Sporulation inhibited secretion in *Aspergillus niger*

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Sporulation inhibited secretion in *Aspergillus niger*

Sporulatie geremde secretie in *Aspergillus niger*
(met een samenvatting in het Nederlands)

Proefschrift

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door

Pauline Krijgsheld

geboren op 30 maart 1984 te Voorburg

Promotor: Prof.dr. H. A. B. Wösten

'Wetenschap is een kunst.'

'Kunst .. zoeken heeft nauwelijks zin, maar vinden wel' – Martin Bril

Voor K

Contents

Chapter 1

1 Development in *Aspergillus*

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Chapter 1

Development in *Aspergillus*

INTRODUCTION

Aspergillus is an anamorph genus, which comprises between 260[1, 2] and 837 species.[3] These species are classified in approximately ten different teleomorph genera.[4] For instance, *Aspergillus nidulans* is part of the teleomorph genus *Emericella*, while *Aspergillus fumigatus* and *Aspergillus flavus* belong to the genera *Neosartorya* and *Petromyces*, respectively. This shows that *Aspergillus* is a diverse group of fungi. Indeed, comparison of the genomic sequences of *A. nidulans* and *A. fumigatus*[5] showed that these fungi are as related to each other as fish to humans. These animals separated about 450 million years ago but diversification in the genus *Aspergillus* is assumed to be restricted to about 200 million years.[5] The large differences in genomic sequence have been proposed to be caused by an accelerated evolutionary rate.[6]

Aspergillus species are among the most abundant fungi worldwide. They are not very selective with respect to abiotic growth conditions (Table 1). For instance, they can grow over a wide range of temperature (6-55 °C) and at relatively low humidity. In fact, *Aspergillus penicilloides* is among the most xerophilic fungi.[7] Moreover, *Aspergillus* species feed on a large variety of substrates including animal faeces and human tissue. Nonetheless, they are predominantly found on complex plant polymers[8] and are considered to be common food spoilage fungi. The success of *Aspergillus* is also explained by their effective dispersal. Spores of this genus are among the most dominant fungal structures in the air, dispersing themselves both short and long distances.[8] Aspergilli are not only known because of their saprobic life style. *Aspergillus niger* has been reported to be a pathogen of *Zingiber officinale* plants.[9] Moreover, a wide variety of aspergilli are opportunistic pathogens of animals and humans. They do not infect healthy individuals but do invade individuals with a compromised immune system.[10, 11] Aspergilli (i.e. *A. fumigatus*, and to a lesser extent species such *A. flavus*, *A. niger*, *Aspergillus terreus*, and *A. nidulans*) cause invasive aspergillosis (involving several organ systems, particularly pulmonary disease), non-invasive pulmonary aspergilloma, and allergic bronchopulmonary aspergillosis.[12, 13]

Aspergillus spp secrete a wide variety of enzymes that degrade polymers within the substrate into molecules that can be taken up to serve as nutrients. For instance, amylases are secreted to degrade starch, xylanases to degrade xylan and pectinases to degrade pectin within plant material. Similarly, elastase is secreted in the human lung to degrade elastin. The capacity to secrete large amounts of proteins (and other metabolites such as organic acids) in combination with established fermentation technology and molecular biology make aspergilli such as *A. niger, Aspergillus oryzae, Aspergillus awamori, Aspergillus sojae,* and *A. terreus* attractive cell factories for the production of homologous and heterologous proteins.[14] The potential of these fungi is exemplified by strains of *A. niger* that produce more than 30 grams per liter of glucoamylase.[15] Of concern, *Aspergillus spp* can form mycotoxins that are toxic for animals and humans. *A. flavus* produces

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

Species	Optimum Temp (°C)	Temp range (°C)	Optimum pH	pH range	Minimal Water activity	Optimum Water activity	Minimum Relative humidity (%)	Optimum Relative humidity (%)	References
A. niger	35-37	6-47	6.0	1.5-9.8	0.77	0.97	88-89	96-98	178-183
A. oryzae	30-37	7-47	6.0-7.5	4-8		0.99			180, 184-186
A. fumigatus	37	10-55	5.5-6.5	3.0-8.0	0.82	0.97	85	98-99	180, 181, 187-189
A. clavatus	20-25	5-42			0.88		88	98	180
A. terreus	37	15-42	5.0		0.78				178, 187, 188
N. fischeri	26-45	11-52				0.98			190-193
A. nidulans	35-37	6-51	7.0	2-12	0.78		80	95	180, 187, 194, 195

aflatoxin, which is one of the most carcinogenic natural molecules. In addition, different aspergilli, including *Aspergillus westerdijkiae*, can form ochratoxin on food products such as coffee and grapes.[16]

This chapter describes the current understanding of development of aspergilli. Germination of spores, formation of a differentiated vegetative mycelium, and formation of asexual and sexual spores are discussed.

VEGETATIVE GROWTH

In nature, aspergilli grow within and on solid substrates. A colony can result from a single sexual or asexual spore but it may also arise after conidia and/or germlings that are in close vicinity to each other have fused. It has been described that fusion in *A. oryzae, A. sojae* and *Aspergillus tamarii* most often occurs between conidia (>80%), while fusions between conidia and germlings and fusion of germlings are much less frequent.[17] Fusion is mediated by fusion bridges that are formed by conidia or germ tubes. They may be similar to the conidial anastomosis tubes that are formed by *Colletotrichum* and *Neurospora*.[18-20] These anastomosis tubes are morphologically and physiologically distinct from germ tubes. They are typically short, thin, and unbranched. Fusion of conidia and germlings have been described to occur within *Aspergillus* strains, between *Aspergillus* strains, between different aspergilli and even between *Aspergillus* and *Penicillium* species.[17] However, fusion between strains and between species often results in heterokaryon incompatibility. For instance, heterokaryon incompatibility is a widespread phenomenon among *A. niger* strains. The underlying mechanism is, however, not known.[21] Fusion of hyphae was reported to be rare when germlings of *A. oryzae, A. sojae* and *A. tamarii* had formed hyphae.[17] Whether this also holds for other aspergilli is not known. At least, fusion of hyphae has been shown to occur in other ascomycetes (see[17]).

Colonies can reach a diameter in the (sub-)milimeter (micro-colonies) to centimeter (macro-colonies) scale depending on the size and the composition of the substrate. For instance, micro-colonies are formed on a wheat kernel, whereas macro-colonies can be formed within the lobes of a lung. In the laboratory, aspergilli are routinely grown on agar media or in liquid media. On agar medium, aspergilli form radial symmetrical macro-colonies. The mycelium of *A. nidulans*[22] and *A. niger* extend their diameter with approximately 0.25 mm per h in excess of nutrients and at a temperature of 37 °C and 30 °C, respectively. Macro-colonies can also be grown between porous polycarbonate membranes on an agar medium.[23-26] Scanning electron microscopy shows that the periphery of a 7-days-old sandwiched macro-colony of *A. niger* consists of a single layer of hyphae (Figure 1AD). A few millimeters behind the periphery this layer becomes thicker and comprises of up to six layers of hyphae growing on top of each other. Notably, three distinct layers are observed another two millimeters towards the centre (Figure 1BE). The upper and lower layer consist of up to five hyphae on top

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

Mycelium can grow dispersed, as clumps or as micro-colonies, also known as pellets, during submerged growth in liquid medium. Clumps are aggregated hyphae that are considered to be an intermediate state between pelleted and dispersed growth. The morphology of the mycelium has an enormous impact on the production of enzymes and primary or secondary metabolites. For instance, micro-colonies are required for the production of citric acid by *A. niger*.[27] It is not clear how morphology exactly affects productivity. It has been proposed that this is due to the effect of the fungal morphology on the viscosity of the medium.[28] Viscosity correlates with the extent of dispersed growth; large micro-colonies thus result in a low viscosity. The center of large pellets may experience

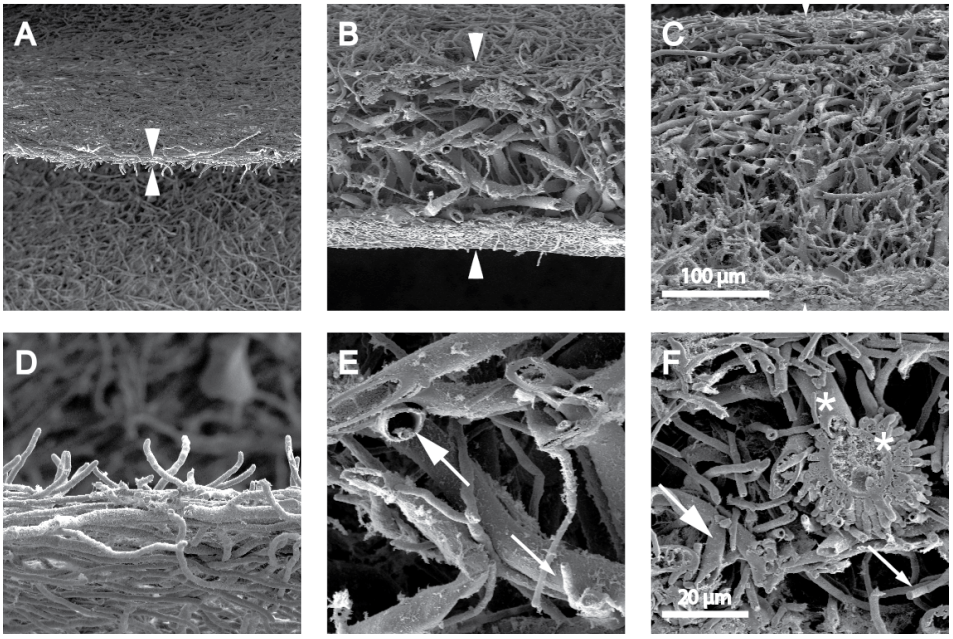

Figure 1. Scanning electron microscopy of cross sections of a 7-days-old sandwiched *A. niger* macro-colony. Cross sections were made at the periphery (A, D), four millimeter behind the periphery (B, E) and at the innermost center (C, F). The thickness of the macro-colony is indicated by the distance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

oxygen starvation and other nutrients may also become limiting in this part of the mycelium. These conditions may also impact productivity of the pellets.

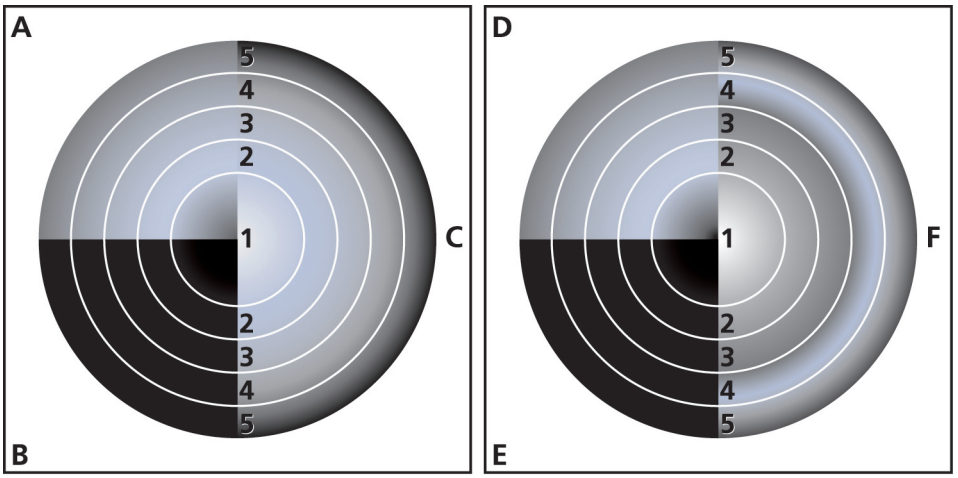

Figure 2. Growth (A, D), protein synthesis (B, E) and protein secretion (C, F) in a 7-days-old xylose grown sandwiched macro-colony of *A. niger* before (A-C) and after transfer (D-F) to fresh medium (Adapted from [25]).

Research in the last two decades has shown that the both macro-colonies and micro-colonies of *Aspergillus* are heterogeneous with respect to gene expression, growth, and secretion. Genome wide expression analysis has shown that the RNA composition of central and peripheral zones of macro-colonies of *A. niger*[25] and *A. oryzae*[26] is different. In the case of 7-days-old macro-colonies of *A. niger*, 25 % of the active genes show a two-fold or more difference in RNA accumulation between the innermost and outermost zone of the mycelium.[25] For instance, RNA levels of the glucoamylase gene *glaA* are 3-fold higher at the periphery of maltose-grown macro-colonies when compared to the center. Similarly, accumulation of transcripts of the ferulic acid esterase gene *faeA* is 5-fold higher at the periphery of xylose grown macro-colonies. Notably, 9% of the genes that are active in a 7-days-old macro-colony are expressed in only one of five concentric zones. For instance, genes related to nitrate metabolism are specifically expressed in the outer zone of the macro-colony, whereas mRNA of the hydrophobin *hfbD* is almost exclusively found in a central zone. Half the variation in RNA profiles is explained by differences in the composition of the medium underlying each zone of the macro-colony, whereas the other half of the variation is caused by medium-independent mechanisms.[25] These findings imply that differentiation occurs within the vegetative mycelium of *Aspergillus.*

The heterogeneity of the mycelium of *A. niger* is also indicated by the fact that distinct zones of the macro-colony grow and secrete proteins.[23-26] Proteins are formed throughout the *A. niger* mycelium[23-25] (Figure 2) but they are mainly

secreted at the periphery. Growth is observed in this outer zone but also in the innermost centre (Figure 2). Spatial growth and protein production is not affected when 6-days-old macro-colonies are transferred to fresh medium for 16 h. However, after transfer protein secretion is not only observed at the periphery of the macro-colony but also in central parts of the mycelium (Figure 2). These data show that non-growing zones of the mycelium abundantly secrete proteins upon transfer to fresh medium.[25] This is a remarkable finding considering the fact that protein secretion is generally assumed to take place in growing hyphae only.[23, 29-31]

The finding that 7-days-old macro-colonies are heterogeneous with respect to RNA accumulation, growth and protein secretion raised the question whether heterogeneity is also observed between and within micro-colonies. Indeed, micro-colonies within liquid shaken cultures are heterogeneous with respect to size and gene expression.[32] A population of small and a population of large micro-colonies can be distinguished by flow cytometry in cultures of *A. niger* that consist of pellets with a maximum diameter of 1 mm. These populations differ 90 μm in diameter. Similarly, two populations of micro-colonies were distinguished when expression of the glucoamylase gene *glaA* and the ferulic acid esterase gene *faeA* were monitored. Notably, the population of lowly expressing micro-colonies is larger than the population of small pellets. This indicates that size of micro-colonies is not the only determinant for expression of genes encoding secreted proteins.[32] It is not yet clear how heterogeneous gene expression is between zones of micro-colonies. At least, the total amount of RNA per hypha is about 50 times higher at the periphery of 1-mm-wide micro-colonies when compared to the center.[32]

Heterogeneous gene expression is not only observed between micro-colonies or between zones of micro- or macro-colonies of *Aspergillus*; it is also observed between hyphae in a particular zone. It has been described that only part of the hyphae at the periphery of macro-colonies of *A. niger* secrete glucoamylase.[23] This observation is explained by heterogeneous expression of the glucoamylase gene *glaA* within this zone.[33] In fact, two populations of hyphae can be distinguished at the outer zone of the macro-colony; those highly and those lowly expressing *glaA*. The hyphae highly expressing *glaA* also highly express other genes encoding secreted proteins.[34] Moreover, these hyphae highly express the glyceraldehyde-3-phosphate dehydrogenase gene *gpdA* and show a high 18S rRNA content. Thus, two populations of hyphae are present at the periphery of a macro-colony; those that are lowly and those that are highly metabolically active. From the fact that the lowly active hyphae have a growth rate similar to that of the highly active hyphae it has been concluded that a "low" activity of hyphae is sufficient to support hyphal growth. However, a "high" metabolism would be needed to support secretion of large amounts of proteins.[34] Recently, it has been described that transcriptionally and translationally highly active and

lowly active hyphae also occur at the periphery of micro-colonies. However, the existence of distinct populations of these types of hyphae seems to be less robust when compared to macro-colonies grown on solid medium.[35] Possibly, signalling between hyphae is involved in maintaining or enhancing heterogeneity. Gradients of signalling molecules cannot be formed between hyphae in liquid shaken cultures, which may explain why heterogeneity is less evident in these cultures.
Single hypha transcriptome analysis indicates that heterogeneity between neighboring hyphae goes beyond two types of hyphae. Individual hyphae each have their own composition of RNA.[36] So far, we can only guess why hyphae are heterogeneous at the macro-colony periphery. The leading hyphae explore the substrate and they may be exposed to rapid changes in the environment. A heterogeneous hyphal population may contribute to the survival under such conditions. Notably, the transcription factor FlbB, which is involved in asexual development (see below), has been shown to accumulate at 60% of the tips of newly formed branches.[37] This is another example of heterogeneity within the *Aspergillus* mycelium.

Heterogeneity within the mycelium is surprising considering the fact that the cytoplasm of a fungal mycelium is assumed to be continuous. This is based on the fact that the septa within and between hyphae are porous allowing streaming of water, (in)organic compounds, proteins and even organelles.[38, 39] Heterogeneity between hyphae would require a certain immobility of molecules. This could be caused by the fact that many proteins are part of large complexes that are immobilized at membranes.[40] For instance, the yeast GPD homologs were found to be part of 17 protein complexes.[40] One of such protein complexes includes two transmembrane proteins that may well decrease the streaming rate by temporally immobilizing the complex at the membrane. In agreement, the streaming rate of a fusion between GFP and GpdA was lower in *A. niger* than that of GFP itself.[41] Closure of septa is another mechanism to maintain differences in composition between hyphae. Septa of vegetative hyphae of *A. oryzae* and *A. niger*[42] and the basidiomycete *Schizophyllum commune*[43, 44] can be in a closed or open state. The incidence of closed septa in *A. niger* and *S. commune* depends on the environmental conditions and is reversible.

ASEXUAL DEVELOPMENT

After a period of vegetative growth, air-exposed macro-colonies of *A. nidulans* and *A. niger* form two types of aerial hyphae (Figure 3). One type is quite similar to vegetative hyphae of these aspergilli and has a diameter of about 2-3 μm. The second type of aerial hyphae has a diameter of about 4-5 and 6-7 μm in the case of *A. nidulans* and *A. niger,* respectively. These so-called stalks can differentiate into conidiophores (Figure 3). The conidiophore stalk of *A. nidulans* extends about 100 μm into the air and is formed from a specialized foot-cell within the substrate mycelium.[45] When the stalk has reached its maximum height,

the tip swells and forms a vesicle with a diameter of 10 µm. In biserate species like *A. nidulans* and *A. niger*, the vesicle surface buds resulting in a layer of primary sterigmata termed metulae. The metulae in turn bud twice. This results in a second layer of sterigmata called phialides. The phialides give rise to chains of mainly uninucleate conidia. As a result, more than 10.000 conidia can be produced per conidiophore. *A. oryzae* can be both uniserate and biserate. In the case of uniserate species, spore producing phialides are positioned directly at the surface of the conidiophore vesicles.

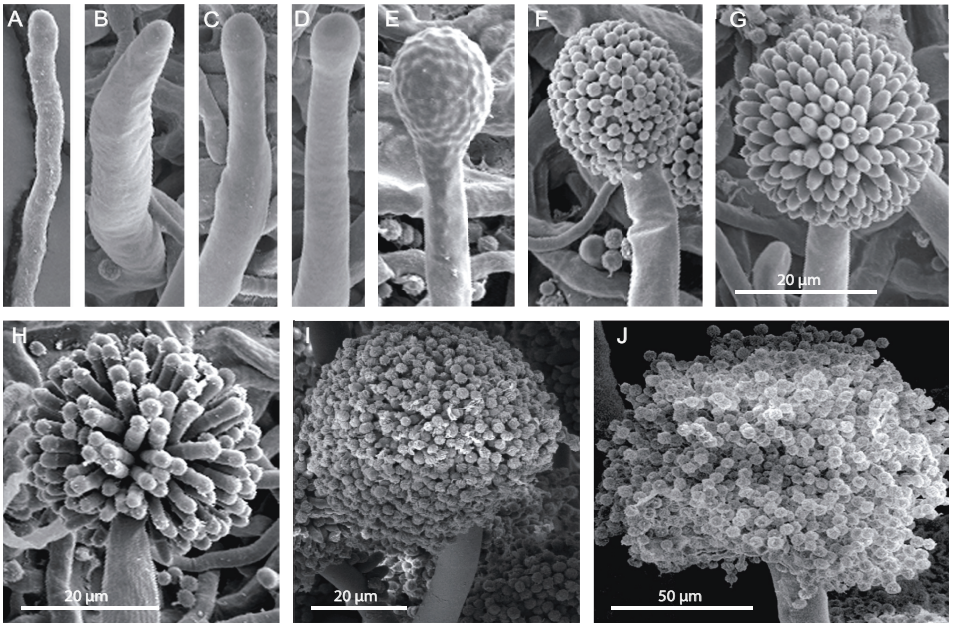

Figure 3. Development of *A. niger* monitored by scanning electron microscopy. The vegetative mycelium forms two types of aerial hyphae. One type is similar to vegetative hyphae (A), while the other type is 2-3 times thicker (B). The tips of the latter aerial hyphae may swell to form a vesicle (C,D).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

The 2-3 µm wide aerial hyphae of *A. nidulans* and *A. niger* are formed about 8 h after inoculation of spores on complete medium. Although timing of this type of aerial hyphae seems to be medium-independent, the density of aerial hyphae is lower in the case of minimal medium. The first stalks of *A. nidulans* and *A. niger* are formed 10 h after spore inoculation on complete medium and growth at 37 °C and 30 °C, respectively. In both cases, conidiophores are formed 20 h post-inoculation. Formation of aerial hyphae in both aspergilli starts in the centre of the macro-colony and moves outwards ending a few millimeters from the edge of the mycelium. This observation implies that the competence of hyphae to form aerial hyphae is acquired faster when a macro-colony gets older.[45] The process of aerial growth has been proposed to involve signaling of the cell density of the

vegetative mycelium.[46-48] The signaling molecule would induce hydrophobin genes. These genes encode proteins that lower the water surface tension to enable hyphae to breach the interface to grow into the air.[49, 50] Which hydrophobin is secreted into the aqueous environment in *Aspergillus* cultures with the aim to lower the water surface tension is not yet known.

A. nidulans can also form conidia in submerged cultures.[45] In this case, conidiation is induced when the culture gets stressed or when nutrients are limited (e.g. limitation of the carbon and the nitrogen source). On the other hand, formation of conidiophores in air-exposed macro-colonies is assumed to be induced by an internal signal that activates a genetic program of sporulation (see below).[45] In both cases, competence to sporulate is acquired in a time-dependent way.[51] Like *A. nidulans, A. niger* can also form conidiophores in submerged conditions. However, these conidiophores do not form spore chains (Figure 1F).

A. nidulans strains in which the *fluG* (*fluffy*) gene is inactivated (i.e. a Δ*fluG* strain) do form aerial hyphae but conidiophores are not being formed in excess of nutrients.[46] During nutrient deprivation, however, some conidiophores are being formed on a solid medium. Similarly, submerged cultures of the Δ*fluG* strain start to sporulate in the absence of a carbon source.[52] These data indicate that FluG is involved in a developmental program of sporulation but not in the stress-related sporulation pathway. Formation of conidiophores in the Δ*fluG* strain can be rescued by growing the mutant next to a wild-type strain. Complementation is also observed when the strains are physically separated by a dialysis membrane with a size exclusion of 6-8 kDa. This indicates that FluG is involved in the production of a low-molecular weight extracellular signaling molecule that is involved in the formation of conidiophores. A similar phenomenon is observed in *Penicillium* species.[53] In this case, an extracellular molecule called conidiogenone induces conidiation. Conidiogenone is a diterpene that accumulates during vegetative growth. At a certain point, a certain threshold level is exceeded and conidiation is initiated.[53]

Regulation of asexual development

Formation of conidiophores has been well studied in *A. nidulans.* Experimental evidence has shown that mechanisms underlying asexual development in *A. fumigatus* and *A. oryzae* are similar but not identical in *A. nidulans* (see below). So far, formation of conidiophores and conidia has not been studied in *A. niger.* However, its genomic sequence predicts that mechanisms of asexual development are also similar, if not identical, to that in *A. nidulans*.[54] About 1300 genes have been found to be up-regulated in whole macro-colonies of *A. nidulans* during asexual reproduction.[55] Recently, RNA was isolated from the vegetative mycelium and from aerial structures (aerial hyphae, conidiophores, and spores) of 7-days-old macro-colonies of *A. niger*. Micro-array analysis showed that 34 genes are found in the top 100 of most highly expressed genes of both the vegetative mycelium

and the aerial structures.[41] These genes include histones, ribosomal proteins, and a hydrophobin homologous to *dewA*. 6 out of 8 predicted hydrophobin genes[54, 56] are within the top 100 of most highly expressed genes in the aerial structures. This top 100 also includes the pigmentation genes *fwnA, olvA* and *brnA*. Seven genes encoding carbohydrate degrading enzymes are in the top 100 of highest expressed genes in the vegetative mycelium. One of these genes is the glucoamylase gene *glaA*.[41]

Regulation by fluG, brlA, abaA, wetA, medA, stuA, and vosA

FluG is believed to be at the start of the developmental program leading to asexual sporulation in *A. nidulans*. Indeed, overexpression of *fluG* in vegetative hyphae is sufficient to cause sporulation under conditions that normally suppress conidia formation.[52] The *fluG* transcripts are present in relatively constant levels during late vegetative growth and conidiation. Notably, a 4-fold higher *fluG* expression level is found in germinating spores during their isotropic growth (3 h after inoculation) when compared to polar growing germlings (5 h after inoculation).[57] This suggests that *fluG* is not only involved in conidiophore formation but also in germination.

FluG activates the *brlA* (*bristle*) gene. A *ΔbrlA* strain of *A. nidulans* forms stalks that do not stop their growth after they have reached a length of 100 μm. These stalks can reach a length 20-30 times longer than those of the wild-type, which results in the characteristic bristle phenotype.[58] Moreover, isotropic growth is not initiated at the apex of the stalks of the *ΔbrlA* strain. As a result, conidiophore vesicles are not being formed. Conidiophore development becomes independent from *fluG* by placing *brlA* under control of an inducible promoter.[58] Similar results have been obtained in *A. oryzae*.[59, 60] Inactivation of *brlA* in *A. oryzae* results in the inability to form conidiophores. In contrast, fully developed conidiophores are formed in submerged culture when the *brlA* gene is expressed under the control of the *amyB* promoter. BrlA is also essential for conidiophore formation in *A. fumigatus*.[61] However, in contrast to *A. nidulans*[58] and *A. oryzae*[59], the *A. fumigatus* gene seems to function earlier in conidiophore development. This is based on the fact that conidiophore development is completely abolished in a Δ*brlA* strain of *A. fumigatus*. The appearance of the macro-colonies of this strain is more similar to that of the fluffy mutants of *A. nidulans* (see below).[61] In addition, the *A. fumigatus* gene seems to function independent from *fluG*. At least, a Δ*fluG* strain of *A. fumigatus* still sporulates in air-exposed cultures. Possibly, *A. fumigatus* has more than one *brlA* activating mechanism.[61] The *brlA* gene of *A. fumigatus* has also been shown to be involved in suppressing ribosomal protein genes during nitrogen stress.[62] This finding conforms to the general starvation response in fungi, which involves both down-regulation of ribosomal protein biogenesis and induction of sporulation.[63-68] However, down-regulation of ribosomal protein encoding genes is not impaired during carbon stress in

A. fumigatus.[62] Nevertheless, these findings suggest that *brlA* of *A. fumigatus* is not only a regulator of formation of conidiophores but also influences the vegetative mycelium by affecting its protein synthesizing capacity.

Transcription of *brlA* in *A. nidulans* results in two transcripts that are called *brlAα* and *brlAβ*. Both transcripts are essential for proper conidiophore development[69] and are controlled at the transcriptional (*brlAα* and *brlAβ*) and translation level (*brlAβ*).[70] Transcript *brlAβ* contains a short upstream ORF (μORF) and a downstream ORF that encodes the same polypeptide as BrlAα but with an N-terminal extension of 23 aa.[69] Both polypeptides contain two C_2H_2 zinc finger DNA binding motifs. The *brlAα* and *brlAβ* transcripts have different functions during asexual development. As mentioned above, inactivation of *brlA* results in indefinitely elongating stalks. In contrast, aberrant primary conidiophores develop in the Δ*brlAβ* strain that can form secondary conidiophores (*i.e.* a conidiophore that develops from another conidiophore). Asexual development proceeds further in the Δ*blrAα* strain but conidia are not produced.[71] So far, it is not known whether transcription of *brlA* of *A. oryzae* and *A. fumigatus* also results in two transcripts.

BrlA activates a central regulatory pathway controlling temporal and spatial expression of conidiation specific genes.[72, 73] This cascade is complex and involves, amongst others, the regulatory genes *abaA*, *wetA, stuA, medA,* and *vosA* (Figure 4). Gene *abaA* (*abacus*) is a regulatory gene that is activated in *A. nidulans* by BrlA during sterigmata differentiation.[57, 72] A Δ*abaA* strain forms metulae that bud apically resulting in chains of cells with metula-like, rather than phialide-like, properties. In other words, phialides are not produced and therefore conidia are not formed.[72, 74, 75] The interaction of AbaA with *brlA* is complex (Figure 4A). Gene *abaA* is activated by BrlA and, in turn, AbaA stimulates formation of *brlAα* transcripts but represses *brlAβ* accumulation.[45, 70, 75, 76] This is caused by AbaA binding to a responsive element in the *brlAβ* locus.[70] The net result of *abaA* inactivation is that *brlA* is over-activated.[77] The positive feedback loop of *brlA* itself is likely to be independent of AbaA because the over-expression of *brlAβ* activates expression of *brlAα* in an *abaA* mutant[70] (Figure 4A). Taken together, both BrlA and AbaA control transcript levels of *brlAα* and *brlAβ*. AbaA regulates several other genes including *abaA* itself, *medA, wetA* (Figure 4A), *vosA* (Figure 4B), and the structural genes *yA* and *rodA* (for their functions see below).[76] Recently *abaA* was identified in *A. oryzae*[59] and *A. fumigatus*.[78] The role of *abaA* in *A. oryzae* is similar to that in *A. nidulans.* In the case of *A. fumigatus abaA* also delays autolysis and cell death.

During the late phase of conidiation, *wetA (wet white)* is activated by *abaA* (Figure 4A). Normal conidiophores are formed by *wetA* mutants. However, the conidia do not form pigments, are not water repellent, and go in autolysis.[75, 79] Gene *wetA* activates a set of genes in phialides and spores (e.g. *wA*), which are involved in making the conidial wall impermeable and mature.[79] In addition,

WetA seems to activate itself[45, 72, 79, 80] and represses *abaA* and *brlA*[78] (Figure 4A). Gene *wetA* of *A. oryzae* [59] has a role similar to that in *A. nidulans.* In the case of *A. fumigatus wetA* seems to have an additional role.[78] It would also function in germ tube formation and reduced hyphal branching.

VosA (*viability of spore*) is a putative transcription factor of the velvet family.[78] This family, which is conserved in filamentous fungi, also includes VeA and VelB of *A. nidulans* (see below). Inactivation of *vosA* results in uncontrolled activation of asexual development, whereas its over-expression blocks sporulation. This may be the result of the observed inhibition of *brlA* by VosA[78] (Figure 4B). It should be noted that *vosA* is lowly expressed in the vegetative mycelium. Yet, these expression levels may be sufficient to control *brlA*. Gene *vosA* is particularly expressed during the formation of conidia and sexual ascospores, where it plays a role in resistance to stress conditions (see below).

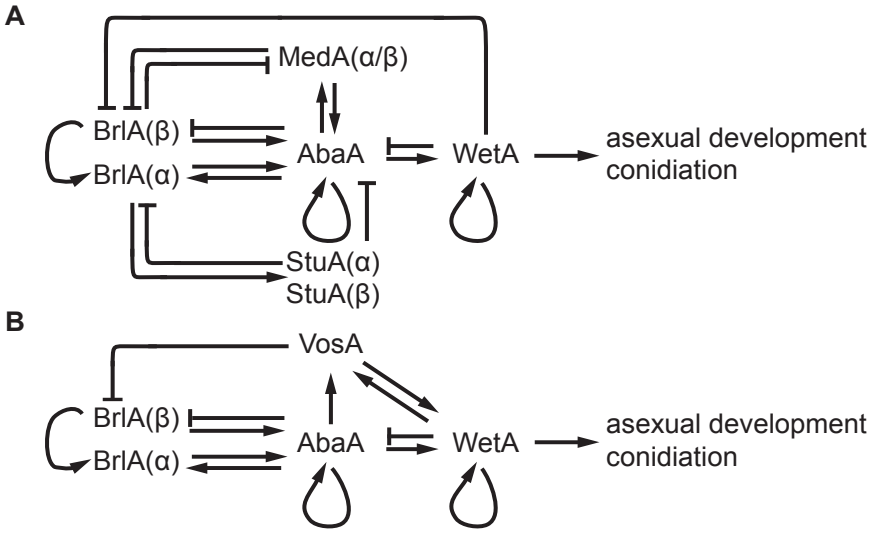

Figure 4. The central regulatory network consisting of BrlA, AbaA and WetA initiates asexual development in *A. nidulans*. StuA and MedA (**A**) and VosA (**B**) are regulators of *brlA, abaA,* and *wetA*.

The *stuA* and *medA* genes are classified as developmental modifiers. Their encoded proteins affect *brlA* and *abaA* expression (Figure 4A). Mutations in *stuA* (*stunted*) of *A. nidulans* results in shortened aerial hyphae, shortened conidiophores and the absence of metulae and phialides. Conidiophores that are formed have reduced vesicles with abnormal numbers of nuclei. Only a few conidia can directly bud from the conidiophore vesicle. Thus, the morphology of the conidiophores is aberrant in *stuA* mutants, but neither temporal development nor conidiophore density is affected.[81] Gene *stuA* has a similar role in asexual development in *A. fumigatus*.[82] StuA is a helix-loop-helix transcription factor

with two transcription start sites. This leads to *stuAα* and *stuAβ* transcripts, of which the former is most important for correct development.[77, 83, 84] Expression of *stuA* depends on *brlA*. As a result, transcript levels of s*tuA* are increased 20-fold in conidiating cultures.[57, 84, 85] In turn, StuA directly or indirectly represses and spatially restricts *brlA* and *abaA* expression (Figure 4A). With this ability *stuA* is involved in proper spatial distribution of AbaA and BrlA.[81, 84] The StuA protein also stimulates *stuA* expression. This seems to be an indirect effect because its responsive elements are absent in the promoter.[81]

The *medA* (*medusa*) gene is conserved in filamentous fungi. Like other regulators, *medA* is transcribed at two initiation sites. While *stuA* of *A. nidulans* is required for proper spatial expression of *abaA* and *brlA, medA* is involved in proper temporal expression of these genes.[45, 85] Accumulation of both *brlA* transcripts is observed earlier in development in a Δ*medA* strain. Moreover, the mutant strain shows higher levels of *brlAβ,* but not *brlAα,* transcripts. As a result, the ratio of *brlA*α and *brlAβ* transcripts is lowered. Gene *medA* thus acts as a repressor of *brlA* expression. In contrast, it is an activator of *abaA* expression. This is concluded from the observation that *abaA* transcription levels are reduced or even absent in the *medA* mutant.[84, 85] The molecular basis of MedA function is still unclear. A Δ*medA* strain forms repeated layers of sterigmata and frequent reinitiated secondary conidiophores.[74, 75] This phenotype resembles that of a strain of *A. nidulans* in which the chitin synthase genes *chsA* and *chsC* genes have been inactivated.[86] In the latter strain, *abaA* expression is reduced. This indicates that *chsA* and *chsC* regulate expression of *abaA*, most probably in an indirect way. The Δ*chsA*Δ*chsC* mutant shows a defective septum formation.[86] Therefore, it was proposed that MedA is involved in septum formation on conidiophore structures. Taken together, conidiophore morphogenesis requires a finely tuned balance of at least BrlA, AbaA, MedA, and StuA[85], and possibly VosA and other velvet complex genes.[72, 80]

Trimeric G-protein signaling

Trimeric G-protein signaling is involved in the decision to grow vegetatively or to start asexual development. Gene *flbA* (*fluffy low brlA expression*) encodes an RGS domain protein, which negatively regulates vegetative growth signaling (Figure 5). It does so by stimulating the intrinsic GTPase activity of the Gα subunit FadA (*fluffy autolytic dominant*) of a heterotrimeric G-protein. As a result, the Gα subunit is converted into the inactive GDP bound state[87-89] (Figure 5). Overexpression of *flbA* in vegetative cells inhibits hyphal growth and stimulates conidiophore development even under conditions that normally prevent sporulation.[22, 52] On the other hand, a mutation in *flbA* results in reduced *brlA* expression and a fluffy phenotype (hence the name *fluffy low brlA expression*). The Δ*flbA* strain does not form conidiophores. Instead, the mycelium proliferates uncontrolled and masses of undifferentiated aerial hyphae are formed. Both the submerged and aerial

hyphae autolyse when colonies mature.[22, 90] The autolytic phenotype of the *flbA* mutant can be partially overcome by deleting a class V endochitinase B (*chiB*). However, reduced cell viability cannot be restored in this way.[91] Inactivation of *fadA* (Δ*fadA* or dominant-interfering *fadA* mutant) can also counteract the autolytic phenotype of the *flbA* mutant. This is in agreement with the function of FlbA to convert FadA into the inactive GDP bound state (Figure 5). A constitutively active *fadA* mutant, *fadA*^{G42R}, results in autolytic mutants similar to the *flbA* mutant.[89, 92] The constitutively active *fadA*^{G42R} mutant phenotype cannot be suppressed by overexpression of *flbA*.[88]

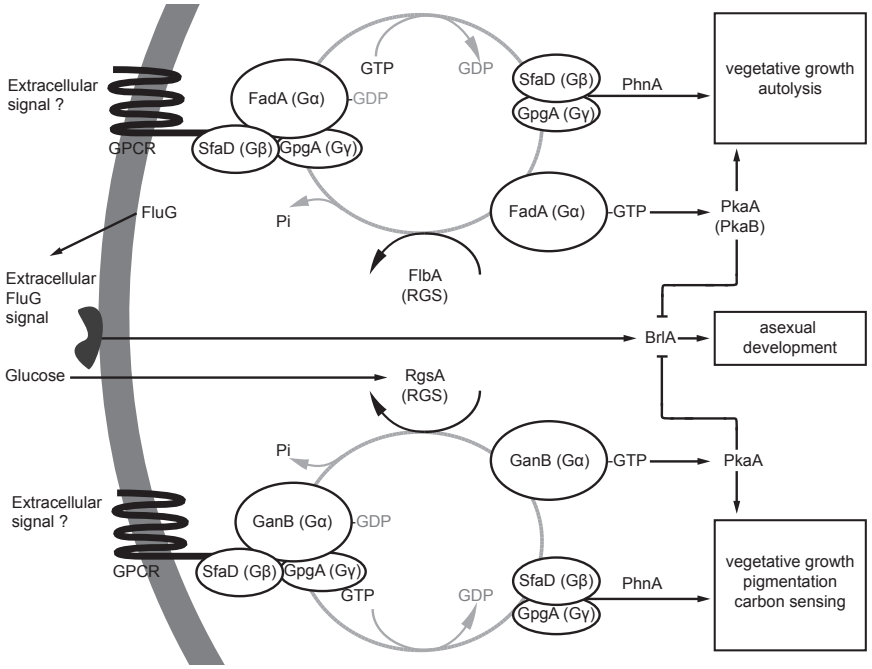

Figure 5. Signaling cascades resulting in vegetative growth or asexual reproduction in *A. nidulans*. Signalling involves FluG (see Figure 6) and independently, two heterotrimeric G-protein complexes, both consisting of SfaD and GpgA (the Gβγ subunits) and the Gα subunits FadA and GanB, respectively. GTP-bound FadA and GanB stimulate vegetative growth via the cAMP PkaA pathway and repress asexual reproduction via *brlA*. The RGS proteins FlbA and RgsA hydrolyze the GTP bound to FadA and GanB, respectively, thereby repressing vegetative growth and promoting asexual development. The SfaD-GpgA dimer also stimulates vegetative growth. This is regulated by PhnA. Adapted from [97].

In its inactive GDP-bound form, FadA of *A. nidulans* forms a heterotrimeric G-protein with the β- and γ-subunits encoded by *sfaD* and *gpgA*, respectively.[88, 89, 93, 94] When FadA becomes GTP bound, this α-subunit dissociates from SfaD and GpgA (Figure 5). Deletion of *sfaD*[94] or *gpgA*[93] suppress the phenotype of the *flbA* mutant. Moreover, in a wild-type background reduced vegetative growth is observed in these deletion strains. Inactivation of *sfaD*[94] but not *gpgA*[93] also

causes hyper-sporulation. The Δ*sfaD*Δ*gpgA* double mutant shows a phenotype identical to those of the Δ*sfaD* mutant.[93] This shows that Δ*sfaD* is epistatic to Δ*gpgA* and that SfaD can induce inappropriate conidiation even in the absence of GpgA. Notably, constitutive activation of *fadA* in the absence of *sfaD* is sufficient to cause proliferation of undifferentiated hyphae.[93, 95] Taken together, FadA and SfaD-GpgA have overlapping functions in stimulating vegetative growth.[93, 94] The phosducin like protein A (PhnA) seems to be involved in positively regulating Gβγ signaling in *A. nidulans*. Deletion of *phnA* results in a phenotype similar to that of a Δ*sfaD* strain.[96] This would agree with the role of phosducin like proteins to act as chaperones for Gβγ assembly.[97] Finally, deletions in *sfaD*, *fadA* or *gpgA* do not suppress conidiation defects in a *fluG* mutant. Therefore, the FadA/SfaD/GpgA vegetative growth-signaling cascade seems to be distinct from that of the FluG pathway.[93]

GTP-bound-FadA promotes vegetative growth and inhibits asexual and sexual development by activating a cAMP-PKA signaling cascade[98] (Figure 5). The cAMP dependent protein kinase A catalytic subunit (PKA) encoded by *pkaA* has a major role in the stimulation of vegetative growth and the repression of conidiation[93, 99-101]. Inactivation of *pkaA* results in hyper-sporulation and reduced radial growth.[98] On the other hand, overexpression of *pkaA* leads to decreased sporulation accompanied by a fluffy-like appearance. Deletion of the other *pka* gene in the genome of *A. nidulans, pkaB*, causes no apparent phenotype.[102] However, over-expression of *pkaB* reduces conidiation and increases vegetative growth on solid medium. Moreover, it complements the reduced radial growth of the Δ*pkaA* strain. Apparently, PkaB functions as a backup for PkaA.[102, 103]

The FlbA/FadA/SfaD/GpgA pathway seems to be conserved in *A. nidulans, A. oryzae* and *A. fumigatus.*[59, 61, 97] Like in *A. nidulans,* the SfaD-GpgA complex is involved in stimulating vegetative growth in *A. fumigatus*.[91] However, there are some differences in the case of the other components. Deletion of *flbA* in *A. nidulans,*[90] *A. oryzae*,[59] or *A. fumigatus*[61] results in low *brlA* expression, and reduced conidiation. In contrast to *A. nidulans* and *A. oryzae,* the autolysis phenotype is missing in *A. fumigatus*. Moreover, in both *A. oryzae* and *A. fumigatus* hyphal proliferation is reduced in the Δ*flbA* strain, blocking formation of aerial hyphae in the case of *A. oryza.*[59] Like in *A. nidulans,* FadA of *A. oryzae*[59] and *A. fumigatus*[61] repress conidiation. Remarkably, in *A. oryzae* it also represses vegetative growth,[59] while in *A. fumigatus* vegetative growth is stimulated.[98, 104]

Apart from FadA, two other Gα subunits are present in *A. nidulans* (GanA and GanB) and *A. fumigatus* (GpaA and GpaB), and three in *A. oryzae* (GanA, GanB, GaoC).[89, 93, 94, 100, 105, 106] In contrast to *ganB,* the functions of *ganA* and *gaoC* have not been established yet.[100, 105, 107] Like FadA, GanB in its inactive form interacts with SfaD and GpgA[93] (Figure 5), which in fact are the only β and γ subunits of trimeric G-proteins in *A. nidulans, A. fumigatus*, and *A. oryzae*.[100] The Δ*ganB* strain shows hyper-sporulation in submerged cultures and earlier

expression of the *brlA* transcripts. Constitutive activation of GanB results in reduced hyphal growth and a severe defect in asexual sporulation.[105] Like FadA, GanB therefore seems to be involved in repression of *brlA* and inhibition of asexual sporulation.[105]

RgsA (*regulator of G protein signaling*) is a repressor of GanB signaling. Colonies of the Δ*rgsA* strain are reduced in size, form more aerial hyphae, and accumulate dark brown pigments.[107] Expression of a constitutively active RgsA results in hyper-sporulation in submerged cultures and earlier expression of the *brlA* transcripts.[107] The presence of glucose is claimed to result in the increase of *rgsA* mRNA levels, and this would result in down-regulation of GanB mediated signaling. In cases of stress or unfavorable carbon sources, *rgsA* levels decrease and as a consequence GanB-GTP signaling is activated.[107] In *A. fumigatus* the outcome of the signaling pathway involving the GanB orthologue GpaB is different. Growth and conidium formation of a Δ*gpaB* strain is slightly decreased.[104] This and other data show that GpaB signaling in *A. fumigatus* promotes asexual sporulation via PKA. However, the PkaC1 cascade in *A. fumigatus* is complex, since it also promotes vegetative growth, when activated by GpaA.[106]

Upstream activators of brlA

FluG activates sporulation by a derepression pathway that involves the SfgA protein (Figure 6). Gene *sfgA* (*suppressor of fluG*) is predicted to encode a transcription factor with a Gal4-type $Zn(II)_2Cys_6$ binuclear cluster DNA binding motif.[108] Mutations in *sfgA* bypass the need for FluG during asexual development. The Δ*sfgA* strain shows hyperactive sporulation in liquid submerged cultures. Overexpression of *sfgA* results in delayed and reduced levels of *brlA* mRNA, and in macro-colonies with reduced conidiation. Apparently, the primary role of FluG is to remove the repressive effects of SfgA.[108] The low FluG levels in young macro-colonies would result in *sfgA*-mediated repression of conidiation. Once the FluG factor has accumulated above a certain threshold, it inhibits the repression of conidiation by SfgA.[108] SfgA has also been proposed to negatively regulate FlbA (Figure 6). By this, the repression of SfgA by FluG will result in both activation of conidiation and inhibition of FadA-mediated stimulation of growth.

Apart from *flbA*, four other regulatory genes, *flbB*, *flbC, flbD,* and *flbE,* have been identified that act upstream of *brlA*.[90] All *flb* mutant strains show low *brlA* expression and a fluffy phenotype.[90] The mutants grow indeterminately and produce masses of aerial hyphae. The *flb* mutants can restore conidiation in *fluG* loss-of-function mutants.[90] These results indicate that the *flb* genes are involved in responding to the diffusible signaling molecule, produced by FluG, which is necessary for conidiation.[90] SfgA acts downstream of *fluG* but upstream of *flbA-D*. As mentioned above, FluG is assumed to repress *sfgA* thus releasing the repression of the *flb* genes. Notably, *flb* genes are involved in regulation of *fluG* expression.[109] A repressing function of FlbA on *fluG* expression is indicated by a 7-fold higher

fluG expression in a Δ*flbA* strain. In contrast, *fluG* expression is stimulated by FlbB and FlbC. For instance, a Δ*flbC* shows no *fluG* mRNA accumulation.[109]

The *flbC* gene encodes a transcriptional regulator containing two C_2H_2 zinc finger DNA binding domains.[110] The Δ*flbC* strain shows delayed and reduced conidiation, while overexpression causes restricted hyphal growth and delayed conidiation. In wild-type mycelium, FlbC is localized in nuclei of vegetative hyphae and in conidiophores (i.e. not in conidia). Here, FlbC activates *brlA, abaA,* and *vosA* but not *wetA*. Overexpression of *flbC* not only inhibits vegetative growth in a wild-type strain but also in a Δ*abaA* or Δ*brlA* background. Thus, FlbC plays a direct role in repressing vegetative growth, independent of *brlA* or *abaA* (Figure 6). The deletion of *flbC* also results in highly enhanced sexual fruiting body formation (see below).[110] Taken together, FlbC has a repressive role in sexual development, but positively regulates germination and asexual development.[110] FlbC acts in a pathway parallel to that of *flbB, flbD*, and *flbE*.[111, 112] Absence of FlbC does not affect expression of *flbB* or *flbE* and vice versa. Moreover, double mutants cause additive effects, resulting in a prolonged delay in conidiation.[110] It has been proposed that FlbC coordinates activation of *brlA* together with a FlbB/FlbD transcriptional complex[112, 113] (Figure 6). Promoter binding regions of FlbC and FlbB/FlbD may overlap.[70, 112]

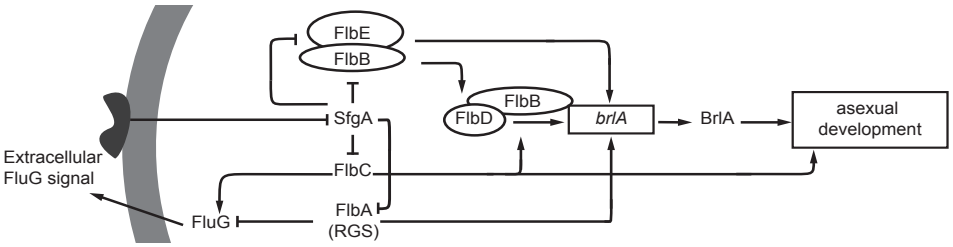

Figure 6. Model of upstream regulation of *brlA*. FluG is involved in the formation of an extracellular factor that activates an unknown receptor. At a certain concentration of FluG, the general suppressor SfgA is inhibited removing the repression of the *flb* genes. FlbB and FlbE form a complex that activates *brlA* leading to asexual development. FlbC activates *brlA* together with the FlbB/FlbD transcription complex. FlbC also activates *fluG* and regulatory genes that act downstream of BrlA. FlbA activates *brlA* by inactivating FadA and probably plays a role in repressing *fluG*. Adapted from [113].

