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Heterogenic expression of genes encoding secreted proteins at the periphery of *Aspergillus niger* colonies

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Summary

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

Introduction

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

Even within a specific zone of a mycelium, not every growing hypha secretes a particular protein. Glucoamylase (GlaA) was found to be secreted by a subset of hyphae at the periphery of the *A. niger* colony (Wösten *et al*., 1991). This heterogeneity was explained by differences in the expression of *glaA* (Vinck *et al*., 2005). Two types of hyphae were distinguished; those that highly and those that lowly express *glaA*, each making up about 50% of the hyphal population. This was a remarkable finding considering the fact that the highly and lowly active hyphae in the outer zone of the *A. niger* colony experienced similar environmental conditions. We here present evidence that hyphae highly expressing *glaA* also highly express other genes encoding secreted proteins. In fact, the data indicate that the high expressers are hyphae with a high transcriptional and translational activity.

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Table 1. Strains used in this study.

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

Results

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

Previously, *A. niger* strains have been described that express the *sGFP*(S65T) gene from the *gpdA* or the *glaA* promoter (Siedenberg *et al*., 1999; Lagopodi *et al*., 2002). Here, *A. niger* was transformed with constructs containing *sGFP*(S65T) under control of the promoter of *aguA* (de Vries *et al*., 2002), *faeA* (de Vries *et al*., 1997) or *aamA* (Boel *et al*., 1990). The xylanolytic genes *aguA* and *faeA* are repressed by glucose and induced by xylose (de Vries *et al*., 1999). In contrast, the amylolytic genes *aamA* and *glaA* are repressed by xylose and induced by maltose (Fowler *et al*., 1990). In all cases, fluorescent transformants carried multiple copies of the transforming construct. Single integrations did not result in a fluorescence strong enough for fluorescence microscopy. Therefore, two representative strains of each transformation were selected for further analysis: AV11.3 and AV11.4 (P*aguA_sGFP*); UU-A005.4 and UU-A005.6 (P*faeA_sGFP*) and AW001.08 and AW001.10 (P*aamA_sGFP*) (Table 1).

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

Expression of *sGFP*(S65T) under control of the *gpdA, aamA*, *glaA, aguA* and *faeA* promoter was analysed at the colony level in 5-day-old sandwiched cultures (i.e. colonies grown in between porous polycarbonate membranes; see *Experimental procedures*). The main advantage of sandwiched cultures is that the colony is forced to grow nearly two-dimensionally and that the culture can be easily transferred to a fresh medium without disturbing growth (Wösten *et al*., 1991). The *gpdA*-driven expression resulted in bright GFP fluorescence throughout the mycelium irrespective of the carbon source (data not shown; Vinck *et al*., 2005). Similar results were obtained with the *aamA*, *glaA, aguA* and *faeA* promoter when these strains were grown on the inducing carbon source for 5 days. In contrast, a weak fluorescent signal was observed during growth on the repressing carbon source (data not shown). A 8 h transfer of these colonies to the inducing medium resulted in an evenly distributed fluorescence in the case *sGFP*(S65T) was expressed from the *aguA* promoter. In contrast, fluorescence was most intense at the periphery of the colony in the case of *aamA*- or *glaA*-driven expression, whereas *faeA*-driven expression resulted in the most intense fluorescence within the colony centre (Fig. 1).

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

Strains expressing *sGFP*(S65T) from the *gpdA*, *glaA*, *aamA*, *aguA* or *faeA* promoter were grown for 5 days on inducing medium or were transferred to this medium for 8 h after growth for 5 days on the repressing medium. In all cases, all hyphae within the outer zone of the colonies were fluorescent (for an example see Fig. 2A and B). The fluorescence of the individual hyphae was quantified, normalized and subjected to statistic analyses. The

