intensities (AU) of individual hyphae (a and b) from panels A and B are shown (C). Scale bar represents 10 μm (A and B).

Table 2
95% CI of the mean assuming a bimodal fluorescence distribution of *glaA* driven dTomato and 2-NBDG uptake in leading hyphae of strain CB-A109.1 when incubated for 18 h induced in the presence of 2-NBDG, and of *glaA* driven dTomato expression (6 h induced). *N* = sample size, CI (μ1 lower/upper) and CI (μ2 lower/upper) represent the confidence intervals of the lower and upper limits of population 1 and 2, respectively. CI (p1 lower/upper) represents the confidence interval of the lower- and upper limits of the participation fraction of population 1.

Strain	*N*	Incubation time (h)	CI μ1 lower	CI μ1 upper	CI μ2 lower	CI μ2 upper	CI p1 lower	CI p1 upper
CB-A109.1 (dTomato)	241	18	4.74	7.06	11.82	19.26	0.48	0.86
CB-A109.1 (2-NBDG)	241	18	5.06	7.75	11.70	20.16	0.48	0.92

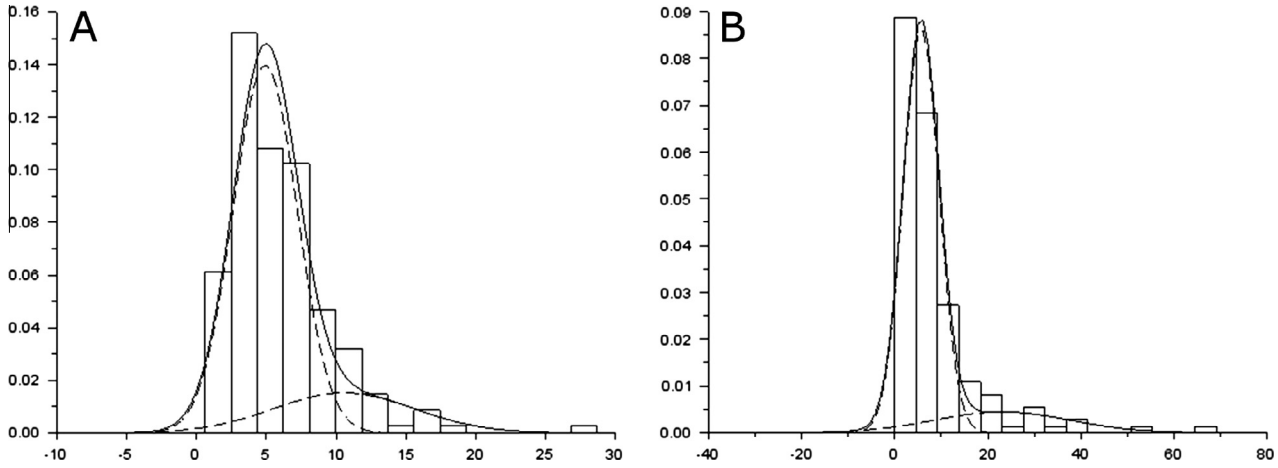

Fig. 8. Heterogeneity of *glaA* expression and glucose uptake. Bimodal fluorescence intensity distributions of dTomato (A) and 2-NBDG (B) within leading hyphae from strain CB-A109.1 that had been incubated with 2-NBDG for 18 h. In all cultures *glaA* had been induced for 6 h on 25 mM maltose. $N > 200$.

colony. Sectors that are exposed to a non-nutritious substrate would benefit from transport of nutrients from the center of the colony or from sectors of the periphery that are colonizing a nutritious substrate. This proposed bi-directional translocation phenomenon was observed at least for the transport of a nitrogen source in the basidiomycete *Phanerochaete velutina* (Tlalka et al., 2007).

Recently, it has been shown that septal plugging maintains hyphal heterogeneity (Bleichrodt et al., 2012; Bleichrodt, 2012). Here, we demonstrated that septal plugging is not a barrier for glucose transport within the colony. It is tempting to speculate that other molecules are transported via transporters that reside in the plasma membrane lining the cross-walls. The regulated insertion or removal of such transporters would thus provide a mechanism for selective inter-hyphal or inter-compartmental transport of nutrients. The origin of hyphal heterogeneity is not known. It may be that the capacity to take up the carbon source glucose is key in the establishment of heterogeneity. The fact that hyphae with high transcriptional activity take up more glucose than hyphae with a lower transcriptional activity is consistent with this idea.

References

Bistis, G.N., Perkins, D.D., Read, N.D., 2003. Different cell types in *Neurospora crassa*. Fungal Genet. Newsl. 50, 17–19.