The *flbB* gene encodes a fungal specific bZIP-type transcription factor.[114] A Δ*flbB* strain shows defective branching patterns, delayed conidiation with a fluffy appearance and, under stress conditions, a high autolysis rate. Overexpression of *flbB* results in reduced conidiophore vesicle formation, and a reduced number of metulae.[37, 114] FlbB is located within the cytoplasm at the hyphal apex during early vegetative growth. In contrast, it preferentially accumulates at the most apical nucleus during later stages of growth.[114] The repressor SfgA may be a key

intermediate in the process of nuclear localization of FlbB. This is indicated by the fact that FlbB is found in all nuclei of Δ*sfgA* hyphae, rather than only at the hyphal tip as observed in the wild-type strain. Gene *flbE* encodes a protein without any known conserved domain.[115] Expression of *brlA* and *vosA* is delayed in the Δ*flbE* strain.[110] This is accompanied by absence of conidiophore formation, a fluffy appearance of the macro-colonies, accelerated vegetative growth, and accelerated autolysis and cell death. FlbE is localized at hyphal tips. In fact, it co-localizes with FlbB. Localization of these proteins at the hyphal tip depends on the presence of F-actin. This is concluded from the fact that disintegration of the actin filaments causes mis-localization of FlbB and FlbE. Localization of FlbB and FlbE was also lost in a Δ*flbE and* a Δ*flbB* strain, respectively. These results indicate that these proteins depend on each other for proper localization at the hyphal apex. It has also been shown that FlbB stability is affected by the absence of a functional form of FlbE.[115] FlbE may thus protect FlbB from proteolytic degradation. It may do so by physical interaction with FlbB. At least, such an interaction was shown *in vivo*.[115] Taken together, FlbE and FlbB function in close association with each other and are functionally interdependent.[115]

The FlbB/FlbE complex is a requisite for *flbD* expression in the wild-type[112] (Figure 6). FlbD is a c-Myb transcription factor. Deletion of its encoding gene results in delayed conidiation and a fluffy phenotype.[90, 111] Overexpression causes sporulation in liquid submerged cultures. This is due to inappropriate activation of *brlA*.[111] As mentioned above, the FlbB/FlbE complex is found at hyphal apices.[115] In contrast, FlbD is found in all nuclei of vegetative hyphae of *A. nidulans*. Thus, other transcription factors seem also to be involved in the regulation of *flbD* expression.[112, 113, 115] Not only depends *flbD* expression on the presence of FlbB, FlbD also interacts with this protein.[112, 113, 115] The underlying mechanism is so far unknown but might involve a translationally modified form of FlbB.[112] Both FlbD and the FlbB/FlbE complex seem to activate *brlA* expression[112] (Figure 6). Overexpression of *flbD* restores the conidiation defect in the Δ*flbE* strain,[110] suggesting an additive effect of both pathways. Interestingly *flbB* and *flbD* transcripts disappear shortly after *brlA* activation.[111, 114] However, the mechanism underlying this effect is independent of *brlA* levels.[112]

The *flb* genes are conserved in *A. fumigatus, A. oryzae* and *A. nidulans*.[59, 110] The phenotypes of the Δ*flb* strains of *A. oryzae* are similar to those of *A. nidulans*. These results indicate that the functions of these regulatory genes are conserved between *A. oryzae* and *A. nidulans*.[59] The *A. fumigatus flbE* could complement the Δ*flbE* phenotype in *A. nidulans*,[110] suggesting a similar role for these orthologues. Indeed, FlbE in *A. fumigatus* is necessary for proper control of conidiation and *brlA* and *vosA* expression. However, deletion of *flbE* does not cause an elevated vegetative proliferation or accelerated autolysis or cell death in *A. fumigatus*.[110] Inactivation of *flbB* in *A. fumigatus* results in delayed and reduced sporulation, and precocious cell death. Moreover, expression of *brlA* and *abaA* is

affected. In contrast to *A. nidulans,* the FlbB protein is in *A. fumigatus* encoded by two transcripts, *flbBα* and *flbBβ*. The longest transcript, *flbBβ,* is constitutively expressed, while the *flbBα* transcript is found during progression of conidiation.[116] Both transcripts are needed for proper asexual development. The *flbB* gene of *A. nidulans,* encoding one transcript, only partially complements the Δ*flbB* strain of *A. fumigatus.* FlbC and FlbD functions in *A. fumigatus* are probably similar to *A. nidulans,* but characterization is ongoing at the moment.[116]

MAPK pathways and downstream targets

A mitogen activated protein kinase (MAPK) module consists of three kinases known as MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. MAPKKK is phosphorylated in response to external stimuli. Active (i.e. phosphorylated) MAPKKK phosphorylates MAPKK, which in turn phosphorylates MAPK. Phosphorylated MAPK enters the nucleus and activates transcription factors by phosphorylation.[117] A MAPK pathway has been shown to repress asexual development and to stimulate sexual development in *A. nidulans* (see below). This pathway includes MAPKKK SteC.[118] Growth of the *ΔsteC* strain on minimal medium results in reduced growth and hyphae are more curled and more branched than the wild-type. Conidiophore development is initiated as in wild-type, but the length distribution of the stalks is affected. Metulae and phialide morphology is normal in the majority of the conidiophores. In about 2 % of the conidiophores, however, only a few metulae arise from the vesicle and these metulae do not differentiate normally. In addition, secondary conidiophores grow out of the vesicle. Finally, the mutant produces 1–2 % of very large conidia in addition to wild-type-like spores. A translational fusion of SteC and GFP localizes in metulae, phialides and young conidia but not in conidiophore stalks. The targets of SteC during asexual development have not yet been identified. A functional study of MAPKK genes in *A. nidulans* is lacking and inactivation of the MAPK gene, *mpkB*, does not affect sporulation.[119] Similarly, the putative target of *mpkB, steA,* is not involved in asexual development.[120] In contrast, a potential other target of *mpkB, nsdD* (*never in sexual development*), does affect conidiation.[121] A Δ*nsdD* strain starts to conidiate earlier. On the other hand, over-expression of *nsdD* suppresses formation of conidiophores. Similarly, deletion of the transcription factor gene *nsdC* results in derepression of conidiation. In this case, the effect depends on the carbon source. Derepression is observed on carbon sources favoring sexual development. This indicates that NsdC acts as a repressor of asexual development under those conditions.[122]

SEXUAL DEVELOPMENT

About one-third of the described species of *Aspergillus* have a known sexual stage.[4] However, analysis of genome sequences suggests that most, if not all, *Aspergillus* species should be able to reproduce sexually.[123] Indeed, *A. fumigatus*,

A. flavus, Aspergillus parasiticus, and *Aspergillus nomius* have been shown to have a sexual stage.[124-127] In the case of *A. fumigatus*, this was 145 years after this fungus was described for the first time. Clearly, the conditions under which sexual reproduction takes place differs between aspergilli; some of which being more restrictive (e.g. *A. fumigatus*) than others (*A. nidulans*).

Aspergillus species with a sexual state can be either homothallic or heterothallic. Homothallic species undergo sexual reproduction without the need to cross with a compatible partner. *A. nidulans*[119] and *Neosartorya fischeri*[128] are known to be homothallic. It should be noted that *A. nidulans* can also be heterothallic and can thus cross with a partner. In contrast, *A. flavus*,[126] *A. parasiticus*,[125] *A. fumigatus,*[124] and *A. nomius*[127] have been shown to be exclusively heterothallic. The genomic sequences of *A. niger*[54] and *A. oryzae*[129] predict that these fungi are either heterothallic or truly asexual.[130] The sequenced *A. niger* strains only contain the *MAT1-1* mating type locus (see below) but otherwise seem to emcompass a complete set of genes enabling sexual development.

Sexual development in *A. nidulans* is a highly complex process, ultimately resulting in fruiting bodies of 125–200 μm in diameter that are called cleisthotecia. Sexual development is influenced by both environmental and intrinsic signals, and occurs only when all prerequisites are met. The process of sexual development starts after 40-50 h of cultivation at 37 °C in the center of the macro-colony and mature cleistothecia can be found after approximately 96 h.[131] The production of Hülle cells (see below) represents the first sign of sexual development. Subsequently, hyphae fuse to form a dikaryon. As mentioned, in the case of *A. nidulans* these hyphae may originate from the same macro-colony (self-fertilization) or from another individual (out-crossing). The hyphae that fuse are morphologically similar. Thus, male antheridia and female ascogonia cannot be distinguished.[132] A population of dikaryotic cells originates from a single cell fusion event, and gives rise to a single cleistothecium.[133] The Hülle cells surround the dikaryotic hyphae and form an increasingly packed "nest". They differentiate into thick-walled globose cells[134, 135] that are believed to provide protection and nutrition.[136] An intertwined network of ascogenous dikaryotic hyphae develops within the cleistothecial shell. Nuclear fusion takes place inside the ultimate branches of the dikaryotic hyphae, which represent the young asci. This is immediately followed by meiosis and a post-meiotic mitosis, thus resulting in eight nuclei. These nuclei are then separated from each other by membranes, giving rise to eight red-pigmented spores in each of the 10.000 asci within one fruiting body.[135] A second post-meiotic mitosis makes that mature ascospores are binucleate.[137]

Regulation of sexual development

Mating type loci

Strains of heterothallic ascomycetes contain either a *MAT1-1* or a *MAT1-2*

mating type locus. These loci are not related in sequence, and are therefore called ideomorphs. The *MAT1-1* locus contains a gene encoding an α-domain transcription factor, while the *MAT1-2* encodes a high mobility group-domain (HMG) transcription factor.[138] The homothallic species *A. nidulans* contains both the *MAT1-1* and *MAT1-2* mating type loci, which reside on different chromosomes.[119] Inactivation of *MAT1-1* or *MAT1-2* does not affect vegetative growth or asexual sporulation. Moreover, Hülle cells and cleistothecia are formed under conditions inducing the sexual cycle. However, the cleistothecia are lower in number and smaller than those of the wild-type strain.[119] In addition, no ascospores are formed within the cleistothecia. This and the fact that fruiting body development can be induced by placing both transcriptional activator genes under an inducible promoter shows that *MAT1-1* and *MAT1-2* are the master switches of sexual reproduction.

In filamentous ascomycetes, the *MAT* loci determine cell type identity and may confer nuclear recognition and proliferation.[139, 140] In addition, *MAT* loci regulate expression of a pheromone-signaling system. This system, which is generally involved in the detection of a mating partner, has been best studied in *Saccharomyces cerevisiae*.[141] In *S. cerevisae* the a- and α-cells each produce a peptide pheromone and a receptor for the pheromone of the other partner. Binding of the pheromone to the receptor that is produced by the compatible partner triggers G protein-mediated signaling via a mitogen activated protein kinase (MAPK) cascade. As a result, a homeodomain transcription factor gene is activated that induces cell cycle arrest, and activates the mating process. Components of a pheromone-signaling pathway similar to that of *S. cerevisae* have been detected in *A. nidulans* and other sequenced *Aspergillus* species, including pheromone and pheromone receptor genes.[130] Notably, expression of the pheromone and pheromone receptor genes is not affected when the *MAT* genes are inactivated in *A. nidulans*. Apparently, these genes are not under control of the mating type loci.[119] They may be induced by environmental conditions that stimulate sexual reproduction. In contrast, *MAT1-1* of *A. fumigates* does stimulate the pheromone gene *ppgA,* while it is repressed by *MAT1-2*.[142]

Pheromones, pheromone receptors, and intracellular signalling

In *A. nidulans* only one putative pheromone gene has been identified, which has homology to the gene encoding the α-pheromone of *S. cerevisae*.[143] Until now, it has not been confirmed that the encoded product binds to a pheromone receptor(s). Alternatively, psi factor (see below) may bind to the receptor protein(s).[131] Receptor proteins have been identified in *A. nidulans* on basis of homology with the pheromone receptor genes of *S. cerevisae*.[131] Inactivation of these genes, *gprA* and *gprB* (*G protein receptor*), does not affect growth rate, Hülle cell formation, and asexual sporulation. However, Δ*gprA* and Δ*gprB* strains produce less cleistothecia that are smaller than wild-type fruiting bodies under homothallic conditions. The

Δ*gprA* and Δ*gprB* cleistothecia produce only 5 % of the ascospores when compared to the wild- type but viability of these spores is not affected. The Δ*gprA*Δ*gprB* strain does not form cleistothecia at all under homothallic conditions but Hülle cells are still formed. Notably, sexual development is not impaired under heterothallic conditions; i.e. when strains are crossed in which either or both *gprA* and *gprB* have been inactivated. Heterokaryon formation in this case can be selected by using parental strains that have different auxotrophic markers. Taken together, pheromone receptors genes, and therefore the pheromone signaling pathway, is only required for homothallic sexual development.[131] Genes *gprA* and *gprB* represent two out of sixteen genes encoding seven-transmembrane-spanning G protein coupled receptor proteins (GPCR).[100] Gene *gprD* is another GPCR gene. Inactivation of *gprD* results in a strain with impaired vegetative growth and exhibiting extremely enhanced sexual development.[107, 144] Deletion of *gprA* and *gprB* rescue the growth defects of the Δ*gprD* strain and fruiting bodies are no longer formed. Thus, GprD functions downstream of GprA/B.[107, 144]

In general, binding of pheromones sensitizes the pheromone receptors. As a result, the receptors physically interact with a heterotrimeric G protein. This induces the exchange of GDP for GTP on the α-subunit of the heterotrimeric G-protein, resulting in its dissociation from the βγ-subunits (Figure 7). Genes *sfaD* and *gpgA* are the only β and γ subunit encoding genes in the genome of *A. nidulans*. Inactivation of *sfaD* or *gpgA* abolishes and severely affects cleistothecia formation under homothallic and heterothallic conditions, respectively. Moreover, under both conditions more Hülle cells are formed.[93, 94] The genome of *A. nidulans* contains three α-subunit encoding genes; *ganA, ganB*, and *fadA* (see above). It has not fully been established which of the α-subunits is the cognate Gα protein of GprA and GprB. A role of GanA and GanB in sexual development, if any, has not yet been reported. On the other hand, the role of *fadA* in sexual development has been studied. Cleistothecia formation is not affected in a Δ*fadA* strain under heterothallic conditions but *fadA* is essential for homothallic sexual development. Under homothallic conditions, cleistothecia formation is abolished in the Δ*fadA* strain but Hülle cell formation is not affected.[93, 94] The RGS protein FlbA that controls FadA-mediated signaling (see above) also plays a role in fruiting body development.[121] Inactivation of *flbA* completely abolishes cleistothecia and Hülle cell formation under homothallic conditions. The developmental modifier genes *medA* and *stuA*, which play a crucial role in asexual development, also function in sexual differentiation (Figure 7). The *ΔmedA* strain only forms unorganized masses of Hülle cells and does not differentiate cleistothecia or ascogenic tissue.[85] Sexual development is delayed in the Δ*stuA* strain and it forms only 1 % of the cleistothecia that are formed by the wild-type. Moreover, the cleistothecial shell of the Δ*stuA* strain is much more fragile when compared to the wild-type.[81] Thus, *medA* and *stuA* are activators of sexual development.

Activation of the heterotrimeric G-protein by pheromone binding

results in activation of a mitogen activated protein kinase pathway (MAPK). As mentioned above, the MAPK module consists of three kinases known as MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. The MAPKKK SteC is involved in formation of cleistothecia.[118] The Δ*steC* strain grows slower, forms more branched hyphae, and has an altered conidiophore morphology. Moreover, heterokaryon formation is inhibited. This is probably due to the inability of hyphae to fuse, possibly by the fact that hyphal recognition is blocked.[118] The

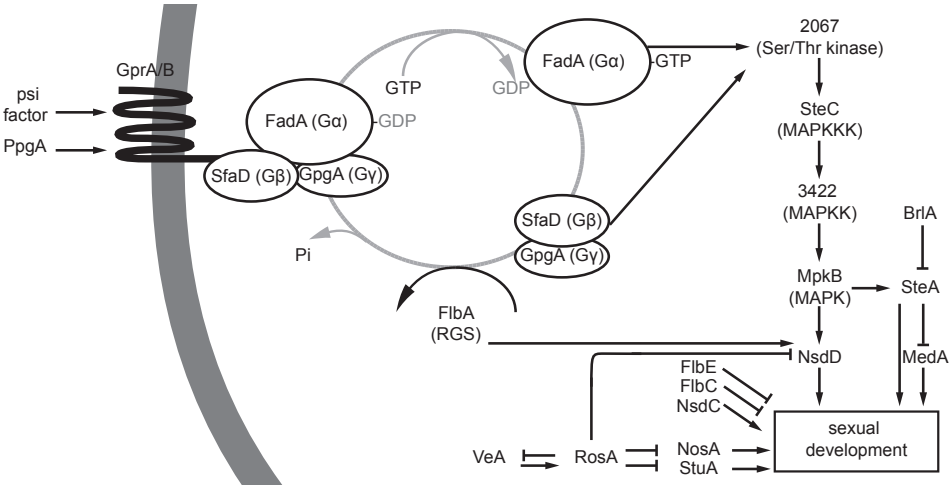

Figure 7. GprA/B mediated signalling resulting in sexual development of *A. nidulans*. Signalling involves the heterotrimeric G-protein consisting of SfaD, GpgA and FadA. FadA bound to GTP activates a MAP kinase cascade (hypothetical protein AN2067.2, SteC, hypothetical protein AN3422.2 and MpkB). This in turn, activates the regulators NsdD and SteA that induce sexual development. In addition, SteA inhibits MedA that is also involved in activating sexual development. StuA, NsdC, and NosA also activate the sexual development program, while FlbC and FlbE are repressors of this pathway. RosA is a transcriptional inhibitor of *veA, nsdD, nosA* and *stuA*. Adapted from [97, 131].

Δ*steC* strain also has a block in cleistothecium development. Only a few small nest-like structures and single Hülle cells are being produced by this strain. The fact that deletion of both *gprA* and *gprB* results in a phenotype similar to that of the Δ*steC* strain strongly indicates that a MAPK cascade functions in GprA/B mediated signaling (Figure 7). This would be similar to that found in the yeast mating response. In fact, the *A. nidulans* genome is predicted to contain all the major components of such a yeast MAPK cascade[97, 131] (Figure 7). However, so far the role of all these genes has not yet been confirmed. For instance, a functional study of MAPKK genes in *A. nidulans* is lacking. However, it has been shown that inactivation of the MAPK gene, *mpkB*, abolishes sexual reproduction. Both Hülle cells and cleistothecia are not formed.[119] This indicates that MpkB is part of the GprA/B mediated signaling pathway that results in formation of cleistothecia and ascospores.

Transcriptional activators

A target of MpkB seems to be SteA.[120] This homeodomain-C_2H_2-zinc finger transcription factor is homologous to Ste12, which in *S. cerevisae* is activated by the MAPK signaling pathway involved in mating. A Δ*steA* strain does neither form cleistothecia nor primordial structures but Hülle cell formation is not affected. Expression of *steA* does not depend on *medA* and *stuA*. In contrast, *medA* but not *stuA* is repressed by *steA*. This suggests that *steA* and *stuA* are part of independent pathways regulating sexual development and that *medA* acts downstream of *steA*[120] (Figure 7). Another target of MpkB could be *nsdD* (*never in sexual development*). This gene encodes a putative GATA-like transcription factor with a type IVb zinc finger DNA-binding domain.[121] Inactivation of *nsdD* abolishes formation of cleistothecia. In contrast, over-expression of the gene results in increased formation of fruiting bodies on solid medium and Hülle cells are formed even in submerged culture. As a result of over-expression the phenotype of the *veA1* mutation (see below) is overruled. This indicates that *nsdD* acts downstream of this gene or functions in an overlapping pathway.[121] The *nsdD* gene of *A. fumigatus* has a similar function as its counterpart in *A. nidulans.* The *ΔnsdD* mutant of *A. fumigatus* accumulates dark pigments and sexual development is abolished.[142] A third gene encoding a transcriptional regulator involved in sexual development is *nsdC* (*never in sexual development*).[122] This gene encodes a putative transcription factor carrying a novel type of zinc-finger DNA-binding domain consisting of two C_2H_2's and a C_2HC motif. Inactivation of *nsdC* completely abolishes fruiting body formation. On the other hand, overexpression of the gene promotes cleistothecia formation, even under repressing stress conditions.[122] The phenotype of the Δ*nsdC* strain is not affected by over-expression of *steA* or *nsdD.* These results suggest that NsdC, NsdD, and SteA are non-redundant proteins and thus are all essential for fruiting body development.

Gene *nosA* (*number of sexual spores*)[145] and *rosA* (*repressor of sexual development*) [146] encode putative $Zn(II)_2Cys_6$ transcription factors that share 43 % amino acid identity. Transcript levels of these genes are usually very low but the genes are up-regulated during carbon starvation and during asexual development. RosA is an inhibitor and NosA an activator of sexual development. Asexual and sexual development is not affected under standard laboratory conditions when *rosA* is inactivated in a strain with *a veA1* mutation (see below). However, the number of cleistothecia increases when compared to the wild-type strain when the Δ*rosA* strain contains a fully functional *veA* gene.[146] These data indicate that RosA function depends on VeA. The difference in the number of cleistothecia between the wild-type strain and the Δ*rosA* strain is even higher under low-glucose or high-osmolarity conditions. When grown submerged, Hülle cells are produced in the Δ*rosA* strain, while sexual development in the wild-type is completely absent under this condition.[146] Asexual development is not affected in a Δ*nosA* strain with a *veA1* mutation. However, this strain was unable to undergo

sexual development.[145] Asexual development in the light is also not affected when the Δ*nosA* strain contains a fully functional *veA* gene. On the other hand, sexual development in the dark is blocked in the primordial stage in this mutant strain. Differentiated cells such as ascus mother cells and Hülle cells are hardly formed and only a few very small cleistothecia (about 30 mm diameter instead of 300 mm) are produced. These fruiting bodies do produce viable ascospores. Expression of *nosA* was increased in a Δ*rosA* strain.[145] Similarly *nsdD, veA,* and *stuA* are up-regulated in this mutant strain.[146] Taken together, RosA represses sexual development, whereas NosA is required for primordium maturation. The balance between these two $Zn(II)_2Cys_6$ transcription factors determines whether vegetative hyphae undergo sexual development.

THE BALANCE OF SEXUAL AND ASEXUAL DEVELOPMENT

There is a balance between sexual and asexual development. *A. nidulans* forms conidia in the light, whereas ascospores are preferentially produced in the dark.[45, 147-149] Several light sensors and regulatory proteins are involved in light-regulated development. Moreover, the balance between sexual and asexual reproduction is the result of psi factor and cross-talk between regulatory pathways that are involved in these developmental pathways. The light-regulated pathway, the regulation by psi factor, and the cross-talk between regulatory pathways of asexual and sexual reproduction are part of a complex regulatory network.

The role of SteA, BrlA, FlbC, and FlbE

SteA is assumed to be the transcription factor that is activated by MpkB (Figure 7) thereby promoting sexual development. Expression of *steA* is increased in undifferentiated hyphae of a *ΔbrlA* strain.[120] Repression of *steA* in uninduced hyphae has been related to the BrlAβ protein. The regulatory interaction between *brlA*, *medA*, and *steA* is a clear example of cross-talk between the developmental programs of asexual and sexual sporulation. FlbC and FlbE also play a role in the cross-talk between the asexual and sexual developmental pathway. FlbC and FlbE are upstream activators of *brlA* involved in asexual development (Figure 6) but at the same time repress the sexual pathway (Figure 7).[110, 150] Both a Δ*flbC* and a Δ*flbE* strain show elevated cleitothecium formation and abundant formation of Hülle cells.[110, 150]

The role of psi factor

A. nidulans produces oleic- and linoleic-acid-derived oxylipins that are known as psi factor (precocious sexual inducer). These hormone-like molecules modulate the timing and balance between sexual and asexual spore development.[151] Psi factor is mainly a mixture of secreted hydroxylated linoleic (18 : 2) and oleic (18 : 1) molecules termed psiα and psiβ, respectively.[152, 153] The positioning of the hydroxy groups distinguishes the psi compounds as psiA (5',8'-dihydroxy-), psiB

(8'-hydroxy-), and psiC (designating a lactone ring at the 5' position of psiA).[154, 155] Purified psiAα enhances asexual sporulation. On the other hand, psiBα and psiCα stimulate sexual reproduction and inhibit asexual spore development.[152, 153] It has therefore been proposed that the ratio of psiAα to psiBα and psiCα determines whether asexual or sexual sporulation dominates.[152, 153] Gene *ppoA*,[156] *ppoB*,[157] and *ppoC*[158] encode putative fatty acid oxygenases. The former gene is required for the production of psiBα, while the latter two genes are involved in the synthesis of psiBβ (Figure 8). Deletion of *ppoA* reduces psiBα levels and, as a consequence, increases the ratio of asexual to sexual spore numbers 4-fold. On the other hand, over-expression of *ppoA* results in elevated levels of psiBα and decreases the ratio of asexual to sexual spore numbers 6-fold. An increased ratio of sexual to asexual spore numbers is also observed after deletion of *ppoC* [156], whereas an opposite phenotype is observed after inactivation of *ppoB*.[157] This is unexpected since the *ΔppoB* and *ΔppoC* strains produce similar psiB profiles.[156, 157] Several explanations for this phenomenon have been proposed, one of which is the possibility that the products of the *ppo* genes have more than one substrate. This would generate oxylipins, of unknown nature, that would also affect differentiation.[159] Another explanation may be related to the finding that the products of the fatty acid oxygenases affect the expression of *ppo* genes and thereby impact the composition of psi factor. Gene *ppoC* is up-regulated in a Δ*ppoB* strain, whereas *ppoA* is

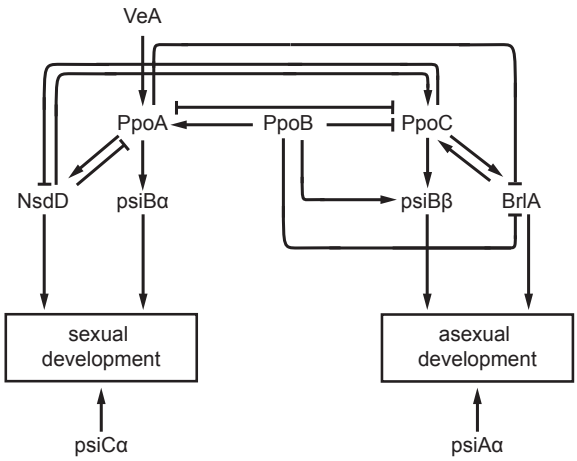

Figure 8. Oxylipins, known as psi factor, regulate timing and balance between sexual and asexual development. The hormone like structures psiBα and psiCα stimulate sexual development, whereas psiAα and psiBβ stimulate asexual development. Psi factor results from *ppoA, ppoB,* and *ppoC* activity. Expression of these genes is regulated by the products resulting from the Ppo proteins and by BrlA and NsdD. In turn, the products resulting from the Ppo genes regulate expression of *brlA* and *nsdD.*

repressed[157] (Figure 8). On the other hand, *ppoA* mRNA levels are higher in a Δ*ppoC* strain, whereas *ppoC* mRNA levels are lower in a strain over-expressing *ppoA*.[156]

Deletion of *ppoB* significantly increases *brlA* expression but has a minor effect on expression of the regulatory genes of sexual reproduction *nsdD* and *veA*[157] (Figure 8). This would explain why the net formation of conidia is up-regulated in a Δ*ppoB* strain when compared to ascospores. Changes in sporulation ratios in the Δ*ppoA* and Δ*ppoC* strains also agree with the relative expression of *brlA* and *nsdD*.[156, 158] Expression of *brlA* is down-regulated in a Δ*ppoA*Δ*ppoB*Δ*ppoC* strain, whereas higher levels of *nsdD* and *veA* transcripts are found. This correlates with the increased number of ascospores when compared with the conidia. Notably, sexual development precedes asexual development in the Δ*ppoA*Δ*ppoB*Δ*ppoC* strain, whereas the opposite is observed in the wild-type. Taken together, *ppo* genes determine timing and ratio of sexual and asexual reproduction.[157] The transcripts of *ppoA* and *ppoC* are developmentally regulated in differentiated tissues.[156, 158] Moreover, PpoA has been localized in metulae of conidiophores and Hülle cells.[158] These observations suggest that Ppo enzymes and/or their substrates are spatially and temporally regulated in reproductive tissues. This would result in a temporal and spatial distribution profile of the oxylipins, which in turn orchestrates the sexual and asexual sporulation schedule.[157] This schedule is associated with two feedback loops (Figure 8). Genes *ppoC* and *ppoA* regulate the expression of *nsdD* and *brlA*. On the other hand, both *brlA* and *nsdD* activate *ppoC,* whereas *nsdD* represses *ppoA*.[156, 158] In the other loop Ppo oxylipin products would antagonistically signal generation of Ppo substrates.[156, 157]

A. niger[160] and *A. fumigatus*[159] produce the same oxylipins as *A. nidulans.* The *A. niger* genome contains the *ppo* genes *ppoA*, *ppoC*, and *ppoD*. A *ppoB* homologue is not present.[160] *A. niger* strains in which the *ppoA* or *ppoD* gene are inactivated are not affected in oxylipin production and sporulation. In contrast, a multi-copy *ppoC* strain has reduced conidia formation. This shows that oxylipins also play a role in *A. niger* development but it could well be that the function of the individual psi factor components is different.

Germination

Three stages can be distinguished during germination of conidia. In the first phase of germination, dormancy is broken by environmental cues such as the presence of water and air either or not in combination with inorganic salts, amino acids or fermentable sugars.[161] Spores grow isotropically in the second phase of germination. This process that is also known as swelling is observed between 2 and 6 h after inoculation of *A. niger* at 25 °C.[162, 163] During this stage, the diameter of the spore increases two fold or more due to water uptake. This is accompanied by a decrease in the microviscosity of the cytoplasm.[164] Moreover, molecules are directed to the cell cortex to enable addition of new plasma membrane and cell wall.[165] In the third phase of germination, a germ tube is formed by polarized growth. To this end, the morphogenetic machinery is redirected to the site of polarization.[165-168] Polarized growth of *A. niger* can be observed 6 h after inoculation at 25 °C.[162, 163]

At a later stage, the growth speed of the germ tube increases.

Transcripts of about one third of the genes can be detected by micro-arrays in dormant conidia of *A. nigeri.*[162, 163] Transcripts representing the functional gene classes protein synthesis and protein fate are enriched in the RNA pool. A strong drop in the amount of RNA is observed in the first two hours of germination.[162, 163] Notably, transcripts belonging to the functional gene classes protein synthesis and its subcategories translation and initiation are over-represented in the up-regulated genes at 2 h. Moreover, the categories transcription (including rRNA synthesis and rRNA processing), energy (respiration), cell cycle & DNA processing are overrepresented in the up-regulated genes at this time point. Up-regulation of genes involved in protein synthesis has also been shown in germinating conidia of *A. fumigatus.*[169] The importance of protein synthesis in early stages of germination is also indicated by the fact that the protein synthesis inhibitor cycloheximide prevents isotropic growth, while inhibitors of the cytoskeleton and DNA- and RNA synthesis do not affect this process.[170] The total number of genes that are expressed in germinating conidia of *A. niger* gradually increase between 2 and 8 h after inoculation at 25 °C.[162] After 4 h of germination, the functional categories metabolism and cell cycle and DNA processing are over-represented in the up-regulated genes. The latter suggests that the conidium prepares itself for mitosis, which occurs a few hours later. No functional gene classes are over- or under-represented in the differentially expressed genes at 6 h and 8 h.[162]

Upon activation of conidia of *A. nidulans* and *A. niger,* the compatible solute trehalose is converted to glucose.[163, 171] Similarly, mannitol levels quickly drop during the first 2-3 h of germination. Intracellular trehalose is degraded by the action of the neutral trehalase TreB.[171] The Δ*treB* strain of *A. nidulans* still contains 1.2 pg of trehalose after 3 h of germination and also shows a reduction in the degradation of mannitol when compared to the wild-type. Germ tube formation is not affected in the Δ*treB* strain of *A. nidulans* in the presence of an external C-source. However, it is delayed in the case the concentration of the external C-source is very low. Apparently, degradation of trehalose is needed to generate energy during germination. Interestingly, germinating Δ*treB*-spores resist a heat shock of 50 °C for 30 min, whereas more than 80 % of the wild-type spores have died after this treatment. This suggests that trehalose has a protective effect. However, experiments with a Δ*tpsA* strain that is not able to synthesize trehalose show that the situation is more complex. Three-hours-old germlings of this mutant strain show accumulation defects of trehalose after subjection to heat stress or oxidative stress. The isotropically growing wild-type spores accumulate 0.8 pg trehalose as a response to the stress, but the Δ*tpsA* strain was not able to do so. Remarkably, there was no effect on the sensitivity of these germlings for a second heat shock at 50 °C.[172]

Regulation of germination of conidia

cAMP and RasA signalling

Initiation and completion of germination requires the sensing of external signals. To this end, conidia of *A. nidulans* use signaling via cAMP/protein kinase A (Figure 9), and independently, signaling via RasA. In general, signalling via cAMP/protein kinase A (PKA) is initiated by an external signal through a heterotrimeric G-protein. Sensing of the external signal leads to the activation of the Gα-subunit of the heterotrimeric G-protein, which activates adenylate cyclase. This enzyme produces cAMP that can bind to the regulatory subunit of PKA. As a result the regulatory subunit dissociates from the catalytic subunit (PKAc). Active PKAc phosphorylates proteins and in this way controls their activity. As described, the heterotrimeric G protein GanB-SfaD-GpgA represses conidia formation in *A. nidulans* (Figure 5), but this heterotrimeric G-protein is also a carbon source sensor involved in early cAMP-dependent germination in *A. nidulans*.[99] Conidia of a Δ*ganb* strain show a reduced rate of swelling and germ tube formation.[105] Wild-type conidia start to swell 2 h after inoculation. 8 % and >90 % of them have formed germ tubes 4 and 6 h post- inoculation, respectively. Germ tubes are hardly observed after 4 h in the case of the Δ*ganB* strain, and less than 50 % of these spores have formed a germ tube after 10 h of inoculation. In contrast, expression of a constitutive active version of GanB ($GanB^{Q208L}$) promotes germ tube formation. In this case, germination of conidia even takes place in the absence of carbon source.[105] Gene *ganB* is also involved in the germination process

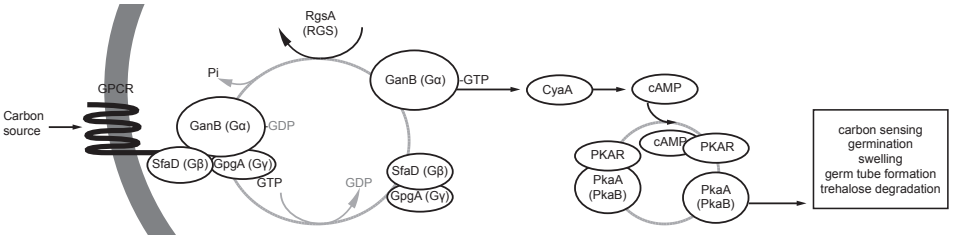

Figure 9. The cAMP/protein kinase A signaling pathway involved in germination of spores in *A. nidulans*. The presence of a carbon source is sensed by a GPCR that activates the Gα subunit GanB. GanB-GTP activates adenylate cyclase CyaA that produces cyclic adenosine-monophosphate (cAMP). cAMP binds to the regulatory subunit of PKA (PKAR), thus releasing the catalytic subunit PkaA. Active PkaA phosphorylates downstream targets resulting in swelling, germ tube formation and trehalose degradation. PkaA and PkaB have an overlapping role in spore germination in the presence of glucose but an opposite role in germination in the absence of a carbon source.

of ascospores. Ascospores of the Δ*ganB* strain germinate very poorly, whereas expression of $GanB^{Q208L}$ results in precocious ascospore germination, even in the absence of carbon source. Taken together, GanB plays a positive role during germination, probably through carbon source sensing. The RgsA protein of *A.*

nidulans negatively regulates GanB signaling[144] (Figure 9). Like a strain expressing GanB^{Q208L}, conidia of the Δ*rgsA* strain germinate even in the absence of a carbon source. This effect is not observed in a Δ*ganB*Δ*rgsA* or a Δ*sfaD*Δ*rgsA* strain.[99, 144]

The Δ*ganB* strain of *A. nidulans* shows a >3-fold reduction in trehalose degradation during spore germination [99]. Breakdown of this disaccharide is a direct outcome of activation of the cAMP/PKA pathway during early germination.[171] A role of GanB in cAMP/PKA signaling has been proven by measuring cAMP levels in germinating spores. Addition of glucose to dormant wild-type spores results in a rapid and transient increase in cAMP levels. This increase is not observed in the Δ*ganB* strain.[99] Thus, GanB regulates cAMP synthesis in response to glucose at the start of germination. As described above GanB forms a heterotrimer G-protein together with SfaD and GpgA (Figure 9). Spore germination and trehalose degradation is also affected in conidia of the Δ*sfaD* and Δ*gpgA* strains, although not as strong as observed in the Δ*ganb* strain.[99] A role of RgsA in GanB signaling is further supported by the finding that trehalose degradation in the Δ*rgsA* strain is increased. This effect is abolished by inactivation of *ganB* in this strain but also by inactivation of *sfaD*.[99] These data indicate that glucose-stimulated activation of the cAMP/PKA pathway by GanB requires a functional G-protein formed by GanB, SfaD, and GpgA (Figure 9).

CyaA represents the adenylate cyclase of the cAMP/PKA pathway that is regulated by GanB (Figure 9). In contrast to the wild-type, mycelium of the Δ*cyaA* strain is completely devoid of cAMP.[173] This indicates that *cyaA* encodes the unique adenylate cyclase during mycelial growth. Conidia of the Δ*cyaA* strain do not degrade trehalose during the onset of germination. Moreover, germ tube outgrowth is affected. As mentioned above, cAMP produced by adenylate cyclase activates the catalytic subunit of PKA (PKAc) by releasing the regulatory subunit. In the case of *A. nidulans, pkaA* encodes the primary PKAc. Trehalose breakdown is reduced in the Δ*pkaA* conidia and germ tube outgrowth is affected, but not as strong as in the Δ*cyaA* conidia.[173] Germination of conidia is also affected in *A. fumigatus*[104] and *A. niger.*[174] when their closest homologue of *pkaA* is inactivated. *A. nidulans* contains a secondary *pka* gene, *pkaB*, which encodes a catalytic subunit of PKA. The Δ*pkaB* strain does not have an apparent phenotype.[102] However, the Δ*pkaA*Δ*pkaB* strain is lethal, indicating that PkaB is involved in hyphal growth and/or spore germination. Approximately 10-fold up-regulation of *pkaB* mRNA levels rescues the defects in germination of conidia of the Δ*pkaA* strain in the presence of glucose (note that the level of up-regulation is important for the phenotypic outcome). In contrast, up-regulation of *pkaB* completely abolishes spore germination in the absence of an external carbon source. Taken together, these data indicate that PkaA and PkaB have an overlapping role in spore germination in the presence of glucose but an opposite role in germination in the absence of a carbon source.[102] Other Ser/Thr protein kinases also contribute to spore germination in *A. nidulans.* Inactivation of the Ser/Thr protein kinase gene,

schA, in the Δ*pkaA* background results in a phenotype similar to that of the Δ*cyaA* conidia. This indicates that PkaA and SchA are activated by cAMP produced by CyaA.

The Ras signaling pathway operates independently from the cAMP/PKA signaling pathway during germination of conidia of *A. nidulans*.[173] Conidia of *A. nidulans* strains expressing a dominant active form of RasA ($RasA^{G17V}$) do not proceed to polarized growth. Instead, swelling continues resulting in giant swollen spores.[175] This suggests that high RasA activity prevents the switch from isotropic to polarized growth. There are indications that the RasA activity is regulated by a GTPase-activating protein GapA. By stimulating hydrolysis of the GTP bound to RasA it loses its activity.[176] Both wild-type and Δ*gapA* conidia germinate in the presence of glucose. In contrast, whereas Δ*gapA* conidia also swell in the absence of a carbon source, the wild-type does not. A similar phenotype has been reported for *A. nidulans* expressing $RasA^{G17V}$. This suggests that RasA plays a role in carbon source sensing during conidiation,[170] and that this is regulated by GapA. RasA has been suggested to function via activation of a mitogen-activated protein kinase pathway.[173] This pathway may well include the mitogen-activated protein kinase MpkA since conidia of the Δ*mpkA* strain have a defect in germination.[177]

Regulation by stuA and flbC

Genes *fluG, brlA, abaA, wetA, medA, stuA,* and *vosA* play a central role in conidiophore and conidia formation in aspergilli (Figure 4). The developmental modifier StuA is a transcriptional regulator involved in proper spatial distribution of AbaA and BrlA.[81, 84] Conidiophores of the Δ*stuA* strain of *A. fumigatus* are extremely malformed and the number of conidia that are formed is strongly reduced.[82] Moreover, these conidiophores are twice the size of wild-type spores. Interestingly, they germinate faster but the underlying mechanism is not known yet. Several transcriptional activators act upstream of BrlA in the regulation of asexual development (Figure 6). One of these regulators is FlbC, which also has a role in germination.[110] Polarized growth has taken place for up to 40 % and 100 % of wild-type conidia 4 h and 6 h post-inoculation, respectively. In contrast, spores of the Δ*flbC* strain only show swelling after 4 h, whereas up to 40 % of the spores have formed germ tubes 6 h after inoculation. These findings show that FlbC has a role in germination.

OUTLINE OF THE THESIS

The genus *Aspergillus* represents a diverse group of fungi that are among the most abundant fungi in the world. The success of aspergilli is explained by the fact that they are not very selective with respect to their abiotic growth conditions, that they can degrade a wide variety of organic molecules, and by the fact that they produce high numbers of asexual and sexual spores that are dispersed over short and long distances. We have now a solid framework of understanding of molecular mechanisms underlying growth and development of *Aspergillus* but the picture is far from complete. There is clear evidence that these regulatory processes are similar but not identical between *Aspergillus* species.

Expression profiles of conidiophores, conidia, ascocarps, ascospores, germlings, and the vegetative mycelium are clearly distinct. Heterogeneity in gene expression is also found between micro-colonies of a liquid culture of *Aspergillus*. Heterogeneity is even observed between and within zones of the vegetative mycelium. The aim of this Thesis was to study spatial secretion patterns in macro-colonies of the cell factory *A. niger* and to identify genes that are involved in zonal secretion. To this end, xylose (Chapter 2, 3,and 4) and maltose (chapter 5) were used as carbon source. Both carbon sources are industrially relevant. Maltose is industrially relevant for it is one of the products of starch degradation. Xylan is one of the major compounds of hemicellulose, which is present in plant cell walls. Xylanases of *A. niger* are used by the industry to convert xylan into xylose. These sugars molecules can be converted by the industry into products like bio-ethanol.

A. niger releases enzymes into the culture medium, from which they can be easily isolated. Spatial release of proteins into the culture medium of *A. niger* was determined in **Chapter 2**. To this end, the secretome of 5 concentric zones of 7-days-old xylose-grown colonies was determined with quantitative stable isotope dimethyl labeling. Fifty nine proteins were found in the secretome of at least one of the zones of the colony. A major part of the secreted proteins are retained in the cell wall after their secretion and are slowly released in the growth medium. Therefore, proteins present in the medium underlying a central or intermediate zone of a colony may have been secreted when this part of the mycelium represented the colony periphery. To discriminate between newly produced and previously produced secreted proteins, xylose-grown colonies were treated for 24 h with the translation inhibitor cycloheximide. Unexpectedly, protein release was increased by cycloheximide in the intermediate and central zones of the mycelium when compared to non-treated colonies. Electron microscopy indicated that this is due to partial degradation of the cell wall. In total, 124 proteins were identified in cycloheximide-treated colonies, of which 19 secreted proteins had not been identified before. The composition of the secretome in each of the 5 concentric zones differed. For instance, a total of 70 proteins was ≥4-fold more abundant

in zone 1 of cycloheximide treated colonies, while only 4 proteins were ≥4-fold more abundant in zone 5. This study thus describes spatial release of proteins in *A. niger*, which is instrumental in understanding how fungi degrade complex substrates in nature.

In **Chapter 3** it is shown that the sporulating zone of the colony overlaps with the non-secreting sub-peripheral zone. This indicates that sporulation inhibits protein secretion. To address this, genes *flbA* and *brlA* were inactivated. Strain Δ*flbA* only formed aerial hyphae, whereas strain Δ*brlA* formed aerial hyphae as well as stalks that did not further develop into conidiophores. These genes thus have a role in development similar to that of *A. nidulans*. The Δ*flbA* strain secreted proteins throughout the colony, while the ΔbrlA strain did not show altered spatial secretion. The secretome of 5 concentric zones of 7-days-old xylose-grown Δ*flbA* colonies was assessed by quantitative proteomics. In total 138 proteins with a signal sequence for secretion were identified in the medium of the Δ*flbA* colonies. Of these, 18 proteins had never been reported to be part of the secretome of *A. niger*, while 101 proteins had not been identified in the culture medium of xylose-grown wild-type colonies (**Chapter 2**). Taken together, inactivation of *flbA* results in spatial changes in secretion and in a more complex secretome. The latter may be explained by the fact that strain Δ*flbA* has a thinner cell wall compared to the wild-type, enabling efficient release of proteins.

The results of **Chapter 3** are of interest to improve *A. niger* as a cell factory. The *flbA* strain was further studied in **Chapter 4** by performing a whole genome wide expression analysis. mRNA from concentric zones of wild-type and ΔflbA colonies was hybridized to Affymetrix *A. niger* whole genome microarrays. It is shown that 1,152 genes had changed their expression ≥2 fold when wild-type and ΔflbA colonies are compared. This gene set contains 38 genes encoding transcriptional regulators. These genes may act downstream of FlbA in controlling differentiation processes in the mycelium, among which spatial secretion and cell wall morphogenesis.

The *fluG* gene acts upstream of *flbA* in development in *A. nidulans*. Inactivation of *fluG* in *A. nidulans* resulted in a colony with a phenotype similar to that of *flbA*. The impact of inactivation of *fluG* in *A. niger* on development, growth and secretion was studied in **Chapter 5**. The Δ*fluG* colonies formed fully developed conidiophores but sterigmata also developed at the stalk of these reproductive structures. 7-days-old Δ*fluG* colonies had formed 20-60 % more biomass and growth was observed in the center and not at the periphery when 6-days-old colonies had been transferred to fresh maltose medium for 24 h. Secretion of

proteins in wild-type and Δ*fluG* colonies mainly took place at the periphery. Seventy-two and 45 proteins were identified in the culture medium of wild-type and Δ*fluG* colonies, respectively. Of these proteins, 29 and 8 were differentially released into the culture medium by the different zones of the colony. These data show that inactivation of *fluG* results in aberrant sterigmata formation on conidiophores, and reduced secreteome complexity and secretion heterogeneity. Moreover, FluG seems to be a repressor of growth.