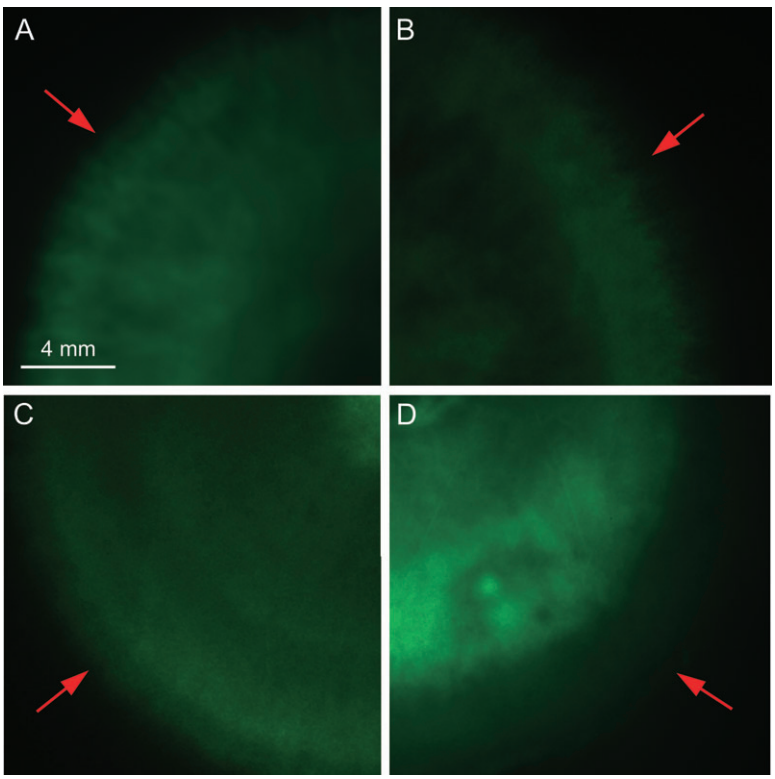

Fig. 1. Expression of *glaA* (A), *aamA* (B), *aguA* (C) and *faeA* (D) in 5-day-old sandwiched colonies of *A. niger* that had been transferred for 8 h from a repressing to inducing medium. Expression was monitored by using sGFP as a reporter. Scale bar represents 4 mm. Arrows indicate edge of the colony.

Kolmogorov–Smirnov test (henceforth KS-test) showed that fluorescence intensity was normally distributed in the strain that expresses *sGFP*(S65T) from the *gpdA* promoter (Table 2). Similar results were obtained with one of the strains that expressed *GFP* from the *faeA* promoter when it had been grown for 5 days on inducing medium. In all other cases, the distribution of fluorescence intensities of the exploring hyphae deviated from normality (Table 2). The fluorescence intensity distributions could be explained by assuming that they are composed of a weighted mixture

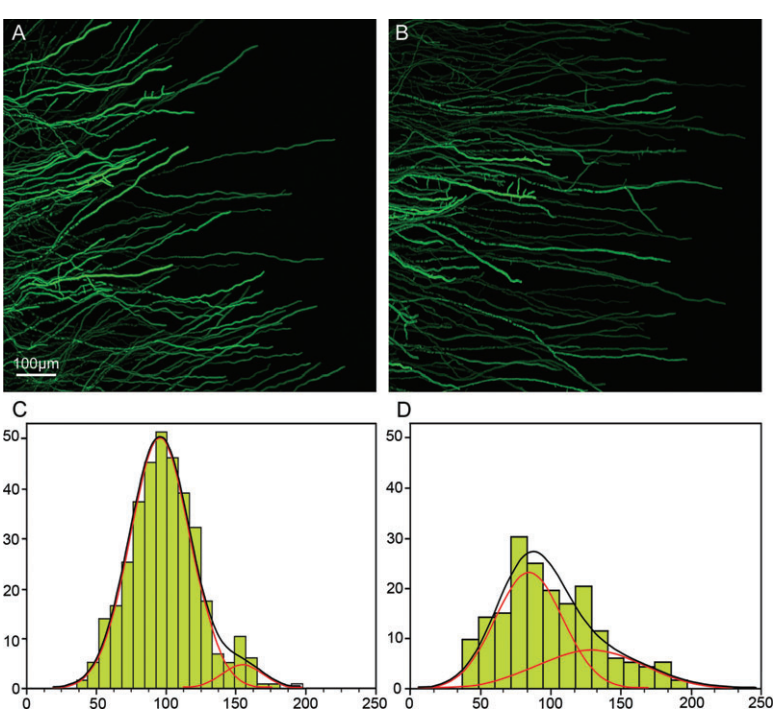

Fig. 2. CLSM images (A and B) and the respective fluorescence intensity distributions (C and D) of hyphae in the exploration zone of colonies of strain AV11#3 and AW001.10 that express s*GFP* from the *aguA* and *aamA* promoter respectively. The strains were grown for 5 days on 50 mM glucose followed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

Table 2. Descriptive statistics of the fluorescence intensities of hyphae in the exploring mycelium of *A. niger*.