Bleichrodt, R., 2012. Intercompartmental streaming in *Aspergillus*. PhD thesis. University of Utrecht.
Bleichrodt, R.J., van Veluw, G.J., Recter, B., Maruyama, J., Kitamoto, K., Wösten, H.A.B., 2012. Hyphal heterogeneity in *Aspergillus oryzae* is the result of dynamic closure of septa by Woronin bodies. Mol. Microbiol. 86, 1334–1344.
Bleichrodt, R.J., Hulsman, M., Wösten, H.A.B., Reinders, M.J., 2015. Switching from a unicellular to multicellular organization in an *Aspergillus niger* hypha. MBio 6, e00111.
Bos, C.J., Debets, A.J., Swart, K., Huybers, A., Kobus, G., Slakhorst, S.M., 1988. Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*. Curr. Genet. 14, 437–443.
de Bekker, C., Wiebenga, A., Aguilar, G., Wösten, H.A.B., 2009. An enzyme cocktail for efficient protoplast formation in *Aspergillus niger*. J. Microbiol. Methods 76, 305–306.
de Bekker, C., Bruning, O., Jonker, M.J., Breit, T.M., Wösten, H.A.B., 2011a. Single cell transcriptomics of neighboring hyphae of *Aspergillus niger*. Genome Biol. 12, R71.
de Bekker, C., van Veluw, G.J., Vinck, A., Wiebenga, L.A., Wösten, H.A.B., 2011b. Heterogeneity of *Aspergillus niger* microcolonies in liquid shaken cultures. Appl. Environ. Microbiol. 77, 1263–1267.
Etxebeste, O., Herrero-Garcia, E., Araújo-Bazán, L., Rodriguez-Urra, A.B., Garzia, A., Ugalde, U., Espeso, E.A., 2009. The bZIP-type transcription factor FlbB regulates distinct morphogenetic stages of colony formation in *Aspergillus nidulans*. Mol. Microbiol. 73, 775–789.
Forment, J.V., Flipphi, M., Ramón, D., Ventura, L., Maccabe, A.P., 2006. Identification of the *mstE* gene encoding a glucose-inducible, low affinity glucose transporter in *Aspergillus nidulans*. J. Biol. Chem. 281, 8339–8346.
Goosen, T., Bloemheuvel, G., Gysler, C., de Bie, D.A., van den Broek, H.W., Swart, K., 1987. Transformation of *Aspergillus niger* using the homologous orotidine-5′-phosphate-decarboxylase gene. Curr. Genet. 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. Mol. Gen. Genet. 219, 282–288.
Hickey, P.C., Jacobson, D., Read, N.D., Glass, N.L., 2002. Live-cell imaging of vegetative hyphal fusion in *Neurospora crassa*. Fungal Genet. Biol. 37, 109–119.
Jedd, G., Chua, N.H., 2000. A new self-assembled peroxisomal vesicle required for efficient resealing of the plasma membrane. Nat. Cell Biol. 2, 226–231.
Jennings, D.H., 1994. Translocation in mycelia. In: Wessels, J.G.H., Meinhardt, F. (Eds.), The Mycota, Part I. Springer Verlag, Berlin, pp. 163–173.
Kasuga, T., Glass, N.L., 2008. Dissecting colony development of *Neurospora crassa* using mRNA profiling and comparative genomics approaches. Eukaryot. Cell 7, 1549–1564.
Knaus, H., Blab, G.A., Agronskaia, A.V., van den Heuvel, D.J., Gerritsen, H.C., Wösten, H.A.B., 2013. Monitoring the metabolic state of fungal hyphae and the presence of melanin by Nonlinear Spectral Imaging. Appl. Environ. Microbiol. 79, 6345–6350.
Krijgsheld, P., Altelaar, A.F., Post, H., Ringrose, J.H., Müller, W.H., Heck, A.J., Wösten, H.A.B., 2012. Spatially resolving the secretome within the mycelium of the cell factory *Aspergillus niger*. J. Proteome Res. 11, 2807–2818.
Krijgsheld, P., Nitsche, B.M., Post, H., Levin, A.M., Müller, W.H., Heck, A.J., Ram, A.F., Altelaar, A.F., Wösten, H.A.B., 2013. Deletion of *flbA* results in increased secretome complexity and reduced secretion heterogeneity in colonies of *Aspergillus niger*. J. Proteome Res. 12, 1808–1819.
Lagopodi, A.L., Ram, A.F., Lamers, G.E., Punt, P.J., van den Hondel, C.A.J.J.M., Lugtenberg, B.J., Bloemberg, G.V., 2002. Novel aspects of tomato root colonization and infection by *Fusarium oxysporum* f. sp. *radicis-lycopersici* revealed by confocal laser scanning microscopic analysis using the green fluorescent protein as a marker. Mol. Plant Microbe Interact. 15, 172–179.
Levin, A.M., de Vries, R.P., Conesa, A., de Bekker, C., Talon, M., Menke, H.H., van Peij, N.N., Wösten, H.A.B., 2007a. Spatial differentiation in the vegetative mycelium of *Aspergillus niger*. Eukaryot. Cell 6, 2311–2322.
Levin, A.M., de Vries, R.P., Wösten, H.A.B., 2007b. Localization of protein secretion in fungal colonies using a novel culturing technique; the ring-plate system. J. Microbiol. Methods 69, 399–401.