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

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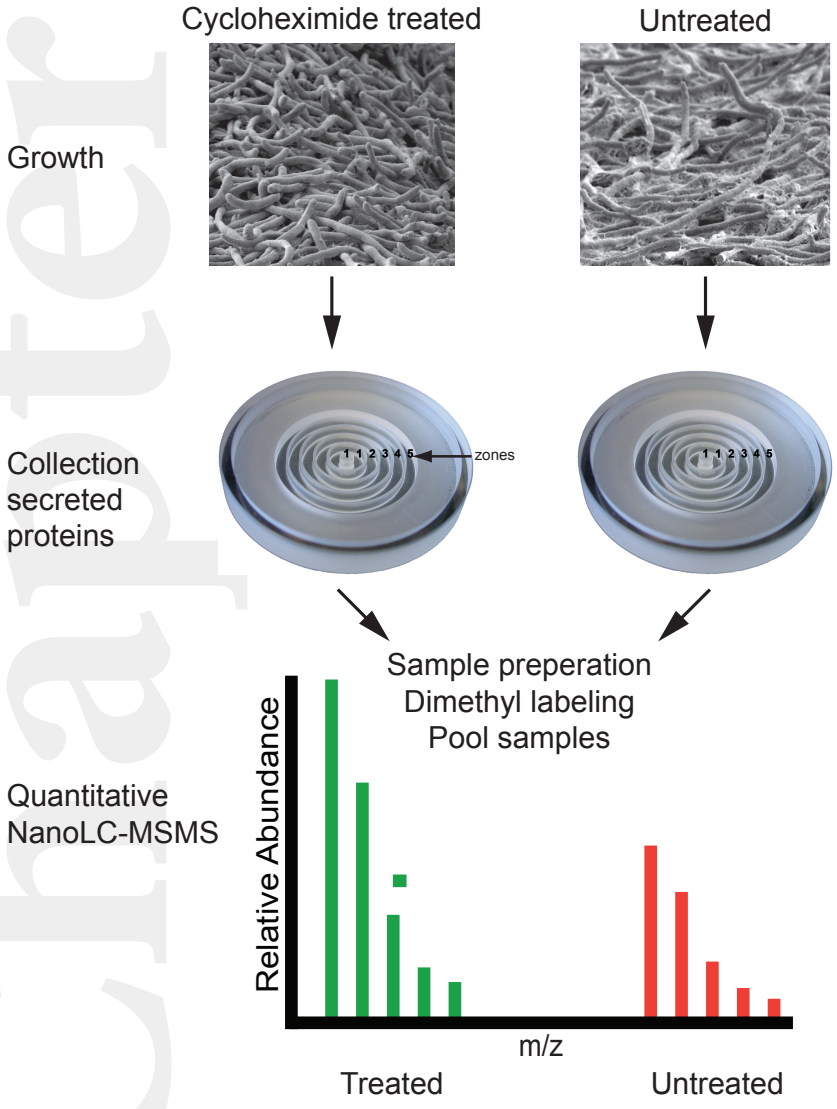
Chapter 2

Spatially resolving the secretome within the mycelium of the cell factory *Aspergillus niger*

This Chapter is based on Krijgsheld P, Altelaar AFM, Post H, Ringrose JH, Müller WH, Heck AJR, and Wösten HAB.(2012). Spatially resolving the secretome within the mycelium of the cell factory *Aspergillus niger. Journal of Proteome Research* 11: 2807–2818. doi: 10.1021/pr201157b.

Graphical Abstract



ABSTRACT

Aspergillus niger is an important cell factory for the industrial production of enzymes. These enzymes are released into the culture medium, from which they can be easily isolated. Here, we determined with stable isotope dimethyl labeling the secretome of 5 concentric zones of 7-days-old xylose-grown colonies of *A. niger* that had either or not been treated with cycloheximide. As expected, cycloheximide blocked secretion of proteins at the periphery of the colony. Unexpectedly, protein release was increased by cycloheximide in the intermediate and central zones of the mycelium when compared to non-treated colonies. Electron microscopy indicated that this is due to partial degradation of the cell wall. In total, 124 proteins were identified in cycloheximide-treated colonies, of which 19 secreted proteins had not been identified before. Within the pool of 124 proteins, 53 secreted proteins were absent in non-treated colonies, and additionally, 35 proteins were released ≥ 4-fold in the central and sub-peripheral zones of cycloheximide-treated colonies when compared to non-treated colonies. The composition of the secretome in each of the 5 concentric zones differed. This study thus describes spatial release of proteins in *A. niger*, which is instrumental in understanding how fungi degrade complex substrates in nature.

INTRODUCTION

The genus *Aspergillus* comprises species that are among the most abundant fungi in the world. They contribute to element recycling in nature by degrading dead organic material. As such, they can cause spoilage of food and feed. *Aspergillus* species are also pathogens of plants, animals, and humans, and can affect quality of the indoor environment.[1, Chapter 1] On the other hand, aspergilli are beneficial for mankind. They are used at large scale in the production of food, metabolites and enzymes. For instance, *Aspergillus niger* is used in the industry for the production of organic acids[2, 3]and a wide variety of enzymes. [4, 5] The production capacity of this fungus is illustrated by strains that secrete more than 30 g L^{-1} glucoamylase.[6]

A. niger grows by means of hyphae that grow at their tips and that branch sub-apically. As a result, a network of interconnected hyphae is formed that is called a mycelium or a colony. The centre and periphery of the colony represent the oldest and youngest parts of the mycelium, respectively. The size of the colony depends on the growth conditions. For instance, centimeter-scale macro-colonies are formed on agar medium, whereas (sub)-millimeter micro-colonies are formed in a submerged liquid culture. Both colony types of *A. niger* are heterogenic with respect to gene expression. [7-11] Twenty-five percent of the expressed genes show a ≥ 2-fold change in expression when the periphery and the center of macro-colonies are compared. [10] Differential gene expression in *A. niger* is accompanied by heterogeneity in growth and protein secretion. [10, 12, 13] Two growth zones are distinguished in macro-colonies. The primary growth zone is located at the periphery of the colony, whereas the secondary growth zone is located in the colony center. Newly synthesized secreted proteins are released by these two growth zones. [13] The majority of these proteins is retained in the cell wall, and are subsequently released into the growth medium. [12] Thus, proteins released by the central zone of a macro-colony were for a large part produced during the primary growth phase at the colony periphery.

The secretome of *Aspergillus* depends on the culture method and the composition of the medium. [14-17] For instance, β-glucosidase and α-amylase of *Aspergillus oryzae* are released in the substrate during solid state fermentation but are trapped in the cell wall during submerged cultivation. [14] The carbon source[15] and the pH[17] also have a profound effect on the secretome. So far, proteomic studies have focused on whole cultures. Here, we describe the quantitative analysis of the secretome of 5 concentric zones of 7-days-old xylose-grown colonies of *A. niger*. It is shown that the protein profiles are qualitatively similar but differ quantitatively. Colonies were treated with the protein synthesis inhibitor cycloheximide to distinguish between secreted proteins that had been recently produced and those that had been produced earlier and that are slowly released from the cell wall. Unexpectedly, a higher number and quantity of proteins was released into the medium upon cycloheximide treatment. Treatment with this compound may thus

be used in industrial fermentation to improve protein yield.

MATERIALS AND METHODS

Strain and Culture Conditions

A. niger N402 was grown at 30 ˚C using 25 mM xylose or 25 mM maltose as a carbon source. Static cultures were grown on minimal agar medium (MM) [18] as sandwiched cultures. [13] To this end, a perforated polycarbonate (PC) membrane (0.1 µm pores, 76 mm diameter; Profiltra, Almere, Netherlands) was placed on the agar medium and topped with a 0.45 mm layer of 1.25% agarose. A 1 mm plug of mycelium was positioned in the center of the agarose layer and covered with another 76 mm wide PC membrane. After 7 days of growth, colonies were transferred for 24 h to a ring plate. [12] The five concentric wells in this plate were filled with liquid MM.

Liquid cultures were grown in transformation medium (TM). [19] After 16 h of growth at 250 rpm, the mycelium was harvested using a Büchner funnel and washed with 250 ml 0.85% NaCl (Merck, Darmstadt, Germany). Ten gram of wet weight mycelium was transferred to a 1 l Erlenmeyer containing 150 ml MM. After growing for 6 or 24 h 0.1 mg ml^{-1} cycloheximide (Sigma C7698, Zwijndrecht, Netherlands), 0.1 mg ml^{-1} hygromycin (Invivogen Europe Cayla, Toulouse, France) or 50 µg ml^{-1} phleomycin (Invivogen Europe Cayla Toulouse, France) were either or not added to the culture medium and incubation was prolonged for 66 and 48 h, respectively.

Localization of Protein Synthesis and Protein Secretion

Protein synthesis and secretion were monitored as described. [13] Sandwiched cultures were labeled for 4 h with 185 kBq ^{14}C-amino acids (Perkin Elmer, NEC-445E amino acid mixture, L-[$^{14}C(U)$]- specific activity 1.94 GBq $milliatom^{-1}$). A polyvinylidene difluoride (PVDF) membrane (Immobulon-P, Millipore, Bedford, USA) was placed in between the sandwiched colony and the agar medium to immobilize secreted proteins. Label was placed on top of the colony after absorbing it to a piece of rice paper (Steicher & Schuell, Dassel, Germany) with the size of the colony. Colonies were fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 1 h at room temperature. Sandwiched colonies and PVDF membranes were washed 3 times 60 min with 1% casamino acids (Becton, Dickinson and company, Le-Pont-De-Claix, France). After drying overnight at room temperature, colonies and PVDF membranes were exposed to Kodak Biomax XAR film (Sigma, Saint Louis, USA).

Protein Detection on SDS-PAGE

Proteins in medium samples were precipitated with 5 volumes of acetone (Merck, Darmstadt, Germany) for 16 h at -20 °C. Samples were centrifuged at 10.000 g for 15 min and dissolved in 2x SDS sample buffer (20% glycerol [LPS Benelux,

Rosmalen, The Netherlands], 4% SDS [JT Baker, Deventer, The Netherlands], 100 mM Tris-HCL pH 6.8 [Roche, Mannheim, Germany], 0.01% bromophenol blue [Across Organics, Geel, Belgium], and 5% β-mercaptoethanol [Sigma-Aldrich, St. Louis, France]). Samples were analyzed on 12% SDS-PAA gels and stained with 0.1% Coomassie Brilliant Blue G250 (Sigma-Aldrich, Steinheim, Germany) in 25% methanol and 10% acetic acid (Merck, Darmstadt, Germany). Low Molecular Weight Marker (14,000-70,000 Da) (Sigma-Aldrich, St. Louis, France) was used as marker.

Sample Preparation and Dimethyl Labeling for Mass Spectrometry

Medium samples were concentrated 10-fold with ultra-concentration columns (4 ml Amicon ultra centrifugal filter units, 10 kDa cut-off; Milipore, Amsterdam, Netherlands) (Figure 1). To this end, 500 µl of culture medium was transferred to the column and subjected to centrifugation for 30 min at 4000 g in a swing out rotor. The concentrated samples were diluted twice with 2.5 ml PBS (Sigma-Aldrich, Zweindrecht, The Netherlands) followed by centrifugation for 30 min at 4000 g. Proteins in the concentrated sample (final volume of 50 µl) were subjected to in-solution-digestion. [20] To this end, 80 µl of 8 M urea (Merck, Darmstadt, Germany) in 400 mM ammonium bicarbonate (Fluka, Steinheim, Germany) was added. Proteins were reduced for 30 min at 50 ˚C in the presence of 2 mM DTT (Sigma-Aldrich, Zweindrecht, The Netherlands) and subsequently alkylated for 30 min at room temperature by adding 4.5 mM iodoacetamide (Sigma-Aldrich, Zweindrecht, The Netherlands). Digestion was performed with 0.1 µg Lys-C (lysyl endopeptidase 129-02541, 10 Au, Wako Pure Chemical Industry, Osaka Japan) by incubation at 37 ˚C for 4 h. Samples were diluted 4-fold with MQ and treated with 0.1 µg trypsin (V528A, Trypsin-Gold-Mass Spec Grade, Porcine Sp. Act 15,000 u mg^{-1} Promega, Madison, USA) for 16 h at 37 ˚C. Samples were desalted using µElution plates (Oasis Waters, Eschborn, Germany). To this end, the wells were washed twice with 100% acetonitrile (Merck, Darmstadt, Germany) containing 10% formic acid (FA, Merck, Darmstadt, Germany). Formic acid was added to the sample to a final concentration of 10% and loaded in the wells of the µElution plates in aliquots of 150 µl. The Waters Extraction plate manifold was used for vacuum extraction of the samples through the wells (Waters, En Yvelines Cedex, France). Wells were washed three times with 10% FA before eluting with 5% FA in 50% acetonitrile (Merck, Darmstadt, Germany). Samples were dried *in vacuo* and reconstituted in 20 µl 100 mM triethylammonium bicarbonate (Sigma-Aldrich, Zweindrecht, The Netherlands) before dimethyl labeling. Stable isotope dimethyl labeling was performed as previously described in a total volume of 50 µl. [21, 22] Labeling efficiency was tested by analyzing an aliquot of the labeled samples on a regular LC MS/MS run and comparing overall peptide signal intensities. In all cases labeling efficiency was > 98%.

Nano LC-LTQ-Orbitrap-MS

Stable isotope dimethyl labeling was performed in a final volume of 50 µl (see above). The labels used for each of the samples are listed in Supplementary Table 1. Two µl of a sample was mixed with 2 µl of (an)other samples. The volume was adjusted to 10 µl and the final concentration of formic acid was 10%. 4µl of the mixture was used for MS. Samples were analyzed on an LTQ-Orbitrap (Thermo Fischer Scientific, Bremen, Germany) that was connected to an Agilent 1200 HPLC system. Samples were delivered to a trap column (ReproSil-Pur C18- AQ, 3 mm [Dr Maisch, Ammerbuch, Germany]; 20 mm × 100 µm inner diameter, packed in house) at 5 µL min^{-1} in 100% solvent A (0.1 M acetic acid in water). Next, peptides were eluted from the trap column onto an analytical column (ReproSil-Pur C18- AQ, 3 mm [Dr Maisch, Ammerbuch, Germany]; 40 cm × 50-µm inner diameter, packed in-house) at 100 nL min^{-1} in a 120 min stepped gradient from 0-28% solvent B (0.1 M acetic acid in 8:2 v/v ACN/water) in 60 min and 28-50% solvent B in 25 min. The eluent was sprayed via distal coated emitter tips connected to the analytical column. The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS. Full-scan MS spectra (from m/z 350 to 1500) were acquired in the Orbitrap with a resolution of 60,000 at m/z 400 after accumulation to a target value of 500,000 in the linear ion trap. The three most intense ions were selected for collision-induced fragmentation in the linear ion trap at a normalized collision energy of 35% after accumulation to a target value of 10,000.

Protein Identification and Quantification

Raw files were searched with Thermo Proteome Discoverer 1.2.0.207 (Thermo Fischer Scientific) and a Mascot search engine (version 2.2.1) software platform (Matrix Science, London, UK) using the DSM protein database of *A. niger* CBS 513.88. [23] The following settings of Mascot were used: trypsin with 2 missed cleavages, mass tolerance of fragment ions of 0.6 Da and carbamidomethyl (C) as fixed modification. Oxidation (M), light dimethyl (K- and N-term), intermediate-dimethyl (K- and N- term) and heavy-dimethyl (K- and N- term) were set as variable modifications. The following settings of Proteome discoverer were used: peptide score filter: Mascot (ion score) > 20, maximum peptide rank =1, peptide length: 7-35 and a peptide mass deviation 12 ppm, 8 ppm and 10 ppm for untreated samples, cycloheximide treated samples and cycloheximide treated-non-treated samples, respectively. Magellan storage files (.mgf) were loaded in Thermo Proteome Discoverer 1.2 simultaneously and heavy/light, medium/heavy, medium/light Quan values were calculated. A technical control experiment was performed with zone 3 that had been labeled with two different labels (Figure 1). The ^{2}log ratios for each protein should be 0. Indeed, this was the case for ≥90% of the proteins (Supplemental Figure 1). Since proteins can be identified in one sample zone only, files were also loaded with the option replaced Missing Quan

values, which allows quantification of 'on/off' situations. Proteins found in both experiments with at least one peptide were included in the analysis. Protein release was considered differential when the average ^{2}log ratio value was higher than 2 or lower than -2 and at least higher than 1 or lower than -1 in both biological replicates. To calculate the False Discovery Rate (FDR), spectra were searched against a reverse protein database. The spectra of the quantification of concentric zones in non-treated and cycloheximide treated colonies had an FDR of < 2%, whereas the non-treated-cycloheximide treated quantification had an FDR of < 2.7%.

Transmission Electron Microscopy

Parts (2 mm diameter) of sandwiched colonies were cut with a biopsy punch (Grieshaber, Hünenberg, Switzerland) and placed into 200 µm aluminum planchets. A 2 µl droplet of 1% low melting agarose was placed on top of the sample. Planchets were closed with 300 µm aluminum planchets and samples were high-pressure frozen. Planchets were subsequently separated under liquid nitrogen. To enable easy separation, planchets had been coated with 10% lecithin (Fluka, St. Louis, MO, USA) in ethanol (w/v) prior to use. Colony parts attached to one of the planchets were transferred to a 2.0-ml Saf-T-seal free-standing tube (Biozym Scientific, Hessisch Oldendorf, Germany) with substitution medium (1% OsO_4 [Electron Microscopy Sciences, Hatfield, USA] , 3% glutaraldehyde (Agar Scientific, Essex, UK], 0.3 % uranyl acetate (Merck, Darmstadt, Germany) and 0.05% filtered ruthenium red (Gurr Microscopy Materials BDH, Poole, England) in anhydrous methanol (Merck, Darmstadt, Germany). Tubes were placed into a CS-auto substitution apparatus[24] at −88 °C for 7 days, after which the temperature was raised to 0 °C at a speed of 5 °C h^{-1}. The freeze-substitution medium was exchanged for methanol, methanol: anhydrous acetone (1:1; 1:2), and anhydrous acetone (i.e. acetone containing 1% (v/v) acidified 2,2-dimethoxypropane (Merck, Darmstadt, Germany). The samples were infiltrated stepwise with 25% (v/v) Spurr's resin (Electron Microscopy Sciences, Hatfield, USA) for 4 hours, 50% (v/v) Spurr's resin overnight at 4 °C, 75% (v/v) Spurr's resin for 1 day, 95% Spurr's resin for 2 days at 4 °C and 100% Spurr's resin (two times each 1 day). [25] After infiltrating with freshly prepared Spurr's resin, samples were polymerized at 65 °C for 48 h in BEEM capsules (EMS, Hatfield, PA, USA). Sections of 90 nm were cut with a diamond knife (Diatome, Hatfield, PA, USA) by the use of an Ultracut E ultramicrotome (Leica Microsystems, Vienna, Austria). Sections were picked up with formvar-coated, carbon-stabilized copper grids (hexagonal 150 mesh Veco grids, EMS, Hatfield, PA, USA) and contrasted with 4% (w/v) aqueous uranyl acetate (Merck, Darmstadt, Germany) for 10 min and 0.4% (w/v) aqueous lead citrate (Merck, Darmstadt, Germany) for 2 min. [26] The thin sectioned and stained samples were viewed with a transmission electron microscope Tecnai 12 (FEI Europe, Eindhoven, The Netherlands) at an acceleration voltage of 100 kV. Cross

sections of hyphea were used to measure the thickness of the cell wall and the hyphal diameter of treated and untreated colonies. Four measurements (north, south, east, and west) per hyphae were made to measure the cell wall thickness, whereas two measurements were made to measure hyphal diameter (north-south, west-east). Statistical analysis was performed with PASW statistics 18.0.0 (SPSS

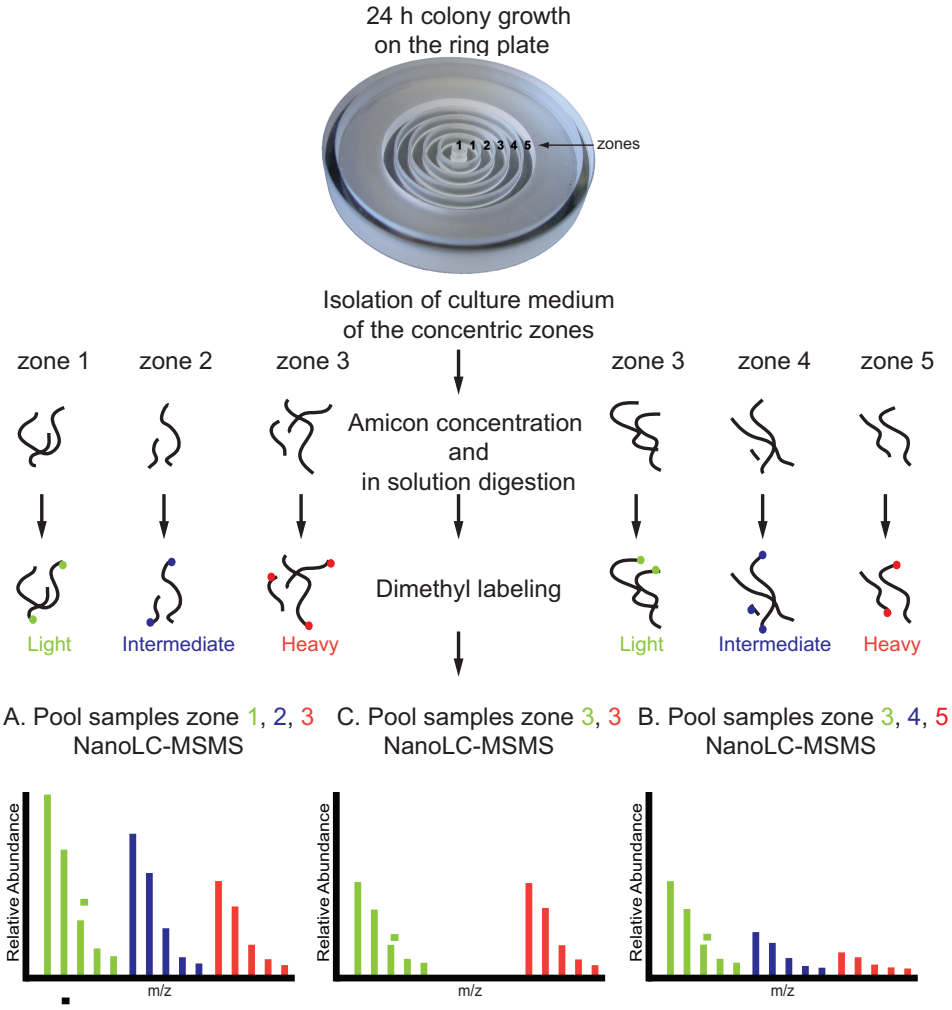


Figure 1. Sample preparation to determine the secretome of concentric zones of colonies of *A. niger*. Sandwiched colonies were grown for 7 days on agar medium and then transferred for 24 h to a ring plate. The medium in each concentric well was collected, concentrated with Amicon columns and digested in solution with LysC and trypsin. Peptides from samples of zone 1 and 3 were labeled via dimethyl labeling with a light label, zone 2 and 4 with an intermediate label and zone 3 and 5 with a heavy label. Labeled peptides from zones 1, 2 and 3 (A) and zones 3, 4 and 5 were pooled (B). As a control, samples of zone 3 with a light and heavy label were pooled (C). The pools were analyzed with nano LC-MS/MS. In a duplo experiment, labels were swapped using peptides from a biological duplicate. This resulted in a light labeled zone 3 and 5, an intermediate labeled zone 1 and 3, and a heavy labeled zone 2 and 4.

statistics; IBM, Waltham MA, USA). Mean hyphal diameter and cell wall thickness of central and peripheral hyphae were statistically analysed with a confidence of $p \leq 0.005$ using a Welch's t-test (accounting for unequal error variances) and a student's *t*-test respectively.

RESULTS

Spatial Protein Secretion by *A. niger*

Spatial protein secretion was monitored in colonies of *A. niger* that had been grown on xylose-containing medium. To this end, 7-days-old sandwiched colonies were transferred for 24 h to a ring plate (Figure 1). This ring plate consists of 5 concentric wells that are filled with liquid medium. [12] Proteins isolated from the medium of each of the concentric wells were digested in solution with lys-C and trypsin and subsequently labeled with isotopmeric dimethyl labels. The addition of a light-label, an intermediate label or a heavy label results in a mass shift of 28, 32 and 36 Da per primary amine of a peptide, respectively. [21] Labeled peptides from the five concentric zones of the colonies were analyzed with LC-MS/MS. Peptides of zones 1, 2 and 3 were mixed (each sample containing a different label) and analyzed in a single LC-MS/MS run. Similarly, a mixture of peptides of zones 3, 4, and 5 was analyzed (Figure 1). By including peptides of zone 3 in both runs, peptides in zones 1, 2, 4, and 5 could be quantitatively compared to zone 3. This procedure was repeated using a biological duplicate combined with a label swap (see Supplemental Table 1).

Table 1. Number of representatives of protein classes in concentric zones of 7-days-old xylose-grown *A. niger* colonies that had either or not been treated with 0.1 mg ml^{-1} cycloheximide. For details see Supplemental Tables 2 and 4.

Protein Class	Not treated with cycloheximide	Treated with cycloheximide
Cellulases	10	20
Xylanases	9	15
Mannosidases	0	2
Pectinases	5	6
Amylases	2	5
Degradation of other carbohydrates	0	6
Proteases	9	17
Phytase	1	4
Fungal cell wall modelling	9	14
Other enzymes	7	23
Unknown	6	8
Intracellular	1	4
Total	59	124

A total of 187 proteins were identified in all runs (Data not shown). Of these, 98 passed our filtering criteria as described in the Material and Methods. Thirty-nine out of the 98 proteins were found in only one biological experiment and were therefore rejected from the analysis (Supplemental Table 2). Thus, 59 proteins were found in the secretome of at least one of the zones in a biological duplicate of xylose grown *A. niger* colonies (Table 1; Supplemental Table 3). Of these proteins, 6 have not been identified in the secretome of *A. niger* before[15, 16, 27, 28] (Supplemental Table 2). Only 1 out of the 59 proteins (An14g01685) did not have a predicted signal sequence according to Pel *et al.*, and Braaksma *et al.*, [16, 23] (Supplemental Tables 2, 3). This protein may be secreted by a non-classical secretion pathway. Taken together, it is concluded that most, if not all, proteins in the culture medium of 7-days-old xylose grown colonies are actively secreted into the medium and are not released due to lysis of hyphae.

Almost half (i.e. 26) of the 59 proteins detected in the culture medium of *A. niger* colonies function in plant polysaccharide degradation (Table 1). Twenty-four of them are related to xylan, cellulose, and pectin degradation, while 2 are involved in starch degradation. Out of the 59 detected proteins, 6 were at least 4-fold more abundant in the outer zone 5 (^{2}log ratio ≥ 2 at the periphery) when compared to the intermediate zone 3, whereas 10 proteins were at least 4-fold less abundant (^{2}log ratio ≤ -2 at the periphery) (Table 2, Supplemental Table 3). Eight proteins were at least 4-fold less abundant in the most central zone 1 when compared to the intermediate zone 3.

XlnR is the xylanolytic and cellulolytic regulator of *A. niger*.[29-31] It regulates the activity of *xlnB*, *xlnC* and *xlnD* that encode endoxylanase B, endoxylanase C, and β-xylosidase D, respectively, [29-31] as well as accessory enzymes and hemicellulolytic and cellulolytic enzymes[29-36]. The products of 13 out of the 16 XlnR-regulated genes were found in the culture medium of 7-days-old xylose-grown colonies (Table 3). Only endo-1,4-beta-xylanase XlnC, α-glucuronidase AguA, and ferulic acid esterase FaeA were not identified in the secretome. Abundance of the products of the genes regulated by XlnR was heterogeneous in the concentric zones of the colony. For instance, the endoxylanase XlnB, the arabinofuranosidases AbfA and the α-galactosidase AglB were most abundant in the peripheral zone 4 (and 5) (Table 2; Supplemental Table 3). On the other hand, the cellobiohydrolases CbhA and CbhB, and the endoglucanase AglA were most abundant in zone 2, 3, and 4 (Table 2; Supplemental Table 3), whereas the β-xylosidase XlnD was most abundant in the central zone 2 (Supplemental Table 3). Products of genes regulated by XlnR were not found to be most abundant in the central zone 1.

Thirty-three out of the 59 quantified proteins in the secretome have a function other than plant polysaccharide degradation (Table 1). These proteins include (putative) enzymes involved in cell wall modeling or morphogenesis (9), (putative) proteases (9), a phytase, proteins with another enzymatic activity (7)

Table 2. Proteins with a ^{2}log fold change ≥ 2 in at least one of the concentric zones of 7-days-old xylose grown sandwiched colonies. Green and red shading indicate increased and decreased abundance when compared to zone 3.

Accession	Description	^{2}log ratio zones			
		zone 1/3	zone 2/3	zone 4/3	zone 5/3
	xylanolytic enzymes - xylan				
An01g14600	endo-1,4-beta-xylanase precursor - Aspergillus niger	-1.2	-0.4	0.3	-2.8
An01g00780	endo-1,4-beta-xylanase B precursor xlnB - Aspergillus niger	-0.6	-0.3	2.0	-0.5
An12g05010	acetyl xylan esterase axeA - Aspergillus niger	-2.0	-0.6	1.1	-1.3
	xylanolytic enzymes - xyloglucan xylan galactomannan				
An01g00330	alpha-N-arabinofuranosidase A precursor abfA - Aspergillus niger	0.0	-0.8	2.8	2.3
An06g00170	alpha-galactosidase aglA - Aspergillus niger	-1.6	-0.5	-0.1	-2.3
An02g11150	alpha-galactosidase aglB - Aspergillus niger	0.0	0.0	1.9	2.1
	cellulases				
An16g06800	strong similarity to endo-beta-1,4-glucanase B eglB - Aspergillus niger	-3.3	-0.1	1.0	0.1
An18g03570	Probable beta-glucosidase A precursor bglA - Aspergillus niger	0.1	1.3	1.3	2.0
An07g09330	1,4-beta-D-glucan cellobiohydrolase A precursor cbhA - Aspergillus niger	-1.4	-0.2	0.3	-2.4
An01g11660	1,4-beta-D-glucan cellobiohydrolase B precursor cbhB - Aspergillus niger	-1.4	-0.1	0.2	-4.9
	putative cellulases				
An14g02670	strong similarity to endoglucanase IV egl4 - Trichoderma reesei	-2.7	-0.5	0.0	0.0
An08g10780	strong similarity to hypothetical protein T16K5.230 - Arabidopsis thaliana	0.0	0.0	2.0	0.1
An09g00840	similarity to plastic-degradation enzyme within SEQ ID NO:6 from patent WO2004038016-A1 - Aspergillus oryzae	-1.4	0.2	0.3	-4.8
	pectinolytic enzymes				
An14g04200	rhamnogalacturonase rhgB - Aspergillus niger	-4.7	-0.4	0.3	-1.7
	starch degrading enzymes				
An03g06550	glucan 1,4-alpha-glucosidase glaA - Aspergillus niger	-2.2	0.3	1.2	0.4
	cell wall/ morphogenesis enzymes				
An01g12450	strong similarity to hypothetical glucan beta-1,3 exoglucanase exgS - Aspergillus phoenicis	-0.3	0.3	-0.2	-2.5
An08g03580	strong similarity to 1,3-beta-glucanosyltransferase bgt1 - Aspergillus fumigatus	0.0	0.0	3.3	4.4
	phytases				
An08g11030	acid phosphatase aph, 3-phytase phyB - Aspergillus niger	0.1	1.0	-3.6	-5.9
	putative proteases				
An14g02470	strong similarity to protein PRO304 from patent WO200104311-A1 - Homo sapiens	0.0	0.0	4.3	5.2
	related to other enzymes				
An07g08940	similarity to acetyl-esterase I from patent WO9502689-A - Aspergillus aculeatus	-3.9	-2.0	1.2	-4.2
An13g01880	strong similarity to cephalosporin esterase - Rhodosporidium toruloides	-2.0	-0.4	0.4	-2.2
An07g01320	strong similarity to antifungal protein precursor paf - Penicillium chrysogenum	-1.8	-0.2	3.7	3.1
	related to unknown				
An16g00670	similarity to hypothetical protein AAO51454.1 - Dictyostelium discoideum	0.5	1.0	-0.1	-2.5
An15g02250	hypothetical protein	-2.7	-0.7	0.0	0.0

or that are related to proteins with an unknown function (6) and the protein that is not predicted to have a signal sequence. Some of these 33 proteins are also heterogeneously distributed in the culture medium underlying the sandwiched colony (Table 2; Supplemental Table 3). The phytase PhyB was most abundant in zone 2, whereas the proteases PepA and Epr were most abundant in zone 4. In contrast a putative protease (An14g02470) was highly abundant in the most outer zone 5.

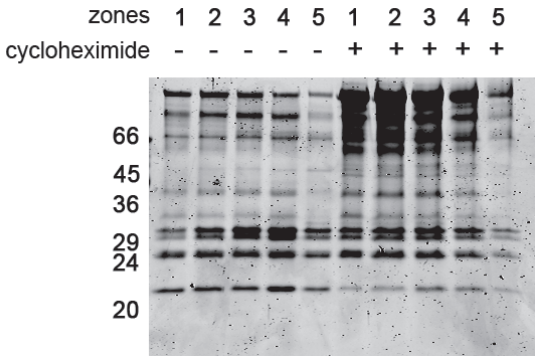


Figure 2. SDS-PAGE of the secretome of the concentric zones of 7-days-old xylose-grown colonies that had either (+) or not (-) been treated for 24 h with 0.1 mg ml^{-1} cycloheximide. Gels were stained with Coomassie Brilliant Blue. Zone 1 represents the innermost zone, whereas zone 5 represents the outermost zone of the colony.

Addition of Cycloheximide Enhances Protein Release in the Medium

A major part of the secreted proteins are retained in the cell wall after their secretion and are slowly released in the growth medium. [12] Therefore, proteins present in the medium underlying a central or intermediate zone of a colony may have been secreted when this part of the mycelium represented the colony periphery. Seven-days-old xylose-grown sandwiched colonies were treated for 24 h with the translation inhibitor cycloheximide to discriminate between newly produced and previously produced secreted proteins. The effectiveness of the inhibitor was monitored by labeling colonies with ^{14}C-labeled amino acids. Autoradiography showed that protein secretion was blocked in the presence of 0.1 mg ml^{-1} of cycloheximide (Supplemental Figure 2). In agreement, SDS PAGE showed that a lower amount of protein was released in the outer zone 5 in the presence of cycloheximide (Figure 2). Unexpectedly, the amount of protein released in zones 1-4 of the colony was much higher when 7-days-old xylose-grown colonies had been treated with cycloheximide (Figure 2). A similar result was obtained with maltose grown colonies (Supplemental Figure 3). The pH of the culture medium of xylose-grown cultures changed from 6 to 4 after 24 h of incubation in the presence of cycloheximide, while a pH of 3 was detected in colonies that had not been treated with cycloheximide.

To analyze whether the increase in protein release in the culture medium was restricted to the sandwiched culture method, colonies were grown as liquid shaken cultures. Spore-inoculated cultures were grown overnight in nutrient rich medium (TM), and the resulting pellets were transferred to minimal medium

(MM) with xylose or maltose as carbon source. In this case, the pH of the culture medium did not differ between cycloheximide treated and untreated colonies. The pH dropped after 6 hours to pH of 3 and increased to 7 in the next 66 h. No proteins were detected by SDS-PAGE and CBB staining after 6 h of growth in MM medium with xylose or maltose as carbon source. Addition of 0.1 mg ml^{-1} cycloheximide to these cultures for up to 66 h completely inhibited protein release into the medium (Supplemental Figure 4AC). In contrast, in the absence of cycloheximide proteins accumulated into the medium and they were clearly detectable after 24 h of growth in minimal medium with xylose or maltose as carbon source. Addition of cycloheximide to cultures that had grown in MM for 24 h hardly affected the amount of protein in a 6-72 h period when compared to the control in the case of xylose (Supplemental Figure 4B). However, increase of proteins was observed in the case of maltose-grown colonies (Supplemental Figure 4D). Taken together, these data indicate that cycloheximide dissociates secreted proteins from the cell wall. To investigate whether other antibiotics have the same influence on the secretome, colonies were grown in the presence of the aminoglycoside antibiotic hygromycin and the glycopeptide antibiotic phleomycin. Both antibiotics had a clear inhibitory effect on the secretome of *A. niger* grown as sandwiched colonies

Table 3. MS/MS detection of XlnR regulated proteins in concentric zones of 7-days-old xylose-grown *A. niger* colonies that had either or not been treated with 0.1 mg ml^{-1} cycloheximide.

Accession	Description XlnR regulated genes	Not treated with cycloheximide	Treated with cycloheximide
	Xylanases		
An01g00780	endo-1,4-xylanase xlnB - Aspergillus niger	x	x
An03g00940	endo-1,4-beta-xylanase A precursor xlnC - Aspergillus niger		x
An01g09960	xylosidase xlnD - Aspergillus niger	x	x
	Accessory enzymes		
An12g05010	acetyl xylan esterase axeA - Aspergillus niger	x	x
An03g00960	1,4-beta-D-arabinoxylan arabinofuranohydrolase axhA - Aspergillus niger	x	x
An01g00330	alpha-l-arabinofuranosidase a precursor abfA - Aspergillus niger	x	x
An15g02300	arabinofuranosidase B abfB - Aspergillus niger	x	x
An14g05800	alpha-glucuronidase aguA - Aspergillus niger		x
An02g11150	alpha-galactosidase aglB - Aspergillus niger	x	x
An01g12150	beta-galactosidase lacA - Aspergillus niger	x	x
An09g00120	ferulic acid esterase A faeA - Aspergillus niger		x
	Hemicellulolytic and cellulolytic enzymes		
An14g02760	endoglucanase A eglA - Aspergillus niger	x	x
An07g08950	endoglucanase B eglB - Aspergillus niger	x	x
An16g06800	strong similarity to endoglucanase eglB - Aspergillus niger	x	x
An07g09330	1,4-beta-D-glucan cellobiohydrolase A precursor cbhA - Aspergillus niger	x	x
An01g11660	1,4-beta-D-glucan cellobiohydrolase B precursor cbhB - Aspergillus niger	x	x

or as liquid shaken cultures (Data not shown).

Proteins released in a 24 h period in the presence of cycloheximide were quantitatively assessed as described above. A total of 216 proteins was identified in both runs (Data not shown). Of these, 148 passed our filtering criteria and 124 were found in at least one zone of both biological replicates (Supplemental Table 4). Four of these proteins have no signal peptide according to Pel *et al.* and Braaksma *et al.* [16, 23] and are therefore classified as intracellular proteins (Table 1; Supplemental Table 4). However, it cannot be excluded that they are secreted. Three proteins were missing in cycloheximide treated colonies that were detected in non-treated colonies, one of which being rhamnogalacturonase B (Supplemental Table 2). Conversely, 53 out of the 124 proteins had not been identified in non-treated colonies (Supplemental Table 4). These proteins comprised proteases, cellulases, hemicellulolytic enzymes and proteins with unknown function. For instance, the three xylanolytic enzymes that were not found in the untreated colonies were identified after cycloheximide treatment. Thus, the complete set of xylanolytic enzymes was identified after cycloheximide treatment (Table 3). Almost all proteins found in cycloheximide-treated colonies were ≥4-fold lower secreted at the periphery compared to the intermediate zones of these colonies (Supplemental Table 4). 12 proteins were most abundantly released in the colony center (^{2}log ratio ≥ 2). Of these 12 proteins, 11 were not found in the secretome of un-treated colonies. They are involved in carbohydrate degradation (5), cell wall morphogenesis (1), and are enzymes involved in different and unknown processes (6). In the next analysis, the quantity of released proteins upon cycloheximide treatment was compared to that found in untreated colonies. To this end, proteins from the culture medium of zone 1 of treated and untreated colonies were labeled with different labels (Supplemental Table 1), mixed, and quantified by MS/MS. The same was done for the intermediate zone 3, and the peripheral zone 5. In this case, 115 proteins were identified in the total analysis. 39 of them were only identified in the cycloheximide treated colonies shown by a ^{2}log ratio of ≥6.6 (Figure 3; Supplemental Table 5), while 2 proteins were only found in the non-treated colonies. A total of 70 and 65 proteins were ≥4-fold more abundant in zones 1 and 3, respectively, in cycloheximide treated colonies (Supplemental Tables 5 and 6). In contrast, only 4 proteins were ≥4-fold more abundant in zone 5. The latter is explained by the fact that the mycelium in zone 5 of non-treated colonies could continue growth, while cycloheximide treated colonies could not.
In our mass spectrometry experiments (see above; Figure 3A-C) 25 proteins (19 with a signal peptide) have not been identified in the secretome of *A. niger* before[15, 16, 27, 28] (Table 4; Supplemental Table 4 and 6). Six of these proteins were also found in the secretome of un-treated colonies. The other 19 proteins comprise of carbohydrate degradation enzymes (5), cell wall modification enzymes (2), other proteins (7), and proteins without a predicted SigP (5) (Supplemental Table 4 and 6).

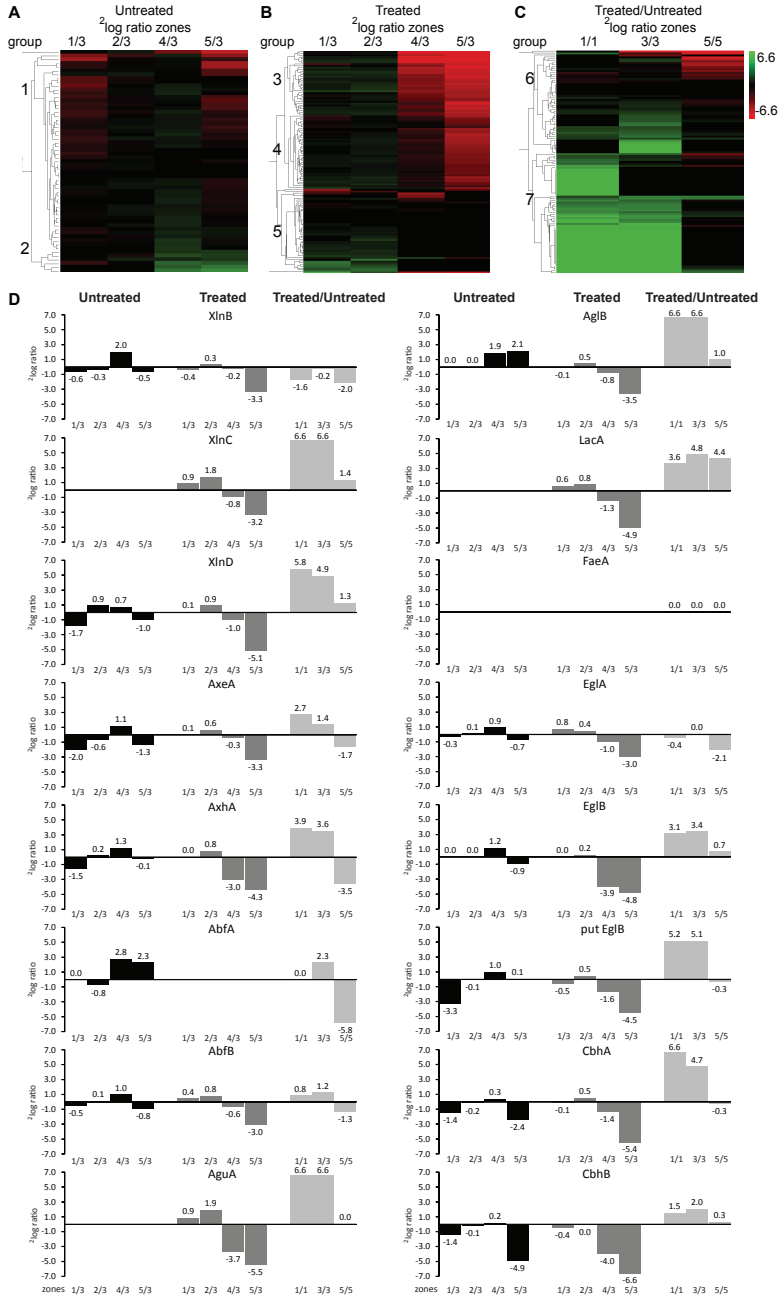


Figure 3. Heat map (A-C) and bar diagram (D) of ^{2}log ratio of all secreted proteins (A-C) and secreted proteins encoded by genes that are regulated by XlnR (D) in concentric zones of colonies that had or had not been treated with 0.1 mg ml^{-1} cycloheximide. Zone 1 represents the most central zone, whereas zone 5 represents the periphery of the colony. (A-C) Group 1 represents proteins that are highly abundant within the secretome of the intermediate zone 3, whereas proteins of group 2 are mainly present in the peripheral zones 4 and 5. Group 3 and 4 represent proteins that are mainly found in the intermediate and the central zones 2 and 3. Group 5 represents proteins that are found in a high abundance in the central zones 1 and 2. Group 6 and 7 represent proteins that were less and more abundant in cycloheximide-treated colonies, respectively. (D)

Table 4. MS/MS detection revealed proteins in concentric zones of 7-days-old xylose-grown colonies that had not been identified in the secretome of *A. niger* before. Colonies had either or not been treated with 0.1 mg ml^{-1} cycloheximide. *This protein is predicted not to have a signal sequence. (x^a) indicate proteins identified by Mascot, but were not identified in replicate experiments.

Accession	Description proteins not identified before	Not treated with cycloheximide	Treated with cycloheximide
	Carbohydrate degrading enzymes		
An01g14600	endo-1,4-beta-xylanase precursor - *Aspergillus niger*	x	x
An17g00300	strong similarity to bifunctiona xylosidase-arabinosidase xarB - *Thermoanaerobacter ethanolicus* [putative frameshift]		x
An01g11670	strong similarity to endo-beta-1,4-glucanase A eglA - *Aspergillus nidulans*		x
An04g08550	strong similarity to endoglucanase IV EGIV - *Trichoderma reesei*		x
An07g07630	strong similarity to avenacinase - *Gaeumannomyces graminis*		x
An16g00540	similarity to large secreted protein - *Streptomyces coelicolor* [truncated ORF]		x
An01g01920	similarity to beta-N-acetylhexosaminidase - *Vibrio furnissii*		x
	related to other enzymes		
An07g00550	strong similarity to salicylate hydroxylase nahG - *Pseudomonas putida*	(x^a)	x
An01g15200	strong similarity to mature penicillin V amidohydrolase PVA from patent US5516679-A - *Fusarium oxysporum* [truncated ORF]		x
An15g01940	strong similarity to antifungal protein from patent WO9813478-A - *Arabidopsis thaliana*		x
An18g06360	similarity to mycelial surface antigen Csa1 - *Candida albicans*		x
	related to unknown		
An14g02980	weak similarity to mucin-like glycoprotein 900 GP900 - *Cryptosporidium parvum*	x	(x^a)
An15g02250	hypothetical protein	x	x
An08g04630	strong similarity to hypothetical protein Afu2g15420 - *Aspergillus fumigatus*	x	x
An15g07790	similarity to hypothetical protein encoded by An11g02730 - *Aspergillus niger*	x	x
An16g02760	strong similarity to hypothetical protein BH0842 - *Bacillus halodurans*		x
An06g01000	strong similarity to hypothetical protein AN5357.2 - *Aspergillus nidulans*		x
An01g00210	strong similarity to hypothetical protein AAO27753.1 - *Fusarium sporotrichioides*		x
An02g11890	strong similarity to hypothetical protein encoded by An14g01330 - *Aspergillus niger*		x
	related to intracellular proteins		
An14g01790*	strong similarity to alpha-galactosidase Afu4g03580 - *Aspergillus fumigatus*		x
An03g06870*	strong similarity to cytidine deaminase - *Homo sapiens*		x
An14g01685*	strong similarity to hypothetical acetyltransferase Afu4g03600 - *Aspergillus fumigatus*	x	x
An13g01830*	weak similarity to SAK-b serine-threonine kinase from patent CA2150789-A - *Mus musculus*		x
An01g00610*	strong similarity to hypothetical protein BAC69773.1 - *Streptomyces avermitilis*		x
An02g03900*	hypothetical protein		x

Influence of Cycloheximide on the Cell Wall

The effect of cycloheximide on the cell wall was studied using transmission electron microscopy (TEM) (Figure 4). The diameter of the hyphae in the central part of the colony was not statistically different between treated and non-treated mycelium (3.4 ± 0.2 μm and 4.2 ± 0.3 μm, respectively) (Figure 4E). A similar

result was obtained at the periphery of the colony. The diameter of treated and untreated colonies was 2.5 ± 0.6 µm and 2.4 ± 0.5 µm, respectively. Cell walls of hyphae in the center of untreated colonies were thicker than those of treated colonies (204 ± 12.9 nm and 145 ± 4.7 nm, respectively) (Figure 4F). In the case of treated colonies, fluffy material was surrounding the hyphae, indicative of cell wall degradation (Figure 4B, D). This was not observed at the periphery of cycloheximide-treated colonies (Data not shown). Moreover, the cell wall of hyphae at the periphery of untreated colonies was thinner than that of treated colonies (105 ± 37 nm and 131 ± 28 nm, respectively) (Figure 4F). Taken together, these data indicate that cycloheximide induces cell wall degradation in the center of the colony but promotes cell wall thickness at the colony periphery.