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

a. This is a lower bound of the true significance.
N, sample size; KS, *P*-value of Kolmogorov–Smirnov test (with Lilliefors significance correction); $\mu_{1,2}$, mean of component 1 and 2 respectively; $SD_{1,2}$, standard deviation of component 1 and 2 respectively; pf_1, participation frequency of component 1; CI, 95% confidence interval of pf1; n.a., not applicable.

of two normally distributed components, representing a population that highly expresses the reporter gene and a population that lowly expresses the reporter gene (Table 2; Fig. 2C and D). For instance, 84% and 74% of the hyphae were part of the population that expresses *aamA* at a low level when the recombinant strains AW001.08 and AW001.10 were grown continuously on the inducing maltose medium. When these transformants were induced for 8 h 55% and 65% of the hyphae belonged to this population, whereas 45% and 35% were expressing *aamA* at a high level.

Sandwiched cultures are routinely inoculated with 1.5 µl of a 1.10^8 spores ml^{-1} solution. Cultures of strain AR9.2 were inoculated with micro-colonies originating from a single spore or a single hypha (see *Experimental procedures*) to address whether heterogeneity in gene expression is caused by heterogeneity within the inoculum. The KS-test showed that fluorescence intensity of hyphae at the periphery of sandwiched colonies deviated from normality when cultures had been inoculated with numerous spores or with micro-colonies originating from a single spore or a single hypha (Table S1). These experiments show that hyphae become heterogenic during formation of the mycelium and that heterogeneity does not depend on the inoculum used.

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

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

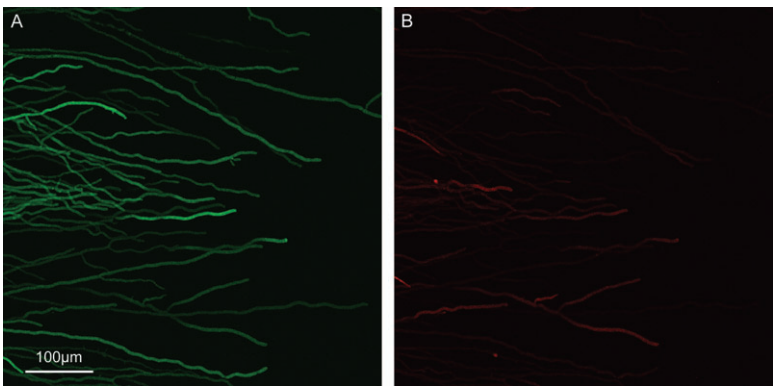

Fig. 3. CLSM images of hyphae in the exploration zone of colonies of strain CB-115.9 that expresses s*GFP*(S65T) behind the *faeA* promoter (A) and *dTomato* behind the a*guA* promoter (B).

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

Strain CB-A112.11 that expresses *sGFP*(S65T) from the *glaA* promoter was transformed with a construct containing the *dTomato* gene under regulation of the amylolytic regulatory gene *amyR* (Petersen *et al*., 1999). The intensity of GFP and dTomato fluorescence was determined in hyphae within the exploration zone of the colonies that had been transferred to the inducing medium for 8 h. A strong positive linear correlation (0.73–0.86; Table 3) was found between *glaA* and *amyR* expression. A similar approach to correlate expression of *aguA* and *faeA* with their regulator *xlnR* (van Peij *et al*., 1998) did not succeed. This was due to the fact that the expression from the *xlnR* promoter was below detection level.

Strains CB-A109.1 and CB-A109.2 that express the *dTomato* gene under regulation of the *glaA* promoter of *A. niger* were hybridized with an 18S rRNA PNA probe. The distribution of dTomato and 18S rRNA was analysed using confocal laser scanning microscopy (CLSM) (Fig. 4). Image analysis showed that *glaA* expression and 18S rRNA distribution exhibit a strong positive correlation (0.81–0.85; Table 3). In general, fluorescein fluorescence, indicative for rRNA distribution, decreased rapidly towards the tips. As a consequence, tips only showed the red fluorescence of dTomato (Fig. 4C).