Lew, R.R., 2005. Mass flow and pressure-driven hyphal extension in *Neurospora crassa*. Microbiology 151, 2685–2692.
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*. Appl. Microbiol. Biotechnol. 71, 881–891.
McLean, K.M., Prosser, J.I., 1987. Development of vegetative mycelium during colony growth of *Neurospora crassa*. Trans. Br. Mycol. Soc. 88, 489–495.
Merzlyak, E.M., Goedhart, J., Shcherbo, D., Bulina, M.E., Shcheglov, A.S., Fradkov, A.F., Gaintzeva, A., Lukyanov, K.A., Lukyanov, S., Gadella, T.W., Chudakov, D.M., 2007. Bright monomeric red fluorescent protein with an extended fluorescence lifetime. Nat. Methods 4, 555–557.
Moore, R.T., McAlear, J.H., 1962. Fine structures of mycota. Observations on septa of ascomycetes and basidiomycetes. Am. J. Bot. 49, 86–94.
Moukha, S.M., Wösten, H.A.B., Mylius, E.J., Asther, M., Wessels, J.G.H., 1993a. Spatial and temporal accumulation of mRNAs encoding two common lignin peroxidases in *Phanerochaete chrysosporium*. J. Bacteriol. 175, 3672–3678.
Moukha, S.M., Wösten, H.A.B., Asther, M., Wessels, J.G.H., 1993b. In situ localization of the secretion of lignin peroxidases in colonies of *Phanerochaete chrysosporium* using a sandwiched mode of culture. J. Gen. Microbiol. 139, 969–978.
Nelissen, B., De Wachter, R., Goffeau, A., 1997. Classification of all putative permeases and other membrane plurispanners of the major facilitator superfamily encoded by the complete genome of *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 21, 113–134.
Oliveira, J., Tavares, R.M., Gerós, H., 2002. Utilization and transport of glucose in *Olea Europaea* cell suspensions. Plant Cell Physiol. 43, 1510–1517.
Punt, P.J., van den Hondel, C.A.M.J.J., 1992. Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. Methods Enzymol. 216, 447–457.
Shatkin, A.J., Tatum, E.L., 1959. Electron microsocpy of *Neurospora crassa* mycelia. J. Biophys. Biochem. Cytol. 6, 423–426.
Simonin, A., Palma-Guerrero, J., Fricker, M., Glass, N.L., 2012. Physiological significance of network organization in fungi. Eukaryot. Cell 11, 1345–1352.
Steele, G.C., Trinci, A.P.J., 1975. Morphology and growth kinetics of hyphae of differentiated and undifferentiated mycelia of *Neurospora crassa*. J. Gen. Microbiol. 91, 362–368.
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*. J. Biol. Chem. 286, 30455–30461.
Teertstra, W.R., Lugones, L.G., Wösten, H.A.B., 2004. In situ hybridisation in filamentous fungi using peptide nucleic acid probes. Fungal Genet. Biol. 41, 1099–1103.
Tlalka, M., Bebber, D.P., Darrah, P.R., Watkinson, S.C., Fricker, M.D., 2007. Emergence of self-organised oscillatory domains in fungal mycelia. Fungal Genet. Biol. 44, 1085–1095.
vanKuyk, P.A., Benen, J.A.E., Wösten, H.A.B., Visser, J., de Vries, R.P., 2012. A broader role for AmyR in *Aspergillus niger*: regulation of the utilisation of D-glucose or D-galactose containing oligo- and polysaccharides. Appl. Microbiol. Biotechnol. 93, 285–293.
van Veluw, G.J., Teertstra, W.R., de Bekker, C., Vinck, A., van Beek, N., Muller, W.H., Arentshorst, M., van der Mei, H.C., Ram, A.F., Dijksterhuis, J., Wösten, H.A.B., 2013. Heterogeneity in liquid shaken cultures of *Aspergillus niger* inoculated with melanised conidia or conidia of pigmentation mutants. Stud. Mycol. 74, 47–57.
Vishniac, W., Santer, M., 1957. The thiobacilli. Bacteriol. Rev. 21, 195–213.
Vinck, A., Terlou, M., Pestman, W.R., Martens, E.P., Ram, A.F., van den Hondel, C.A.M.J.J., Wösten, H.A.B., 2005. Hyphal differentiation in the exploring mycelium of *Aspergillus niger*. Mol. Microbiol. 58, 693–699.
Vinck, A., de Bekker, C., Ossin, A., Ohm, R.A., de Vries, R.P., Wösten, H.A.B., 2011. Heterogenic expression of genes encoding secreted proteins at the periphery of *Aspergillus niger* colonies. Environ. Microbiol. 13, 216–225.
Wösten, H.A.B., Moukha, S.M., Sietsma, J.H., Wessels, J.G.H., 1991. Localization of growth and secretion of proteins in *Aspergillus niger*. J. Gen. Microbiol. 137, 2017–2023.
Wösten, H.A.B., van Veluw, G.J., de Bekker, C., Krijgsheld, P., 2013. Heterogeneity in the mycelium: implications for the use of fungi as cell factories. Biotechnol. Lett. 35, 1155–1164.