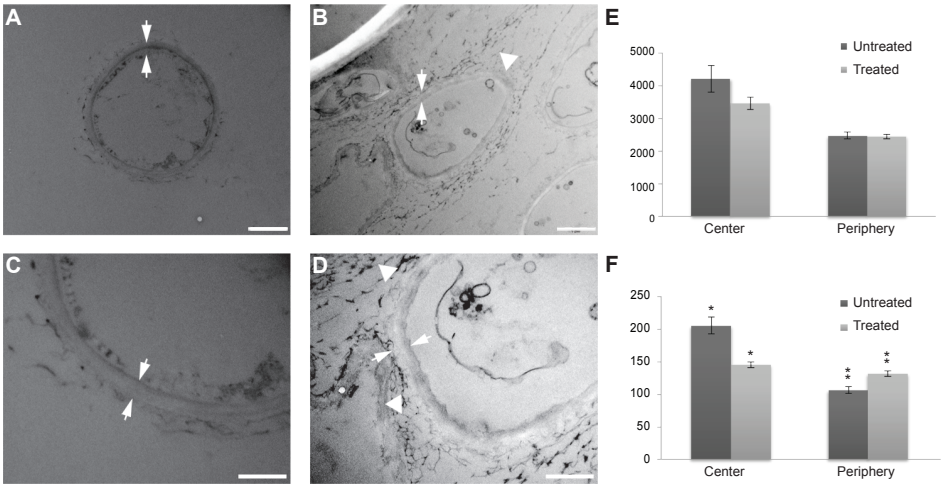


Figure 4. Transmission electron microscopy of hyphea from the centre of cycloheximide treated (B, D) and untreated sandwiched colonies (A, C). The diameter of untreated and treated colonies is similar both at the periphery and the center of the colony (E), but the cell walls of treated colonies are thicker and thinner, respectively, at the outer and inner part of the colony (F) (* and ** significant difference, * Welch's t-test $p \leq 0.005$; **Students t-test $p \leq 0.005$). Bars in A, B and C, D represent 1 µm, and 500 nm respectively. Hyphal diameter (E) and cell wall width (F) are given in nm. White arrows indicate the outer and inner part of the cell wall. White arrow heads point to fluffy material surrounding the cell wall of cycloheximide treated mycelium.

DISCUSSION

Quantitative secretome analysis is a tool to understand how fungi interact with their environment. For instance, it provides insight how substrates are degraded by saprobic fungi such as *A. niger.* A complete secretome determination is key to this understanding as well as information about spatial and temporal release of the proteins. In this study, we show for the first time that treatment with cycloheximide results in the release of cell wall-associated proteins into the culture medium, enabling a comprehensive secretome analysis. Moreover, by using ring plates we show for the first time that the composition of the secretome of central,

intermediate and peripheral zones of the mycelium is different.

It was shown previously that a large part of secreted proteins is associated with the cell wall of *A. niger*.[12] Here, the protein synthesis inhibitor cycloheximide was used to discriminate between proteins that were immediately extruded into the culture medium after their secretion and proteins that first associated with the cell wall before they were slowly released into the culture medium. We assumed that cycloheximide would impact release of the former but not of the latter group of proteins. As expected, protein release was reduced at the periphery of the colony when cycloheximide was added. Unexpectedly, protein content in the culture medium underlying intermediate and central zones of the colony was much higher. This was not explained by cell lysis since more than 95% of the proteins found after cycloheximide treatment are known to or predicted to have a signal sequence for secretion. How can we explain this phenomenon? It was previously shown that cycloheximide does not affect chitin synthesis in the aquatic fungus *Blastocladiella emersonii*.[37] Cell wall synthesis was also reported not to be affected in *Aspergillus nidulans*.[38] Elongation of hyphae of *A. nidulans* was not observed, but cell walls were found to be thicker. We also observed this phenomenon at the periphery of colonies of *A. niger.* However, cycloheximide exposure resulted in a reduced thickness of the cell walls in the center of the colony. This and the fluffy material surrounding the hyphae in the colony center indicate that the cell wall is degraded during cycloheximide treatment. It may be that this is caused by degrading enzymes that reside in the cell wall and that normally function in cell wall synthesis together with cell wall synthases. (Indirect) inhibition of the cell wall synthases in the colony centre may result in a net degradation activity within the cell wall. The fact that this happens in the center and not at the periphery may be explained by the fact that different cell wall synthases are active at the periphery and centre of colonies of *A. niger.*[10] The net degradation of the cell wall in the colony center would result in a more porous structure allowing proteins to diffuse into the culture medium.

A total of 124 proteins were detected in the culture medium upon cycloheximide treatment, of which 53 proteins had not been identified in non-treated colonies. Apparently, a major part of the proteins are associated with the cell walls of *A. niger.* For instance, 13 and 16 out of 16 secreted enzymes encoded by XlnR regulated genes were found in the secretome with and without cycloheximide treatment, respectively. Proteases, cellulases, hemicellulolytic enzymes and enzymes with unknown function were among the other proteins that were only detected after treatment with the antibiotic. Over and above this, an additional 35 proteins were ≥ 4-fold more abundant in the central and intermediate zones in cycloheximide treated colonies when compared to untreated colonies. Taken together, cycloheximide can be used to obtain a near complete secretome of *A. niger.* Moreover, the total amount of protein is increased upon treatment with this antibiotic. It is tempting to speculate that the protein mixture resulting from

cycloheximide treatment shows improved degradation of natural substrates. The fact that proteins have been detected in this study that had not been identified in the secretome of *A. niger* before[15, 16, 27, 28] is also of interest from a biotechnological point of view. Previously, the secretome of *A. niger* has been determined during growth on D-maltose or D-xylose[15, 28, 17] sorbitol, galacturonic acid, [16] glucose, glycerol, locus bean gum, pectin, xylan, sorbitol[27] or during carbon starvation. [16] Over 200 proteins have been identified in these studies, each using different culture techniques. In this study, 19 proteins with a signal sequence for secretion were detected for the first time in the secretome of *A. niger.* Six of them were also detected in cultures that had not been treated with cycloheximide.

Transfer of sandwiched colonies to a ring plate with concentric wells filled with liquid medium enables a temporal and spatial assessment of release of proteins into the medium. By adding cycloheximide to the wells only proteins will be detected that had been formed before transfer and that had been associated to the cell wall. Under normal conditions, these proteins are slowly released into the environment. By comparing the profiles of proteins in the medium of cycloheximide treated and untreated colonies, degradation of complex natural substrates by fungi can be predicted. For instance, xylan is known to be degraded by 12 enzymes that are regulated by XlnR (i.e. the xylanases and accessory enzymes in Table 3). [39] XlnB, AbfA AxhA, AbfB, AxeA were more abundantly released at the periphery in non-treated colonies. In other words, these enzymes were actively secreted by the hyphae that explore the substrate. The other 7 enzymes were especially found in central zones of cycloheximide treated colonies. These enzymes are thus slowly released into the medium under normal conditions. Taken together, these results indicate that xylan would be first attacked by XlnB, AbfA AxhA, AbfB, AxeA. These enzymes cleave the xylan backbone into smaller oligosaccharides (XlnB), release arabinose from the xylan side chains (AbfA, AbfB, and AxhA) and remove acetyl groups from the xylose backbone (AxeA). In the second step, glucuronic acid, ferulic acid and galactose residues are removed from the main and side chains of xylan.

Taken together, this study has shown that the secretome of concentric zones of the *A. niger* colony differs in composition. This agrees with previous work showing that the vegetative mycelium of fungi is heterogenic with respect to gene expression, [7-10, 40-44] growth and protein secretion. [13, 44] A major part of the secreted proteins remains associated with the cell wall, from which it is slowly released. The release can be accelerated by adding cycloheximide, which seems to be related to cell wall degradation. These findings are instrumental to deduce how fungi degrade complex organic substrates. Moreover, the use of cycloheximide at the end of a fermentation might provide us with improved enzyme mixtures to degrade plant cell wall material for industrial processes such as bioethanol production.

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ASSOCIATED CONTENT

Supplemental Table 1. Labels added to the proteins contained in the wells of the ring plate (see Figure 1) underlying the central zone (pooled zones 1 and 2), the middle zone (zone 3) and the peripheral zone (pooled zones 4 and 5) of sandwiched colonies. L=light label, I= Intermediate label, H= Heavy label. Experiment 1 (A, B) and experiment 2 (C,D) represent a biological duplo with a label swap.

Quantitative analysis of proteins in the medium underlying concentric zones of 7-days-old xylose-grown colonies of *A. niger*						
Biological experiment 1	**Zone 1**	**Zone 2**	**Zone 3a**	**Zone 3b**	**Zone 4**	**Zone 5**
Label	Light	Intermediate	Heavy	Light	Intermediate	Heavy
Mass shift (Da)	28	32	36	28	32	36
# MS/MS Run	1	1	1, 2	2, 3	3	3
Biological experiment 2	**Zone 1**	**Zone 2**	**Zone 3a**	**Zone 3b**	**Zone 4**	**Zone 5**
Label	Intermediate	Heavy	Light	Intermediate	Heavy	Light
Mass shift (Da)	32	36	28	32	36	28
# MS/MS Run	1	1	1, 2	2, 3	3	3
Quantitative analysis of proteins in the medium underlying concentric zones of 7-days-old xylose-grown cylcoheximide (CHX) treated and untreated colonies of *A. niger*						
Biological experiment 1	**Zone 1**	**Zone 3**	**Zone 5**	**Zone 1 + CHX**	**Zone 3 + CHX**	**Zone 5 + CHX**
Label	Light	Heavy	Heavy	Intermediate	Light	Light
Mass shift (Da)	28	36	36	32	28	28
# MS/MS Run	1	2	3	1	2	3
Biological experiment 2	**Zone 1**	**Zone 3**	**Zone 5**	**Zone 1 + CHX**	**Zone 3 + CHX**	**Zone 5 + CHX**
Label	Intermediate	Light	Light	Light	Heavy	Heavy
Mass shift (Da)	32	28	28	28	36	36
# MS/MS Run	1	2	3	1	2	3

Supplemental Table 2. Quantitative analysis of the secretome of concentric zones of sandwiched colonies of *A. niger* that had not been treated with cycloheximide. This material is available free of charge via the Internet at http://pubs.acs.org

Supplemental Table 3. Quantitative secretome analysis of 7-days-old xylose grown sandwiched colonies. Heat map indicates the ^{2}log fold change of a protein compared to the intermediate zone 3. Green and red shading indicate increased and decreased abundance when compared to zone 3. *This protein is predicted not to have a signal sequence (see Supplemental Table 2).

Accession	Description - Supplemental Table 3	^{2}log ratio zones			
	xylanolytic enzymes - xylan	zone 1/3	zone 2/3	zone 4/3	zone 5/3
An01g14600	endo-1,4-beta-xylanase precursor - Aspergillus niger	-1.2	-0.4	0.3	-2.8
An01g00780	endo-1,4-beta-xylanase B precursor xlnB - Aspergillus niger	-0.6	-0.3	2.0	-0.5
An01g09960	exo-1,4-beta-xylosidase xlnD - Aspergillus niger	-1.7	0.9	0.7	-1.0
An03g00960	alpha-L-arabinofuranosidase axhA - Aspergillus niger	-1.5	0.2	1.3	-0.1
An12g05010	acetyl xylan esterase axeA - Aspergillus niger	-2.0	-0.6	1.1	-1.3
	xylanolytic enzymes - xyloglucan xylan galactomannan				
An01g00330	alpha-N-arabinofuranosidase A precursor abfA - Aspergillus niger	0.0	-0.8	2.8	2.3
An15g02300	alpha-N-arabinofuranosidase B precursor abfB - Aspergillus niger	-0.5	0.1	1.0	-0.8
An06g00170	alpha-galactosidase aglA - Aspergillus niger	-1.6	-0.5	-0.1	-2.3
An02g11150	alpha-galactosidase aglB - Aspergillus niger	0.0	0.0	1.9	2.1
	cellulases				
An14g02760	endoglucanase A eglA - Aspergillus niger	-0.3	0.1	0.9	-0.7
An07g08950	endoglucanase B eglB - Aspergillus niger	0.0	0.0	1.2	-0.9
An16g06800	strong similarity to endo-beta-1,4-glucanase B eglB - Aspergillus niger	-3.3	-0.1	1.0	0.1
An18g03570	Probable beta-glucosidase A precursor bglA - Aspergillus niger	0.1	1.3	1.3	2.0
An07g09330	1,4-beta-D-glucan cellobiohydrolase A precursor cbhA - Aspergillus niger	-1.4	-0.2	0.3	-2.4
An01g11660	1,4-beta-D-glucan cellobiohydrolase B precursor cbhB - Aspergillus niger	-1.4	-0.1	0.2	-4.9
	putative cellulases				
An03g05530	strong similarity to endo-beta-1,4-glucanase EGIII-like from patent WO9931255-A2 - Emericella desertoru	-0.3	0.5	1.0	-0.3
An14g02670	strong similarity to endoglucanase IV egl4 - Trichoderma reesei	-2.7	-0.5	0.0	0.0
An08g10780	strong similarity to hypothetical protein T16K5.230 - Arabidopsis thaliana	0.0	0.0	2.0	0.1
An09g00840	similarity to plastic-degradation enzyme within SEQ ID NO:6 from patent WO2004038016-A1 - Aspergillus oryzae	-1.4	0.2	0.3	-4.8
	pectinolytic enzymes				
An04g09700	strong similarity to endo-xylogalacturonan hydrolase xghA - Aspergillus tubingensis	-0.9	0.3	0.6	-1.6
An02g10550	strong similarity to endo-alpha-1,5-arabinanase abnA - Aspergillus niger	-0.2	0.3	0.7	-1.1
An04g09690	strong similarity to pectin methylesterase pme1 - Aspergillus aculeatus	-0.9	-0.4	0.0	0.0
An01g14670	polygalacturonase E precursor pgaE - Aspergillus niger	0.0	0.0	1.2	-0.8
An14g04200	rhamnogalacturonase rhgB - Aspergillus niger	-4.7	-0.4	0.3	-1.7
	starch degrading enzymes				
An03g06550	glucan 1,4-alpha-glucosidase glaA - Aspergillus niger	-2.2	0.3	1.2	0.4
An11g03340	acid alpha-amylase - Aspergillus niger	-0.8	-0.1	1.0	0.8
Accession	*cell wall/ morphogenesis enzymes*				
An03g05290	glucan endo-1,3-beta-glucosidase eglC precursor - Aspergillus niger	-0.2	0.5	0.7	-0.5
An01g12450	strong similarity to hypothetical glucan beta-1,3 exoglucanase exgS - Aspergillus phoenicis	-0.3	0.3	-0.2	-2.5
An08g03580	strong similarity to 1,3-beta-glucanosyltransferase bgt1 - Aspergillus fumigatus	0.0	0.0	3.3	4.4

Accession	Description - Supplemental Table 3 *(continued)*	^{2}log ratio zones			
		zone 1/3	zone 2/3	zone 4/3	zone 5/3
An01g11010	strong similarity to cell wall protein Crh1 - Saccharomyces cerevisiae	0.0	0.6	1.4	-0.3
An01g04560	strong similarity to mixed-linked glucanase precursor MLG1 - Cochliobolus carbonum	0.0	0.0	0.0	0.0
An14g01820	strong similarity to hypothetical cell wall protein binB - Aspergillus nidulans	-1.5	-0.4	0.6	-0.3
	putative cell wall/ morphogenesis enzymes				
An02g01550	strong similarity to secreted serine protease 19 kDa CS antigen CS-Ag - Coccidioides immitis	-1.3	0.3	1.0	-1.3
An04g01230	strong similarity to hypothetical ECM33 homolog SPCC1223.12c - Schizosaccharomyces pombe	-0.2	0.6	0.9	-0.7
An09g00670	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3) glucanosyltransferase gel3 - Aspergillus fumigatus	-0.5	0.8	0.7	-1.9
	phytases				
An08g11030	acid phosphatase aph, 3-phytase phyB - Aspergillus niger	0.1	1.0	-3.6	-5.9
	proteases				
An14g04710	aspartic proteinase aspergillopepsin I pepA - Aspergillus niger	-1.0	0.3	1.4	0.8
An03g05200	strong similarity to carboxypeptidase S1 - Penicillium janthinellum	0.3	0.6	0.3	-1.1
An08g04490	endoprotease Endo-Pro precursor EPR -Aspergillus niger	-0.3	-0.2	1.4	-0.1
An02g13750	strong similarity to glutaminase A gtaA - Aspergillus oryzae	-0.8	0.3	0.7	-1.3
An01g01750	similarity to lysosomal protease CLN2 - Rattus norvegicus	-0.1	0.2	0.0	-0.7
	putative proteases				
An14g02470	strong similarity to protein PRO304 from patent WO200104311-A1 - Homo sapiens	0.0	0.0	4.3	5.2
An02g04690	strong similarity to serine-type carboxypeptidase I cdpS - Aspergillus saitoi	-1.0	0.3	0.0	0.0
An15g06280	strong similarity to aspartic proteinase aspergillopepsin I pepA - Aspergillus niger [truncated ORF]	-1.0	-0.3	0.9	-0.4
An08g04640	strong similarity to hypothetical lysosomal pepstatin insensitive protease CLN2 - Canis lupus	0.1	0.7	0.8	-1.1
	related to other enzymes				
An07g08940	similarity to acetyl-esterase I from patent WO9502689-A - Aspergillus aculeatus	-3.9	-2.0	1.2	-4.2
An13g01880	strong similarity to cephalosporin esterase - Rhodosporidium toruloides	-2.0	-0.4	0.4	-2.2
An10g00800	strong similarity to purine nucleoside permease NUP - Candida albicans	-1.6	0.0	0.0	0.0
An01g14940	similarity to nonhemolytic phospholipase C PC-PLC - Burkholderia pseudomallei	-1.2	0.1	1.3	-0.9
An01g10580	strong similarity to ribonuclease T2 precursor rntB - Aspergillus oryzae	-0.3	0.1	1.8	0.3
An07g01320	strong similarity to antifungal protein precursor paf - Penicillium chrysogenum	-1.8	-0.2	3.7	3.1
An14g02660	strong similarity to hypothetical necrosis and ethylene inducing protein BH0395 - Bacillus halodurans	0.0	-0.4	1.1	0.6
	related to unknown				
An14g02980	weak similarity to mucin-like glycoprotein 900 GP900 - Cryptosporidium parvum	-0.5	0.3	0.0	0.0
An11g00040	weak similarity to cDNA for 59-kDa readthrough protein RT - Sorghum chlorotic spot virus	0.0	0.0	1.3	-0.4
An16g00670	similarity to hypothetical protein AAO51454.1 - Dictyostelium discoideum	0.5	1.0	-0.1	-2.5
An02g13650	strong similarity to hypothetical protein Afu4g00380 - Aspergillus fumigatus	-1.8	-0.1	0.6	-1.0
An15g07790	similarity to hypothetical protein encoded by An11g02730 - Aspergillus niger	0.0	0.0	0.0	0.0
An15g02250	hypothetical protein	-2.7	-0.7	0.0	0.0
	related to intracellular proteins				
An14g01685*	strong similarity to hypothetical acetyltransferase Afu4g03600 - Aspergillus fumigatus	-0.4	0.3	0.0	0.0

Supplemental Table 4. Quantitative analysis of the secretome of concentric zones of sandwiched colonies of *A. niger* that had been with cycloheximide. This material is available free of charge via the Internet at http://pubs.acs.org

Supplemental Table 5. Quantitative secretome analysis of the outer zone 5, the intermediate zone 3 and the central zone 1 of 7-days-old xylose-grown sandwiched colonies that had either or not been treated with 0.1 mg ml^{-1} cycloheximide. Green and red shading indicate increased and decreased abundance in cycloheximide-treated colonies. The ^{2}log ratio of proteins found in treated/untreated is indicated here (i.e. a number ≥ 2, shows a 4-fold increase in treated colonies, whereas a number ≤ 2 shows a 4-fold increase in untreated colonies). Proteins with an * are predicted not to have a signal sequence (see Supplemental Table 6).

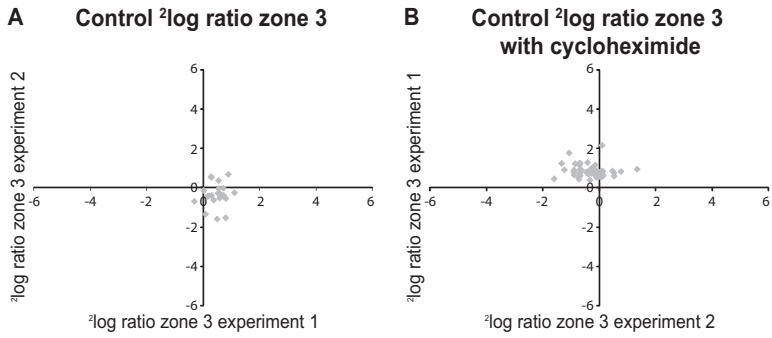
Accession	Description - Supplemental Table 5	^{2}log ratio cycloheximide treated/ untreated colonies		
	xylanolytic enzymes - xylan	*zone 1*	*zone 3*	*zone 5*
An01g00780	endo-1,4-beta-xylanase B precursor xlnB - *Aspergillus niger*	-1.6	-0.2	-2.0
An01g09960	exo-1,4-beta-xylosidase xlnD - Aspergillus niger	5.8	4.9	1.3
An03g00940	endo-1,4-beta-xylanase C precursor xlnC - Aspergillus niger	≥6.6	≥6.6	1.4
An12g05010	acetyl xylan esterase axeA - Aspergillus niger	2.7	1.4	-1.7
An14g05800	alpha-glucuronidase aguA - Aspergillus niger	≥6.6	≥6.6	0.0
An09g03300	strong similarity to alpha-xylosidase XylS - Sulfolobus solfataricus	≥6.6	≥6.6	0.0
An03g00960	alpha-L-arabinofuranosidase axhA - Aspergillus niger	3.9	3.6	-3.5
	xylanolytic enzymes - xyloglucan xylan galactomannan			
An06g00170	alpha-galactosidase aglA - Aspergillus niger	≥6.6	≥6.6	0.0
An02g11150	alpha-galactosidase aglB - Aspergillus niger	≥6.6	≥6.6	1.0
An14g01800	strong similarity to alpha-galactosidase - Cyamopsis tetragonoloba	≥6.6	≥6.6	3.2
An01g00330	alpha-N-arabinofuranosidase A precursor abfA - Aspergillus niger	0.0	2.3	-5.8
An15g02300	alpha-N-arabinofuranosidase B precursor abfB - Aspergillus niger	0.8	1.2	-1.3
	putative xylan/ hemicellulose degrading enzymes			
An08g01900	strong similarity to xylan 1,4-beta-xylosidase - Butyrivibrio fibrisolvens	≥6.6	0.0	0.0
An17g00300	strong similarity to bifunctiona xylosidase-arabinosidase xarB - Thermoanaerobacter ethanolicus [putative frameshift]	≥6.6	≥6.6	0.0
	mannosidases			
An05g01320	strong similarity to mannase man1 - Aspergillus aculeatus	0.0	1.3	0.0
An11g06540	beta-mannosidase mndA - Aspergillus niger	≥6.6	≥6.6	0.0
	cellulases			
An14g02760	endoglucanase A eglA - Aspergillus niger	-0.4	0.0	-2.1
An07g08950	endoglucanase B eglB - Aspergillus niger	3.1	3.4	0.7
An03g01050	similarity to endo-beta-1,4-glucanase - Bacillus polymyxa	0.0	0.0	0.0
An07g09330	1,4-beta-D-glucan cellobiohydrolase A precursor cbhA - Aspergillus niger	≥6.6	4.7	-0.3
An01g11660	1,4-beta-D-glucan cellobiohydrolase B precursor cbhB - Aspergillus niger	1.5	2.0	0.3
An01g11670	strong similarity to endo-beta-1,4-glucanase A eglA - Aspergillus nidulans	≥6.6	5.2	0.0
An16g06800	strong similarity to endo-beta-1,4-glucanase B eglB - Aspergillus niger	5.2	5.1	-0.3
An18g03570	Probable beta-glucosidase A precursor bglA - Aspergillus niger	≥6.6	≥6.6	4.2
An11g00200	Probable beta-glucosidase M precursor bglM - Aspergillus niger	≥6.6	≥6.6	0.0
	putative cellulases			
An08g05230	strong similarity to hypothetical endoglucanase IV - Trichoderma reesei	≥6.6	6.4	0.0
An14g02670	strong similarity to endoglucanase IV egl4 - Trichoderma reesei	0.0	4.1	0.0
An04g08550	strong similarity to endoglucanase IV EGIV - Trichoderma reesei	0.0	≥6.6	0.0

Accession	Description - Supplemental Table 5 *(continued)*	^{2}log ratio cycloheximide treated/ untreated colonies		
	xylanolytic enzymes - xylan	*zone 1*	*zone 3*	*zone 5*
An03g05530	strong similarity to endo-beta-1,4-glucanase EGIII-like from patent WO9931255-A2 - Emericella desertoru	0.0	-3.6	0.0
An01g01870	strong similarity to hypothetical Avicelase III aviII - Aspergillus aculeatus	≥6.6	≥6.6	0.0
An08g01760	strong similarity to cellulase from patent WO9733982-A1 - Acremonium cellulolyticus	0.0	6.3	0.0
An01g01540	strong similarity to alpha,alpha-trehalase treA - Aspergillus nidulans	≥6.6	≥6.6	0.0
An07g07630	strong similarity to avenacinase - Gaeumannomyces graminis	≥6.6	0.0	0.0
An09g00840	similarity to plastic-degradation enzyme within SEQ ID NO:6 from patent WO2004038016-A1 - Aspergillus oryzae	0.0	3.1	0.0
An01g10930	strong similarity to enzyme with sugar transferase activity from patent JP11009276-A - Acremonium sp.	≥6.6	0.0	0.0
	pectinolytic enzymes			
An01g12150	beta-galactosidase lacA - Aspergillus niger	3.6	4.8	4.4
An04g09700	endo-xylogalacturonan hydrolase xghA - Aspergillus tubingensis	1.0	1.2	0.0
An14g04200	rhamnogalacturonase rhgB - Aspergillus niger	3.8	0.5	0.0
An09g00120	ferulic acid esterase A faeA - Aspergillus niger	0.0	0.0	0.0
An02g10550	strong similarity to endo-alpha-1,5-arabinanase abnA - Aspergillus niger	0.2	-0.4	-3.7
An02g12505	similarity to pectin methylesterase pmeA - Aspergillus oryzae	0.0	0.0	0.0
An15g05370	endopolygalacturonase II pgaII - Aspergillus niger	0.0	3.2	0.9
	starch degrading enzymes			
An04g06920	extracellular alpha-glucosidase aglU - Aspergillus niger	≥6.6	≥6.6	0.0
An11g03340	acid alpha-amylase - Aspergillus niger	4.4	4.7	0.1
An03g06550	glucan 1,4-alpha-glucosidase glaA - Aspergillus niger	5.0	3.6	-0.7
	Putative starch degrading enzymes			
An16g00540	similarity to large secreted protein - Streptomyces coelicolor [truncated ORF]	≥6.6	≥6.6	0.0
	(putative) other carbohydrates			
An08g08370	similarity to alpha-1,2-mannosidase aman2 - Bacillus sp.	0.0	≥6.6	0.0
An12g08280	exo-inulinase inu1 - Aspergillus niger	0.0	≥6.6	0.0
An01g12550	strong similarity to mannosyl-oligosaccharide 1,2-alpha-mannosidase msdS - Aspergillus saitoi	≥6.6	0.0	0.0
An08g03060	strong similarity to hypothetical protein CC0533 - Caulobacter crescentus	≥6.6	0.0	0.0
An03g00500	strong similarity to diglycosidase related protein SEQ ID NO:10 from patent WO200018931-A1 - Aspergillus fumigatus	6.0	1.9	-2.2
	cell wall/ morphogenesis enzymes			
An08g03580	strong similarity to 1,3-beta-glucanosyltransferase bgt1 - Aspergillus fumigatus	0.0	0.0	-≥6.6
An01g12450	strong similarity to hypothetical glucan beta-1,3 exoglucanase exgS - Aspergillus phoenicis	0.1	0.7	-1.0
An03g05290	glucan endo-1,3-beta-glucosidase eglC precursor - Aspergillus niger	1.0	1.4	-1.0
An14g01820	strong similarity to hypothetical cell wall protein binB - Aspergillus nidulans	-0.7	-0.7	0.0
An01g11010	strong similarity to cell wall protein Crh1 - Saccharomyces cerevisiae	-1.1	0.9	-2.3
An01g01920	similarity to beta-N-acetylhexosaminidase - Vibrio furnissii	0.0	≥6.6	0.0
An09g02240	strong similarity to beta-N-acetylhexosaminidase from patent WO9839459-A1 - Penicillium chrysogenum	≥6.6	≥6.6	0.0
	putative cell wall/ morphogenesis enzymes			
An02g01550	strong similarity to secreted serine protease 19 kDa CS antigen CS-Ag - Coccidioides immitis	3.0	1.5	-1.5

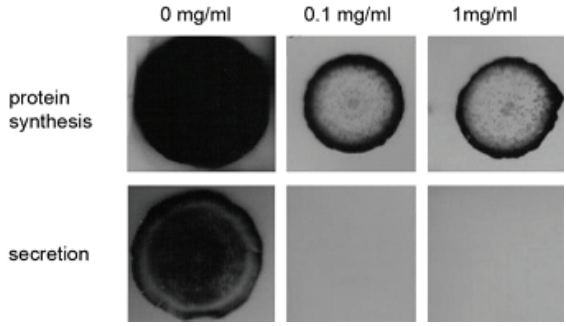
Accession	Description - Supplemental Table 5 *(continued)*	^{2}log ratio cycloheximide treated/ untreated colonies		
		zone 1	*zone 3*	*zone 5*
	xylanolytic enzymes - xylan			
An04g01230	strong similarity to hypothetical ECM33 homolog SPCC1223.12c - Schizosaccharomyces pombe	1.4	2.3	0.4
An09g00670	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3) glucanosyltransferase gel3 - Aspergillus fumigatus	2.2	2.5	2.1
	phytases			
An12g01910	strong similarity to phytase phyA3 - Aspergillus fumigatus	≥6.6	≥6.6	0.0
An08g11030	acid phosphatase aph, 3-phytase phyB - Aspergillus niger	2.4	4.7	4.8
	proteases			
An14g04710	aspartic proteinase aspergillopepsin I pepA - Aspergillus niger	0.2	0.4	-3.9
An15g06280	strong similarity to aspartic proteinase aspergillopepsin I pepA - Aspergillus niger [truncated ORF]	0.0	0.0	-5.0
An07g08030	serine carboxypeptidase pepF - Aspergillus niger	5.1	≥6.6	0.0
An08g04490	endoprotease Endo-Pro precursor EPR -Aspergillus niger	0.1	2.9	0.4
An01g00370	strong similarity to aspergillopepsin apnS - Aspergillus phoenicis	4.7	4.8	0.0
An02g04690	strong similarity to serine-type carboxypeptidase I cdpS - Aspergillus saitoi	4.1	0.0	0.0
An12g05960	strong similarity to dipeptidyl peptidase II DPPII - Rattus norvegicus	≥6.6	0.0	0.0
An06g00190	strong similarity to lysosomal pepstatin insensitive protease CLN2 - Homo sapiens	0.0	≥6.6	0.7
An03g01010	strong similarity to lysosomal pepstatin insensitive protease CLN2 - Homo sapiens	2.1	4.5	0.0
An08g04640	strong similarity to hypothetical lysosomal pepstatin insensitive protease CLN2 - Canis lupus	0.1	1.6	-0.7
An01g01750	similarity to lysosomal protease CLN2 - Rattus norvegicus	2.4	3.2	-0.3
An16g09010	strong similarity to carboxypeptidase I protein from patent WO9814599-A1 - Aspergillus oryzae [putative frameshift]	≥6.6	0.0	0.0
An03g05200	strong similarity to carboxypeptidase S1 - Penicillium janthinellum	2.5	3.7	1.2
An04g01440	strong similarity to precursor of pepsin A3 - Homo sapiens	0.0	5.5	0.0
An14g02150	strong similarity to serine-type carboxypeptidase precursor cpdS - Aspergillus phoenicis	0.0	4.3	0.0
An12g03300	strong similarity to aspartic protease pr1 - Phaffia rhodozyma	2.4	2.8	0.0
An02g13750	strong similarity to glutaminase A gtaA - Aspergillus oryzae	3.9	4.2	0.0
	putative proteases			
An14g02470	strong similarity to protein PRO304 from patent WO200104311-A1 - Homo sapiens	≥6.6	≥6.6	-1.5
	related to other enzymes			
An01g01820	catalase R catR - Aspergillus niger	≥6.6	≥6.6	0.0
An01g01550	strong similarity to catalase cat1 - Aspergillus fumigatus	≥6.6	≥6.6	0.0
An01g13660	strong similarity to laccase I precursor yA - Aspergillus nidulans	0.0	0.0	0.0
An07g02360	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - Arthrobacter oxidans	5.7	0.0	0.0
An08g00490	strong similarity to lactonohydrolase - Fusarium oxysporum	≥6.6	0.0	0.0
An02g09690	strong similarity to lipase I precursor TFLI - Geotrichum fermentans	≥6.6	≥6.6	0.0
An07g08940	similarity to acetyl-esterase I from patent WO9502689-A - Aspergillus aculeatus	≥6.6	5.6	1.9
An13g01880	strong similarity to cephalosporin esterase - Rhodosporidium toruloides	5.0	3.9	1.2
An16g03700	strong similarity to phospholipase B from patent US6146869-A - Aspergillus oryzae	5.2	4.8	0.0
An10g00790	similarity to hypothetical protein SCE6.29 - Streptomyces coelicolor	≥6.6	0.0	0.0
An11g01220	strong similarity to precursor of tannase - Aspergillus oryzae	≥6.6	0.0	0.0

Accession	Description - Supplemental Table 5 *(continued)*	^{2}log ratio cycloheximide treated/ untreated colonies		
An01g14940	similarity to nonhemolytic phospholipase C PC-PLC - Burkholderia pseudomallei	3.9	1.9	-0.4
An01g15200	strong similarity to mature penicillin V amidohydrolase PVA from patent US5516679-A - Fusarium oxysporum [truncated ORF]	≥6.6	≥6.6	0.0
An10g00800	strong similarity to purine nucleoside permease NUP - Candida albicans	0.0	2.8	0.0
An02g00190	similarity to enantiomer-selective amidase amdA - Rhodococcus sp.	≥6.6	≥6.6	0.0
An02g09090	strong similarity to mutarotase enzyme from patent JP07039380-A - Sus scrofa	5.9	5.4	0.0
An11g00040	weak similarity to cDNA for 59-kDa readthrough protein RT - Sorghum chlorotic spot virus	-0.1	-0.7	-5.1
An01g10580	strong similarity to ribonuclease T2 precursor rntB - Aspergillus oryzae	-0.9	-0.2	-3.0
An14g02660	strong similarity to hypothetical necrosis and ethylene inducing protein BH0395 - Bacillus halodurans	0.0	2.3	-3.9
An07g01320	strong similarity to antifungal protein precursor paf - Penicillium chrysogenum	3.9	-1.9	-≥6.6
	related to unknown			
An16g00670	similarity to hypothetical protein AAO51454.1 - Dictyostelium discoideum	-0.9	0.0	0.0
An02g13650	strong similarity to hypothetical protein Afu4g00380 - Aspergillus fumigatus	0.9	-0.4	-3.3
An04g08730	strong similarity to hypothetical conserved protein SPAC12B10.16c - Schizosaccharomyces pombe	≥6.6	≥6.6	0.0
An15g07790	similarity to hypothetical protein encoded by An11g02730 - Aspergillus niger	0.6	0.4	0.0
An02g11890	strong similarity to hypothetical protein encoded by An14g01330 - Aspergillus niger	≥6.6	0.0	0.0
An08g04630	strong similarity to hypothetical protein Afu2g15420 - Aspergillus fumigatus	0.0	0.0	0.0
An15g02250	hypothetical protein	0.0	-≥6.6	-≥6.6
	related to intracellular proteins			
An14g01790*	strong similarity to alpha-galactosidase Afu4g03580 - Aspergillus fumigatus	≥6.6	0.0	0.0
An02g03900*	hypothetical protein	0.0	0.0	0.0
An12g00240*	strong similarity to translational inhibitor uk14 - Homo sapiens [truncated ORF]	≥6.6	≥6.6	0.0
An01g00610*	strong similarity to hypothetical protein BAC69773.1 - Streptomyces avermitilis	≥6.6	0.0	0.0
An03g06870*	strong similarity to cytidine deaminase - Homo sapiens	≥6.6	0.0	0.0
An14g01685*	strong similarity to hypothetical acetyltransferase Afu4g03600 - Aspergillus fumigatus	2.4	2.7	0.0
An13g01830*	weak similarity to SAK-b serine-threonine kinase from patent CA2150789-A - Mus musculus	1.3	0.0	0.0

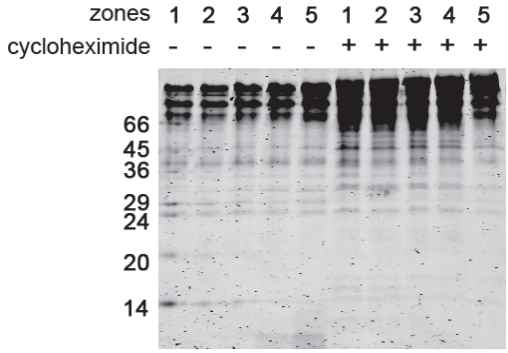
Supplemental Table 6. Quantitative differences between the secretome of cycloheximide treated and untreated colonies of *A. niger*. This material is available free of charge via the Internet at http://pubs.acs.org



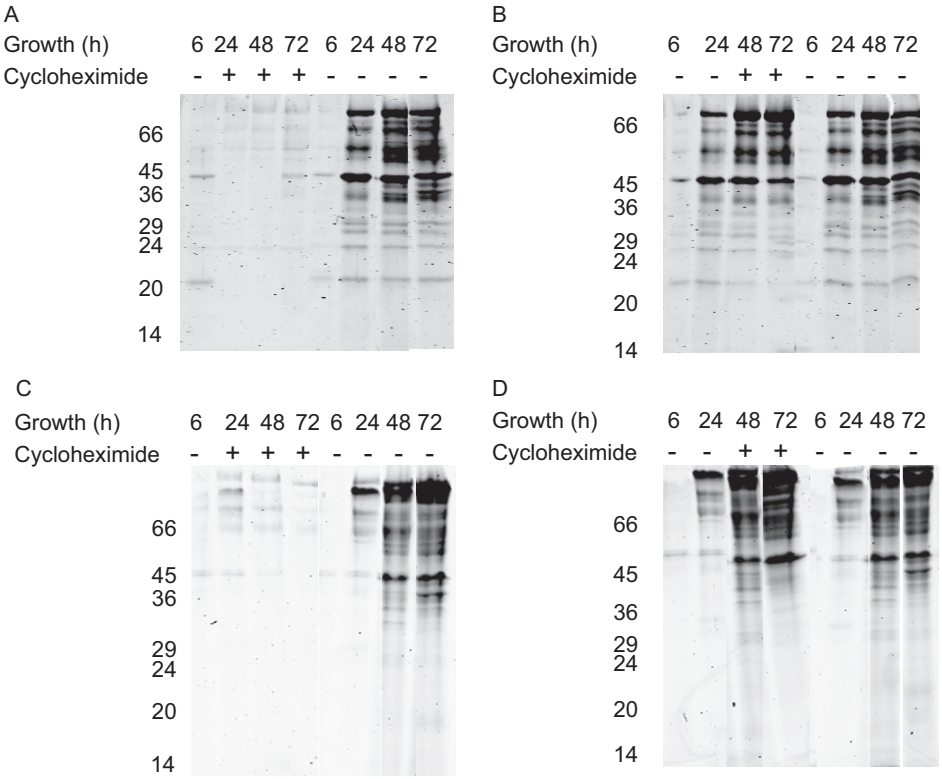
Supplemental Figure 1. 2log ratios of the technical duplicate of individual proteins of zone 3 as obtained by labeling the peptides with two different dimethyl labels (see Figure 1). The data of the biological duplicates are shown.



Supplemental Figure 2. Localization of protein synthesis and protein secretion in sandwiched colonies of *A. niger* that had been grown in the absence or presence of the protein synthesis inhibitor cycloheximide by labeling with ^{14}C-labelled amino-acids. Upper panel shows autoradiograms of the colonies, while the lower panels show autoradiograms of protein binding PVDF membranes that had been placed between the sandwiched colony and the agar medium.



Supplemental Figure 3. SDS PAGE of the secretome of the concentric zones of 7-days-old maltose-grown colonies that had either (+) or not (-) been treated for 24 h with 0.1 mg ml^{-1} cycloheximide. Gels were stained with Coommassie Brilliant Blue. Zone 1 represents the innermost zone, whereas zone 5 represents the outermost zone of the colony.



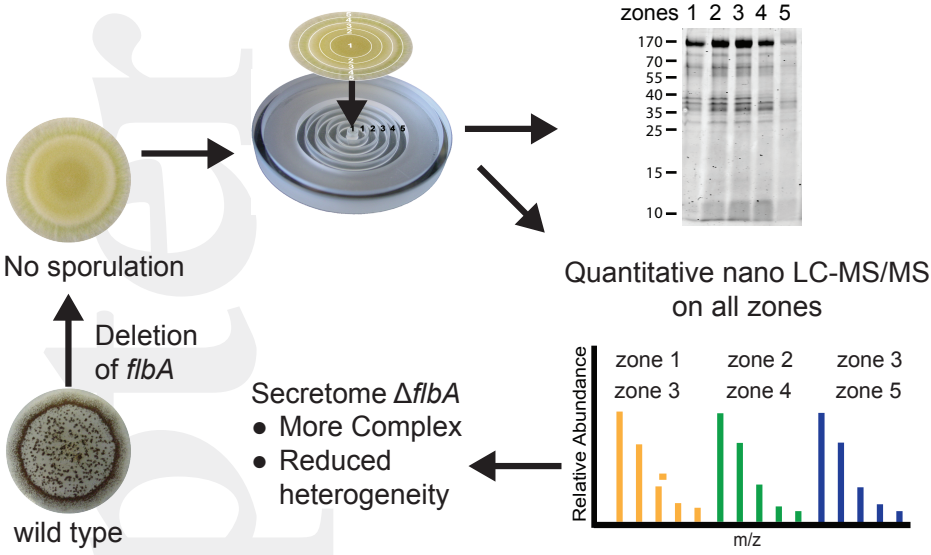
Supplemental Figure 4. Proteins released into the medium of xylose-grown (A, B) and maltose-grown (C, D) liquid shaken cultures after 6, 24, 48, and 72 h of incubation. Cycloheximide was either (+) or not (-) added after 6 h (A, C) or 24 h (B, D) of growth in MM and incubation of the cultures was prolonged up to 72 h.

Chapter 3

Deletion of *flbA* results in increased secretome complexity and reduced secretion heterogeneity in colonies of *Aspergillus niger*

This Chapter is based on Krijgsheld P, Nitsche BM, Post H, Levin AM, Müller WH, Heck AJR, Ram AFJ, Altelaar AFM, and Wösten HAB (2013). Deletion of *flbA* results in increased secretome complexity and reduced secretion heterogeneity in colonies of *Aspergillus niger*. *Journal of Proteome Researc*h 12: 1808-1819. doi: 10.1021/pr301154w.

Graphical Abstract



ABSTRACT

Aspergillus niger is a cell factory for the production of enzymes. This fungus secretes proteins in the central part and at the periphery of the colony when the culture has been transferred to fresh medium. The sporulating zone of the colony overlapped with the non-secreting sub-peripheral zone, indicating that sporulation inhibits protein secretion. Indeed, strain Δ*flbA* that is affected early in the sporulation program secreted proteins throughout the colony. In contrast, the Δ*brlA* strain that initiates but not completes sporulation did not show altered spatial secretion. The secretome of 5 concentric zones of xylose-grown Δ*flbA* colonies was assessed by quantitative proteomics. In total 138 proteins with a signal sequence for secretion were identified in the medium of Δ*flbA* colonies. Of these, 18 proteins had never been reported to be part of the secretome of *A. niger*, while 101 proteins had previously not been identified in the culture medium of xylose-grown wild-type colonies. Taken together, inactivation of *flbA* results in spatial changes in secretion and in a more complex secretome. The latter may be explained by the fact that strain Δ*flbA* has a thinner cell wall compared to the wild-type, enabling efficient release of proteins. These results are of interest to improve *A. niger* as a cell factory.

INTRODUCTION

The genus *Aspergillus* represents species that are among the most abundant fungi in nature. As such, aspergilli play an important role in global recycling of organic material. The genus *Aspergillus* also comprises species that are pathogens of plants, animals, and humans. [Chapter 1, 1] Moreover, it includes species such as *Aspergillus niger* that are used by the industry for the production of enzymes and metabolites. [2]

Germination of the asexual spores of aspergilli, known as conidia, results in a colony. This colony consists of a mycelium of interconnected hyphae. The center and the periphery of the colony represent the oldest and youngest part of the mycelium, respectively. Hyphae at the periphery of the colony are exposed to unexplored organic material, whereas the substrate is (partly) utilized in the colony center. Colonization of the substrate includes the degradation of polymers by secreted enzymes. The breakdown products can be taken up by the fungus to serve as nutrients. Notably, *Aspergillus* colonies show zonal differences in gene expression, growth and protein secretion. [3-8, Chapter 2] This secretion heterogeneity would imply that substrates are broken down in a sequential order. [Chapter 2, 7] It was shown that a major part of the secreted proteins are retained in the cell walls of hyphae of *A. niger*. [4, Chapter 2, 7] These proteins were released in the medium after a cycloheximide treatment. [Chapter 2, 7] So far, it is not known whether cell wall associated enzymes have a role in substrate degradation.