Discussion

Previously, it was shown that the AmyR regulated genes *glaA* and *aamA* and the XlnR regulated genes *aguA* and *faeA* are most highly expressed at the periphery of colonies of *A. niger* (Levin *et al*., 2007). We here monitored expression of these genes using sGFP(S65T) as a reporter. Colonies expressing *sGFP(S65T)* from the *glaA,*

Table 3. Pearson correlation coefficient (r) with standard error of the mean (sem) between the intensity of fluorescence of (1) GFP and dTomato and (2) fluorescein and dTomato in hyphae at the periphery of 5-day-old *A. niger* colonies.

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

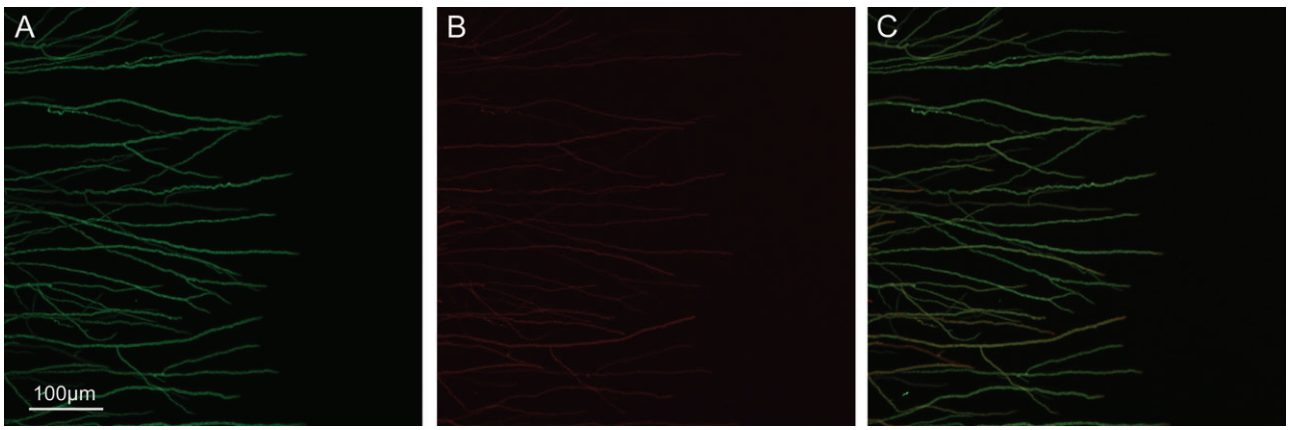

Fig. 4. *In situ* hybridization of 18S rRNA using a fluorescein labelled PNA probe (A) in colonies of strain CB-A109#2 that expresses *dTomato* behind the glaA promoter (B). (C) represents an overlay of the fluorescein and dTomato signals.

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

Expression of *glaA* on inducing maltose medium was not only heterogenic at the colony level but also within the outer zone of the colony (Vinck *et al*., 2005). In the study of Vinck *et al*. colonies were inoculated with multiple spores. We here showed that heterogenic *glaA* expression also occurs at the periphery of colonies that originated from a single spore or a single hypha. This strongly indicates that the hyphae that highly and lowly express *glaA* arise during vegetative growth and are not due to heterogeneity in spores produced in the conidiophores.

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

The high correlation coefficient between *glaA* and *amyR* and between *glaA* and *aamA* suggests a causative role of AmyR in heterogenic expression of the amylolytic genes at the periphery of the *A. niger* colony. However, the correlation between expression of the amylolytic

genes and the xylanolytic genes suggests a higher level of regulation. This is strengthened by the correlation of the expression of the xylanolytic gene *faeA* and the constitutively expressed gene *gpdA* and by the strong correlation between expression of *glaA* and the abundance of 18S rRNA. We propose that at least two populations of hyphae exist at the periphery of the *A. niger* colony that can be discriminated by their transcriptional and translational activity. Intuitively, one would assume that a lower transcriptional and translational activity would result in a lower growth rate. Yet, this is not the case. The reporter proteins that were monitored were formed in an eight-hour period. During this time the outer zone had expanded 0.6 mm. In our analysis, the apical zones (100 μm) of the outermost hyphae were visualized. Therefore, these hyphae must have had a similar growth rate. Taking this into account, we assume that the transcriptional and translational activity in the low-expressing hyphal population is sufficient to support growth. The higher activity in the high-expressing hyphal population may result in a higher secretory activity. It is known that filamentous fungi secrete a significant part of their proteins into their environment. Considering the fact that this is executed by a minority of the hyphae in the colony, one would indeed expect increased cellular activity in the secreting hyphae. Future studies will be devoted to assess gene expression in high and low expressing hyphae at the periphery of the colony. This should provide further insight into the mechanism of heterogeneity. Moreover, it may reveal why colonies send out exploring hyphae that are heterogenic with respect to gene expression.