At a certain moment, colonies of *Aspergillus* form conidiophores that produce conidia. [Chapter 1,1] Conidiophore development includes the formation of a stalk, a vesicle bud, metulae, phialides, and conidia. This process has been well studied in *A. nidulans*. [Chapter 1, 1, 9] The *brlA* gene is the central regulator of conidiophore development. [10, 11] The Δ*brlA* strain of *A. nidulans* has a bristle phenotype caused by stalks that are 20-30 times longer than those of wild-type. [12] Moreover, vesicle formation is not initiated in a Δ*brlA* strain. Gene *brlA* is activated by the production of an extracellular signal that is released by the FluG protein. [13, 14] This extracellular signal releases the repression of SfgA on several *flb* genes that activate *brlA* expression (Figure 1). [Chapter 1, 1, 15] For instance, the protein encoded by *flbA* activates *brlA* expression and negatively regulates vegetative growth, thereby allowing asexual development. The Δ*flbA* strain of *A. nidulans* has a fluffy phenotype (hence the name *fluffy low brlA expression*). Its mycelium proliferates uncontrolled and masses of undifferentiated aerial hyphae are formed. When Δ*flbA* colonies mature, both submerged and aerial hyphae start to autolyse. [14, 16] Gene *flbA* encodes an RGS domain protein that stimulates the intrinsic GTPase activity of the Gα-subunit FadA. This Gα-subunit is part of a heterotrimeric G-protein complex that also contains a Gβ- (SfaD) and Gγ-subunit (GpgA) (Figure 1). The activated Gα-subunit and the Gβ-Gγ dimer both stimulate vegetative growth. The protein product of *flbA* converts the active Gα-subunit-GTP to its inactive GDP bound stage, thereby repressing vegetative

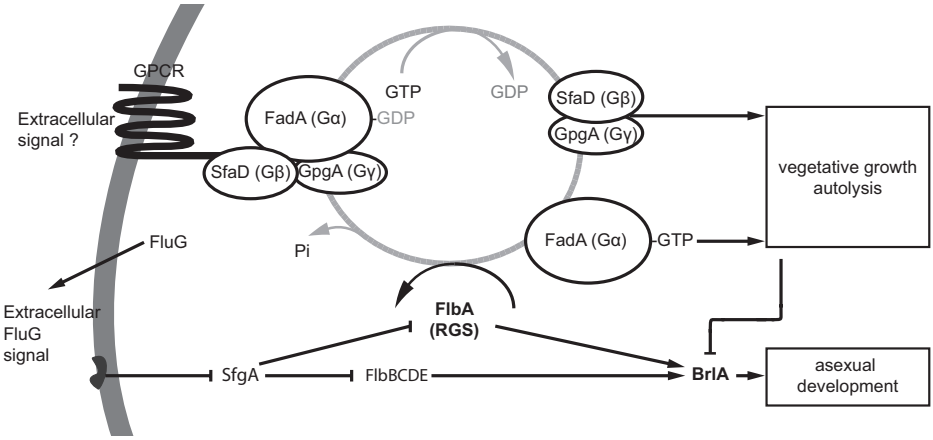


Figure 1: Model of the sporulation pathway in *Aspergillus*. FluG is involved in the synthesis and / or secretion of an extracellular factor that releases the inhibition of SfgA on Flb proteins. These proteins in turn activate *brlA* that induces asexual development. FlbA is an RGS protein that regulates the signaling of the Gα-subunit FadA. GTP-bound FadA stimulates vegetative growth. FlbA enhances the intrinsic activity of GTP hydrolysis, converting FadA-GTP to the inactive hetrotrimeric FadA-SfaD-GpgA GDP-bound G-protein complex (Adapted from [1, Chapter 1]).

growth and promoting asexual development.

Here we show that *brlA* and *flbA* of *A. niger* have a similar role in asexual development as in *A. nidulans*. We also show that zones that are actively sporulating do not secrete proteins. This can be abolished by deletion of *flbA* but not *brlA*. The reduced secretion heterogeneity between zones of the Δ*flbA* colonies is accompanied by a more complex secretome. It includes proteins that had not been reported in the secretome of *A. niger* before. These results and the fact that the Δ*flbA* strain forms a thinner cell wall are of interest to improve *A. niger* as a cell factory.

MATERIAL AND METHODS

Strain and Culture Conditions

A. niger N402 and the derived Δ*brlA* and Δ*flbA* strains were grown at 30 ˚C. Static cultures were grown as sandwiched cultures[6] on minimal agar medium (MM) [17] with 1.5 % agar and 25 mM xylose as a carbon source. To this end, a perforated polycarbonate (PC) membrane (0.1 µm pores, 76 mm diameter; Profiltra, Almere, The Netherlands) was placed on the agar medium and topped with a 0.45 mm layer of 1.25 % agarose. A 1 mm plug of mycelium was positioned in the center of the agarose layer and covered with another 76 mm wide PC membrane. After 7 days of growth, colonies were transferred for 24 h to fresh agar medium or to a ring plate. [4] The five concentric wells in the ring plate were filled with liquid MM supplemented with 25 mM xylose.

Plasmid construction

Upstream and downstream flanking regions of *brlA* (An01g10540) and *flbA* (An02g03160) were amplified from chromosomal DNA of *A. niger* N402[18] using Taq polymerase and primer pairs 1 and 2, and 3 and 4, respectively (Table 1). In the case of the *brlA* deletion construct, the primers introduced a *Bgl*II and a *Nhe*I site at the 5' and 3' ends of the upstream flank, respectively, and a *Hin*dIII and *Xba*I site at the 5' and 3' ends of the downstream flank. The PCR fragments were cloned in pGEMTeasy (Promega, Maddison, MI, USA) and transformed to *E. coli* DH5α. The *brlA* downstream flank was digested with *Hin*dIII and *Xba*I and ligated into pAN7-1[19] that had been digested with the same enzymes. The resulting construct was digested with *Bgl*II and *Nhe*I and ligated with the *Bgl*II-*Nhe*I upstream fragment of *brlA*. This resulted in the *brlA* deletion construct PKO-*brlA* consisting of the hygromycine resistance cassette flanked by the upstream and downstream sequences of *brlA*. In the case of the *flbA* deletion construct, the upstream and downstream flanks were blunt end ligated in pJET1.2 (Fermentas, Thermo Fischer Scientific, Waltham, MA, USA). A *Not*I, and *Xba*I/*Xho*I site had been introduced at the 5' and 3' end of the upstream flank, respectively, and a *Xho*I and *Kpn*I site at the 5' and 3' end of the downstream flank. The resulting plasmids were digested with *Not*I and *Xho*I and *Xho*I and *Kpn*I, respectively. This was followed by a three point ligation with pBluescriptII SK(+) that had been digested with *Not*I and *Kpn*I. The *Xho*I/*Xba*I fragment of pAN7-1 containing the hygromycine resistance cassette was inserted in the vector containing both flanks of *flbA*. This resulted in the *flbA* deletion construct BNO-*flbA*. The deletion constructs PKO-*brlA* and BNO-*flbA* were digested with *Bgl*II and *Hind*III and

Table 1. Primers used in this study.

Primer	Primer description	Sequence '5- '3
Primer 1a (PK001)	5'flank *brlA* FW	gagatctATCCCTCCGCTGCAAC
Primer 1b (PK002)	5'flank *brlA* REV	gctagGCAACGACTTCGGTCTTC
Primer 2a (PK003)	3'flank *brlA* FW	gtctagaACCCTTGGGCACCTGAG
Primer 2b (PK004)	3'flank *brlA* REV	gaagcttGACTCACCGCTGGAAGG
Primer 3a (BN018)	5'flank *flbA* FW	ataagaatgcggccgcCGGTCGCCTGTAGGAACCTTAC
Primer 3b (BN019)	5'flank *flbA* REV	ccgctcgagttaatcctagtctagaCAAGACGGAGTGGGAGGAAAG
Primer 4a (BN020)	3'flank *flbA* FW	ccgctcgagGCAGCATGTGTGTCATGCAG
Primer 4b (BN021)	3'flank *flbA* REV	cggggtaccCTGATCGTGAAGAGGACAACCCTC

*Not*I and *Kpn*I, respectively, and the flanking sequences interspersed with the antibiotic resistance marker were introduced in *A. niger* N402. Gene replacement by homologous recombination was confirmed by Southern analysis (data not shown).

Transformation of *Aspergillus niger*

Transformation of *A. niger* was performed as described by Meyer et al. [20] using 40 mg lysing enzymes (L-1412, Sigma-Aldrich, Zwijndrecht, The Netherlands) per g wet weight of mycelium. Transformants were isolated from MMS plates[21] supplemented with 200 µg ml^{-1} hygromycine and 50 µg ml^{-1} caffeine.

Localization of Protein Synthesis and Protein Secretion

Protein synthesis, secretion, and growth were monitored as described. [6, 7, Chapter 2] Sandwiched cultures were labeled for 4 h with 185 kBq ^{14}C-amino acids (NEC-445E amino acid mixture, L-[^{14}C(U)]- specific activity 1.94 GBq $milliatom^{-1}$; Perkin Elmer, Waltham MA, USA) to visualize protein synthesis. A polyvinylidene difluoride (PVDF) membrane (Immobulon-P, Millipore, Bedford, USA) was placed in between the sandwiched colony and the agar medium to immobilize secreted proteins. Growth was visualized by labeling for 10 min with 185 kBq ^{14}C-N-acetylglucosamine (CFA485 N-acetyl-D-[1-14C] glucosamine, specific activity 1.85–2.29 GBq $mmol^{-1}$; GE Healthcare, Westborough, MA, USA,). Label was placed on top of the colony after absorbing it to a piece of rice paper (Steicher & Schuell, Dassel, Germany) with the size of the colony. Colonies were fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 1 h at room temperature. For the visualization of protein synthesis and secretion, sandwiched colonies and PVDF membranes were washed 3 times 60 min with 1% casamino acids (Becton, Dickinson and company, Le-Pont-De-Claix, France). For monitoring growth, sandwiched colonies were washed 3 times 60 min with 0.44 mM N-acetyl-glucosamine in water. After drying overnight at room temperature, colonies and PVDF membranes were exposed to Kodak Biomax XAR film (Sigma-Aldrich, Saint Louis, USA).

SDS-PAGE

SDS-PAGE and Coomassie staining was performed as described before.[Chapter 2, 7] Gels were imaged with an Odyssey Infrared Imaging System (Licor, Bad-Homburg, Germany) using the 700 nm channel.

Sample Preparation and Dimethyl Labeling for Mass Spectrometry

Medium samples were concentrated 10-fold with ultra-concentration columns (4 ml Amicon ultra centrifugal filter units, 10 kDa cut-off; Millipore, Amsterdam, Netherlands). To this end, 500 µl of culture medium of each zone was transferred to the column and centrifuged for 30 min at 4000 g in a swing out rotor. The retentate was washed twice with 2.5 ml PBS (Sigma-Aldrich, Zweindrecht, The Netherlands). Proteins in the concentrated sample (final volume of 50 µl) were then subjected to in-solution-digestion. To this end, samples were lys-C and trypsin-digested, and concentrated and desalted with µElution plates as described before. [Chapter 2, 7] Stable isotope dimethyl labeling was performed in a total volume

of 50 µl. [Chapter 2,7, 22] Zone 1 and 3 were labeled with the light dimethyl label, zone 2 and 4 with the intermediate dimethyl label and zone 3 and 5 with the heavy dimethyl label. In the biological duplicate dimethyl labels were swapped resulting in an intermediate-labeled zone 1 and 3, a heavy-labeled zone 2 and 4, and a light-labeled zone 3 and 5. Labeling efficiency was ≥ 97% as was shown by running an aliquot of the labeled samples on a regular LC MS/MS and comparing the number of dimethyl labeled peptides versus the total number of peptides.

Nano LC-LTQ-Orbitrap-MS

Stable isotope dimethyl labeling was performed as described before. [Chapter 2, 7] Labeling was performed in a final volume of 50 µl. Two µl aliquots of samples were mixed and the volume was adjusted to 10 µl taking into account a final concentration of formic acid of 10% (v/v). Samples (4 µl) were analyzed on an LTQ-Orbitrap XL (Thermo Fischer Scientific, Bremen, Germany) that was connected to an Agilent 1200 HPLC system. Samples were loaded on a trap column (ReproSil-Pur C18-AQ, 20 mm × 100 µm inner diameter, packed in house; Dr Maisch, Ammerbuch, Germany) at 5 µL min^{-1} in 100% solvent A (0.1 M acetic acid in water). Peptides were eluted from the trap column onto an analytical column (ReproSil-Pur C18-AQ, 40 cm × 50 µm inner diameter, packed in-house) at 100 nL min^{-1} during 120 min using a gradient from 0-28% solvent B (0.1 M acetic acid in 8:2 v/v ACN/water) in 60 min and 28-50% solvent B in 25 min. The eluent was sprayed via distal coated emitter tips connected to the analytical column. The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS. Full-scan MS spectra (from m/z 350 to 1500) were acquired in the Orbitrap with a resolution of 60,000 at m/z 400 after accumulation to a target value of 500,000 in the linear ion trap. The three most intense ions were selected for collision-induced fragmentation in the linear ion trap at an normalized collision energy of 35% after accumulation to a target value of 10,000.

Protein Identification and Quantification

The database that was used to assign peptides consisted of 14,166 *A. niger* CBS 513.88 sequences[23], 14,166 reversed *A. niger* sequences, 256 common contaminant sequences, and 256 reversed contaminant sequences. Thermo Proteome Discoverer 1.2.0.207 (Thermo Fischer Scientific, Bremen, Germany) was used in combination with a Mascot search engine (version 2.2.1) software platform (Matrix Science, London, UK). The following settings of Mascot were used: trypsin with 2 missed cleavages, precursor mass tolerance of 50 ppm, fragment ions mass tolerance of 0.6 Da and carbamidomethyl (C) as fixed modification. Oxidation (M), light-dimethyl (K- and N-term), intermediate-dimethyl (K- and N- term) and heavy-dimethyl (K- and N- term) were set as variable modifications. The following settings of Proteome discoverer were used: peptide

score filter: Mascot (ion score) > 20, maximum peptide rank = 1, peptide length: 7-35 and a peptide mass deviation 10 ppm. In Thermo Proteome Discoverer 1.2 simultaneously heavy/light, medium/heavy, medium/light Quan values were calculated. A technical control experiment was performed with zone 3 that had been labeled with two different labels (Supplemental Figure 1). The ^{2}log ratio for most of the proteins was expected to be 0. This was indeed the case for ≥ 90% of the proteins (Supplemental Figure 1). Since proteins can be identified in one zone of the colony only, files were also loaded with the option replaced Missing Quan values, which allows quantification of 'on/off' situations. Proteins with at least one peptide in both biological replicates were included in the analysis. Protein release was considered differential when the average ^{2}log ratio value was higher than 2 or lower than -2 in one sample or at least higher than 1 or lower than -1 in both biological replicates. To calculate the False Discovery Rate (FDR), spectra were searched against a reverse protein database. The spectra of the quantification of concentric zones of Δ*flbA* colonies had an FDR of < 1.5%.

Scanning Electron Microscopy

Sandwiched colonies were grown in the absence of the agarose layer on a PC membrane. Samples (8 x 5 mm) were cut from the periphery and the center of the colony and fixed with 3 % glutaraldehyde (Agar Scientific, Essex, UK) in 0.1 M PBS for one day at 4 °C. Samples were rinsed 3 times in PBS and 3 times in distilled water and post-fixed in 1 % (w/v) aqueous osmium tetroxide (EMS, Hatfield, PA, USA) for 16 h at 4 °C. Samples were washed 3 times for 20 min with distilled water, and gradually dehydrated for 15 min in 25 % (v/v) ethanol, 50 % (v/ v) ethanol, 70 % (v/ v) ethanol, 80 % (v/v) ethanol, 90 % (v/v) ethanol, 95 % (v/ v) ethanol, 100 % (two times) ethanol, ethanol: anhydrous acetone (1:1; 1:2), and finally in anhydrous acetone (i.e. acetone containing 1 % (v/v) acidified 2,2-dimethoxypropane). Samples were carefully transferred under anhydrous acetone into the chamber of a Balzers CPD 030 (Bal-Tec, Schalksmülhe, Germany), and critical point dried at 40 °C and 85 bar. Samples were mounted on stubs and coated with Pt/Pd by the use of a Cressington sputter coater 208HR (Cressington Scientific Instruments, Watford, England). Mycelium was viewed with a Field Emission Scanning Electron Microscope (FEI, Eindhoven, The Netherlands) at an acceleration voltage of 5 kV and a working distance of about 5 mm.

Transmission Electron Microscopy

Sample preparation and transmission electron microscopy (TEM) was performed as described.[Chapter 2,7] Samples were viewed with a transmission electron microscope Tecnai 12 (FEI, Eindhoven, The Netherlands) at an acceleration voltage of 100 kV. The thickness of the cell wall and the hyphal diameter of treated and untreated colonies were determined in cross sections. Four measurements (north, south, east, and west) per hyphae were used to measure cell wall thickness, whereas two

measurements were made to measure hyphal diameter (north-south, west-east). Statistical analysis was performed with IBM Statistics 20 (SPSS statistics; IBM, New York USA). Mean hyphal diameter and cell wall thickness of central and peripheral hyphae were statistically analyzed with a confidence of $p \leq 0.05$ using a Welch's *t*-test (accounting for unequal error variances).

RESULTS

Sporulation inhibits secretion

Sandwiched colonies of the wild-type strain were grown for 7 days on solid minimal medium with 25 mM xylose as carbon source. Five concentric zones were distinguished in these colonies. The most inner zone is referred to as zone 1, whereas the most outer zone is called zone 5 (Figure 2). Labeling with ^{14}C-*N*-acetylglucosamine showed that growth of the wild-type colony took place in zone 5 (Figure 2A). Labeling with a mixture of ^{14}C-amino acids demonstrated that proteins were formed throughout the mycelium (Figure 2B), while secretion only occurred at the periphery of the colony (Figure 2C). When colonies were transferred to fresh xylose medium, spatial growth and protein production was not affected (Figure 2DE). Protein secretion (Figure 2F), however, was not only observed at the periphery (ring 5) but also in central parts of the mycelium (zones 1, 2 and 3). Interestingly, zone 4 did not secrete proteins. This zone started to sporulate when the upper polycarbonate membrane was removed from the sandwiched culture (Figure 2G). This was irrespective of the fact whether the colony had been transferred to fresh medium or not. Yet, sporulation was more abundant in transferred colonies. Taken together, these results indicate that the capacity to sporulate inhibits secretion in the vegetative mycelium.

Inactivation of *flbA* but not of *brlA* affects secretion

Wild-type N402 colonies form stalks that further develop into conidiophores that produce chains of asexual spores (Figure 3AB). Genes *brlA* and *flbA* were deleted in *A. niger* to assess their role in conidiophore development. The N402Δ*flbA* and N402Δ*brlA* strains showed a *fluffy* and *bristle* phenotype, respectively. Scanning electron microscopy of the Δ*brlA* strain on agar plates showed that this mutant formed stalks with infinite growth (Figure 3EF). These stalks reached a length of up to 1000 μm, which is 20-30 times longer than that of wild-type stalks. The Δ*flbA* mutant formed aerial hyphae but conidiophore stalks were not formed (Figure 3CD). Colonies of the wild-type strain and those of the Δ*flbA* and Δ*brlA* strains had formed a similar biomass on xylose after 3, 5, and 7 days of growth (Figure 3G).

Growth, protein synthesis and protein secretion in Δ*brlA* was similar to wild-type colonies, irrespective whether the colonies had or had not been transferred to fresh medium (Supplemental Figure 2). Interestingly, deletion of *flbA* did affect spatial secretion (Figure 2JM). Protein secretion in the Δ*flbA*

strain before transfer (Figure 2J) was most pronounced in the center and was also observed at the periphery of the colony. After transfer, secretion in Δ*flbA* occurred throughout the colony (Figure 2M) but the amount of secreted protein was lower when compared to the center and the periphery of the non-transferred colony. Growth in Δ*flbA* colonies was observed throughout the colony before (Figure 2H) and after transfer (Figure 2K) to fresh medium. This is in contrast to that observed in the wild-type (Figure 2A) and the Δ*brlA* strain (Supplemental Figure 2). Aerial hyphae were formed throughout the colony when the upper membrane of a sandwiched Δ*flbA* colony was removed (Figure 2N).

Quantification of the Δ*flbA* secretome

Protein profiles released by the concentric zones of 7-days-old xylose-grown sandwiched colonies were monitored by SDS PAGE. To this end, the colonies were transferred for 24 h to a ring plate. [4, 7, Chapter 2] The ring plate consists of five concentric wells that were filled with xylose-containing liquid minimal medium.

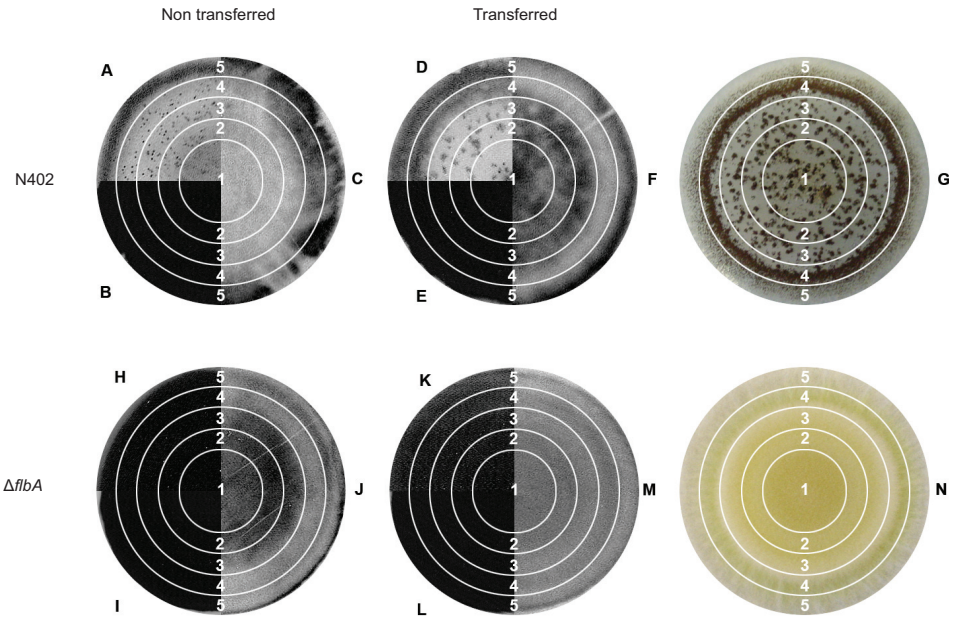


Figure 2: Growth, (ADHK), protein synthesis (BEIL), and secretion (CFJM) in 7-days-old xylose-grown wild-type (A-G) and Δ*flbA* (H-N) colonies. Colonies were analyzed before (ABC, HIJ) and after transfer (DEF, KLM) to fresh xylose medium. Growth was detected with 14C-labeled N-acetylglucosamine. Protein synthesis and secretion were monitored by incorporation of ^{14}C-labeled amino acids. Secreted proteins were immobilized by a PVDF membrane that had been placed underneath the colony. Conidia forming conidiophores were observed after 24 hours of growth in the sub-peripheral zone 4 of the wild-type when the upper membrane of a sandwiched was removed at day 7 (G), while the Δ*flbA* strain formed aerial hyphae throughout the colony (N).

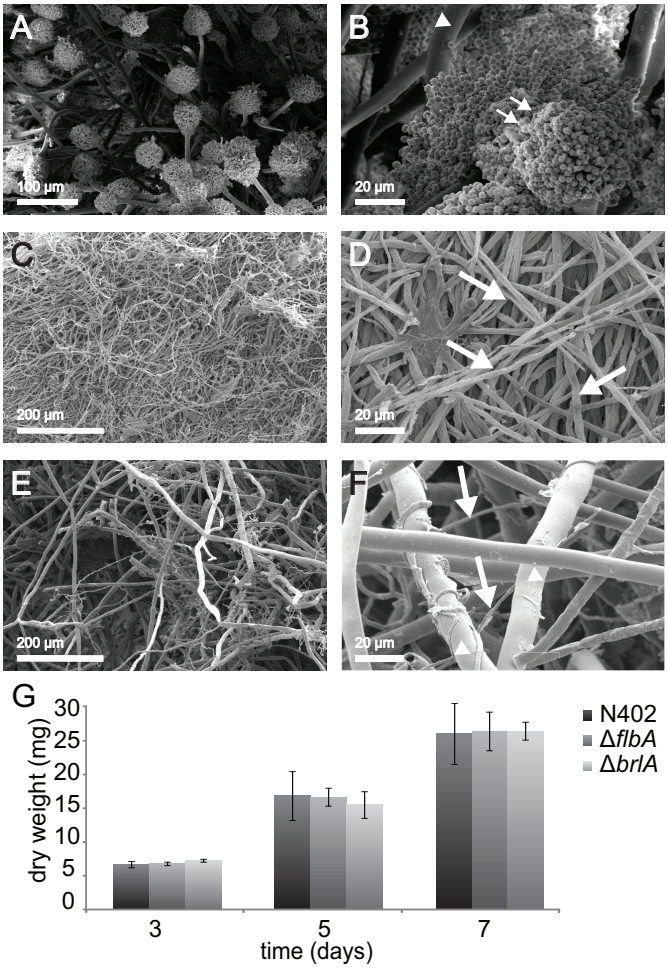


Figure 3: Scanning electron microscopy (A-F) of 7-days-old *A. niger* wild-type (N402) (A,B), Δ*flbA* (C,D), and Δ*brlA* (E,F), colonies and their biomass after 3, 5 and 7 days of growth (G). Wild-type colonies formed masses of conidia (thin arrows). The Δ*flbA* strain formed abundant aerial hyphae (thick arrows), while the Δ*brlA* strain produced both aerial hyphae (thick arrows) and stalks (arrow heads).

The innermost zone of the colony released proteins in zone 1, whereas the outermost part of the colony secreted proteins in zone 5 of the ring plate. The protein profiles of each of the zones were the result of proteins that had been formed and released after transfer to the ring plate and proteins that had been formed before transfer and that slowly diffused out of the cell wall in which they had been trapped. [4, 7, Chapter 2] It was previously shown that cycloheximide treatment induces release of trapped proteins from the cell wall. [7, Chapter 2] The Δ*brlA* colonies showed a similar protein pattern as the wild-type in the absence and presence of cycloheximide during incubation on the ring plate (Figure 4AB). The *ΔflbA* (Figure 4C) colonies did show a different protein profile in the medium when

compared to wild-type colonies. Moreover, based on the SDS PAGE profiles, cycloheximide treatment did not increase release of protein in the medium of the *ΔflbA* strain. In fact, the amount of some of the proteins was reduced as shown by the lower intensity after staining the SDS PAGE gel.

Table 2. Number of representatives of protein classes in the secretome of 7-days-old xylose grown Δ*flbA* colonies. Numbers are compared to the proteins found in wild-type with or without cycoheximide treatment. [Chapter 2, 7]

	Δ*flbA*	wild-type[7]	wild-type cycloheximide treatment[7]
Cellulases	16	10	20
Xylanases	16	9	15
Mannosidases	1	0	2
Pectinases	7	5	6
Amylases	3	2	5
Other carbohydrates	3	0	6
Proteases	14	9	17
Phytases	1	1	4
Fungal cell wall remodeling	23	9	14
Other enzymes			
superoxide dismutases / oxidases	13	0	7
lipases	3	0	1
phospholipases	6	0	2
other	14	7	13
Unknown	18	6	8
Proteins without SigP	33	1	4
Total	**171**	**59**	**124**

Since the Δ*flbA* strain showed a different protein profile when compared to the wild-type, we decided to analyse the secretome of this mutant strain by quantitative MS/MS. To this end, 7-days-old xylose-grown colonies of the Δ*flbA* strain were transferred to a ring plate for 24 h. Proteins contained in each of the wells of the ring plate were isolated, concentrated, digested with lys-C and trypsin, and labeled with different isotopomeric dimethyl labels. [22] The addition of the light-dimethyl-label, the intermediate-dimethyl-label, and the heavy-dimethyl-label results in a mass shift of 28, 32, and 36 Da per primary amine of a peptide, respectively. [22] Labeled peptides of the three innermost zones 1, 2 and 3 (containing the light, intermediate and heavy label, respectively) were mixed and analyzed in a single LC-MS/MS run. Similarly, peptides of zones 3, 4, and 5 (containing the light, intermediate and heavy label, respectively) were mixed and analyzed by LC-MS/MS. By including the zone 3 sample in both runs, the peptides of each of the zones could be quantitatively compared. To prevent

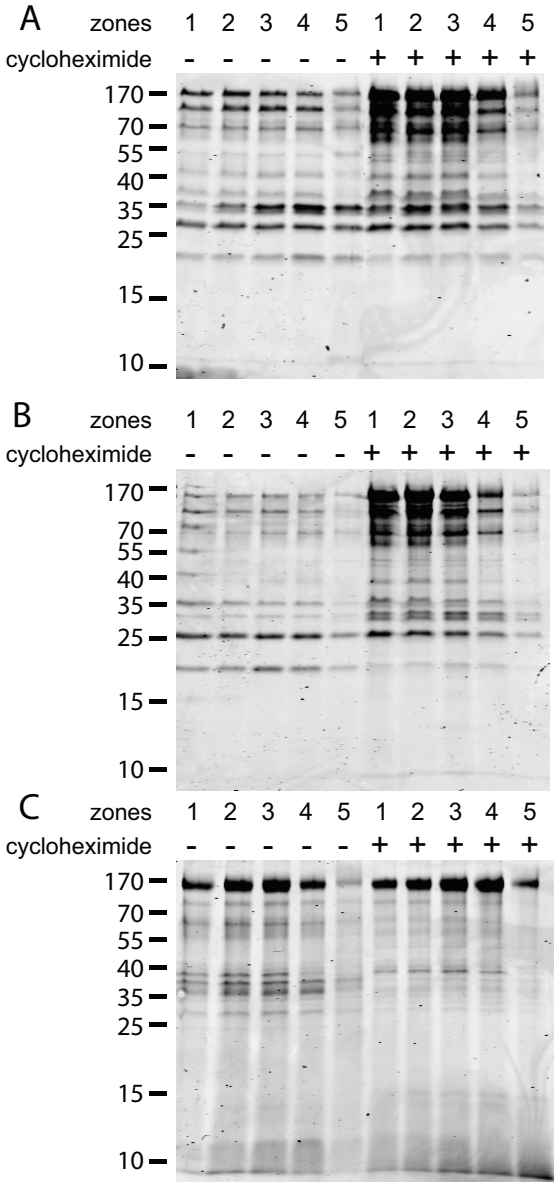


Figure 4: SDS-PAGE of the secretome of concentric zones of 7-days-old xylose-grown colonies of wild-type (A), Δ*brlA* (B), and Δ*flbA* (C) that had either (+) or not (-) been treated with cycloheximide. Gels were stained with coomassie brilliant blue. Zone 1 and 5 represent the most central and peripheral zone, respectively.

label-specific peptide modifications, a biological duplicate was performed with a label swap on the peptides of the different concentric zones (see Material and Methods). The peptides in the Δ*flbA* secretome were assigned to 723 sequences in the *A. niger* database (data not shown). Of these, 300 passed the filter criteria (see Material and Methods), and 171 of them were present in at least one of the zones of both biological duplicates. These 171 proteins were used for quantification (Supplemental Table 1). This selection comprised 138 proteins with a signal

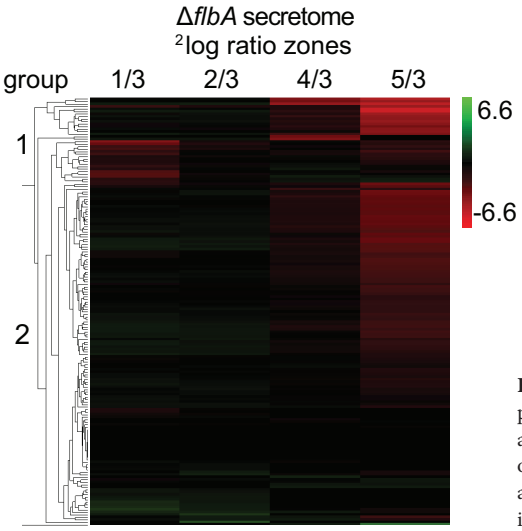


Figure 5: Heat map of ^{2}log ratios of secreted proteins in xylose grown Δ*flbA* colonies. Zone 1 and 5 represent the innermost and outermost zone of the colony, respectively. Green shading indicates a ^{2}log fold change of ≤2, whereas red shading indicates a ^{2}log fold change in ratio of ≤-2.

Table 3. Proteins identified in concentric zones of 7-days-old xylose grown Δ*flbA* colonies that had not been identified in the secretome of *A. niger* before.

Accession	Description
	(putative) cellulases
An15g04900	probable endoglucanase D eglD - Aspergillus niger
An11g06090	strong similarity to beta-glucosidase2 BGL2 - Saccharomycopsis fibuligera
An03g05380	similarity to cellulase FI-CMCase - Aspergillus aculeatus
	(putative) cell wall/ morphogenesis enzymes
An07g01160	strong similarity to cell wall protein Utr2 - Saccharomyces cerevisiae
An16g06120	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanosyltransferase gel3 - Aspergillus fumigatus
An03g06220	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanosyltransferase gel3 - Aspergillus fumigatus
	(putative) proteases
An07g03880	serine proteinase pepC - Aspergillus niger
An05g02170	strong similarity to serine-type carboxypeptidase F CPD-II - Aspergillus niger
	related to other enzymes
An02g08560	similarity to hypothetical dioxygenase SCOEDB - Streptomyces coelicolor
An03g05210	strong similarity to reticuline oxidase bbe1 - Eschscholzia californica
An11g06480	weak similarity to antigenic protein f86.aa from patent WO9859071 - Borrelia burgdorferi
An13g02510	strong similarity to allergen rAsp f 9 - Aspergillus fumigatus
An11g07730	similarity to mucin MUC4 - Homo sapiens
An10g00950	strong similarity to tannase precursor from patent JP08080196-A - Aspergillus oryzae
	related to unknown
An14g02350	weak similarity to protein belonging to the presenilin superfamily protein S182 - Mus musculus
An12g07430	similarity to homeostasis assocoiated protein sequence SEQ ID NO:380 from patent WO200100842-A2 - Corynebacterium glutamicum
An09g01580	weak similarity to RING finger protein rngB - Dictyostelium discoideum
An01g13600	strong similarity to mitochondrial phosphate transport protein Mir1 - Saccharomyces cerevisiae

sequence and 33 proteins that did not have a signal sequence for secretion (Table 2; Supplemental Table 1).[23, 24] SecretomeP predicted that 18 out of these 33 proteins contain a motif for non-classical secretion (Supplemental Table 2). Within the pool of proteins with a signal sequence, 101 had not been identified in the secretome of 7-days-old xylose-grown wild-type colonies [7, Chapter 2], and 18 had even never been reported to be part of the secretome of *A. niger* (Table 3; Supplemental Table 1).[7, Chapter 2, 24-26] On the other hand, 20 proteins were absent in the Δ*flbA* strain that were present in the secretome of 7-days-old xylose-grown wild-type colonies.[7, Chapter 2] Out of the 138 proteins with a signal sequence within the secretome of the Δ*flbA* strain, 51 proteins were released ≥ 4-fold lower in the peripheral zone 5 when compared to the intermediate zone 3 of the colony (Table 4; Figure 5; Supplemental Table 1). Out of 4 proteins that were less abundantly released in zone 4 when compared to zone 3, 3 were also released to a lower extent in zone 5. In the central zone 1, 4 proteins were released 4-fold lower compared to zone 3. In total, only 4 proteins (i.e. 1 in zone 1, 2 in zone 2, and 1 in zone 5) were 4-fold more abundant in another zone when compared to zone 3.

Table 4. Secreted proteins released in zones 1, 2, 4 and 5 of 7-days-old xylose grown sandwiched Δ*flbA* colonies that show a ^{2}log fold change ≥ 2 in abundance when compared to zone 3. Proteins are shown with a ^{2}log fold change ≥ 2 in one biological sample and at least a ^{2}log fold change ≥ 1 in the biological duplicate. Green and red shading indicate increased and decreased abundance respectively. y*,y**, and y indicate proteins found by SigP3.0 prediction[24] SigP2.0 prediction[23], and proteins found in both SigP databases, respectively.

Accession	Description - Table 4	^{2}log ratio zones				SigP
	xylanolytic enzymes - xylan	zone 1/3	zone 2/3	zone 4/3	zone 5/3	
An01g00780	endo-1,4-beta-xylanase B precursor xlnB - Aspergillus niger	0.1	1.0	0.6	-2.0	y
An01g09960	exo-1,4-beta-xylosidase xlnD - Aspergillus niger	0.1	0.3	-1.0	-2.9	y
An14g05800	alpha-glucuronidase aguA - Aspergillus niger	-0.1	0.0	-0.6	-2.5	y
An09g03300	strong similarity to alpha-xylosidase XylS - Sulfolobus solfataricus	1.0	0.8	-1.4	-6.3	y
	xylanolytic enzymes - xyloglucan xylan galactomannan					
An02g11150	alpha-galactosidase aglB - Aspergillus niger	0.7	0.7	-0.1	-2.0	y
An09g00260	alpha-galactosidase aglC - Aspergillus niger [truncated ORF]	0.3	0.1	-1.6	-3.1	y
An01g00330	alpha-N-arabinofuranosidase A precursor abfA - Aspergillus niger	1.1	0.6	-0.7	-2.7	y
An09g01190	endo 1,5-alpha-arabinanase abnA - Aspergillus niger	1.0	0.5	-0.6	-3.2	y
	putative xylan/ hemicellulose degrading enzymes					
An08g01900	strong similarity to xylan 1,4-beta-xylosidase - Butyrivibrio fibrisolvens	0.1	0.0	-0.3	-2.0	y
An17g00300	strong similarity to bifunctiona xylosidase-arabinosidase xarB - Thermoanaerobacter ethanolicus [putative frameshift]	0.6	0.3	-1.7	-4.5	y
	Cellulases					
An14g02760	endoglucanase A eglA - Aspergillus niger	0.8	0.7	0.0	-2.0	y
An07g09330	1,4-beta-D-glucan cellobiohydrolase A precursor cbhA - Aspergillus niger	0.3	-0.1	-1.0	-2.8	y
An18g03570	Probable beta-glucosidase A precursor bglA - Aspergillus niger	0.4	0.4	-1.0	-3.1	y
	putative cellulases					
An12g02220	Probable 1,4-beta-D-glucan cellobiohydrolase C - Aspergillus niger	-0.7	-0.2	-0.4	-2.8	y

Accession	Description - Table 4 *(continued)*	^{2}log ratio zones				SigP
		zone 1/3	zone 2/3	zone 4/3	zone 5/3	
An14g01770	strong similarity to beta-glucosidase bgln - Candida molischiana	0.9	0.6	-1.0	-2.1	y
An01g10930	strong similarity to enzyme with sugar transferase activity from patent JP11009276-A - Acremonium sp.	-0.4	0.1	-1.4	-3.6	y
An01g01540	strong similarity to alpha,alpha-trehalase treA - Aspergillus nidulans	0.7	0.5	-0.7	-2.0	y
	starch degrading enzymes					
An15g07800	strong similarity to precursor of alpha-amylase A - Aspergillus niger	0.5	0.4	-1.2	-2.8	y
An03g06550	glucan 1,4-alpha-glucosidase glaA - Aspergillus niger	0.9	0.7	-0.2	-2.1	y
	(putative) other carbohydrates					
An11g06540	beta-mannosidase mndA - Aspergillus niger	0.4	0.1	-0.6	-2.0	y
An07g07700	similarity to hypothetical glycosyl hydrolase BAC68337.1 - Streptomyces avermitilis	0.1	0.6	-0.9	-3.4	y*
Accession	**cell wall/ morphogenesis enzymes**					
An01g12450	strong similarity to hypothetical glucan beta-1,3 exoglucanase exgS - Aspergillus phoenicis	0.1	0.1	-0.6	-2.0	y
An01g11010	strong similarity to cell wall protein Crh1 - Saccharomyces cerevisiae	0.9	0.7	-0.6	-2.0	y
An11g01540	strong similarity to cell wall protein Crh1 - Saccharomyces cerevisiae	0.2	0.5	-0.4	-2.8	y
An09g02240	strong similarity to beta-N-acetylhexosaminidase from patent WO9839459-A1 - Penicillium chrysogenum	1.0	0.6	-2.0	-4.0	y
	putative cell wall/ morphogenesis enzymes					
An18g04100	probable glucan 1,3-beta-glucosidase A exgA	-0.2	0.1	-1.1	-2.9	y
An14g02100	strong similarity to cell wall antigen MP1 - Penicillium marneffei	-2.0	0.1	-0.7	-1.4	y
An01g03090	strong similarity to 1,3-beta-glucanosyltransferase gel1 - Aspergillus fumigatus	0.2	0.5	-0.9	-3.1	y
An02g03310	strong similarity to hypothetical phosphatidylglycerol/ phosphatidylinositol transfer protein pltpao - Aspergillus oryzae	-2.0	-0.6	-0.4	-1.6	y
An02g01550	strong similarity to secreted serine protease 19 kDa CS antigen CS-Ag - Coccidioides immitis	-0.2	0.2	-0.7	-2.3	y
An02g11620	strong similarity to hypothetical cell wall protein Afu3g08110 - Aspergillus fumigatus	-2.6	0.0	0.1	-0.7	y
	phytases					
An12g01910	strong similarity to phytase phyA3 - Aspergillus fumigatus	0.9	0.8	-0.8	-3.3	y
	Proteases					
An14g04710	aspartic proteinase aspergillopepsin I pepA - Aspergillus niger	0.3	-0.4	-1.0	-4.2	y
An08g04490	probable endoprotease Endo-Pro precursor EPR -Aspergillus niger	0.0	-0.1	-0.2	-2.2	y
An03g05200	strong similarity to carboxypeptidase S1 - Penicillium janthinellum	0.7	0.4	-0.9	-2.5	y
An02g13750	strong similarity to glutaminase A gtaA - Aspergillus oryzae	0.2	0.3	-1.0	-4.3	y
An02g07210	aspartic protease pepE - Aspergillus niger	0.0	0.0	-0.9	-3.0	y
An07g03880	serine proteinase pepC - Aspergillus niger	-2.7	-0.4	0.2	-0.1	y
	putative proteases					
An03g01660	strong similarity to vacuolar aminopeptidase Y Ape3 - Saccharomyces cerevisiae	-0.5	-0.1	-0.7	-2.1	y
	related to other enzymes					
	superoxide/dismutases					
An01g01550	strong similarity to catalase cat1 - Aspergillus fumigatus	0.4	0.1	-1.0	-6.3	y
An01g14740	glucose oxidase precursor goxC - Aspergillus niger [putative sequencing error]	2.0	1.0	0.0	-0.5	y
An08g00490	strong similarity to lactonohydrolase - Fusarium oxysporum	0.4	0.3	-1.0	-3.0	y*

Accession	Description - Table 4 *(continued)*	²log ratio zones				SigP
		zone 1/3	zone 2/3	zone 4/3	zone 5/3	
	(putative) Lipases					
An16g08870	strong similarity to triacylglycerol lipase lipl - Geotrichum candidum	0.5	0.5	-1.2	-4.6	y
	(putative) phospholipases					
An16g03700	strong similarity to phospholipase B from patent US6146869-A - Aspergillus oryzae	0.0	0.0	-0.7	-2.5	y
An02g13220	strong similarity to lysophospholipase phospholipase B - Penicillium notatum	0.8	1.0	-0.6	-2.6	y
	related to other enzymes					
An10g00790	similarity to hypothetical protein SCE6.29 - Streptomyces coelicolor	0.1	0.2	-0.9	-2.9	y
An02g09090	strong similarity to mutarotase enzyme from patent JP07039380-A - Sus scrofa	0.4	0.3	-1.0	-2.7	y
An01g06280	strong similarity to IgE-binding protein - Aspergillus fumigatus	1.0	2.0	0.0	0.0	y
An11g07730	similarity to mucin MUC4 - Homo sapiens	-0.4	0.2	-1.0	-4.2	y**
An03g05260	similarity to chitosanase csnA - Aspergillus oryzae	-0.3	-0.1	-0.1	-2.0	y
An07g08640	strong similarity to mutanase mutA - Penicillium purporogenum	1.0	1.1	-4.5	-5.8	y
An10g00950	strong similarity to tannase precursor from patent JP08080196-A - Aspergillus oryzae	0.0	2.9	0.0	0.0	y
An02g06720	strong similarity to glutaminase A gtaA - Aspergillus oryzae	-0.4	0.4	-1.0	-4.9	y
	related to unknown					
An10g00800	strong similarity to purine nucleoside permease NUP - Candida albicans	0.4	0.3	-0.7	-2.1	y
An06g00160	strong similarity to hypothetical protein AN1813.2 - Aspergillus nidulans	1.0	0.9	-1.1	-2.6	y
An04g08730	strong similarity to hypothetical conserved protein SPAC12B10.16c - Schizosaccharomyces pombe	0.2	0.2	-0.8	-2.2	y
An07g02730	strong similarity to SUN family protein su1p - Schizosaccharomyces pombe	0.0	0.0	0.9	4.1	y
An09g01580	weak similarity to RING finger protein rngB - Dictyostelium discoideum	0.0	0.0	-3.4	0.0	y*
An14g04240	strong similarity to hypothetical conserved protein CC0533 - Caulobacter crescentus	0.1	0.3	-0.8	-2.2	y*
An04g07160	similarity to hypothetical protein MLD14.3 - Arabidopsis thaliana	0.0	0.0	-3.6	-4.5	y

The 138 proteins with a signal sequence in the secretome of 7-days-old xylose-grown Δ*flbA* colonies comprised 46 proteins involved in carbohydrate degradation (Table 2). They are involved in hydrolysis of cellulose (16), xylan (16), pectin (7), starch (3) and other carbohydrates (4). Out of the 46 proteins involved in carbohydrate degradation, 21 were ≥ 4-fold less abundant in zone 5 when compared to all other zones (Table 4). The products of 15 out of the 16 XlnR-regulated genes were found in the culture medium of 7-days-old xylose-grown colonies of the Δ*flbA* strain (Table 5). Only the endo-glucanase EglB (An16g06800) was not identified in the secretome. Unlike the wild-type secretome [7] products of the genes regulated by XlnR were homogenously released in the various zones of the colony. For instance, the endoxylanases XlnB, XlnC, and XlnD, the acetyl xylan esterase AxeA, the α-glucuronidase AguA, the arabinofuranohydrolase AxhA, the arabinofuranosidase AbfA, and the α-arabinase AbnA are equally abundant in central zones 1-4. Only in the peripheral zone 5, a 4-fold lower amount of protein is seen (Table 5; Supplemental Table 1). This can be explained by the fact that hyphae colonized this zone during the transfer to the ring plate, thus yielding a

Table 5. Presence of proteins encoded by XlnR regulated genes in the secretome of 7-days-old xylose-grown Δ*flbA* colonies. Numbers are compared to the proteins found in wild-type *A. niger* colonies that had either or not been treated with cycloheximide. [Chapter 2, 7]

Accession	Description	Δ*flbA*	wild-type[7]	wild-type cycloheximide treated[7]
	Xylanases			
An01g00780	endo-1,4-xylanase xlnB - *Aspergillus niger*	x	x	x
An03g00940	endo-1,4-beta-xylanase A precursor xlnC - *Aspergillus niger*	x		x
An01g09960	xylosidase xlnD - *Aspergillus niger*	x	x	x
	Accessory enzymes			
An12g05010	acetyl xylan esterase axeA - *Aspergillus niger*	x	x	x
An03g00960	1,4-beta-D-arabinoxylan arabinofuranohydrolase axhA - *Aspergillus niger*	x	x	x
An01g00330	alpha-l-arabinofuranosidase a precursor abfA - *Aspergillus niger*	x	x	x*
An15g02300	arabinofuranosidase B abfB - *Aspergillus niger*	x	x	x
An14g05800	alpha-glucuronidase aguA - *Aspergillus niger*	x		x
An02g11150	alpha-galactosidase aglB - *Aspergillus niger*	x	x	x
An01g12150	beta-galactosidase lacA - *Aspergillus niger*	x	x	x
An09g00120	ferulic acid esterase A faeA - *Aspergillus niger*	x		x
An14g02760	endoglucanase A eglA - *Aspergillus niger*	x	x	x
	Hemicellulolytic and cellulolytic enzymes			
An16g06800	strong similarity to endoglucanase eglB - *Aspergillus niger*		x	x
An03g05290	glucan endo-1,3-beta-glucosidase eglC - *Aspergillus niger*	x	x	x
An07g09330	1,4-beta-D-glucan cellobiohydrolase A precursor cbhA - *Aspergillus niger*	x	x	x
An01g11660	1,4-beta-D-glucan cellobiohydrolase B precursor cbhB - *Aspergillus niger*	x	x	x
	putative XlnR regulated			
An01g11670	strong similarity to endo-beta-1,4-glucanase A eglA - *Aspergillus nidulans*	x		x
An01g14600	endo-1,4-beta-xylanase precursor - *Aspergillus niger*	x	x	x

low density mycelium in this part of the colony.

The majority (i.e. 92 out of 138) of the secreted proteins in the secretome of 7-days-old xylose grown Δ*flbA* colonies are involved in other processes than plant polysaccharide degradation. These proteins include a phytase (1), proteases (10), superoxide dismutases and oxidases (13), lipases (3), phospholipases (6), enzymes involved in cell wall synthesis and morphogenesis (23), other enzymes (14) or enzymes with unknown function (18). One protein (An10g00950) with unknown function is found 8-fold higher in the center of Δ*flbA* (Table 4). Other proteins (39) are found in relatively low amount in the periphal zone 5 as shown by a negative ^{2}log ratio in 5/3.

Influence of cycloheximide on the cell wall of Δ*flbA* hyphae

The presence of cycloheximide results in thinner cell walls in the central zones of a 7-days-old wild-type colony. In contrast, the peripheral zones of the colony

showed thicker cell walls compared to untreated colonies.[7, Chapter 2] Here, the effect of cycloheximide on the cell wall of 7-days-old colonies of the Δ*flbA* strain was studied with transmission electron microscopy. The diameter of hyphae at the periphery and at the center of a Δ*flbA* colony that had or had not been treated with cycloheximide was not statistically different. The diameter of the hyphae in the centre of Δ*flbA* colonies was 3.5 ± 0.16 µm and 3.1 ± 0.15 µm in the absence and presence of cycloheximide, respectively. These values were 3.9 ± 0.27 µm and 3.6 ± 0.17 µm for hyphae at the periphery of the Δ*flbA* colony (Figure 6). Cycloheximide treatment did also not affect the width of the cell wall at the colony center. The width was 101.0 ± 5.9 and 118.6 ± 7.9 nm with and without cycloheximide treatment. In contrast, cell walls at the periphery of untreated colonies were thinner than those that had been treated with cycloheximide (91.0 ± 4.9 nm and 120 ± 9.8 nm, respectively).

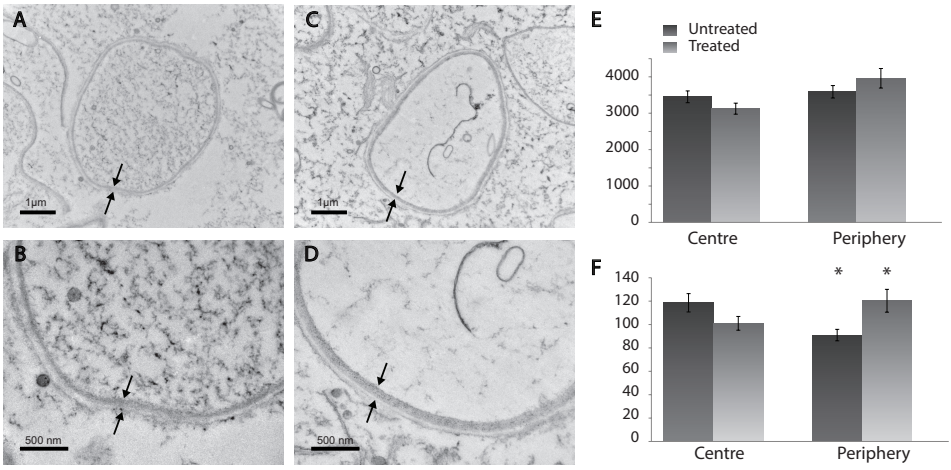


Figure 6. Transmission electron microscopy of hyphae from the center of a Δ*flbA* colony that had not (A, B) or had (C, D) been treated with cycloheximide and analysis of hyphal diameter (E) and width of cell walls (F) of hyphae from the center and the periphery of a Δ*flbA* colony. Bars in A, B and C, D represent 1 µm and 500 nm, respectively. Black arrows indicate the outer and inner parts of the cell wall. Hyphal diameter and cell wall width in E and F are given in nm. * represents significant difference, $p \leq 0.05$.

DISCUSSION

Colonies of *A. niger* are heterogeneous with respect to growth and secretion. Until recently it was believed that only growing hyphae secrete proteins. This was partly based on the finding that protein secretion and growth were mainly observed at the periphery of sandwiched colonies.[6] Levin et al.[3] and this study, however, showed that protein secretion can also occur in non-growing zones of a sandwiched colony. Growth was still localized at the periphery when 7-days-old xylose-grown colonies were transferred for 24 hours to fresh medium, while proteins were secreted in the center as well. Notably, the non-growing sub-peripheral zone (zone 4) still did not secrete proteins. Here, we showed that this

zone started to sporulate when the upper membrane of the sandwiched colony was removed. This indicates that sporulation represses secretion of the vegetative mycelium. To test this hypothesis spatial secretion of proteins was studied in *A. niger* strains that had a deletion of the sporulation genes *flbA* or *brlA*. It was shown that inactivation of *flbA,* but not *brlA,* affects the spatial secretion pattern and that the secretome of the Δ*flbA* strain is more complex when compared to the wild-type. These results indicate that the Δ*flbA* strain is a good candidate strain for industrial fermentations.

Asexual development of aspergilli has been best studied in *A. nidulans.* Experimental evidence has shown that mechanisms underlying this developmental process in *A. fumigatus* and *A. oryzae* are similar but not identical to that of *A. nidulans*.[23, 27-29] Until now, no experimental data were available for the regulatory program underlying asxual reproduction in *A. niger*. Its genome sequence predicts that this program is similar to that of *A. nidulans*.[23] In *A. nidulans*, the Δ*brlA* strain forms stalks that do not stop their growth after they have reached a length of 100 μm. In contrast, the Δ*brlA* strain of *A. fumigatus* does not initiate conidiophore development at all.[29] We here showed that the *A. niger* Δ*brlA* strain has a similar phenotype with respect to asexual reproduction when compared to *A. nidulans* This indicates that BrlA acts earlier in asexual development in *A. fumigatus* when compared to *A. nidulans* and *A. niger*. Deletion of *flbA* in *A. nidulans*, *A. oryzae*, and *A. fumigatus* results in a low *brlA* expression, abundant formation of aerial hyphae and absence of conidiation.[13, 16, 27-30] Inactivation of *flbA* in *A. niger* also resulted in abundant aerial hyphae and abolished formation of conidiophores.
Location of growth and secretion was similar in the Δ*brlA* strain when compared to the wild-type strain of *A. niger.* In contrast, more zones of the colony were active in growth and protein secretion in the Δ*flbA* strain when compared to the wild-type. Colonies of the Δ*flbA* strain that had not been transferred to fresh medium secreted proteins both at the periphery and in the colony center. Thus, the spatial release of proteins was similar to that of wild-type and Δ*brlA* colonies that had been transferred to fresh medium. Transferred Δ*flbA* colonies on the other hand secreted proteins throughout the colony. Mass spectrometry analysis showed that the differences in quantitiative composition of the secretome of the four inner zones of the Δ*flbA* colonies are smaller than that observed in the wild-type.[7, Chapter 2] The peripheral zone 5 of the Δ*flbA* colony secreted less protein but this can be explained by the fact that the mycelium had not fully colonized this part of the medium. Taken together, it is concluded that *flbA* has a role in heterogeneity of growth and protein secretion in *A. niger* colonies. This is not the case for *brlA*. This can be explained by the fact that *brlA* acts more downstream in the developmental pathway of sexual reproduction.

Inactivation of *flbA* resulted in a different protein secretion profile when compared to the wild-type and the Δ*brlA* strain. This change in the protein profile may be partly due to pH changes. The Δ*flbA* mutant maintained its pH at 6 during

the 24 h transfer on the ring plate. In contrast, the pH dropped to as low as 2 in the case of xylose-grown wild-type colonies (data not shown). However, the differences in the secretome cannot fully be attributed to changes in pH. This is based on the finding that α-galactosidase, cellobiohydrolase, and xylosidase activity were higher in the Δ*flbA* strain than in the wild-type when the pH of the medium was kept at pH 5.0 by using a stronger buffer (data not shown).

The secretome of Δ*flbA* colonies consisted of 138 protein with a signal sequence. Of these, 18 had not been reported in any secretome study[Chapter 2, 7, 24, 26, 31, 32] and 101 had not been identified in the secretome of 7-days-old xylose grown wild-type colonies that had not been treated with cycloheximide.[7, Chapter 2] Cycloheximide not only inhibits protein synthesis, it also induces partial cell wall degradation.[7, Chapter 2] As a consequence, secreted proteins that are associated to the cell wall of the wild-type strain are effectively released. Indeed, cycloheximide treatment reduced the number of proteins absent in the wild-type secretome [Chapter 2, 7] but present in the Δ*flbA* strain from 101 to 70. For instance, the products of the XlnR regulated genes *xlnC, aguA* and *faeA* were absent in the secretome of wild-type colonies that had not been treated with cycloheximide but were found in the secretome of the Δ*flbA* strain and cycloheximide treated wild-type colonies.[7, Chapter 2] These data suggest that absence of FlbA affects the cell wall. Indeed, the cell wall of hyphae of the Δ*flbA* strain is thinner than that in the wild-type, especially in the colony centre. In the case of peripheral hyphae the cell wall of the Δ*flbA* strain is 91 nm, while it is 106 nm in the case of the wild-type. [7, Chapter 2] These values are 119 nm and 205 nm for hyphae in the center, respectively. Moreover, unlike the wild-type,[7, Chapter 2] cycloheximide treatment did not affect the width of the cell wall in the centre of Δ*flbA* colonies. Of interest, glucanosyltransferase GelD and BgtE, both annotated as homologues of cell wall morphogenesis and synthesis proteins, were found in the secretome of the Δ*flbA* mutant but not in the wild-type. These proteins may affect cell wall composition of the mutant strain.

Submerged and aerial hyphae of the Δ*flbA* strain of *A. nidulans* go in autolysis when colonies get older. [14, 16, 27, 28] However, this was not observed in *A. fumigatus*. [29] We also do not have definite proof for autolysis in sandwiched cultures of the *A. niger* Δ*flbA* strain. The secretome of the *A. niger* Δ*flbA* strain contained 33 proteins without a signal sequence, of which 18 were predicted to be released via the non-classical secretion pathway. So, only a limited number of intracellular proteins were observed in the culture medium. On the other hand, 3 predicted intracellular proteases were contained in the secretome of the Δ*flbA* strain that were absent in the wild-type. These proteases (i.e. lysine aminopeptidase apsA (An04g03930), dipeptidyl peptidase III (An04g00410), and vacuolar aminopeptidase Ysci (An09g06250) may point to autolytic activity. However, a semi-tryptic Mascot search assigned only twelve extra proteins (i.e. 8.5 %) in the culture medium. This indicates that proteinases are not highly active on the proteins in the culture medium of the Δ*flbA* strain. The most strong indication for

autolysis is the presence of chitinase *chiB* (An02g07020) in the secretome of the *A. niger* Δ*flbA* strain. This enzyme has been related to autolysis of the mycelium of the Δ*flbA* strain in *A. nidulans*.[33] Inactivation of *chiB* in *A. nidulans* did not suppress the developmental defects of the Δ*flbA* strain but abolished autolysis. It would be of interest to assess whether inactivation of *chiB* also reduces release of intracellular proteins in the Δ*flbA* strain of *A. niger* and whether the Δ*flbA*Δ*chiB* strain is a preferred strain for use as a cell factory.

ACKNOWLEDGEMENTS

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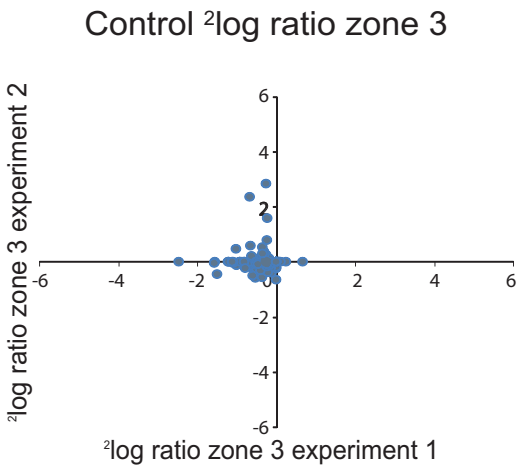
ASSOCIATED CONTENT

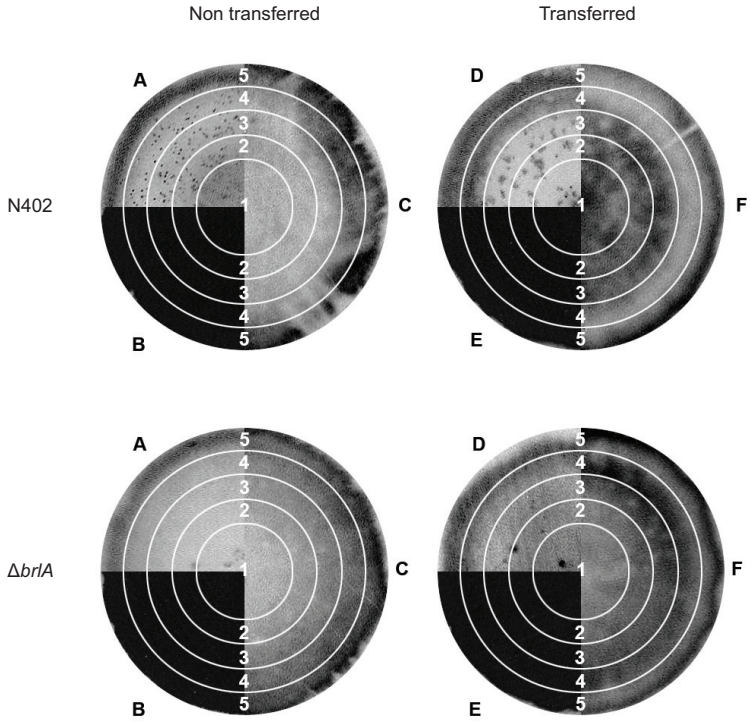
Supplemental Table 1. Quantitative analysis of the secretome of concentric zones of sandwiched colonies of the Δ*flbA* strain of *A. niger*. This material is available free of charge via the Internet at http://pubs.acs.org.

Supplemental Table 2. Proteins of the secretome of 7-days-old xylose grown sandwiched colonies of the Δ*flbA* strain of *A. niger* that are predicted to be released into the culture medium via the non-classical secretion pathway by the SecretomeP prediction tool. The abundance (expressed as the ^{2}log fold change) of these proteins in zones 1, 2, 4 and 5 is related to zone 3. Green and red shading indicate increased and decreased abundance, respectively, when compared to zone 3.

Accession	Quantitative analysis Δ*flbA* secretome	^{2}log ratio zones			
	related to non-classical secreted proteins	zone 1/3	zone 2/3	zone 4/3	zone 5/3
An02g07020	chitinase chiB - Aspergillus niger	0.1	0.2	-3.9	-5.4
An12g00240	strong similarity to translational inhibitor uk14 - Homo sapiens [truncated ORF]	-0.5	0.0	-0.5	-2.2
An01g00370	aspergillopepsin apnS - Aspergillus niger	0.0	0.0	0.0	0.0
An09g00270	alpha-galactosidase aglC - Aspergillus niger [truncated ORF]	0.2	0.3	-1.0	-3.1
An02g01830	cytochrome c cyc - Aspergillus niger	0.0	0.0	-0.7	-2.3
An09g02830	strong similarity to acylaminoacyl-peptidase DPP V - Aspergillus fumigatus	0.7	0.7	-3.9	0.0
An15g05960	strong similarity to hypothetical protein Afu2g15710 - Aspergillus fumigatus	-1.3	-0.4	-0.9	-3.4
An11g01120	strong similarity to NADPH-dependent aldehyde reductase - Sporobolomyces salmonicolor	-1.5	-0.1	0.7	1.1
An12g08570	similarity to type 2 peroxiredoxin PrxII - Brassica napus	-2.6	-0.3	1.0	0.5
An04g06510	strong similarity to polyubiquitin 5 Ubi4 - Saccharomyces cerevisiae	-3.3	-1.1	-0.1	-1.2
An01g02500	strong similarity to thioredoxin - Aspergillus nidulans	-2.3	-0.8	0.0	-1.1
An02g08660	strong similarity to hypothetical protein H04M03.4 - Caenorhabditis elegans	-0.8	-0.5	0.2	-1.5
An16g08760	similarity to zinc metalloprotease ZmpB from patent WO9964610-A1 - Streptococcus pneumoniae	0.0	0.0	-0.4	-2.4
An07g05830	strong similarity to formamidase fmdS - Aspergillus nidulans	-0.8	0.0	0.0	0.0
An03g06870	strong similarity to cytidine deaminase - Homo sapiens	-1.9	-1.0	-1.0	-4.7
An14g02250	strong similarity to flavocytochrome b2 L-lactate dehydrogenase CYB2 - Pichia anomala	0.0	0.0	0.1	0.0
An18g04590	strong similarity to rho GDP dissociation inhibitor Rdi1 - Saccharomyces cerevisiae	0.0	0.0	1.0	1.0
An07g01100	similarity to glutathione S-transferase omega - Sus scrofa	0.0	0.0	-0.4	0.0



Supplemental Figure 1: ^{2}log ratios of the technical duplicate of proteins of zone 3 as obtained by labeling the peptides with two different dimethyl labels



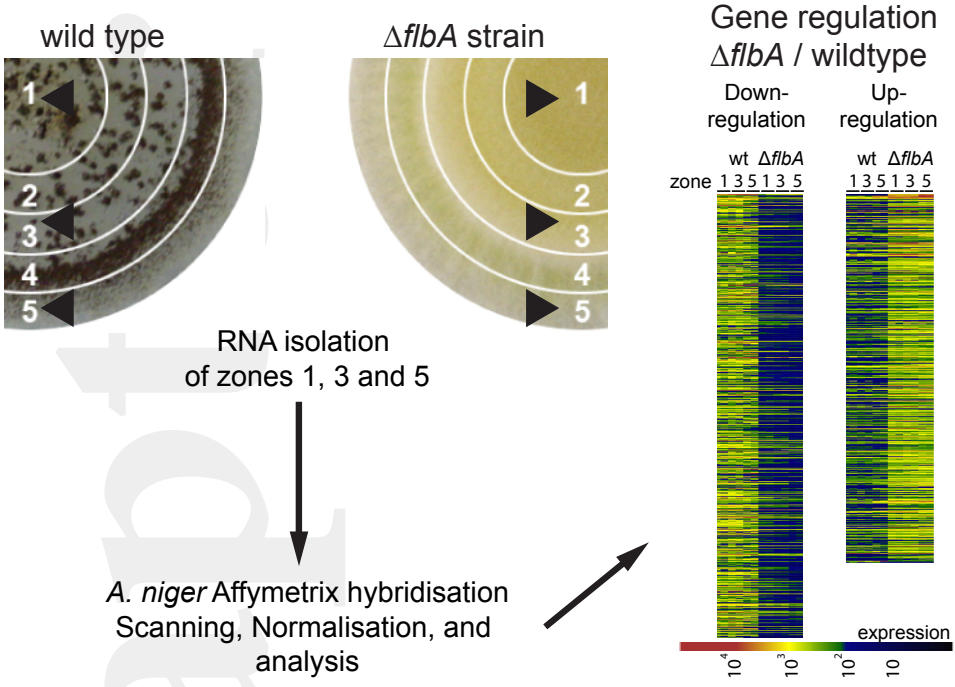
Supplemental Figure 2. Growth, (ADGJ), protein synthesis (BEHK), and secretion (CFIL) in 7-days-old xylose-grown wild-type (A-F) and Δ*brlA* (G-L) colonies. Colonies were analyzed before (ABC, GHI) and after transfer (DEF, JKL) to fresh xylose medium. Growth was detected with ^{14}C-labeled N-acetylglucosamine. Protein synthesis and secretion were monitored by incorporation of ^{14}C-labeled amino acids. Secreted proteins were immobilized by a PVDF membrane that had been placed underneath the colony.

Chapter 4

Transcriptome analysis of zones of colonies of the Δ*flbA* strain of *Aspergillus niger*

This Chapter is based on Krijgsheld P and Wösten HAB. Transcriptome analysis of zones of colonies of the Δ*flbA* strain *of Aspergillus niger. Fungal Genomics & Biology* Submitted.

Graphical Abstract



ABSTRACT

Wild-type colonies of *Aspergillus niger* grow and secrete enzymes at their periphery when they are grown continuously on an agar medium. It has been shown that sporulation inhibits secretion in such colonies. Inactivation of the sporulation gene *flbA* results in colonies that not only secrete proteins at their periphery but also in central zones. This is accompanied by a more complex secretome, growth throughout the mycelium and by thinner cell walls. Here, gene expression was studied at the periphery, an intermediate zone, and the centre of wild-type and Δ*flbA* colonies using whole genome microarrays. Heterogeneity in gene expression was not reduced in the Δ*flbA* colonies when compared to wild-type colonies despite decreased heterogeneity in zonal secretion and growth. It was shown that 1152 genes had a fold change in expression ≥ 2 when the averaged expression profiles of the zones of the wild-type were compared with those of the Δ*flbA* colonies. This gene set contained 13 genes predicted to be involved in reproduction, 12 genes involved in cell wall biosynthesis, modification and degradation, 345 genes encoding secreted proteins, and 38 genes encoding transcriptional regulators. These genes may account for the differences between wild-type and Δ*flbA* colonies in zonal growth and secretion, the complexity of the secretome and the thickness of the cell wall. The set of differentially expressed genes, in particular the genes encoding transcriptional regulators, may be instrumental to improve *Aspergillus niger* as a cell factory for the production of enzymes.

INTRODUCTION

Aspergillus species are among the most abundant fungi worldwide. They feed on a large variety of organic substrates, in particular on plant material.[1] To this end, enzymes are secreted that degrade the organic polymers within the substrate into small molecules that can be taken up as nutrients. The capacity of *Aspergillus* species to secrete proteins is enormous. For instance, some strains of *Aspergillus niger* secrete more than 30 grams per liter of glucoamylase.[2] This and the fact that a variety of secreted enzymes of aspergilli are used in the industry or as pharmaceutical proteins makes these fungi important cell factories.

Aspergilli form colonies that consist of hyphae that grow at their tips and that branch subapically.[3, Chapter 1] Growth and protein secretion mainly occur at the periphery of the colony when they are grown continuously on an agar medium.[4, 5] Hyphae at the periphery of the colony are thus exposed to unexplored organic

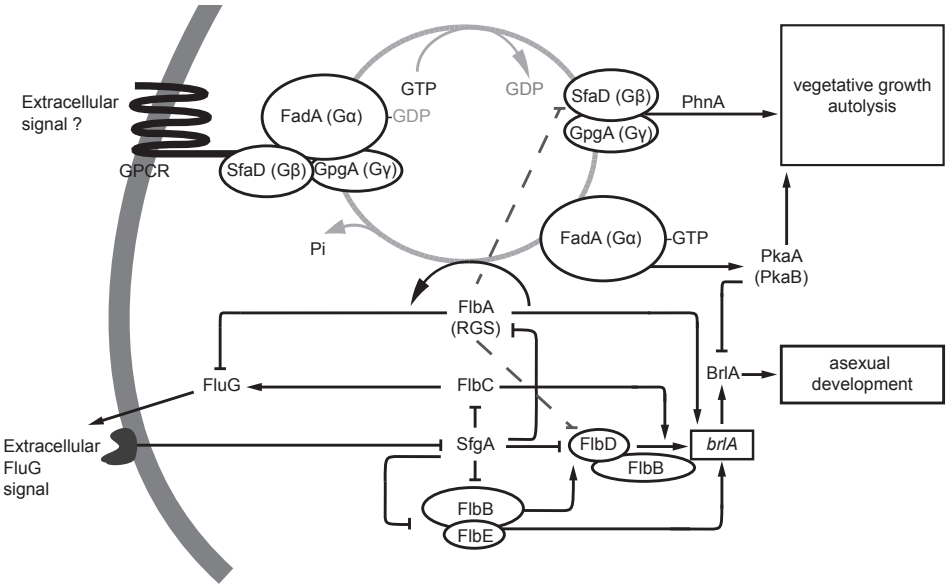


Figure 1. Model of the role of FlbA in the sporulation pathway of Aspergillus. FlbA is an RGS protein regulating signaling of the Gα-subunit FadA. GTP-bound FadA stimulates vegetative growth. FlbA enhances the intrinsic activity of GTP hydrolysis, converting FadA-GTP to the inactive hetrotrimeric FadA-SfaD-GpgA GDP-bound G-protein complex. FlbA also inhibits *fluG* and activates *brlA*. The latter may be a direct effect or the result of inactivation of FadA. Stippled lines represent regulation of FlbA in *A. niger* based on the transcriptional analysis described in this study. (Adapted from 3).

material, whereas hyphae in the intermediate and central zones are confronted with a (partly) utilized substrate. The composition of the substrate explains about 50 % of the variation in gene expression between different zones of the colonies.[6] The other half of the variation is caused by differentiation processes in the vegetative mycelium.

Recently, it was shown that zones that have the capacity to sporulate hardly secrete proteins.[7, Chapter 2] This suggested that secretion by vegetative hyphae is repressed by the sporulation process. Indeed, a Δ*flbA* strain that does not sporulate, not only secrete proteins at the periphery of the colony but also within central zones of the mycelium. This is accompanied by a secretome with increased complexity, a reduced width of cell walls and growth throughout the mycelium.[8, Chapter 3] Gene *flbA* encodes an RGS domain protein that stimulates the intrinsic GTPase activity of the Gα-subunit FadA.[9] This Gα-subunit is part of a heterotrimeric G-protein complex that also consists of the Gβ- and Gγ-subunits SfaD and GpgA, respectively (Figure 1). The activated Gα-subunit and the Gβ-Gγ dimer both stimulate vegetative growth. FlbA converts the active Gα-subunit-GTP into its inactive GDP bound stage, thereby repressing vegetative growth and promoting asexual development.[10-12] Taken together, these data indicate that FlbA stimulates sporulation and at the same time represses vegetative growth and secretion by the vegetative mycelium. Moreover, it promotes cell wall synthesis, thus increasing the width of this outer layer of hyphae. The underlying molecular mechanisms are not yet known.

Here, the impact of inactivation of *flbA* on spatial gene expression in the *A. niger* colony was assessed. A total of 1152 genes had changed their expression ≥2 fold when RNA profiles of wild-type and Δ*flbA* colonies were compared. This set includes genes involved in reproduction and cell wall synthesis, and genes encoding transcriptional regulators and secreted proteins and is of interest to improve *A. niger* as a cell factory.

MATERIAL AND METHODS

Plasmids, fungal strains and growth conditions

The wild-type *A. niger* strain N402[13] and its derivative N402Δ*flbA*[8, Chapter 3] were used in this study. These strains were grown as sandwiched colonies between porous polycarbonate membranes (0.1 µm pores, 76 mm diameter; Profiltra, Almere, The Netherlands) placed on top of solidified minimal medium with 25 mM xylose as a carbon source.[4-6] Cultures were inoculated with a 1 mm mycelial plug and grown at 30 °C .

RNA preparation and analysis

RNA was isolated from a biological duplicate of concentric zones of 7-days-old colonies. Zone 1 represented the most central zone, zone 3 an intermediate zone, and zone 5 the peripheral zone.[6] Mycelium of zones was harvested from three colonies, frozen in liquid nitrogen, and ground with a TissueLyser (Qiagen, Venlo, The Netherlands) in a 2 ml Eppendorf tube with two metal balls (4.76 in diameter) for 1 min at 25 Hz. The frozen material was taken up in 1 ml TRIzol reagent (Invitrogen, Bleiswijk, Netherlands) by vortexing. Samples were incubated for 2 min after mixing with 200 µl chloroform. This was followed by centrifugation at

10000 g for 10 min. RNA was purified using an RNA clean up column (Machery Nagel, Düren, Germany) after addition of 1 volume 70 % EtOH to the water phase. After loading the sample on the column, it was centrifuged for 30 sec at 10000 rpm. This was followed by addition of 600 µl RA3 buffer (provided by the RNA-clean up kit). After 2 min of centrifugation at 10000 g, 250 µl RNA3 was added, followed by another 2 min centrifugation at 10000 g. RNA was eluted after a 10 min incubation in two steps with 40 µl and 50 µl RNAse free water. The eluted RNA was pooled and 1 µl was checked for concentration and purity using the nanodrop ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, USA) and the Bioanalyser 2100 (Agilent Technologies, Santa Clara, USA), respectively.

cDNA labeling, microarray hybridization and data analysis

cDNA labeling, microarray hybridization, and scanning were performed at ServiceXS (Leiden, The Netherlands) according to Affymetrix protocols. From each RNA sample 100 ng was used to synthesize biotin-labeled cRNA with the Affymetrix 3' IVT-Express Labeling Kit. Quality of the cRNA was checked with the nano-lab-on-a chip Bioanalyzer 2100 (Agilent Technology, Santa Clara, CA, USA). 15 µg cRNA was fragmented and half of it was used for hybridization on Affymetrix *A. niger* GeneChips. After an automated process of washing and staining, absolute values of expression were calculated from the scanned array using the Affymetrix Command Console v1 software. The Affymetrix probe sets on the chip represent 14,259 annotated ORFs and genetic elements of *A. niger* (see [14]; GEO [15] under accession no. GPL6758). The *A. niger* array data of this study have been deposited in the GEO database under accession number GSE44391. MAS5.0 was used to determine the number of expressed genes in colonies and its zones. Absent/present calls showed that on average 50 % and 48 % of the genes were expressed in colonies of the wild-type and the Δ*flbA* strain, respectively. Genedata Expressionist and Genedata Analyst were used for normalization and statistical analysis of the arrays (Genedata, Basel, Switzerland). The arrays were condensed with the RMA algorithm and normalized on the quantile. Statistical assessment of differential expression was performed with *t*-test ($p \leq 0.01$, using a false discovery rate (BHQ) of ≤ 0.05) or by ANOVA with $p \leq 0.01$ and a BHQ ≤ 0.05. Differentially expressed genes (change in expression $\geq$ 2-fold) were clustered using K-Means clustering. A differentially expressed set of genes obtained by ANOVA was used for a maximal paired contrast analysis to find the zone or strain where the highest mean-difference was observed. The Functional Catalogue (FunCat, Munich Information Center for Protein Sequence, Munich, Germany)[16] was used for functional classification of genes. Fisher's exact test was used to identify over- or under-represented functional classes in the sets of differentially expressed genes ($p \leq 0.001$, BHQ $\leq$ 0.05).

RESULTS

Five concentric zones can be distinguished in 7-days-old sandwiched colonies of *A. niger*.[4, 5] Zone 1, 3, and 5 represent the most inner zone, an intermediate zone, and the most outer zone of the colony, respectively. Gene expression was assessed in these three zones of xylose-grown colonies of the wild-type and the Δ*flbA* strain of *A. niger* using Affymetrix microarrays. Principal component analysis (PCA) showed that the transcriptomes of the wild-type zones and those of the Δ*flbA* strain cluster in the first component of the analysis. This component accounts for 54 % of the variation in the datasets (Figure 2). The second component of the analysis accounted for 27 % of the variation and separates the transcriptomes from zones 1 and 3 of the wild-type and the Δ*flbA* strain from zone 5 of these strains (Figure 2). Expression in the central zone 1 and the intermediate zone 3 of the wild-type correlated to a high extent (Pearson's $r^2 \geq 0.97$) when compared to the duplicates of these zones (Pearson's $r^2 \geq 0.97$ and ≥ 0.98, respectively). Wild-type zone 5 was more distinct from zone 3 and 1 (Pearson's $r^2 = 0.91$ and 0.93). The expression profile of zone 5 was also most different within the Δ*flbA* colony (Figure 2). The fact that zone 5 has the most distinct expression profile was also illustrated by the number of genes with a fold change in expression ≥ 2 between the zones of wild-type and Δ*flbA* colonies (Figure 3). The number of differentially expressed genes between zone 1 and zone 3 of the wild-type colony was 5, between zone 3 and zone 5 119, and between zone 1 and zone 5 325. These numbers were

A

zones	WT-1	WT-3	WT-5	ΔflbA-1	ΔflbA-3	ΔflbA-5
WT-1	0.97	0.98	0.91	0.92	0.91	0.86
WT-3		0.98	0.93	0.91	0.90	0.86
WT-5			0.98	0.89	0.89	0.90
ΔflbA-1				0.99	0.99	0.95
ΔflbA-3					0.99	0.97
ΔflbA-5						1.00

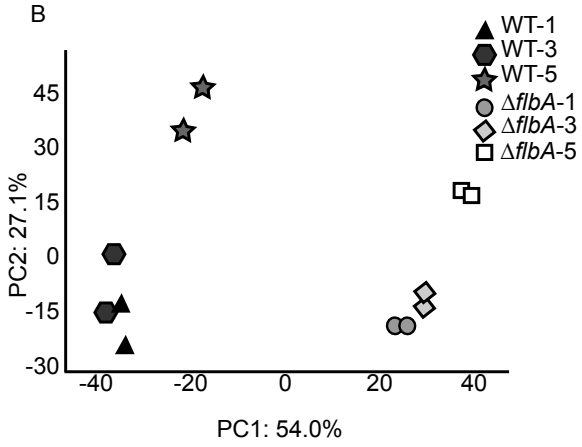


Figure 2. Pearson correlation (r^2) (A) and principal component analysis (B) of the transcriptomes of zone 1, 3, and 5 of wild-type (▲⬣★) and Δ*flbA* (●◆■) colonies.

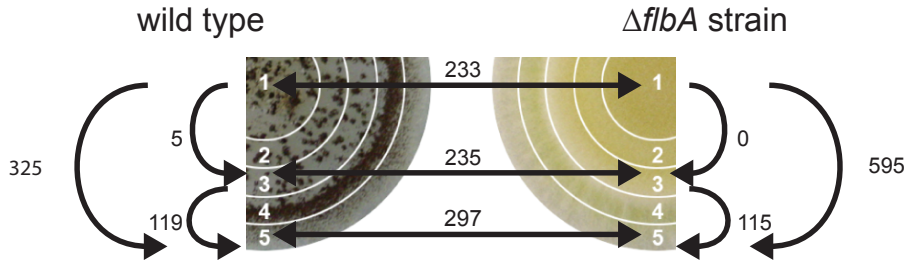


Figure 3. Differentially expressed genes that show 2-fold lower or higher expression between zones of wild-type and / or Δ*flbA* colonies.

0, 115, and 595 for the Δ*flbA* strain, respectively (Figure 3). Of these differentially expressed genes only 138 were found in both the wild-type and the Δ*flbA* strain.

In the next analysis, the number of genes was determined that are differentially expressed when zones of the wild-type and the Δ*flbA* strain were compared (Figure 3). The number of genes differentially expressed in zone 1 of the wild-type and the Δ*flbA* strain was 233. Of these, 104 and 129 were up- and down-regulated in the Δ*flbA* strain, respectively. A total of 235 genes were found to be differentially expressed in zone 3. Of these, 89 were up-regulated in Δ*flbA*, whereas 149 were up-regulated in the wild-type. In zone 5, 297 genes were differentially expressed, of which 126 and 153 were up- and down-regulated in the Δ*flbA* strain, respectively.

Expression analysis of functional gene classes.

Fisher's exact test was used to determine whether functional gene classes[16] were

Table 1. Over- (O) and under- (U) representation of functional gene classes in the pool of genes that are differentially expressed in the intermediate and central zone when compared to the periphery of the wild-type and Δ*flbA* strain.

FunCat	wt up intermediate/ center	wt up periphery	Δ*flbA* up Intermediate/ center	Δ*flbA* up periphery
01 METABOLISM	O	O	O	O
01.01.10 amino acid degradation (catabolism)	O			O
01.03.19 nucleotide transport				O
01.05.01 C-compound and carbohydrate utilization				O
02 ENERGY		O		O
02.11.05 accessory proteins of electron transport and membrane-associated energy conservation		O		
03 CELL CYCLE AND DNA PROCESSING			U	
04 TRANSCRIPTION		U	U	U
04.05.01 mRNA synthesis		U		U
05 PROTEIN SYNTHESIS		O	U	
08 CELLULAR TRANSPORT AND TRANSPORT MECHANISMS				O
40 SUBCELLULAR LOCALISATION				U
99 UNCLASSIFIED PROTEINS	U	U	U	U

over- or underrepresented in the set of genes that are differentially expressed in zones 1 and 3 compared to zone 5 of the wild-type and the Δ*flbA* strain (Table 1). The functional classes metabolism and amino acid degradation were overrepresented in the up-regulated genes of zones 1 and 3 of the wild-type colony (Table 1), whereas the functional classes metabolism, energy, accessory proteins of electron transport and membrane associated energy conservation, and protein synthesis were overrepresented in the peripheral wild-type zone 5 (Table 1). The functional classes transcription, mRNA synthesis, and unclassified proteins were under-represented in this cluster of genes, while only the latter functional group was underrepresented in zone 1 and 3 of the wild-type. In the up-regulated genes in the central and intermediate zone of the Δ*flbA* strain, the functional class metabolism was over-represented, whereas cell cycle and DNA processing, transcription, protein synthesis, and unclassified proteins were under-represented (Table 1). Metabolism, amino acid degradation, nucleotide transport, C-compound and carbohydrate utilization, energy and cellular transport and transport mechanisms were over-represented in the up-regulated genes in the peripheral zone of Δ*flbA* colonies (Table 1). On the other hand, transcription, mRNA synthesis, subcellular localization and unclassified proteins were under-represented.

Table 2. Over- (O) and under- (U) representation of functional gene classes in the pool of genes that are ≥2 fold up- or down-regulated in the Δ*flbA* strain when compared to the wild-type.

FunCat	zone 1 Up	zone 1 Down	zone 3 Up	zone 3 Down	zone 5 Up	zone 5 Down
01 METABOLISM	O	O	O	O		O
01.01.07 amino acid transport	O					
01.05.01 C-compound and carbohydrate utilization						O
03 CELL CYCLE AND DNA PROCESSING						U
06.13.99 other proteolytic degradation						O
08 CELLULAR TRANSPORT AND TRANSPORT MECHANISMS					O	
99 UNCLASSIFIED PROTEINS	U	U		U		U

Funcat analysis was performed on the differentially expressed genes between the zones of the wild-type and the Δ*flbA* colonies (Table 2). Up-regulated genes in zone 1 of Δ*flbA* compared to wild-type zone 1 were overrepresented in metabolism and amino acid transport, whereas unclassified proteins were underrepresented. Metabolism and unclassified proteins were also over- and underrepresented, respectively, in the down-regulated genes in zone 1 of Δ*flbA* compared to wild-type zone 1. The same was observed in the down-regulated genes in zone 3, whereas only the gene class metabolism was overrepresented in the up-regulated genes of this zone. Cellular transport and transport mechanisms were overrepresented in the up-regulated genes of the peripheral zone 5 of Δ*flbA* colonies. In contrast, the down-regulated genes in this zone showed an over-

representation of genes of the functional classes metabolism, C-compound and carbohydrate utilization, and other proteolytic degradation, whereas cell cycle and DNA processing and unclassified proteins were underrepresented (Table 2).

Expression analysis of specific functional gene classes

The expression profiles of zones 1, 3, and 5 of the wild-type were pooled and compared with the pooled profiles of zones 1, 3, and 5 of the Δ*flbA* strain. In other words, for each gene the mean expression within the wild-type colony was compared to the mean expression within the Δ*flbA* strain. This was done to increase the statistical power of the comparison (comparing 6 instead of 2 arrays in each case). The number of genes up-regulated in the Δ*flbA* colonies was 520, while 632 genes were down-regulated. Funcat analysis showed that the up-regulated genes are overrepresented in the functional categories metabolism and cellular transport and transport mechanisms, whereas transcription and unclassified proteins were underrepresented (Table 3). Down-regulated genes were overrepresented in metabolism and C-compound and carbohydrate utilization, and underrepresented in energy, cell cycle and DNA processing, mitotic cell cycle and cell cycle control, protein synthesis, subcellular localisation, and unclassified proteins (Table 3).

Table 3. Over- (O) and under- (U) representation of functional gene classes in the pool of genes that are 2 fold up- or down-regulated between the mean expression values of the Δ*flbA* strain and the mean expression values of the wild-type strain.

FunCat	up-regulated in Δ*flbA*	Down-regulated in Δ*flbA*
01 METABOLISM	O	O
01.01.07 amino acid transport		
01.05.01 C-compound and carbohydrate utilization		O
02 ENERGY		U
03 CELL CYCLE AND DNA PROCESSING		U
03.03.01 mitotic cell cycle and cell cycle control		U
04 TRANSCRIPTION	U	
05 PROTEIN SYNTHESIS		U
08 CELLULAR TRANSPORT AND TRANSPORT MECHANISMS	O	
40 SUBCELLULAR LOCALISATION		U
99 UNCLASSIFIED PROTEINS	U	U

Twenty-six out of the 50 genes with the highest up-regulation (≥ 2-fold) in Δ*flbA* compared to wild-type are predicted to encode proteins with a signal sequence for secretion (sigP) (Table 4). This number was 22 in the top 50 of genes with the highest down-regulation in Δ*flbA* colonies. Among the top 50up-regulated and down-regulated genes 47 and 43 genes are not yet characterized, respectively, of which 18 and 23 belong to the family of unclassified proteins. These gene sets also contain genes involved in development, cell wall biosynthesis

/ remodelling and genes encoding transcriptional regulators. Up- and down-regulated (≥ 2-fold) genes of these classes[17] were analyzed in more detail and are described below.

Transcriptional changes associated to asexual and sexual development
Of the 68 genes implicated in asexual and / or sexual reproduction in *A. niger*, 13 are found to be differentially expressed between the wild-type and Δ*flbA* strain (Table 5; Supplemental Table 1). Of these genes, 6 are down-regulated in the Δ*flbA* strain, and 7 are up-regulated. Of the genes indicated in Figure 1, *flbD* and *sfaD* are ≥ 2-fold higher expressed in the Δ*flbA* strain.

Transcriptional changes associated to cell wall synthesis
A total of 102 genes are predicted to be involved in cell wall synthesis.[17] Of these genes, 10 and 2 were found to be up- and down-regulated in the Δ*flbA* strain when compared to the wild-type, respectively (Table 6; Supplemental Table 1). Among the up-regulated genes are three glucanosyltransferase genes, two chitin synthase genes, the chitinase gene *chiB*, two glucanase genes, and two glycosylphosphatidylinositol-anchored endo-mannanase genes. One of these glucanosyltransferases, a *gelD* homologue of *A. nidulans* was 48-fold up-regulated in Δ*flbA* compared to wild-type. In contrast, the annotated *gelD* gene of *A. niger* was found to be 40-fold down-regulated in Δ*flbA*. The other down-regulated gene in the Δ*flbA* strain is the glucan beta-1,3 exoglucanase gene *exsG*.

Transcriptional changes associated to proteins with a signal sequence for secretion
As mentioned above, wild-type and Δ*flbA* were grown on minimal medium supplemented with xylose. This carbon source activates XlnR, a transcriptional regulator that controls 16 genes encoding xylanolytic enzymes.[18-24] Of these, none were higher expressed in the Δ*flbA* strain, whereas 6 (*xlnB*, *xlnC*, *axhA*, *aglB*, *eglB*, *cbhA*, *chbB*) were found to be 2-fold lower expressed when compared to the wild-type (Table 7, Supplemental Table 1). In fact, *xlnC*, *xlnB*, and *axhA* are among the highest expressed genes at the periphery of wild-type colonies, while they are hardly expressed in the Δ*flbA* strain (Table 4). Thirty-two other carbohydrases are down-regulated in the Δ*flbA* strain (e.g. the glucoamylase gene *glaA*) and 8 carbohydrolasess are found to be up-regulated in this strain. Among these genes are the α-galactosidase gene *aglC*, α-glucan synthase *agsE*, pectin lysase *pelB*, and the pectin esterase *pmeA* (Table 7).

Of the total number of 2612 genes with a predicted signal sequence for secretion, 156 and 189 were up- and down-regulated, respectively (Supplemental Table 1, Supplemental Table 2). The up-regulated genes are mainly uncharacterized proteins, but include some carbohydrases (see above), a putative cytochrome P450 reductase, and two putative proteases. Among the down-regulated are also several carbohydrate degrading enzymes (see above, Supplemental Table 1, Supplemental

Table 4. Average hybridization values of top 50 up- and down-regulated genes in zones 1, 3, and 5 of the Δ*flbA* strain when compared to wild-type. *Gene name or its closest homologue. A. nid: *A. nidulans*; A. ory: *A. oryzae*.