Experimental procedures

Strains and plasmids

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

Sandwiched colonies of A. niger

A. niger was grown as sandwiched colonies (Wösten *et al*., 1991) at 30°C. To this end, *A. niger* was grown in a 0.2 mm thin layer of 1.25% agarose (D-1 agarose, type low EEO, Hispanagar, Spain) between two perforated polycarbonate membranes (diameter 76 mm, pore size 0.1 μm; Osmonics, GE Water Technologies, Trevose, PA, USA) placed on top of solidified (1.5% agar) minimal medium (MM) (see below). The sandwiched cultures were routinely inoculated with small mycelial plugs from the periphery of pre-grown sandwiched cultures. Alternatively, cultures were inoculated with a micro-colony originating from a single conidium or single hypha. In the former case, sandwiched colonies were inoculated with 50 μl of a spore solution containing 20 spores ml^{-1}. Resulting 3-day-old micro-colonies were used as an inoculum for fresh sandwiched colonies. To obtain micro-colonies originated from a single hypha, sandwiched colonies were grown with the modification that there was no agarose layer between the two membranes and that the upper PC-membrane was replaced by a Lumox membrane. The latter membrane was taken from a Lumox dish (Greiner Bio-One, Kremsmünster, Austria), and the hydrophobic side was placed such that it faced the PC membrane. After 5 days of growth, the Lumox membrane was removed and parts of the periphery of the colony, still sticking to the PC membrane, were transferred to a 1.0 PEN MembraneSlide (Carl Zeiss MicroImaging, München, Germany). Subsequently, individual hyphae were dissected using a PALM system that was operated with PALM RoboSoftware V4.0 (Carl Zeiss MicroImaging). The apical part (75–150 μm) of the hyphae at the periphery of the colony was dissected. These hyphal fragments that had a diameter of 4 to 6 μm were catapulted into the lid of a 0.5 ml Eppendorf tube that contained 50 μl MM with 25 mM glucose as a carbon source. The medium containing the hypha was then transferred to a 2 ml Eppendorf tube containing 0.4 ml MM with 25 mM glucose as a carbon source. After 3 days of growth at 150 r.p.m., a small pellet had formed that was used to inoculate sandwiched colonies.

Medium composition

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

Construction of GFP reporter constructs

The primers used for PCR are presented in Table 4. The *A. niger aguA* promoter was amplified by PCR from plasmid pIM3243 (de Vries *et al*., 2002) with primers FPraguABam and RPraguANco. The resulting 550 bp BamHI–NcoI fragment was cloned in pAN52-10S65TGFPn/s (Siedenberg *et al*., 1999). To this end, pAN52-10S65TGFPn/s was partially digested with NotI and the overhangs were blunted with Klenow. This was followed by digestion with NcoI to remove

Table 4. Primers used in this study.

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

The restriction sites are underlined. A second restriction site present in the primer is indicated in bold.

the 4.1 kb *glaA* promoter. The 550 bp BamHI–NcoI fragment of the *aguA* promoter of which the BamHI site was blunted with Klenow was then inserted. This resulted in construct P*aguA*_sGFP+ that contains the *sGFP*(S65T) gene (Chiu *et al*., 1996) under the regulation of the *aguA* promoter of *A. niger*.

A 3.5 kb genomic *Sma*I fragment from pIM3207 (de Vries *et al*., 1997) containing the *faeA* open reading frame and 2000 and 300 bp 5′ and 3′ flanking sequences, respectively, was cloned in pGEM7 resulting in construct pRV421. The construct pHB65-16 was derived from pRV421 by removing the open reading frame of *faeA* by outward PCR using primers pFAEANcoNot and tFAEANotPst thus introducing NcoI and NotI sites. An NcoI–NotI fragment containing the coding sequence of *sGFP*(S65T) was introduced in the respective sites of pHB65-16, resulting in construct pHB68-28.