Annotation	Gene Name*	Description - Table 4	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation	sigP
Top 50 down-regulated genes in the Δ*flb*A strain compared to wildtype										
An05g01730	*uncharacterized*	hypothetical protein	11442 ± 3316	14891 ± 315	4511 ± 2021	42 ± 7	37 ± 0	27 ± 1	Down	y
An09g00840	*uncharacterized*	weak similarity to antigenic cell wall galactomannoprotein MP1 - Aspergillus fumigatus	3153 ± 2599	10725 ± 146	3462 ± 103	29 ± 11	35 ± 22	14 ± 2	Down	y
An18g05480	*uncharacterized*	strong similarity to alcohol oxidase AOX1 - Pichia pastoris	7168 ± 839	7322 ± 2676	3105 ± 1353	46 ± 2	44 ± 3	42 ± 5	Down	n
An16g05920	*uncharacterized*	weak similarity to surface recognition protein PTH11 - Magnaporthe grisea	4282 ± 1762	6936 ± 1040	5405 ± 665	48 ± 1	47 ± 11	44 ± 1	Down	n
An07g06460	*uncharacterized*	similarity to C-7 hydroxycephem methyltransferase coupling protein of patent WO9529253-A1 - Streptomyces lactamdurans	1807 ± 56	3006 ± 541	4779 ± 1295	29 ± 5	24 ± 5	28 ± 0	Down	n
An16g05930	*uncharacterized*	strong similarity to predicted protein An08g06890 - Aspergillus niger	5034 ± 1765	7101 ± 440	6203 ± 1646	63 ± 18	58 ± 11	58 ± 9	Down	n
An01g06890	*uncharacterized*	similarity to hypothetical peptide synthetase pesA - Metarhizium anisopliae	1497 ± 1410	2785 ± 1060	5197 ± 1630	88 ± 10	29 ± 2	18 ± 0	Down	n
An16g05910	*uncharacterized*	similarity to cholesterol 7alpha-hydroxylase CYP7 - Sus scrofa	902 ± 636	1646 ± 590	2001 ± 725	17 ± 3	25 ± 5	18 ± 2	Down	y
An03g00690	*uncharacterized*	hypothetical protein	1261 ± 1329	2284 ± 1815	2321 ± 1481	21 ± 7	28 ± 1	26 ± 2	Down	n
An08g02330	*uncharacterized*	strong similarity to multidrug resistance protein MLP-2 - Rattus norvegicus	2009 ± 37	2116 ± 179	1500 ± 529	45 ± 2	29 ± 3	22 ± 4	Down	n
An07g06480	*uncharacterized*	similarity to cytochrome 4F8 cyp4F8 - Homo sapiens	420 ± 130	586 ± 213	753 ± 142	9 ± 1	10 ± 2	9 ± 1	Down	y
An05g01710	*uncharacterized*	strong similarity to hypothetical protein An16g07720 - Aspergillus niger	1738 ± 1486	2310 ± 357	387 ± 170	19 ± 2	18 ± 2	19 ± 3	Down	n
An01g00530	*pepB*	proteinase aspergillopepsin II - Aspergillus niger	682 ± 649	3677 ± 2802	6268 ± 3005	44 ± 5	35 ± 1	34 ± 0	Down	y
An01g06860	*uncharacterized*	strong similarity to hypothetical Fum9p protein - Gibberella moniliformis	1135 ± 1295	2702 ± 926	5766 ± 2392	67 ± 10	36 ± 3	31 ± 0	Down	n
An01g06870	*uncharacterized*	strong similarity to hypothetical protein Fum8p - Gibberella moniliformis	581 ± 599	1632 ± 396	3427 ± 1234	50 ± 2	26 ± 1	21 ± 6	Down	n
An02g08300	*uncharacterized*	strong similarity to the hypothetical protein encoded by An11g06450 - Aspergillus niger	842 ± 71	710 ± 230	2085 ± 860	30 ± 1	21 ± 2	24 ± 8	Down	n
An15g07700	*protD*	strong similarity to aspergillopepsin II precursor (acid proteinase A) - Aspergillus niger	649 ± 452	5044 ± 2370	9630 ± 101	75 ± 5	76 ± 5	65 ± 2	Down	y
An09g00670	*gelD*	strong similarity to beta (1-3) glucanosyltransferase Gel3p - Aspergillus fumigatus	1596 ± 565	2244 ± 607	2063 ± 821	45 ± 1	39 ± 6	59 ± 10	Down	y
An16g06570	*uncharacterized*	hypothetical protein	7069 ± 619	3576 ± 3514	1162 ± 1292	62 ± 4	52 ± 8	62 ± 4	Down	y
An02g00090	*uncharacterized*	strong similarity to prolidase - Aureobacterium esteraromaticum	6755 ± 2149	4420 ± 2149	707 ± 467	134 ± 13	58 ± 3	36 ± 11	Down	n

Annotation	Gene Name*	Description - Table 4 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation	sigP
An05g02450	*uncharacterized*	similarity to halogenase bhaA from patent DE19926770-A1 - Amycolatopsis mediterranei	663 ± 62	1035 ± 59	469 ± 33	25 ± 6	18 ± 2	18 ± 1	Down	y
An07g08950	*eglC*	endoglucanase B eglB - Aspergillus niger	2511 ± 570	11775 ± 532	7793 ± 1370	52 ± 29	366 ± 244	422 ± 77	Down	y
An08g02300	*uncharacterized*	weak similarity to enniatin synthetase - Fusarium scirpi[truncated ORF]	1123 ± 198	1542 ± 488	1754 ± 1024	79 ± 8	53 ± 1	22 ± 5	Down	n
An01g06900	*sm to amyR-A. ory*	weak similarity to transcription activator amyR - Aspergillus oryzae	758 ± 96	1056 ± 406	654 ± 233	34 ± 8	25 ± 1	21 ± 1	Down	n
An12g01320	*ppoD*	strong similarity to linoleate diol synthase - Gaeumannomyces graminis	1123 ± 612	1043 ± 345	132 ± 72	18 ± 2	20 ± 3	14 ± 2	Down	y
An01g06880	*uncharacterized*	similarity to dihydroflavonol 4-reductase BAA12723.1 - Rosa hybrid cultivar	625 ± 534	1769 ± 746	3403 ± 1745	110 ± 3	36 ± 3	27 ± 6	Down	n
An02g08330	*uncharacterized*	strong similarity to sequence 253 from Patent WO0100804 - Corynebacterium glutamicum	627 ± 234	624 ± 177	877 ± 309	27 ± 2	25 ± 1	24 ± 6	Down	y
An01g06840	*uncharacterized*	strong similarity to acid-CoA ligase Fat2p - Saccharomyces cerevisiae	419 ± 441	1295 ± 731	3234 ± 1795	52 ± 0	34 ± 1	29 ± 4	Down	n
An03g06670	*uncharacterized*	weak similarity to myosin-like protein MLP1 - Saccharomyces cerevisiae	5998 ± 1898	6281 ± 1029	1761 ± 437	171 ± 1	171 ± 7	118 ± 9	Down	n
An01g06830	*uncharacterized*	similarity to ketosphinganine reductase Tsc10p - Saccharomyces cerevisiae	418 ± 405	1031 ± 265	2067 ± 700	46 ± 1	33 ± 6	24 ± 3	Down	n
An01g06850	*uncharacterized*	similarity to 4-hydroxybutyrate dehydrogenase - Alcaligenes eutrophus	967 ± 1081	1917 ± 437	4224 ± 1181	112 ± 1	54 ± 2	44 ± 4	Down	n
An03g00640	*uncharacterized*	similarity to neutral amino acid permease mtr - Neurospora crassa[truncated ORF]	2950 ± 1037	2645 ± 295	2479 ± 1469	149 ± 28	122 ± 9	57 ± 2	Down	n
An01g11670	*eglA*	strong similarity to endo-beta-1,4-glucanase A eglA - Emericella nidulans	638 ± 408	2367 ± 12	589 ± 232	21 ± 2	42 ± 24	58 ± 1	Down	y
An07g08940	*uncharacterized*	similarity to acetyl-esterase I of patent WO9502689-A - Aspergillus aculeatus	242 ± 13	1268 ± 407	914 ± 553	20 ± 1	31 ± 7	26 ± 0	Down	y
An11g07020	*uncharacterized*	strong similarity to the hypothetical protein encoded by An07g00200 - Aspergillus niger	3237 ± 1203	2067 ± 1234	1620 ± 142	125 ± 37	97 ± 1	54 ± 11	Down	n
An07g09330	*cbhA*	cellulose 1,4-beta-cellobiosidase cbhA from patent WO9906574-A1- Aspergillus niger	1157 ± 1121	7883 ± 2929	3034 ± 1406	67 ± 16	130 ± 62	170 ± 76	Down	y
An09g06200	*uncharacterized*	strong similarity to PTH11 transmembrane protein - Magnaporthe grisea strain 4091-5-8	5976 ± 1928	5351 ± 2683	3632 ± 1622	442 ± 58	211 ± 45	82 ± 12	Down	y
An04g09990	*uncharacterized*	strong similarity to 2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase linC - Sphingomonas paucimobilis	749 ± 308	422 ± 138	106 ± 4	36 ± 6	34 ± 6	34 ± 5	Down	y
An04g03840	*uncharacterized*	similarity to microtubule binding protein D-CLIP-190 - Drosophila melanogaster	365 ± 35	373 ± 52	257 ± 17	16 ± 0	14 ± 0	12 ± 3	Down	n
An05g01720	*uncharacterized*	strong similarity to hypothetical protein An06g00950 - Aspergillus niger	1552 ± 1286	2068 ± 211	368 ± 231	47 ± 7	42 ± 7	41 ± 1	Down	n
An09g02990	*uncharacterized*	similarity to probable Sua5 protein APE2397 - Aeropyrum pernix	618 ± 224	781 ± 58	344 ± 112	25 ± 6	26 ± 0	24 ± 2	Down	n
An04g06750	*uncharacterized*	similarity to hypothetical transmembrane protein - Candida albicans	491 ± 201	386 ± 153	489 ± 169	25 ± 4	20 ± 2	19 ± 2	Down	n

Annotation	Gene Name*	Description - Table 4 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation	sigP
An07g06490	*uncharacterized*	strong similarity to insulin-degrading enzyme IDE - Rattus norvegicus	213 ± 37	361 ± 124	474 ± 112	17 ± 0	17 ± 0	16 ± 2	Down	n
An12g05390	*uncharacterized*	weak similarity to integral membrane protein PTH11 - Magnaporthe grisea	2079 ± 127	1355 ± 14	84 ± 32	38 ± 0	31 ± 2	30 ± 3	Down	y
An16g02910	*uncharacterized*	strong similarity to hypothetical protein CC0533 - Caulobacter crescentus	865 ± 51	1033 ± 82	613 ± 238	52 ± 1	40 ± 8	42 ± 10	Down	y
An08g11680	*uncharacterized*	strong similarity to 2,5-dicloro-2,5-cyclohexadiene-1,4-diol dehydrogenase linC - Pseudomonas paucimobilis	320 ± 4	214 ± 41	125 ± 4	56 ± 0	68 ± 11	61 ± 1	Down	y
An01g06930	*uncharacterized*	strong similarity to polyketide synthase FUM5 - Gibberella moniliformis	714 ± 704	1442 ± 246	2506 ± 866	110 ± 1	60 ± 2	46 ± 1	Down	n
An14g03130	*uncharacterized*	hypothetical protein	1109 ± 560	752 ± 164	262 ± 94	49 ± 11	29 ± 4	25 ± 2	Down	y
An08g03760	*uncharacterized*	similarity to hypothetical protein Rv3472 - Mycobacterium tuberculosis	191 ± 86	197 ± 37	843 ± 343	16 ± 1	16 ± 1	23 ± 5	Down	n
An08g05230	*uncharacterized*	strong similarity to putative endoglucanase IV - Trichoderma reesei	1152 ± 1281	2411 ± 2729	648 ± 116	55 ± 5	58 ± 11	46 ± 5	Down	y
Top 50 up-regulated genes in the Δ*flb*A strain compared to wildtype										
An08g08490	*uncharacterized*	similarity to the calcium-independent phospholipase A2 2 - Homo sapiens	143 ± 8	118 ± 7	85 ± 3	728 ± 0	847 ± 91	775 ± 76	Up	n
An07g06240	*uncharacterized*	strong similarity to ferrioxamine B permease sit1 - Saccharomyces cerevisiae	88 ± 24	87 ± 8	94 ± 8	323 ± 28	397 ± 32	1915 ± 383	Up	n
An03g03620	*uncharacterized*	strong similarity to multidrug resistance protein atrD - Aspergillus nidulans	18 ± 1	22 ± 2	26 ± 10	83 ± 5	110 ± 1	378 ± 123	Up	y
An08g10830	*uncharacterized*	strong similarity to geranylgeranyl pyrophosphate synthase ggpps - Gibberella fujikuroi	65 ± 5	58 ± 0	68 ± 18	412 ± 9	388 ± 31	555 ± 30	Up	n
An01g12200	*uncharacterized*	similarity to hypothetical protein F10B6.29 - Arabidopsis thaliana	112 ± 53	68 ± 23	94 ± 12	741 ± 26	726 ± 82	429 ± 36	Up	n
An09g00270	*aglC*	alpha-galactosidase C aglC - Aspergillus niger[truncated ORF]	89 ± 18	85 ± 22	760 ± 428	879 ± 73	1158 ± 119	1805 ± 251	Up	nc
An07g04900	*uncharacterized*	strong similarity to mRNA sequence of cDNA clone 2589 - Aspergillus niger	1065 ± 70	929 ± 155	364 ± 239	5638 ± 245	5796 ± 687	3483 ± 369	Up	y
An11g09170	*uncharacterized*	similarity to the secreted aspartic proteinase SAP8 - Candida albicans	48 ± 5	55 ± 11	42 ± 1	316 ± 18	441 ± 6	290 ± 41	Up	y
An14g01840	*uncharacterized*	similarity to hypothetical temperature-shock induced protein TIR3 - Saccharomyces cerevisiae	227 ± 122	258 ± 66	426 ± 36	1679 ± 739	2311 ± 995	2553 ± 877	Up	y
An11g02600	*uncharacterized*	strong similarity to PT2/PHT4 Phosphate transporter - Arabidopsis thaliana	50 ± 15	60 ± 10	104 ± 14	483 ± 47	653 ± 132	394 ± 127	Up	n
An16g07040	*sm to btgE -A. nid*	similarity to beta-1,3-glucanosyltransferase BGT1 - Aspergillus fumigatus[truncated ORF]	502 ± 224	676 ± 353	2375 ± 144	5982 ± 202	6554 ± 1346	7358 ± 380	Up	y
An17g00120	*uncharacterized*	strong similarity to major facilitator superfamily transporter protein mfs1 - Botrytis cinerea	30 ± 6	26 ± 0	24 ± 2	237 ± 3	221 ± 13	140 ± 13	Up	n

Annotation	Gene Name*	Description - Table 4 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation	sigP
An16g08360	*uncharacterized*	weak similarity to cytochrome c1 of ubiquinol--cytochrome-c reductase - Paracoccus denitrificans[truncated ORF]	64 ± 10	75 ± 12	158 ± 35	610 ± 68	591 ± 130	852 ± 12	Up	n
An11g01810	*uncharacterized*	weak similarity to probable membrane protein YBR005w - Saccharomyces cerevisiae	55 ± 6	62 ± 5	96 ± 20	438 ± 9	541 ± 62	564 ± 34	Up	n
An02g07930	*ppoC*	strong similarity to linoleate diol synthase precursor - Gaeumannomyces graminis	180 ± 34	126 ± 52	38 ± 2	983 ± 279	747 ± 19	464 ± 32	Up	n
An09g01240	*uncharacterized*	strong similarity to phospholipase B - Penicillium notatum	78 ± 7	63 ± 9	104 ± 3	499 ± 142	727 ± 111	627 ± 178	Up	y
An01g06280	*uncharacterized*	strong similarity to IgE-binding protein - Aspergillus fumigatus	267 ± 123	341 ± 167	136 ± 4	2010 ± 28	2634 ± 280	1002 ± 145	Up	y
An13g01250	*uncharacterized*	strong similarity to the yeast siderophore-iron transporter for enterobactin Enb1 - Saccharomyces cerevisiae.	37 ± 2	35 ± 3	36 ± 3	183 ± 64	331 ± 79	415 ± 58	Up	n
An14g07130	*uncharacterized*	strong similarity to neutral amino acid permease mtr - Neurospora crassa	95 ± 8	70 ± 4	93 ± 8	481 ± 32	602 ± 135	1245 ± 123	Up	n
An11g04810	*aox1*	alternative oxidase Aox1 - Aspergillus niger	43 ± 8	54 ± 32	47 ± 20	279 ± 254	470 ± 353	890 ± 194	Up	n
An15g03940	*uncharacterized*	strong similarity to monosaccharide transporter Mst-1 - Amanita muscaria	244 ± 17	202 ± 47	2589 ± 838	3196 ± 21	3689 ± 943	8423 ± 210	Up	y
An13g00510	*uncharacterized*	strong similarity to hexokinase 1 hxk1 - Schizosaccharomyces pombe	199 ± 43	202 ± 28	67 ± 10	3335 ± 329	2920 ± 299	223 ± 64	Up	n
An18g01290	*uncharacterized*	strong similarity to predicted protein An13g01340 - Aspergillus niger	26 ± 2	24 ± 2	38 ± 10	233 ± 53	218 ± 65	405 ± 13	Up	y
An01g07000	*uncharacterized*	strong similarity to C-14 sterol reductase ERG24 - Saccharomyces cerevisiae	89 ± 14	77 ± 2	270 ± 166	498 ± 24	1009 ± 531	3138 ± 50	Up	y
An10g00680	*uncharacterized*	strong similarity to H+-ATPase V0 domain 17 KD subunit, vacuolar, CUP5 - Saccharomyces cerevisiae	443 ± 342	220 ± 116	119 ± 50	2783 ± 380	2761 ± 26	1102 ± 42	Up	y
An16g09040	*uncharacterized*	strong similarity to N-acetylglucosamine-6-phosphate deacetylase CaNAG2 - Candida albicans	629 ± 51	516 ± 71	81 ± 7	6612 ± 950	6653 ± 1009	637 ± 136	Up	n
An01g00390	*uncharacterized*	hypothetical protein	45 ± 2	47 ± 2	319 ± 99	542 ± 59	685 ± 35	1891 ± 74	Up	n
An16g01850	*uncharacterized*	similarity to blastomyces yeast phase-specific protein 1 bys1 - Ajellomyces dermatitidis	2984 ± 2759	1197 ± 780	166 ± 54	12215 ± 313	12035 ± 1137	3132 ± 648	Up	y
An16g01880	*lipanI*	strong similarity to lysophospholipase - Aspergillus foetidus	648 ± 245	795 ± 248	454 ± 202	10644 ± 663	10585 ± 2214	2676 ± 160	Up	y
An14g02940	*uncharacterized*	strong similarity to L-sorbose dehydrogenase, FAD dependent - Gluconobacter oxydans	212 ± 101	153 ± 71	139 ± 92	2065 ± 372	1565 ± 349	1690 ± 279	Up	n
An07g03570	*uncharacterized*	strong similarity to sorbitol utilization protein sou2 - Candida albicans	83 ± 7	85 ± 17	1534 ± 228	1757 ± 42	1613 ± 167	6032 ± 312	Up	n
An18g03360	*uncharacterized*	similarity to the proteophosphoglycan ppg1 - Leishmania major	32 ± 19	46 ± 26	27 ± 5	473 ± 19	503 ± 4	242 ± 2	Up	y
An14g06980	*uncharacterized*	strong similarity to delta-12 fatty acid desaturase - Mortierella alpina	33 ± 3	36 ± 2	301 ± 112	465 ± 89	750 ± 251	1939 ± 15	Up	n

Annotation	Gene Name*	Description - Table 4 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation	sigP
An05g00790	*uncharacterized*	strong similarity to nodulin GmNOD53b - Glycine max[truncated ORF]	25 ± 15	20 ± 4	12 ± 1	213 ± 57	341 ± 30	161 ± 9	Up	n
An03g06660	*uncharacterized*	strong similarity to peptide transporter ptr2 - Arabidopsis thaliana	2166 ± 1123	1709 ± 1392	80 ± 15	12416 ± 598	10787 ± 865	3842 ± 47	Up	n
An18g01320	*uncharacterized*	strong similarity to extracellular protease precursor BAR1 - Saccharomyces cerevisiae	148 ± 76	129 ± 56	328 ± 31	2146 ± 67	2897 ± 61	2501 ± 161	Up	y
An05g00800	*uncharacterized*	similarity to nodulin GmNOD53b - Glycine max[truncated ORF]	47 ± 21	31 ± 11	22 ± 1	395 ± 130	667 ± 69	333 ± 6	Up	n
An12g09870	*uncharacterized*	hypothetical protein	50 ± 0	57 ± 1	56 ± 0	571 ± 232	432 ± 82	2224 ± 250	Up	n
An11g00100	*uncharacterized*	strong similarity to triacylglycerol lipase LIP5 - Candida rugosa	71 ± 4	64 ± 3	59 ± 8	800 ± 192	866 ± 397	2265 ± 77	Up	y
An01g01630	*uncharacterized*	strong similarity to hypothetical protein An09g00510 - Aspergillus niger	69 ± 71	36 ± 26	96 ± 40	1151 ± 99	830 ± 115	802 ± 17	Up	y
An01g07730	*uncharacterized*	weak similarity to TcSL-2 protein precursor - Toxocara cani	118 ± 74	64 ± 3	63 ± 5	1697 ± 86	1478 ± 312	1257 ± 12	Up	y
An15g03550	*uncharacterized*	weak similarity to protopectinase patent WO9806832-A1 - Bacillus subtilis	57 ± 44	57 ± 45	20 ± 0	954 ± 34	1066 ± 21	330 ± 22	Up	y
An16g06950	*uncharacterized*	strong similarity to carboxylic acid transport protein JEN1 - Saccharomyces cerevisiae	31 ± 8	26 ± 0	23 ± 5	837 ± 53	725 ± 9	357 ± 20	Up	n
An03g01770	*uncharacterized*	strong similarity to the EST an_3645 - Aspergillus niger	758 ± 657	336 ± 176	55 ± 12	10029 ± 207	8451 ± 0	1933 ± 247	Up	y
An03g05360	*uncharacterized*	strong similarity to neutral amino acid permease mtr - Neurospora crassa	39 ± 0	36 ± 2	31 ± 2	1673 ± 148	1362 ± 88	771 ± 2	Up	y
An14g04210	*uncharacterized*	similarity to hypothetical protein An07g05660 - Aspergillus niger	30 ± 0	35 ± 7	31 ± 0	931 ± 174	1386 ± 104	2511 ± 5	Up	y
An03g06220	*sm to gelD- A. nid*	strong similarity to beta (1-3) glucanosyltransferase GEL3 - Aspergillus fumigatus	42 ± 3	48 ± 11	58 ± 3	1406 ± 256	1874 ± 54	5043 ± 547	Up	y
An02g08560	*uncharacterized*	similarity to probable dioxygenase SCOEDB - Streptomyces coelicolor	22 ± 3	23 ± 3	19 ± 0	797 ± 22	1048 ± 83	1682 ± 135	Up	y
An02g08130	*uncharacterized*	similarity to hypothetical protein 2SCG18.24 - Streptomyces coelicolor	46 ± 3	48 ± 9	41 ± 5	2045 ± 64	2532 ± 185	3306 ± 224	Up	y
An09g05050	*uncharacterized*	questionable ORF	25 ± 1	24 ± 4	22 ± 3	3522 ± 1024	2872 ± 506	8559 ± 471	Up	y

Table 5. Average hybridization values of 13 genes in zones 1, 3, and 5 that are implicated in asexual and / or sexual reproduction and that are differentially expressed in wild-type when compared to the Δ*flbA* strain of *A. niger*. The table is based on Pel et al.[17] with the addition of FlbE (An08g07210), FlbB (An15g03710) and FlbC (An12g08230). *Gene name or its closest homologue. Sm: similar; A. fum: *A. fumigatus*.

Annotation	Gene Name*	Description - Table 5	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An02g03160	*flbA*	strong similarity to developmental regulator flbA - Emericella nidulans	147 ± 11	166 ± 25	77 ± 24	21 ± 2	24 ± 4	25 ± 3	Down
An04g05880	*ppoA*	strong similarity to linoleate diol synthase - Gaeumannomyces graminis	1658 ± 245	1167 ± 359	282 ± 22	301 ± 2	298 ± 6	188 ± 11	Down
An04g06620	*(RAM1/STE16)*	similarity to farnesyl-protein transferase beta chain - Homo sapiens	1125 ± 2	1045 ± 31	831 ± 197	527 ± 31	499 ± 42	378 ± 4	Down
An04g07400	*sm rosA- A. fum*	strong similarity to C6 zinc finger transcription factor PRO1 - Sordaria macrospora [putative sequencing error]	120 ± 28	195 ± 76	562 ± 77	86 ± 3	83 ± 9	43 ± 6	Down
An05g00480	*stuA /phd1*	strong similarity to transcription factor involved in differentiation stuA - Aspergillus nidulans	3804 ± 1205	4031 ± 108	1721 ± 59	1672 ± 63	1531 ± 47	978 ± 7	Down
An12g01320	*ppoD*	strong similarity to linoleate diol synthase - Gaeumannomyces graminis	1123 ± 612	1043 ± 345	132 ± 72	18 ± 2	20 ± 3	14 ± 2	Down
An01g03750	*abaA*	strong similarity to protein abaA - Aspergillus nidulans	61 ± 9	71 ± 12	67 ± 17	113 ± 28	129 ± 3	182 ± 22	Up
An01g04830	*flbD*	strong similarity to myb-like DNA binding protein flbD - Aspergillus nidulans	89 ± 18	91 ± 8	81 ± 20	238 ± 35	220 ± 32	107 ± 5	Up
An02g07930	*ppoC*	strong similarity to linoleate diol synthase precursor - Gaeumannomyces graminis	180 ± 34	126 ± 52	38 ± 2	983 ± 279	747 ± 19	464 ± 32	Up
An14g01820	*phiA /binB*	strong similarity to hypothetical cell wall protein binB - Aspergillus nidulans	2018 ± 1048	3064 ± 430	843 ± 70	8800 ± 3676	14346 ± 1791	4066 ± 653	Up
An15g02740	*apsA*	strong similarity to the anucleate primary sterigmata gene apsA - Aspergillus nidulans	199 ± 47	206 ± 53	252 ± 49	453 ± 29	444 ± 68	560 ± 22	Up
An16g01860	*(STE23)*	strong similarity to protease involved in a-factor processing STE23 - Saccharomyces cerevisiae	99 ± 20	95 ± 10	118 ± 8	192 ± 8	215 ± 7	279 ± 28	Up
An18g02090	*sfaD (STE 4)*	strong similarity to G-protein beta subunit sfaD - Aspergillus nidulans	41 ± 10	43 ± 8	104 ± 4	109 ± 5	113 ± 4	165 ± 13	Up

Table 6. Average expression values of 12 genes in zones 1, 3, and 5 that are implicated in cell wall biosynthesis and that are differentially expressed in wild-type when compared to the Δ*flbA* strain of *A. niger*. The table was adapted from Pel et al.[17]. *Description of gene name or its closest homologue. Sm: similar; A. nid: *A. nidulans*.

Annotation	Gene Name*	Description - Table 6	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An01g12450	*exsG*	strong similarity to putative glucan beta-1,3 exoglucanase - Trichoderma harzianum	3091 ± 197	3667 ± 1573	1008 ± 250	966 ± 4	1053 ± 14	689 ± 27	Down
An09g00670	*gelD*	strong similarity to beta (1-3) glucanosyltransferase Gel3p - Aspergillus fumigatus	1596 ± 565	2244 ± 607	2063 ± 821	45 ± 1	39 ± 6	59 ± 10	Down
An02g02660	*dfgG*	strong similarity to the protein required for filamentous growth, cell polarity, and cellular elongation Dfg5 - Saccharomyces cerevisiae	87 ± 5	110 ± 17	102 ± 21	487 ± 30	648 ± 129	532 ± 98	Up
An03g06220	*sm to gelD- A. nid*	strong similarity to beta (1-3) glucanosyltransferase GEL3 - Aspergillus fumigatus	42 ± 3	48 ± 11	58 ± 3	1406 ± 256	1874 ± 54	5043 ± 547	Up
An04g04670	*sm to chiB- A. nid*	strong similarity to chitinase cts1 - Coccidioides immitis	106 ± 36	104 ± 26	111 ± 1	171 ± 65	308 ± 102	331 ± 185	Up
An07g04650	*sm to btgC- A. nid*	similarity to exo-beta-1,3-glucanase BGL2 - Saccharomyces cerevisiae.	203 ± 14	275 ± 109	184 ± 41	699 ± 119	797 ± 4	319 ± 12	Up
An07g07530	*chrB*	strong similarity to cell wall protein UTR2 - Saccharomyces cerevisiae	176 ± 39	254 ± 48	1452 ± 40	1812 ± 115	1764 ± 453	2719 ± 364	Up
An08g07350	*sm to gelB- A. nid*	strong similarity to glycophospholipid-anchored surface glycoprotein GAS1 precursor - Saccharomyces cerevisiae	226 ± 26	257 ± 8	363 ± 114	674 ± 9	839 ± 71	1001 ± 69	Up
An09g02290	*sm to chsD- A.nid*	strong similarity to chitin synthase chsE - Aspergillus nidulans	308 ± 12	314 ± 57	255 ± 23	779 ± 15	723 ± 100	479 ± 18	Up
An12g10380	*chsF*	strong similarity to chitin synthase C chsC - Aspergillus fumigatus	118 ± 6	137 ± 13	478 ± 239	468 ± 12	527 ± 142	1407 ± 9	Up
An16g07040	*sm to btgE -A. nid*	similarity to beta-1,3-glucanosyltransferase BGT1 - Aspergillus fumigatus [truncated ORF]	502 ± 224	676 ± 353	2375 ± 144	5982 ± 202	6554 ± 1346	7358 ± 380	Up
An16g08090	*dfgE*	strong similarity to hypothetical protein B2J23.120 - Neurospora crassa	114 ± 8	125 ± 5	174 ± 10	229 ± 21	280 ± 11	438 ± 28	Up

Table 7. Average expression values of 46 genes in zones 1, 3, and 5 that are implicated in carbohydrate degradation and that are differentially expressed in wild-type when compared to the Δ*flbA* strain of *A. niger*. The table was adapted from Pel et al.[17]. *Description of gene name or its closest homologue. Sm: similar; A. nid: *A. nidulans*; A. ory: *A. oryzae*.

Annotation	Gene Name*	Description - Table 7	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An01g00780	*xlnB*	xylanase xynB of patent WO9713853-A2 - Aspergillus niger	4528 ± 1905	16891 ± 683	19085 ± 0	953 ± 977	4014 ± 2052	1371 ± 235	Down
An01g01870	*eglC*	strong similarity to hypothetical Avicelase III aviIII - Aspergillus aculeatus	77 ± 47	501 ± 267	400 ± 240	22 ± 0	23 ± 1	23 ± 2	Down
An01g03340	*sm to celA-A.oryzae*	strong similarity to xyloglucan-specific endo-beta-1,4-glucanase - Aspergillus aculeatus	552 ± 60	4966 ± 831	3065 ± 537	151 ± 48	272 ± 34	191 ± 34	Down
An01g06120	*gdbA*	strong similarity to 4-alpha-glucanotransferase / amylo-1,6-glucosidase GDB1 - Saccharomyces cerevisiae	1138 ± 28	1366 ± 280	915 ± 411	446 ± 27	385 ± 41	290 ± 12	Down
An01g11660	*cbhB*	1,4-beta-D-glucan cellobiohydrolase B precursor cbhB of patent WO9906574-A1 - Aspergillus niger	1289 ± 335	8224 ± 845	6037 ± 1558	48 ± 13	430 ± 386	1098 ± 313	Down
An01g11670	*eglA*	strong similarity to endo-beta-1,4-glucanase A eglA - Emericella nidulans	638 ± 408	2367 ± 12	589 ± 232	21 ± 2	42 ± 24	58 ± 1	Down
An01g14600	*uncharacterized*	strong similarity to the endo-1,4-beta-Xylanase B XynB, patent WO9414965 - Aspergillus tubingensis	256 ± 239	364 ± 349	195 ± 92	30 ± 1	34 ± 6	37 ± 4	Down
An02g11150	*aglB*	alpha-galactosidase aglB - Aspergillus niger	390 ± 96	2373 ± 668	3155 ± 569	111 ± 5	160 ± 21	126 ± 14	Down
An02g13240	*agdC*	strong similarity to alpha-1-6-glucosidase glcA - Aspergillus parasiticus	806 ± 223	877 ± 435	313 ± 147	224 ± 5	144 ± 26	77 ± 7	Down
An03g00940	*xlnC*	endo-1,4-beta-xylanase C precursor xlnC - Aspergillus niger	2605 ± 1941	15761 ± 4701	15538 ± 1230	337 ± 232	1058 ± 553	1067 ± 324	Down
An03g00960	*axhA*	1,4-beta-D-arabinoxylan arabinofuranohydrolase axhA - Aspergillus niger	4089 ± 1838	15097 ± 1853	17374 ± 0	456 ± 313	1314 ± 776	1602 ± 429	Down
An03g01050	*Uncharacterized*	similarity to endo-beta-1,4-glucanase - Bacillus polymyxa	137 ± 32	345 ± 202	1027 ± 608	31 ± 8	48 ± 17	89 ± 12	Down
An03g03740	*bgl4*	strong similarity to beta-glucosidase bgl4 - Humicola grisea var. thermoidea	656 ± 278	958 ± 22	587 ± 133	359 ± 34	367 ± 72	312 ± 13	Down
An03g06550	*glaA*	glucan 1,4-alpha-glucosidase glaA - Aspergillus niger	370 ± 102	1497 ± 1144	5690 ± 1251	182 ± 15	171 ± 29	166 ± 8	Down
An04g06920	*agdA*	extracellular alpha-glucosidase aglU - Aspergillus niger	236 ± 29	316 ± 16	651 ± 135	176 ± 8	145 ± 5	176 ± 10	Down
An04g09360	*uncharacterized*	strong similarity to hypothetical protein CC0812 - Caulobacter crescentus	136 ± 33	153 ± 76	90 ± 13	68 ± 0	64 ± 1	38 ± 8	Down
An04g09690	*sm to pmeA- A. nidulans*	strong similarity to pectin methylesterase PME1 - Aspergillus aculeatus	60 ± 19	190 ± 41	174 ± 59	38 ± 3	48 ± 0	47 ± 9	Down
An04g09700	*uncharacterized*	strong similarity to endo-xylogalacturonan hydrolase xghA - Aspergillus tubingensis	60 ± 41	435 ± 272	143 ± 33	28 ± 4	28 ± 0	39 ± 3	Down
An05g02410	*uncharacterized*	strong similarity to beta-glucuronidase GUSB - Canis familiaris	287 ± 18	343 ± 48	338 ± 149	201 ± 2	132 ± 4	104 ± 8	Down
An06g00170	*aglA*	alpha-galactosidase aglA - Aspergillus niger	600 ± 56	678 ± 85	124 ± 23	59 ± 7	32 ± 6	18 ± 3	Down
An07g08950	*eglC*	endoglucanase B eglB - Aspergillus niger	2511 ± 570	11775 ± 532	7793 ± 1370	52 ± 29	366 ± 244	422 ± 77	Down

Annotation	Gene Name*	Description - Table 7 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An07g09330	*cbhA*	cellulose 1,4-beta-cellobiosidase cbhA from patent WO9906574-A1- Aspergillus niger	1157 ± 1121	7883 ± 2929	3034 ± 1406	67 ± 16	130 ± 62	170 ± 76	Down
An08g05230	*uncharacterized*	strong similarity to putative endoglucanase IV - Trichoderma reesei	1152 ± 1281	2411 ± 2729	648 ± 116	55 ± 5	58 ± 11	46 ± 5	Down
An08g10780	*uncharacterized*	strong similarity to hypothetical protein T16K5.230 of A. thaliana	72 ± 40	140 ± 70	1109 ± 145	30 ± 8	26 ± 2	43 ± 0	Down
An09g02160	*rgaeA*	rhamnogalacturonan acetyl esterase rgaeA - Aspergillus niger	318 ± 9	829 ± 168	409 ± 176	165 ± 41	149 ± 17	58 ± 2	Down
An11g02100	*sm to bglR- A. nidulans*	strong similarity to furostanol glycoside 26-O-beta-glucosidase CSF26G1 - Costus speciosus	360 ± 21	1634 ± 301	808 ± 253	105 ± 38	140 ± 12	114 ± 5	Down
An12g04610	*uncharacterized*	similarity to endoglucanase IV egl4 - Trichoderma reesei	966 ± 1072	5636 ± 3072	243 ± 11	76 ± 9	108 ± 31	58 ± 5	Down
An13g03710	*agdD*	strong similarity to alpha-glucosidase AGLU - Bacillus sp. SAM1606	103 ± 18	130 ± 57	77 ± 28	44 ± 4	42 ± 4	47 ± 3	Down
An14g01800	*aglD*	alpha-galactosidase aglD - Aspergillus niger	54 ± 4	109 ± 6	136 ± 18	27 ± 0	33 ± 3	34 ± 2	Down
An14g02670	*uncharacterized*	strong similarity to endoglucanase IV egl4 - Trichoderma reesei	347 ± 273	857 ± 754	737 ± 10	37 ± 3	45 ± 3	37 ± 1	Down
An14g04190	*gbeA*	strong similarity to 1,4-alpha-glucan branching enzyme glc3 - Saccharomyces cerevisiae	1445 ± 19	1453 ± 195	915 ± 221	769 ± 3	603 ± 58	451 ± 20	Down
An14g04200	*rhgB*	rhamnogalacturonase rhgB - Aspergillus niger	83 ± 57	177 ± 184	105 ± 34	33 ± 7	36 ± 5	34 ± 0	Down
An14g05820	*uncharacterized*	strong similarity to beta-galactosidase lacA - Aspergillus niger	126 ± 37	290 ± 21	275 ± 6	60 ± 13	62 ± 12	72 ± 12	Down
An15g04550	*xynA*	strong similarity to xylanase A xynA of patent WO200068396-A2 - Aspergillus niger	55 ± 0	384 ± 304	1176 ± 75	35 ± 4	41 ± 21	34 ± 1	Down
An15g05370	*pgaII*	polygalacturonase pgaII of patent EP421919-A - Aspergillus niger	224 ± 219	767 ± 877	327 ± 240	29 ± 3	25 ± 5	28 ± 5	Down
An16g00540	*uncharacterized*	similarity to putative large secreted protein - Streptomyces coelicolor [truncated ORF]	32 ± 1	89 ± 3	159 ± 14	23 ± 1	25 ± 1	26 ± 6	Down
An16g06800	*eglB*	strong similarity to endoglucanase eglB - Aspergillus niger	154 ± 25	226 ± 100	423 ± 232	87 ± 5	92 ± 5	77 ± 5	Down
An17g00300	*xarB*	strong similarity to bifunctiona xylosidase-arabinosidase xarB - Thermoanaerobacter ethanolicus	156 ± 10	534 ± 22	396 ± 19	88 ± 6	124 ± 19	144 ± 17	Down
An01g01540	*uncharacterized*	strong similarity to alpha,alpha-trehalase treA - Aspergillus nidulans	154 ± 10	161 ± 8	149 ± 13	568 ± 30	607 ± 43	366 ± 5	Up
An01g10350	*sm to lacB- A. niger*	strong similarity to secreted beta-galactosidase lacA - Aspergillus niger	113 ± 5	109 ± 20	206 ± 35	469 ± 36	394 ± 38	499 ± 69	Up
An03g00190	*pelB*	the pectin lyase pelB - Aspergillus niger	56 ± 4	48 ± 4	61 ± 9	338 ± 67	536 ± 67	248 ± 3	Up
An03g06310	*pmeA*	pectinesterase pmeA- Aspergillus niger	33 ± 6	36 ± 6	43 ± 5	65 ± 4	75 ± 14	179 ± 41	Up
An09g00260	*aglC*	alpha-galactosidase C aglC - Aspergillus niger [truncated ORF]	31 ± 6	34 ± 4	143 ± 60	208 ± 5	216 ± 38	370 ± 31	Up
An09g00270	*aglC*	alpha-galactosidase C aglC - Aspergillus niger [truncated ORF]	89 ± 18	85 ± 22	760 ± 428	879 ± 73	1158 ± 119	1805 ± 251	Up

Annotation	Gene Name*	Description - Table 7 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An09g03070	*agsE*	strong similarity to alpha-glucan synthase mok1 - Schizosaccharomyces pombe	174 ± 2	142 ± 40	163 ± 50	544 ± 44	598 ± 19	458 ± 41	Up
An15g03550	*uncharacterized*	weak similarity to protopectinase patent WO9806832-A1 - Bacillus subtilis	57 ± 44	57 ± 45	20 ± 0	954 ± 34	1066 ± 21	330 ± 22	Up

Table 2), a phytase (*phyB*), and six (putative) proteases.

Previously, we identified 138 proteins with a signal sequence for secretion in the secretome of Δ*flbA* colonies when grown on fresh xylose medium.[8, Chapter 3] Of these, 70 were not found in the wild-type secretome (i.e. even after partial degradation of the cell wall by the addition of cycloheximide).[8, Chapter 3] Out of the 70 encoding genes, 23 were differentially expressed (Table 8). One of these genes was down-regulated, whereas 22 were up-regulated in the Δ*flbA* colonies. Most of these genes are uncharacterized, but encode proteins that are putative carbohydrate degrading enzymes (3), proteases (2), cell wall/ morphogenesis enzymes (2), lipases (2), phospholipases (2) oxidases and superoxide-dismutates (5), and other/ unkown proteins (6) (Table 8). Futhermore, out of the 30 proteins contained in the Δ*flbA* secretome[8, Chapter 3] that were absent in the wild-type secretome[7, Chapter 2] and that were predicted to be secreted via non-classical secretion (15) (SecP), or have no prediction for secretion (15),[8, Chapter 3] 2 and 5 genes were up- and down-regulated respectively (Table 8). The secretome of the cycloheximide treated wild-type strain contained 55 proteins[7, Chapter 2] that were absent in the Δ*flbA* secretome,[8, Chapter 3] of which 35 encoding genes were differentially expressed. The two up-regulated genes in the Δ*flbA* strain encode uncharacterized proteins. The 33 down-regulated genes encode carbohydrate degrading enzymes (17), phytases (2), proteases (5), oxidase (1), and other/unknown proteins (5) (Table 8). Of the 37 proteins that were identified in both the wild-type and Δ*flbA* secretome 6 and 14 encoding genes were found to be 2-fold higher and lower expressed in the Δ*flbA* strain, respectively (Table 8). The up-regulated genes consist of putative cell wall/ morphogenesis enzymes (3), carbohydrate degrading enzymes (1), a protease (1), and a superoxide dismutase (1), while the down-regulated genes encode putative carbohydrate degrading enzymes (11), a cell wall/ morphogenesis enzyme (1), a phospholipase (1), and a lipase (1).

Transcriptional changes associated to transcription factors

Pel et al.[17] described 623 *A. niger* transcription factors. Of these, 20 and 18 are found to be up- and down-regulated in the Δ*flbA* strain, respectively (Table 9, Supplemental Table 1). Of these genes, 28 encode transcription factors with an unknown function, while 3 are homologous to transcription factor genes of *A. oryzae* and 1 to *A. fumigatus*. These orthologues encode *atfB* (An14g06250), *xlnR* (An11g06290), *amyR* (An01g06900), and *rosA* (An04g07400), respectively. Moreover, the regulator of extracellular protease *prtT* was found to be down-regulated in the Δ*flbA* strain. In contrast, *pacC* that is activated upon exposure to alkaline pH and *acuB* that activates acetate metabolism are ≥ 2-fold higher expressed in the Δ*flbA* strain. Among the genes encoding transcriptional activators that are up-regulated in Δ*flbA* are *abaA* and *flbD* that are involved in reproductive development (see above).

Table 8. Average expression values of zones 1, 3, and 5 of 87 differentially regulated genes whose protein products were identified in the secretome of xylose-grown sandwiched colonies of the Δ*flbA*[8] strain and / or the wild-type[7] of *A. niger* . *Description of gene name or its closest homologue. Sm: similar; A. nid: *A. nidulans.*

Annotation	Gene Name*	Description - Table 8	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
protein products identified in the secretome of Δ*flbA* that are predicted to be secreted									
An11g06480	*uncharacterized*	weak similarity to antigenic protein f86.aa. of patent WO9859071 - Borrelia burgdorferi	165 ± 5	154 ± 2	119 ± 6	43 ± 9	38 ± 1	43 ± 4	Down
An09g00260	*aglC*	alpha-galactosidase C aglC - Aspergillus niger[truncated ORF]	31 ± 6	34 ± 4	143 ± 60	208 ± 5	216 ± 38	370 ± 31	Up
An03g06310	*pmeA*	pectinesterase pmeA- Aspergillus niger	33 ± 6	36 ± 6	43 ± 5	65 ± 4	75 ± 14	179 ± 41	Up
An15g03550	*uncharacterized*	weak similarity to protopectinase patent WO9806832-A1 - Bacillus subtilis	57 ± 44	57 ± 45	20 ± 0	954 ± 34	1066 ± 21	330 ± 22	Up
An11g00100	*uncharacterized*	strong similarity to triacylglycerol lipase LIP5 - Candida rugosa	71 ± 4	64 ± 3	59 ± 8	800 ± 192	866 ± 397	2265 ± 77	Up
An16g08870	*uncharacterized*	strong similarity to the triacylglycerol lipase I precursor lipI - Geotrichum candidum	139 ± 16	125 ± 28	84 ± 2	395 ± 28	267 ± 13	181 ± 12	Up
An09g01240	*uncharacterized*	strong similarity to phospholipase B - Penicillium notatum	78 ± 7	63 ± 9	104 ± 3	499 ± 142	727 ± 111	627 ± 178	Up
An16g01880	*lipanI*	strong similarity to lysophospholipase - Aspergillus foetidus	648 ± 245	795 ± 248	454 ± 202	10644 ± 663	10585 ± 2214	2676 ± 160	Up
An02g00740	*uncharacterized*	similarity to 6-Hydroxy-D-nicotine oxidase 6-HDNO - Arthrobacter oxidans	31 ± 1	30 ± 2	35 ± 3	61 ± 5	69 ± 11	130 ± 14	Up
An02g08560	*uncharacterized*	similarity to probable dioxygenase SCOEDB - Streptomyces coelicolor	22 ± 3	23 ± 3	19 ± 0	797 ± 22	1048 ± 83	1682 ± 135	Up
An03g00460	*uncharacterized*	strong similarity to the 6-hydroxy-D-nicotine oxidase 6-HDNO - Arthrobacter oxidans	129 ± 69	90 ± 27	102 ± 7	913 ± 19	751 ± 8	198 ± 2	Up
An03g05210	*uncharacterized*	strong similarity to reticuline oxidase bbe1 - Eschscholzia californica	146 ± 47	180 ± 82	308 ± 23	423 ± 2	453 ± 23	767 ± 34	Up
An06g00720	*uncharacterized*	similarity to chloroperoxidase CPO - Caldariomyces fumago	59 ± 24	59 ± 16	40 ± 14	109 ± 12	134 ± 32	109 ± 2	Up
An03g06220	*sm to gelD- A. nid*	strong similarity to beta (1-3) glucanosyltransferase GEL3 - Aspergillus fumigatus	42 ± 3	48 ± 11	58 ± 3	1406 ± 256	1874 ± 54	5043 ± 547	Up
An16g07040	*sm to btgE -A. nid*	similarity to beta-1,3-glucanosyltransferase BGT1 - Aspergillus fumigatus[truncated ORF]	502 ± 224	676 ± 353	2375 ± 144	5982 ± 202	6554 ± 1346	7358 ± 380	Up
An05g02170	*uncharacterized*	strong similarity to serine-type carboxypeptidase F CPD-II - Aspergillus niger	60 ± 17	57 ± 6	70 ± 11	109 ± 12	121 ± 19	240 ± 17	Up
An18g01320	*uncharacterized*	strong similarity to extracellular protease precursor BAR1 - Saccharomyces cerevisiae	148 ± 76	129 ± 56	328 ± 31	2146 ± 67	2897 ± 61	2501 ± 161	Up
An01g06280	*uncharacterized*	strong similarity to IgE-binding protein - Aspergillus fumigatus	267 ± 123	341 ± 167	136 ± 4	2010 ± 28	2634 ± 280	1002 ± 144	Up
An03g00770	*uncharacterized*	strong similarity to allergic bronchopulmonary aspergillosis allergen rAsp f 4 of patent WO9828624-A1 - Aspergillus fumigatus	93 ± 3	98 ± 34	454 ± 19	473 ± 118	437 ± 60	2858 ± 7	Up
An04g07160	*uncharacterized*	similarity to hypothetical protein MLD14.3 - Arabidopsis thaliana	202 ± 34	215 ± 74	246 ± 38	548 ± 65	656 ± 82	666 ± 81	Up

Annotation	Gene Name*	Description - Table 8 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An06g00160	*uncharacterized*	weak similarity to hypothetical cell wall protein binB - Aspergillus nidulans	319 ± 29	330 ± 70	128 ± 21	3226 ± 732	2940 ± 1230	453 ± 184	Up
An07g02730	*uncharacterized*	strong similarity to SUN family protein Psu1 - Schizosaccharomyces pombe	261 ± 96	317 ± 133	1534 ± 12	2045 ± 210	2424 ± 353	3717 ± 85	Up
An09g03650	*uncharacterized*	weak similarity to hypothetical protein Ta0309 - Thermoplasma acidophilum	84 ± 40	52 ± 1	133 ± 16	375 ± 55	488 ± 99	438 ± 1	Up
protein products identified in the Δ*flbA* secreteome that are predicted to be released by non-classical secretion									
An09g02830	*uncharacterized*	strong similarity to acylaminoacyl-peptidase DPP V - Aspergillus fumigatus	385 ± 95	420 ± 131	622 ± 25	251 ± 45	224 ± 14	116 ± 2	Down
An09g00270	*aglC*	alpha-galactosidase C aglC - Aspergillus niger[truncated ORF]	89 ± 18	85 ± 22	760 ± 428	879 ± 73	1158 ± 119	1805 ± 251	Up
proteins-products identified in the Δ*flbA* secretome without signal sequence for secretion									
An01g06970	*ara1*	strong similarity to D-arabinose dehydrogenase ARA1 - Saccharomyces cerevisiae	2370 ± 516	3061 ± 357	2476 ± 456	643 ± 50	627 ± 28	1838 ± 51	Down
An02g11970	*uncharacterized*	strong similarity to 4-nitrophenylphosphatase pho2 - Schizosaccharomyces pombe	85 ± 12	93 ± 21	123 ± 9	164 ± 6	183 ± 36	416 ± 16	Up
An08g10830	*uncharacterized*	strong similarity to geranylgeranyl pyrophosphate synthase ggpps - Gibberella fujikuroi	65 ± 5	58 ± 0	68 ± 18	412 ± 9	388 ± 31	555 ± 30	Up
An09g06250	*uncharacterized*	strong similarity to vacuolar aminopeptidase ysc1 - Saccharomyces cerevisiae	184 ± 42	187 ± 30	405 ± 70	526 ± 16	555 ± 41	430 ± 39	Up
An12g08610	*glkA*	glucokinase GlkA - Aspergillus niger	302 ± 29	324 ± 114	306 ± 96	652 ± 16	622 ± 75	660 ± 65	Up
protein-products identified in wild-type and Δ*flbA* secretome that are predicted to be secreted									
An02g11150	*aglB*	alpha-galactosidase aglB - Aspergillus niger	390 ± 96	2373 ± 668	3155 ± 569	111 ± 5	160 ± 21	126 ± 14	Down
An01g00780	*xlnB*	endo-1,4-beta-xylanase B precursor xlnB - Aspergillus niger	4528 ± 1905	16891 ± 683	19085 ± 0	953 ± 977	4014 ± 2052	1371 ± 235	Down
An03g00940	*xlnC*	endo-1,4-beta-xylanase C precursor xlnC - Aspergillus niger	2605 ± 1941	15761 ± 4701	15538 ± 1230	337 ± 232	1058 ± 553	1067 ± 324	Down
An03g00960	*axhA*	1,4-beta-D-arabinoxylan arabinofuranohydrolase axhA - Aspergillus niger	4089 ± 1838	15097 ± 1853	17374 ± 0	456 ± 313	1314 ± 776	1602 ± 429	Down
An14g01800	*aglD*	alpha-galactosidase aglD - Aspergillus niger	54 ± 4	109 ± 6	136 ± 18	27 ± 0	33 ± 3	34 ± 2	Down
An17g00300	*xarB*	strong similarity to bifunctiona xylosidase-arabinosidase xarB - Thermoanaerobacter ethanolicus	156 ± 10	534 ± 22	396 ± 19	88 ± 6	124 ± 19	144 ± 17	Down
An01g11660	*cbhB*	1,4-beta-D-glucan cellobiohydrolase B precursor cbhB of patent WO9906574-A1 - Aspergillus niger	1289 ± 335	8224 ± 845	6037 ± 1558	48 ± 13	430 ± 386	1098 ± 313	Down
An07g09330	*cbhA*	cellulose 1,4-beta-cellobiosidase cbhA from patent WO9906574-A1- Aspergillus niger	1157 ± 1121	7883 ± 2929	3034 ± 1406	67 ± 16	130 ± 62	170 ± 76	Down
An16g06800	*eglB*	strong similarity to endoglucanase eglB - Aspergillus niger	154 ± 25	226 ± 100	423 ± 232	87 ± 5	92 ± 5	77 ± 5	Down