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

Construction of dTomato reporter constructs

The primers used for PCR are presented in Table 4. The *dTomato* (Shaner *et al*., 2004) coding region was amplified by PCR from plasmid pRO020 with primers tomNcoIFW2 and tomHindIIIRV2. Plasmid pRO020 contains the *dTomato* gene in which the internal NcoI site was removed for cloning purposes (R.A. Ohm, unpubl. data). The resulting 705 bp NcoI-HindIII fragment was used to replace the *GFP* NcoI-HindIII fragment of pGPDGFP (Lagopodi *et al*., 2002) resulting in pCB025.

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

The *A. niger amyR* promoter was amplified by PCR from genomic DNA of strain N402 with primers F-PamyRNotI and R-PamyRNcoI. The resulting 1620 bp NotI-NcoI fragment was cloned in pAN5210dTom that had been digested with the same enzymes, which resulted in construct pAV150. The plasmid pAN5210dTom is a derivative of pAN52-10*Not*I (Siedenberg *et al*., 1999) in which a Klenow blunted NcoI-BamHI fragment consisting of the dTomato coding sequence (of which the internal NcoI-site was removed by site-directed mutagenesis) has been inserted in the NcoI/*Eco*RV sites.

Transformation of A. niger

Protoplast preparation and transformation were performed as previously described (de Bekker *et al*., 2009). Transformants were selected by co-transforming with pAB4.1 (van Hartingsveldt *et al*., 1987) or pGW635 (Kusters-van Someren *et al*., 1991) that both contain the *A. niger pyr* gene and/or pIM2104 (Vankuyk *et al*., 2004) that contains the *A. niger arg* gene. Transformants were purified by repeated streaking of conidia.

In situ *hybridization with PNA probes*

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

Microscopy

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

For detection of fluorescence at the hyphal level, the top PC membrane of sandwiched colonies was removed. Small pieces (~8 mm × 8 mm) of the periphery of the colony were excised, placed on a coverslip (24 × 50 mm) and topped with a block (~1 × 1 × 0.5 cm) of inducing agar medium. This prevents drying out of the samples. GFP fluorescence at the

hyphal level was studied by confocal laser scanning microscopy (CLSM). The laser intensity was kept to a minimum to reduce photobleaching and phototoxic effects. For the single-reporter transformants an inverted Leica TCS SPII system equipped with a PL FLUOTAR 16x/0.5 Imm plan apochromatic objective lens was used. GFP was excited with the 488 nm laser line and fluorescence was detected at 500–550 nm band pass (BP). Images were captured as z-series of optical sections (~1 μm). The data sets were displayed as maximum intensity projections (1024 × 1024 pixels) using Leica LSM software.

Fluorescence of the dual-reporter strains and of strains CB-A109.1 and CB-A109.2 hybridized with the EuUni PNA probe was imaged with an inverted Zeiss LSM 5 system using a Plan-Neofluar 16×/0.5 Imm objective lens. GFP and fluorescein were excited with the 488 nm laser line, whereas a 543 nm laser line was used to excite dTomato. GFP and fluorescein fluorescence was detected at 505–530 nm BP, whereas dTomato fluorescence was monitored at 560 nm long pass. Images were captured as z-series of optical sections (optimal interval 2.02 μm; 4× line average; 8 bit scan depth) using the multi-track scanning mode. The data sets were displayed as maximum intensity projections (1024 × 1024 pixels) using Zeiss software.

Image and data analysis

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

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

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

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Descriptive statistics of the fluorescence intensities of hyphae in the exploring mycelium of *A. niger* strain AR9#2. Sandwiched colonies were grown from a plug inoculum or from micro-colonies originating from a single spore or a single hypha. Each experiment was carried out twice. N = sample size; KS = *P*-value of Kolmogorov–Smirnov test (with Lilliefors significance correction); $\mu_{1,2}$ = mean of component 1 and 2 respectively; $SD_{1,2}$ = standard deviation of component 1 and 2 respectively; pf_1 = participation frequency of component 1; CI = 95% confidence interval of pf1.

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