Annotation	Gene Name*	Description - Table 8 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An08g05230	*uncharacterized*	strong similarity to putative endoglucanase IV - Trichoderma reesei	1152 ± 1281	2411 ± 2729	648 ± 116	55 ± 5	58 ± 11	46 ± 5	Down
An03g06550	*glaA*	glucan 1,4-alpha-glucosidase glaA - Aspergillus niger	370 ± 102	1497 ± 1144	5690 ± 1251	182 ± 15	171 ± 29	166 ± 8	Down
An01g12450	*exsG*	strong similarity to putative glucan beta-1,3 exoglucanase - Trichoderma harzianum	3091 ± 197	3667 ± 1573	1008 ± 250	966 ± 4	1053 ± 14	689 ± 27	Down
An02g09690	*uncharacterized*	strong similarity to lipase I precursor TFL I - Geotrichum fermentans[putative sequencing error]	652 ± 18	1719 ± 762	529 ± 399	195 ± 22	294 ± 108	356 ± 50	Down
An01g14940	*uncharacterized*	similarity to nonhemolytic phospholipase C PC-PLC - Burkholderia pseudomallei	299 ± 58	486 ± 126	203 ± 35	122 ± 2	138 ± 34	57 ± 2	Down
An01g01540	*uncharacterized*	strong similarity to alpha,alpha-trehalase treA - Aspergillus nidulans	154 ± 10	161 ± 8	149 ± 13	568 ± 30	607 ± 43	366 ± 5	Up
An07g07530	*crhB*	strong similarity to cell wall protein UTR2 - Saccharomyces cerevisiae	176 ± 39	254 ± 48	1452 ± 40	1812 ± 115	1764 ± 453	2719 ± 364	Up
An14g01820	*phiA /binB*	strong similarity to hypothetical cell wall protein binB - Aspergillus nidulans	2018 ± 1048	3064 ± 430	843 ± 70	8800 ± 3676	14346 ± 1791	4066 ± 653	Up
An08g07350	*sm to gelB- A. nid*	strong similarity to glycophospholipid-anchored surface glycoprotein GAS1 precursor - Saccharomyces cerevisiae	226 ± 26	257 ± 8	363 ± 114	674 ± 9	839 ± 71	1001 ± 69	Up
An08g00490	*uncharacterized*	strong similarity to lactonohydrolase - Fusarium oxysporum	160 ± 14	237 ± 17	346 ± 177	484 ± 34	516 ± 99	806 ± 14	Up
An14g02470	*uncharacterized*	strong similarity to the protein PRO304 of patent WO200104311-A1 - Homo sapiens	326 ± 177	470 ± 61	384 ± 107	2772 ± 181	3044 ± 235	770 ± 49	Up
Protein products identified in wild-type secretome that are predicted to be secreted									
An01g14600	*uncharacterized*	strong similarity to the endo-1,4-beta-Xylanase B XynB, patent WO9414965 - Aspergillus tubingensis	256 ± 239	364 ± 349	195 ± 92	30 ± 1	34 ± 6	37 ± 4	Down
An06g00170	*aglA*	alpha-galactosidase aglA - Aspergillus niger	600 ± 56	678 ± 85	124 ± 23	59 ± 7	32 ± 6	18 ± 3	Down
An04g06920	*agdA*	extracellular alpha-glucosidase aglU - Aspergillus niger	236 ± 29	316 ± 16	651 ± 135	176 ± 8	145 ± 5	176 ± 10	Down
An04g09690	*sm to pmeA- A. nidulans*	strong similarity to pectin methylesterase PME1 - Aspergillus aculeatus	60 ± 19	190 ± 41	174 ± 59	38 ± 3	48 ± 0	47 ± 9	Down
An07g08940	*uncharacterized*	similarity to acetyl-esterase I of patent WO9502689-A - Aspergillus aculeatus	242 ± 13	1268 ± 407	914 ± 553	20 ± 1	31 ± 7	26 ± 0	Down
An01g11670	*eglA*	strong similarity to endo-beta-1,4-glucanase A eglA - Emericella nidulans	638 ± 408	2367 ± 12	589 ± 232	21 ± 2	42 ± 24	58 ± 1	Down
An07g08950	*eglC*	endoglucanase B eglB - Aspergillus niger	2511 ± 570	11775 ± 532	7793 ± 1370	52 ± 29	366 ± 244	422 ± 77	Down
An01g01870	*eglC*	strong similarity to hypothetical Avicelase III aviIII - Aspergillus aculeatus	77 ± 47	501 ± 267	400 ± 240	22 ± 0	23 ± 1	23 ± 2	Down
An03g01050	*uncharacterized*	similarity to endo-beta-1,4-glucanase - Bacillus polymyxa	137 ± 32	345 ± 202	1027 ± 608	31 ± 8	48 ± 17	89 ± 12	Down
An08g10780	*uncharacterized*	strong similarity to hypothetical protein T16K5.230 of A. thaliana	72 ± 40	140 ± 70	1109 ± 145	30 ± 8	26 ± 2	43 ± 0	Down

Annotation	Gene Name*	Description - Table 8 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An14g02670	*uncharacterized*	strong similarity to endoglucanase IV egl4 - Trichoderma reesei	347 ± 273	857 ± 754	737 ± 10	37 ± 3	45 ± 3	37 ± 1	Down
An08g08370	*uncharacterized*	similarity to the alpha-1,2-mannosidase aman2 - Bacillus sp. M-90	1771 ± 843	2086 ± 211	376 ± 19	179 ± 33	78 ± 14	38 ± 4	Down
An16g00540	*uncharacterized*	similarity to putative large secreted protein - Streptomyces coelicolor[truncated ORF]	32 ± 1	89 ± 3	159 ± 14	23 ± 1	25 ± 1	26 ± 6	Down
An02g02540	*uncharacterized*	strong similarity to acetyl-esterase I from patent R63066 - Aspergillus aculeatus	296 ± 66	375 ± 174	350 ± 139	83 ± 16	94 ± 1	74 ± 12	Down
An04g09700	*uncharacterized*	strong similarity to endo-xylogalacturonan hydrolase xghA - Aspergillus tubingensis	60 ± 41	435 ± 272	143 ± 33	28 ± 4	28 ± 0	39 ± 3	Down
An14g04200	*rhgB*	rhamnogalacturonase rhgB - Aspergillus niger	83 ± 57	177 ± 184	105 ± 34	33 ± 7	36 ± 5	34 ± 0	Down
An15g05370	*pgaII*	polygalacturonase pgaII of patent EP421919-A - Aspergillus niger	224 ± 219	767 ± 877	327 ± 240	29 ± 3	25 ± 5	28 ± 5	Down
An07g02360	*uncharacterized*	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - Arthrobacter oxidans	60 ± 24	49 ± 23	104 ± 15	32 ± 3	31 ± 2	25 ± 4	Down
An08g09850	*uncharacterized*	strong similarity to phosphate-repressible acid phosphatase precursor phoA - Penicillium chrysogenum	424 ± 125	551 ± 364	636 ± 431	245 ± 15	256 ± 8	132 ± 7	Down
An08g11030	*phyB*	acid phosphatase aph, 3-phytase phyB - Aspergillus niger	436 ± 438	975 ± 1113	973 ± 889	54 ± 4	59 ± 5	44 ± 0	Down
An08g04640	*protB*	strong similarity to putative lysosomal pepstatin insensitive protease CLN2 - Canis familiaris	98 ± 32	218 ± 127	827 ± 628	62 ± 13	58 ± 5	59 ± 2	Down
An16g09010	*protH*	strong similarity to carboxypeptidase I protein of patent WO9814599-A1 - Aspergillus oryza	1018 ± 249	1107 ± 340	353 ± 147	312 ± 2	298 ± 3	161 ± 9	Down
An06g00190	*uncharacterized*	strong similarity to lysosomal pepstatin insensitive protease CLN2 - Homo sapiens	305 ± 23	733 ± 87	1175 ± 28	144 ± 2	114 ± 5	58 ± 5	Down
An12g05960	*uncharacterized*	strong similarity to dipeptidyl peptidase II DPPII - Rattus norvegicus	105 ± 22	126 ± 40	207 ± 38	81 ± 22	81 ± 4	47 ± 7	Down
An14g02150	*uncharacterized*	strong similarity to serine-type carboxypeptidase precursor cpdS - Aspergillus phoenicis	49 ± 10	57 ± 12	38 ± 7	23 ± 2	21 ± 1	23 ± 4	Down
An09g00670	*gelD*	strong similarity to beta (1-3) glucanosyltransferase Gel3p - Aspergillus fumigatus	1596 ± 565	2244 ± 607	2063 ± 821	45 ± 1	39 ± 6	59 ± 10	Down
An09g00840	*uncharacterized*	weak similarity to antigenic cell wall galactomannoprotein MP1 - Aspergillus fumigatus	3153 ± 2599	10725 ± 146	3462 ± 103	29 ± 11	35 ± 22	14 ± 2	Down
An01g15200	*uncharacterized*	strong similarity to mature penicillin V amidohydrolase PVA of patent US5516679-A - Fusarium oxysporum[truncated ORF]	1551 ± 24	1626 ± 657	351 ± 58	146 ± 9	92 ± 0	108 ± 31	Down
An13g01880	*uncharacterized*	strong similarity to cephalosporin esterase - Rhodosporidium toruloides	407 ± 104	3000 ± 374	3350 ± 147	96 ± 37	194 ± 69	155 ± 2	Down
An01g00210	*uncharacterized*	hypothetical protein	105 ± 13	174 ± 37	91 ± 31	36 ± 2	37 ± 0	50 ± 8	Down
An02g11890	*uncharacterized*	strong similarity to hypothetical protein An14g01330 - Aspergillus niger	61 ± 14	161 ± 33	124 ± 11	24 ± 1	30 ± 1	29 ± 2	Down
An08g04630	*uncharacterized*	hypothetical protein	361 ± 349	674 ± 706	305 ± 86	51 ± 1	48 ± 13	36 ± 1	Down

Annotation	Gene Name*	Description - Table 8 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An15g02250	*uncharacterized*	hypothetical protein	1220 ± 1202	1162 ± 905	78 ± 37	47 ± 2	49 ± 6	50 ± 9	Down
An06g01000	*uncharacterized*	strong similarity to hypothetical protein AN5357.2 - Aspergillus nidulans	201 ± 49	252 ± 68	397 ± 160	601 ± 188	760 ± 179	892 ± 307	Up
An11g00040	*uncharacterized*	weak similarity to cDNA for 59-kDa readthrough protein RT - Sorghum chlorotic spot virus	780 ± 377	869 ± 597	1222 ± 357	3463 ± 134	3264 ± 0	2983 ± 97	Up
proteins identified in wild-type without signal sequence for secretion									
An14g01790	*uncharacterized*	hypothetical protein	93 ± 2	199 ± 15	220 ± 21	37 ± 4	54 ± 8	47 ± 16	Down
An01g00610	*uncharacterized*	weak similarity to hypothetical protein yukJ - Bacillus subtilis	455 ± 238	550 ± 223	1226 ± 644	47 ± 3	40 ± 6	73 ± 6	Down

Table 9. Average expression values of 38 transcription factor genes[17] in zones 1, 3, and 5 that are differentially expressed in wild-type when compared to the Δ*flbA* strain. Names of transcription factors are indicated that show homology to or that are encoded by *A. niger* genes. *Description of gene or its closest homologue. Sm: similar; A. fum: *A. fumigatus*; A. ory: *A. oryzae*.

Annotation	Gene Name*	Description - Table 9 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An04g08620	*uncharacterized*	similarity to heme activator protein HAP1 - Saccharomyces cerevisiae	1079 ± 14	1118 ± 11	942 ± 47	642 ± 42	512 ± 28	191 ± 21	Down
An12g01870	*uncharacterized*	similarity to positive regulator of the lactose-galactose regulon LAC9 - Kluyveromyces lactis	495 ± 135	1003 ± 137	720 ± 305	254 ± 24	271 ± 9	167 ± 16	Down
An16g08800	*uncharacterized*	similarity to the zinc finger transcription factor ACEI - Trichoderma reesei	543 ± 121	642 ± 119	310 ± 34	77 ± 17	78 ± 5	77 ± 1	Down
An15g00120	*uncharacterized*	similarity to Wilms tumor susceptibility protein WT1 - Homo sapiens	476 ± 307	442 ± 230	580 ± 259	112 ± 11	111 ± 1	65 ± 3	Down
An04g05060	*uncharacterized*	similarity to XFIN protein - Xenopus laevis	3584 ± 911	3946 ± 117	1943 ± 688	1248 ± 6	1042 ± 73	810 ± 91	Down
An04g07400	*sm rosA- A. fum*	strong similarity to C6 zinc finger transcription factor PRO1 - Sordaria macrospora [putative sequencing error]	120 ± 28	195 ± 76	562 ± 77	86 ± 3	83 ± 9	43 ± 6	Down
An13g01370	*uncharacterized*	strong similarity to the hypothetical protein An01g14540 - Aspergillus niger	568 ± 230	603 ± 155	359 ± 47	207 ± 8	157 ± 8	192 ± 26	Down
An11g07610	*uncharacterized*	strong similarity to the hypothetical protein encoded by An15g04740 - Aspergillus niger	226 ± 129	212 ± 48	104 ± 38	54 ± 1	43 ± 2	33 ± 0	Down
An11g06290	*sm to xlnR -A. ory*	strong similarity to transcriptional activator xlnR - Aspergillus niger	145 ± 30	141 ± 35	84 ± 17	54 ± 2	40 ± 4	30 ± 1	Down
An01g13790	*uncharacterized*	weak similarity to homeodomain protein Prep-1 - Homo sapiens	792 ± 126	685 ± 218	368 ± 78	220 ± 10	203 ± 29	317 ± 8	Down
An12g02880	*uncharacterized*	weak similarity to hypothetical protein SPBC530.05 - Schizosaccharomyces pombe	80 ± 51	80 ± 48	39 ± 1	24 ± 1	27 ± 0	31 ± 0	Down
An06g00830	*uncharacterized*	weak similarity to hypothetical transcription regulatory protein SPBC530.08 - Schizosaccharomyces pombe	121 ± 26	146 ± 14	221 ± 35	55 ± 6	67 ± 22	49 ± 8	Down
An08g03770	*uncharacterized*	weak similarity to mucin MUC5AC - Homo sapiens	85 ± 33	77 ± 21	132 ± 63	23 ± 2	19 ± 3	18 ± 2	Down
An07g07370	*uncharacterized*	weak similarity to PC-MYB2 - Arabidopsis thaliana	2629 ± 9	2410 ± 318	1104 ± 502	724 ± 44	649 ± 44	253 ± 2	Down
An04g01430	*uncharacterized*	weak similarity to the chitinase ChiA - Emericella nidulans	14247 ± 1226	12839 ± 0	6031 ± 975	4922 ± 660	3743 ± 121	3693 ± 284	Down
An01g06900	*sm to amyR- A. ory*	weak similarity to transcription activator amyR - Aspergillus oryzae	758 ± 96	1056 ± 406	654 ± 233	34 ± 8	25 ± 1	21 ± 1	Down
An14g06250	*sm to atfB-A. ory*	weak similarity to transcription factor atf1+ - Schizosaccharomyces pombe	269 ± 19	523 ± 113	434 ± 242	67 ± 0	67 ± 5	89 ± 8	Down
An04g06940	*prtT*	similarity to hypothetical transcriptional regulator SPAC1399_1 - Schizosaccharomyces pombe	2945 ± 971	3996 ± 1429	2983 ± 738	1702 ± 175	1499 ± 130	608 ± 77	Down
An08g06580	*acuB / FacB*	DNA binding protein facB - Aspergillus niger	364 ± 29	340 ± 18	400 ± 148	899 ± 47	929 ± 83	656 ± 14	Up
An07g05960	*uncharacterized*	similarity to finger protein msn2 - Saccharomyces cerevisiae	143 ± 21	220 ± 133	537 ± 288	759 ± 389	1226 ± 285	1125 ± 434	Up
An04g06950	*uncharacterized*	similarity to homeobox transcription factor hth - Drosophila melanogaster	102 ± 1	124 ± 6	67 ± 16	288 ± 24	302 ± 50	287 ± 54	Up

Annotation	Gene Name*	Description - Table 9 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An08g06850	*uncharacterized*	similarity to hypothetical C2H2 zinc-finger protein SPBC1105.14 - Schizosaccharomyces pombe	66 ± 3	75 ± 1	129 ± 30	583 ± 676	787 ± 517	791 ± 668	Up
An06g02570	*uncharacterized*	similarity to nitrogen assimilation regulatory protein nit-4 - Neurospora crassa	56 ± 3	56 ± 10	114 ± 11	133 ± 0	144 ± 7	235 ± 27	Up
An03g05170	*uncharacterized*	similarity to sterol regulatory element binding protein-1 srebp-1 - Homo sapiens	352 ± 68	378 ± 126	574 ± 9	654 ± 59	806 ± 134	1485 ± 37	Up
An04g06430	*uncharacterized*	similarity to zinc finger protein BMZF3 of patent WO9962951-A1 - Homo sapiens	43 ± 3	45 ± 6	63 ± 23	103 ± 1	143 ± 1	147 ± 0	Up
An01g05630	*uncharacterized*	strong similarity to cytoplasmic aspartate--tRNA ligase APS - Saccharomyces cerevisiae [possible sequencing error]	70 ± 1	75 ± 5	63 ± 0	189 ± 5	214 ± 24	164 ± 7	Up
An14g05810	*uncharacterized*	strong similarity to histone acetyltransferase GCN5 - Saccharomyces cerevisiae	63 ± 15	58 ± 2	46 ± 1	148 ± 17	140 ± 22	85 ± 2	Up
An01g04830	*flbD*	strong similarity to myb-like DNA binding protein flbD - Aspergillus nidulans	89 ± 18	91 ± 8	81 ± 20	238 ± 35	220 ± 32	107 ± 5	Up
An03g06760	*uncharacterized*	strong similarity to probable transcription activator SPAC139.03 - Schizosaccharomyces pombe	106 ± 0	112 ± 14	57 ± 13	158 ± 8	209 ± 42	193 ± 22	Up
An01g03750	*abaA*	strong similarity to protein abaA - Aspergillus nidulans	61 ± 9	71 ± 12	67 ± 17	113 ± 28	129 ± 3	182 ± 22	Up
An02g07890	*pacC*	transcription factor pacC - Aspergillus niger	263 ± 12	282 ± 35	184 ± 19	552 ± 69	552 ± 118	562 ± 23	Up
An07g09460	*tan1*	transposase Tan1 of patent WO9808960-A1 - Aspergillus niger	17 ± 4	16 ± 3	20 ± 2	38 ± 3	67 ± 10	51 ± 6	Up
An08g06030	*uncharacterized*	weak similarity to putative zinc finger transcription factor stzA - Aspergillus nidulans	232 ± 9	305 ± 46	394 ± 61	668 ± 211	801 ± 153	741 ± 116	Up
An04g00480	*uncharacterized*	weak similarity to the vitellogenin II precursor - Gallus gallus	469 ± 62	309 ± 76	266 ± 36	1222 ± 145	1525 ± 171	947 ± 325	Up
An11g10870	*uncharacterized*	weak similarity to transcription factor CHA4 - Saccharomyces cerevisiae	193 ± 83	180 ± 27	273 ± 6	626 ± 226	622 ± 163	560 ± 43	Up
An14g07040	*uncharacterized*	weak similarity to transcriptional activator of lysine pathway genes LYS14 - Saccharomyces cerevisiae	76 ± 5	71 ± 10	70 ± 1	178 ± 10	217 ± 56	141 ± 8	Up
An17g00800	*uncharacterized*	weak similarity to transcriptional activator prtT of patent WO200020596-A1 - Aspergillus niger	119 ± 48	89 ± 35	69 ± 5	201 ± 38	195 ± 22	142 ± 5	Up
An02g06000	*uncharacterized*	weak similarity to ZMS1 - Saccharomyces cerevisiae	471 ± 242	481 ± 196	683 ± 362	980 ± 41	1338 ± 83	1688 ± 256	Up

DISCUSSION

Colonies of a strain in which *flbA* is inactivated do not sporulate. Instead, they grow throughout the colony and show secretion in a large part of the mycelium. Moreover, cell walls of the Δ*flbA* strain are thinner than those of the wild-type. The molecular mechanisms underlying these phenomena are not known. Here, the impact of inactivation of *flbA* on spatial gene expression in the *A. niger* colony was assessed with emphasis on genes encoding secreted proteins, genes involved in cell wall synthesis, genes involved in asexual and sexual development, and genes encoding transcriptional regulators. The latter genes may control spatial growth and secretion, cell wall synthesis, or may have a role in reproduction.

Genes that could account for a sexual pathway have been identified in *A. niger*.[17] However, so far only asexual reproduction has been shown to occur in this fungus. Asexual development involves the formation of conidia forming conidiophores. This process has been studied in most detail in *A. nidulans*.[25] Based on its genome, *A. niger* is expected to have similar mechanisms underlying conidiophore development. Indeed, the central regulator of conidiophore formation *brlA* as well as *flbA* have a similar role in asexual development in *A. niger* and *A. nidulans*.[26]. Inactivation of *flbA* of *A. niger* does not have a major impact on the expression of genes involved in asexual development. Of the 68 genes implicated in reproduction, only 13 were differentially expressed when compared to the wild-type. Genes *flbD* and *sfaD* (Figure 1) were among the 6 genes that were up-regulated ≥ 2-fold in the Δ*flbA* strain. In contrast, *flbD* and *sfaD* expression was not increased in the Δ*flbA* strains of *A. nidulans* and *A. oryzae*.[27, 28] Transcripts of *brlA* were absent in wild type *A. niger* colonies, while some *brlA* expression was observed in the center of Δ*flbA* colonies. In contrast to *A. niger,* FlbA directly or indirectly stimulates expression of *brlA* in *A. nidulans*[9], *A. oryzae*[28], and *A. fumigatus*.[29] Taken together, it can be concluded that the sporulation pathway of *A. niger* is similar but not identical to that of *A. nidulans, A. oryzae,* and *A. fumigatus*. Our data also indicate that FlbA has repressing activity in *A. niger* colonies in a zone that does no longer have the potential to sporulate. It might be that this also occurs in other aspergilli. This should be studied by extracting RNA of zones rather than the whole mycelium, as is routinely done.

A total of 102 genes are predicted to be involved in cell wall synthesis[17]. Of these genes, only 10 and 2 were found to be up- and down-regulated in the Δ*flbA* strain, respectively. It is tempting to speculate that these genes are involved in the changed spatial distribution of growing hyphae in the colony. The up-regulation of two chitin synthase genes is of particular interest in this respect. The *chsD* homologue of *A. nidulans* is expressed throughout the *A. niger* colony. Upon deletion of *flbA* up-regulation occurs in all zones of the colony. The *chsF* chitin synthase gene is even more interesting. This gene is periphery specific in wild-type colonies, and its expression thus correlates with growth. Expression levels are

similar in the central zones in the Δ*flbA* strain when compared to the periphery of the wild-type strain, and is even higher at the periphery of the Δ*flbA* strain. Genes involved in cell wall synthesis are not only involved in growth but also in architecture and thickness of the cell wall. Cell walls in the central part of wild-type colonies are thicker than those at the periphery (i.e. 200 vs 145 nm).[7, Chapter 2] Cell walls of the Δ*flbA* strain are generally thinner than those of the wild-type (i.e. about 120 nm in width both at the periphery and the centre of the colony).[8, Chapter 3] The differentially expressed genes involved in cell wall synthesis may impact cell wall thickness in the Δ*flbA* strain. This may involve synthesizing activity, cell wall processing activity (i.e. crosslinking), as well as degradation activity.

The secretome of the wild-type and the Δ*flbA* strain was determined by transferring 7-days-old colonies that had been grown on agar medium to a ring plate containing fresh medium. During the 24 h incubation time, proteins were released in the wells of the ring plate that had been formed during the 7 days of growth on the agar plate by slowly diffusing through the cell wall as well as proteins that were formed during the last 24 h and that were immediately released into the culture medium. FlbA was shown to have a more complex secretome when compared to the wild-type.[8, Chapter 3] Out of 138 secreted proteins of the Δ*flbA* strain,[8, Chapter 3] 101 had not been identified in the secretome of wild-type colonies.[8, Chapter 3] Cycloheximide treatment releases proteins into the culture medium that had been trapped in the cell walls of the wild-type strain.[8, Chapter 3] Still, 70 secreted proteins were found in the Δ*flbA* secretome that were absent in the medium of cycloheximide treated wild-type colonies. Of these proteins, 23 were differentially expressed, of which 22 were up-regulated in the Δ*flbA* strain. This shows that part of the differences in the secretome are caused by transcriptional control during the 7-days of growth on the agar medium. Another part of the differences may be explained by induction of genes after transfer to fresh medium and by (post)-translational regulation. Moreover, we can not exclude that part of the proteome is still trapped in or associated with the wild type cell wall after cycloheximide treatment. 55 proteins were identified in the wild-type secretome that were absent in the culture medium of the Δ*flbA* strain.[8, Chapter 3] Of the genes encoding these proteins, 35 were differentially expressed, of which 33 were down-regulated in the Δ*flbA* strain. This shows that transcriptional control during growth on the agar medium has a major impact on genes encoding proteins that are released by the wld-type strain but not by the Δ*flbA* strain. Finally, 20 out of 37 genes encoding proteins that were released both in wild-type and the Δ*flbA* secretome were differentially expressed. Quantitative proteomics should reveal whether this differential expression also results in quantitative differences in levels of these proteins in the medium. In this study, *A. niger* was grown on xylose. XlnR is the xylanolytic regulator that controls expression of at least 16 genes. All encoded proteins were identified in the wild-type secretome after cycloheximide treatment, whereas 15 out of 16 proteins were identified in the Δ*flbA* secretome. 6 out of 16

XlnR regulated genes were shown to be down-regulated in the Δ*flbA* strain. This is not in conflict with the proteomics data per se, since quantitative proteomics was not performed on wild-type versus Δ*flbA*. Moreover, proteomics was performed on colonies that had been transferred to fresh medium (see above).

Spatial growth and secretion and cell wall synthesising, modifying and degrading activities are most probably regulated by transcriptional regulators. In total, 38 transcriptional regulators were found to be differentially regulated in the Δ*flbA* strain. Most of these regulators have not been characterised. However, the list includes 9 genes with a known function or that are homologous to a transcription factor that has been characterized in another *Aspergillus* species. Genes that have homology to *rosA* of *A. fumigatus* and *atfB* of *A. oryzae* (both down-regulated in Δ*flbA*), and to *flbD* and *abaA* of *A. nidulans* (both up-regulated in Δ*flbA*) are involved in development (*rosA, flbD,* and *abaA*) and stress tolerance of conidia (*attB*).[3, 30-32] Genes homologous to *xlnR* and *amyR* of *A. oryzae, prtT*[33]and *acuB*[34, 35] (all down-regulated in Δ*flbA*) are predicted to be involved in degradation of polysaccharides, proteins, and acetate. Gene *prtT* has been shown to be the transcriptional activator of protease genes in *A. niger*.[33] Its down-regulation in Δ*flbA* colonies is in agreement with the finding that only 6 protease genes were up-regulated in Δ*flbA*, while 13 were down-regulated. The transcriptional regulatory gene *pacC* [36] was found to be up-regulated at the periphery of Δ*flbA* colonies. It is not known how *pacC* expression is exactly regulated in *Aspergillus*. The pH does not seem to regulate its expression or that of the six pH-sensing *pal* components.[36] However, PacC protein is abundant in alkaline growth conditions, where it is activated by proteolytical cleavage.[36-38] At alkaline pH, alkaline-associated genes are activated (*palD, prtA, xlnA, acvA, ipnA, pacC, sidA, mirA, mirB*)[39], and acid-associated genes (*pacA*, *xlnB, abfB, gabA,* and *stcU*) are repressed by PacC in *A. nidulans*.[40] In *A. niger* these genes are not fully annotated, but homologues of *mirA* (An02g14190), *mirB* (An03g03560) *sidA* (An05g00220), and *pacC* itself are up-regulated in Δ*flbA*, whereas *xlnB*, putative *gabA* (*An12g10000*), but also *xlnA* are down-regulated when compared to wild-type. This might be explained by the overexpression of PacC, but is in conflict with the extracellular pH of 4 that was observed in the Δ*flbA* strain.[8, Chapter 3] In *A. nidulans* PacC is only minimally processed under these acidic growth conditions.[41] However, PacC processing might be different in *A. niger*, since this strain has the tendency to acidify its culture medium to as low as pH 2. Genes An11g06290 and An01g06900 are homologous to the xylanolytic and amylolytic regulatory genes *xlnR* and *amyR* of *A. oryzae*, respectively. However, they do not encode the XlnR and AmyR regulators of *A. niger* since these functions have been attributed to the *A. niger xlnR* (An15g05810) [19, 23, 24] and *amyR* (An04g06910) genes.[42] The *xlnR* gene of *A. niger* was down-regulated 1.5 fold, which may explain the high number

(i.e. 9 out of 16) of xylanolytic genes that are down-regulated compared to wild-type. Apart from the 9 transcriptional regulatory genes with a predicted function, there are 29 of such genes with an unknown role in growth and development of *A. niger*. One or more of these genes may be involved in spatial growth, spatial secretion, cell wall formation and asexual development. As such, they are of interest to improve *A. niger* as a cell factory.

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ASSOCIATED CONTENT

Supplemental Table 1. Microarray analysis of the central zone (zone 1), an intermediate zone (zone 3) and the periphery (zone 5) of 7-days-old xylose grown wildtype and ΔflbA colonies. This information is available free of charge on http://goo.gl/BH1Kd

Supplemental Table 2. Average expression value of zone 1, 3, and 5 of the 345 differentially expressed genes of the total number of 2610 proteins with a signal sequence for secretion as predicted with SigP2.0 17 (1551) or SigP343(2159). Names of proteins are indicated when they show homology to or are encoded by *A. niger* genes. *Description of gene or of its closest homologue. Sm: similar; *A. nid*: *A.nidulans*; *A. ory*: *A. oryzae*; *A. nig*: *A. niger*.

Annotation	Gene Name*	Description - Suplemental Table 2	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An01g00210	*uncharacterized*	hypothetical protein	105 ± 13	174 ± 37	91 ± 31	36 ± 2	37 ± 0	50 ± 8	Down
An01g00530	*pepB*	proteinase aspergillopepsin II - Aspergillus niger	682 ± 649	3677 ± 2802	6268 ± 3005	44 ± 5	35 ± 1	34 ± 0	Down
An01g00780	*xlnB*	xylanase xynB of patent WO9713853-A2 - Aspergillus niger	4528 ± 1905	16891 ± 683	19085 ± 0	953 ± 977	4014 ± 2052	1371 ± 235	Down
An01g01240	*uncharacterized*	similarity to HC-toxin efflux pump TOXA - Cochliobolus carbonum [truncated ORF]	266 ± 26	406 ± 196	474 ± 206	48 ± 0	53 ± 7	60 ± 3	Down
An01g01870	*eglC*	strong similarity to hypothetical Avicelase III aviIII - Aspergillus aculeatus	77 ± 47	501 ± 267	400 ± 240	22 ± 0	23 ± 1	23 ± 2	Down
An01g03340	*sm to celA-A.oryzae*	strong similarity to xyloglucan-specific endo-beta-1,4-glucanase - Aspergillus aculeatus	552 ± 60	4966 ± 831	3065 ± 537	151 ± 48	272 ± 34	191 ± 34	Down
An01g05060	*uncharacterized*	hypothetical protein	361 ± 116	472 ± 123	812 ± 249	242 ± 18	245 ± 26	151 ± 2	Down
An01g05730	*uncharacterized*	strong similarity to monosaccharide transporter 3 OsMST3 - Oryza sativa	211 ± 8	247 ± 60	99 ± 37	84 ± 5	67 ± 6	45 ± 1	Down
An01g06030	*uncharacterized*	strong similarity to ketohexokinase khk - Homo sapiens	127 ± 20	110 ± 32	156 ± 47	61 ± 3	46 ± 6	61 ± 12	Down
An01g06910	*uncharacterized*	strong similarity to cytochrome P450 CYP94A1 - Vicia sativa	163 ± 2	199 ± \	208 ± 7	93 ± 1	77 ± 3	57 ± 6	Down
An01g07100	*uncharacterized*	strong similarity to subunit TaGST1 of glutathione transferase from patent Y05537 - Triticum aestivum	488 ± 9	553 ± 102	385 ± 188	226 ± 34	160 ± 16	99 ± 25	Down
An01g09590	*uncharacterized*	strong similarity to voltage-gated potassium channel beta subunit Kv beta 1 - Rattus norvegicus	273 ± 8	305 ± 78	226 ± 43	139 ± 9	121 ± 9	119 ± 1	Down
An01g09970	*uncharacterized*	weak similarity to Lactobacillus crispatus silent surface layer protein cbsB - Lactobacillus crispatus	86 ± 46	97 ± 22	42 ± 8	22 ± 2	27 ± 1	43 ± 1	Down
An01g10000	*uncharacterized*	strong similarity to atp-binding cassette transporter abc1 - Schizosaccharomyces pombe	174 ± 0	152 ± 29	92 ± 3	45 ± 1	45 ± 5	53 ± 4	Down
An01g11190	*uncharacterized*	similarity to the plasma membrane protein Pth11 - Magnaporthe grisea	61 ± 12	138 ± 48	185 ± 29	37 ± 5	38 ± 6	36 ± 2	Down
An01g11660	*cbhB*	1,4-beta-D-glucan cellobiohydrolase B precursor cbhB of patent WO9906574-A1 - Aspergillus niger	1289 ± 335	8224 ± 845	6037 ± 1558	48 ± 13	430 ± 386	1098 ± 313	Down
An01g11670	*eglA*	strong similarity to endo-beta-1,4-glucanase A eglA - Emericella nidulans	638 ± 408	2367 ± 12	589 ± 232	21 ± 2	42 ± 24	58 ± 1	Down
An01g11880	*uncharacterized*	strong similarity to O-methylsterigmatocystin oxidoreductase ordA - Aspergillus parasiticus [possible sequencing error]	589 ± 376	681 ± 95	211 ± 22	56 ± 13	60 ± 3	40 ± 2	Down
An01g12240	*uncharacterized*	strong similarity to hypothetical protein An02g05360 - Aspergillus niger	594 ± 383	722 ± 532	1690 ± 424	288 ± 40	194 ± 6	57 ± 8	Down
An01g12440	*uncharacterized*	similarity to hypothetical protein BAA13766.1 (fragment) - Schizosaccharomyces pombe	913 ± 941	1370 ± 1409	911 ± 199	121 ± 6	115 ± 2	100 ± 4	Down

Annotation	Gene Name*	Description- Supplemental Table 2 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An01g12450	*exsG*	strong similarity to putative glucan beta-1,3 exoglucanase - Trichoderma harzianum	3091 ± 197	3667 ± 1573	1008 ± 250	966 ± 4	1053 ± 14	689 ± 27	Down
An01g14600	*uncharacterized*	strong similarity to the endo-1,4-beta-Xylanase B XynB, patent WO9414965 - Aspergillus tubingensis	256 ± 239	364 ± 349	195 ± 92	30 ± 1	34 ± 6	37 ± 4	Down
An01g14940	*uncharacterized*	similarity to nonhemolytic phospholipase C PC-PLC - Burkholderia pseudomallei	299 ± 58	486 ± 126	203 ± 35	122 ± 2	138 ± 34	57 ± 2	Down
An01g15200	*uncharacterized*	strong similarity to mature penicillin V amidohydrolase PVA of patent US5516679-A - Fusarium oxysporum [truncated ORF]	1551 ± 24	1626 ± 657	351 ± 58	146 ± 9	92 ± 0	108 ± 31	Down
An02g01050	*uncharacterized*	strong similarity to salicylate hydroxylase nahG - Pseudomonas putida	219 ± 50	177 ± 54	140 ± 20	46 ± 2	44 ± 1	44 ± 8	Down
An02g02540	*uncharacterized*	strong similarity to acetyl-esterase I from patent R63066 - Aspergillus aculeatus	296 ± 66	375 ± 174	350 ± 139	83 ± 16	94 ± 1	74 ± 12	Down
An02g02870	*uncharacterized*	strong similarity to alcohol dehydrogenase ADHIII - Emericella nidulans	407 ± 22	451 ± 22	341 ± 95	217 ± 5	178 ± 6	153 ± 16	Down
An02g04110	*uncharacterized*	similarity to phenylacetaldehyde dehydrogenase PadA - Escherichia coli	564 ± 64	498 ± 99	343 ± 124	281 ± 19	238 ± 10	167 ± 4	Down
An02g05260	*uncharacterized*	similarity to protein phosphatase 1 binding protein (PTG) - Mus musculus	1160 ± 359	1242 ± 444	566 ± 207	88 ± 2	74 ± 14	89 ± 13	Down
An02g08230	*uncharacterized*	strong similarity to high affinity glucose transporter HGT1 - Kluyveromyces lactis	1271 ± 66	1795 ± 17	609 ± 296	475 ± 47	498 ± 7	290 ± 22	Down
An02g08270	*uncharacterized*	strong similarity to acetylspermidine oxidase ASOD - Candida boidinii	50 ± 4	75 ± 8	136 ± 63	33 ± 2	36 ± 1	47 ± 5	Down
An02g08330	*uncharacterized*	strong similarity to sequence 253 from Patent WO0100804 - Corynebacterium glutamicum	627 ± 234	624 ± 177	877 ± 309	27 ± 2	25 ± 1	24 ± 6	Down
An02g09500	*uncharacterized*	similarity to integral membrane protein Pth11 - Magnaporthe grisea strain G-11	230 ± 74	203 ± 23	606 ± 163	119 ± 3	74 ± 10	26 ± 3	Down
An02g09690	*uncharacterized*	strong similarity to lipase I precursor TFL I - Geotrichum fermentans [putative sequencing error]	652 ± 18	1719 ± 762	529 ± 399	195 ± 22	294 ± 108	356 ± 50	Down
An02g09810	*uncharacterized*	strong similarity to mitochondrial nicotinamide nucleotide transhydrogenase NNTM - Bos taurus	2502 ± 73	2673 ± 98	2312 ± 196	1726 ± 167	1290 ± 69	511 ± 53	Down
An02g11150	*aglB*	alpha-galactosidase aglB - Aspergillus niger	390 ± 96	2373 ± 668	3155 ± 569	111 ± 5	160 ± 21	126 ± 14	Down
An02g11550	*uncharacterized*	strong similarity to hypothetical protein YDCJ - Escherichia coli	371 ± 14	527 ± 235	37 ± 12	25 ± 8	30 ± 5	16 ± 1	Down
An02g11890	*uncharacterized*	strong similarity to hypothetical protein An14g01330 - Aspergillus niger	61 ± 14	161 ± 33	124 ± 11	24 ± 1	30 ± 1	29 ± 2	Down
An02g12680	*uncharacterized*	similarity to acetylornithine deacetylase argE - Myxococcus xanthus	81 ± 59	77 ± 40	45 ± 10	27 ± 0	28 ± 1	28 ± 5	Down
An02g13270	*uncharacterized*	strong similarity to benzoate 4-monooxygenase cytochrome P450 53 bphA - Aspergillus niger	541 ± 145	554 ± 200	293 ± 141	107 ± 14	79 ± 5	81 ± 18	Down
An02g13300	*uncharacterized*	hypothetical protein	167 ± 84	499 ± 228	670 ± 253	48 ± 2	53 ± 4	57 ± 2	Down
An02g13630	*uncharacterized*	weak similarity to the recombinant collagenase from patent EP430635-A - Vibrio alginolyticus	146 ± 21	257 ± 124	89 ± 31	40 ± 3	39 ± 8	36 ± 2	Down

Annotation	Gene Name*	Description- Supplemental Table 2 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An02g13980	*uncharacterized*	strong similarity to trichodiene oxygenase cytochrome P450 CYP58 - Fusarium sporotrichioides	1427 ± 168	1470 ± 74	793 ± 297	413 ± 44	288 ± 21	371 ± 67	Down
An03g00210	*uncharacterized*	similarity to hypothetical protein An16g01170 - Aspergillus niger	135 ± 89	188 ± 106	313 ± 141	32 ± 3	34 ± 6	37 ± 6	Down
An03g00940	*xlnC*	endo-1,4-beta-xylanase C precursor xlnC - Aspergillus niger	2605 ± 1941	15761 ± 4701	15538 ± 1230	337 ± 232	1058 ± 553	1067 ± 324	Down
An03g00960	*axhA*	1,4-beta-D-arabinoxylan arabinofuranohydrolase axhA - Aspergillus niger	4089 ± 1838	15097 ± 1853	17374 ± 0	456 ± 313	1314 ± 776	1602 ± 429	Down
An03g00960	*axhA*	1,4-beta-D-arabinoxylan arabinofuranohydrolase axhA - Aspergillus niger	2493 ± 1177	13393 ± 1012	15612 ± 0	298 ± 225	896 ± 401	989 ± 214	Down
An03g01050	*uncharacterized*	similarity to endo-beta-1,4-glucanase - Bacillus polymyxa	137 ± 32	345 ± 202	1027 ± 608	31 ± 8	48 ± 17	89 ± 12	Down
An03g01140	*uncharacterized*	strong similarity to oxalate decarboxylase (APOXD) of patent WO9842827-A2 - Aspergillus phoenices	563 ± 49	344 ± 83	81 ± 43	39 ± 1	38 ± 1	37 ± 4	Down
An03g01390	*uncharacterized*	similarity to 6-hydroxynicotinic acid mono-oxygenase of patent JP09121864-A - Pseudomonas fluorescens (strain TN5)	73 ± 6	78 ± 6	53 ± 1	28 ± 1	32 ± 1	31 ± 1	Down
An03g01910	*uncharacterized*	strong similarity to the N-acetylglutamate kinase AGK - Neurospora crassa	290 ± 151	336 ± 218	257 ± 208	88 ± 3	72 ± 11	53 ± 8	Down
An03g03930	*uncharacterized*	weak similarity to neurofilament NF-180 - Petromyzon marinus	72 ± 18	66 ± 38	108 ± 53	38 ± 6	35 ± 0	38 ± 5	Down
An03g05560	*uncharacterized*	strong similarity to spherulin 4 precursor SR4 - Physarum polycephalum	415 ± 149	469 ± 281	1013 ± 239	136 ± 18	110 ± 5	83 ± 12	Down
An03g06090	*uncharacterized*	strong similarity to telomeric repeated gene RTM1 - Saccharomyces cerevisiae	532 ± 151	794 ± 19	609 ± 307	160 ± 2	63 ± 7	105 ± 23	Down
An03g06550	*glaA*	glucan 1,4-alpha-glucosidase glaA - Aspergillus niger	370 ± 102	1497 ± 1144	5690 ± 1251	182 ± 15	171 ± 29	166 ± 8	Down
An03g06550	*glaA*	glucan 1,4-alpha-glucosidase glaA - Aspergillus niger	431 ± 121	1483 ± 1174	5129 ± 850	237 ± 2	242 ± 13	221 ± 17	Down
An04g00100	*uncharacterized*	strong similarity to hypothetical protein SPAC15E1.02c - Schizosaccharomyces pombe	1370 ± 405	1828 ± 1113	1183 ± 1060	423 ± 42	322 ± 84	352 ± 109	Down
An04g03180	*uncharacterized*	hypothetical protein	559 ± 180	768 ± 191	2255 ± 409	253 ± 12	271 ± 20	92 ± 2	Down
An04g06920	*agdA*	extracellular alpha-glucosidase aglU - Aspergillus niger	236 ± 29	316 ± 16	651 ± 135	176 ± 8	145 ± 5	176 ± 10	Down
An04g06990	*uncharacterized*	strong similarity to alpha 1,2-mannosidase IC - Homo sapiens	132 ± 29	263 ± 117	531 ± 210	101 ± 3	113 ± 3	92 ± 27	Down
An04g08150	*uncharacterized*	strong similarity to EST an_2637 - Aspergillus niger	2187 ± 1223	1771 ± 1870	72 ± 9	77 ± 2	51 ± 4	38 ± 1	Down
An04g08440	*uncharacterized*	strong similarity to cytochrome P450 monooxygenase TRI11 - Fusarium sporotrichioides	759 ± 21	602 ± 25	236 ± 80	126 ± 2	84 ± 13	33 ± 2	Down
An04g09360	*uncharacterized*	strong similarity to hypothetical protein CC0812 - Caulobacter crescentus	136 ± 33	153 ± 76	90 ± 13	68 ± 0	64 ± 1	38 ± 8	Down
An04g09410	*uncharacterized*	strong similarity to D-arabinitol dehydrogenase AAY56815 of patent JP11332569-A - Bacillus sp. IKD-5A868	157 ± 45	156 ± 48	79 ± 32	78 ± 12	52 ± 5	34 ± 0	Down

Annotation	Gene Name*	Description- Supplemental Table 2 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An04g09690	*uncharacterized*	strong similarity to pectin methylesterase PME1 - Aspergillus aculeatus	60 ± 19	190 ± 41	174 ± 59	38 ± 3	48 ± 0	47 ± 9	Down
An04g09700	*uncharacterized*	strong similarity to endo-xylogalacturonan hydrolase xghA - Aspergillus tubingensis	60 ± 41	435 ± 272	143 ± 33	28 ± 4	28 ± 0	39 ± 3	Down
An04g09990	*uncharacterized*	strong similarity to 2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase linC - Sphingomonas paucimobilis	749 ± 308	422 ± 138	106 ± 4	36 ± 6	34 ± 6	34 ± 5	Down
An04g09990	*uncharacterized*	strong similarity to 2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase linC - Sphingomonas paucimobilis	1422 ± 516	824 ± 344	236 ± 56	52 ± 3	26 ± 7	14 ± 1	Down
An05g01490	*uncharacterized*	strong similarity to hypothetical protein PA3762 - Pseudomonas eruginosa	503 ± 48	498 ± 206	344 ± 149	169 ± 3	114 ± 4	98 ± 5	Down
An05g01730	*uncharacterized*	hypothetical protein	11442 ± 3316	14891 ± 315	4511 ± 2021	42 ± 7	37 ± 0	27 ± 1	Down
An05g02270	*uncharacterized*	strong similarity to hypothetical protein B15I20.50 - Neurspora crassa	536 ± 1	486 ± 67	221 ± 76	171 ± 6	124 ± 14	110 ± 6	Down
An05g02280	*uncharacterized*	similarity to esterase protein patent WO9802556-A2 - Alcaligenes species	1063 ± 402	1434 ± 680	1100 ± 102	119 ± 23	134 ± 25	137 ± 20	Down
An05g02300	*gpi12*	similarity to secreted protein BLAST search protein SEQID no. 127 - Homo sapiens	906 ± 411	1132 ± 486	1070 ± 399	107 ± 44	155 ± 75	150 ± 28	Down
An05g02310	*uncharacterized*	strong similarity to SUR1 protein - Saccharomyces cerevisiae	855 ± 460	961 ± 487	1596 ± 92	155 ± 65	259 ± 140	333 ± 42	Down
An05g02320	*uncharacterized*	similarity to alpha-1,6-mannosyltransferase patent JP09003097-A - Pichia pastoris	294 ± 60	334 ± 126	357 ± 22	91 ± 11	121 ± 47	141 ± 3	Down
An05g02450	*uncharacterized*	similarity to halogenase bhaA from patent DE19926770-A1 - Amycolatopsis mediterranei	663 ± 62	1035 ± 59	469 ± 33	25 ± 6	18 ± 2	18 ± 1	Down
An06g00170	*aglA*	alpha-galactosidase aglA - Aspergillus niger	600 ± 56	678 ± 85	124 ± 23	59 ± 7	32 ± 6	18 ± 3	Down
An06g00190	*uncharacterized*	strong similarity to lysosomal pepstatin insensitive protease CLN2 - Homo sapiens	305 ± 23	733 ± 87	1175 ± 28	144 ± 2	114 ± 5	58 ± 5	Down
An07g02360	*uncharacterized*	similarity to 6-hydroxy-D-nicotine oxidase 6-HDNO - Arthrobacter oxidans	60 ± 24	49 ± 23	104 ± 15	32 ± 3	31 ± 2	25 ± 4	Down
An07g04360	*uncharacterized*	hypothetical protein	319 ± 10	337 ± 87	292 ± 42	16 ± 4	23 ± 4	23 ± 4	Down
An07g04360	*uncharacterized*	hypothetical protein	503 ± 29	590 ± 186	471 ± 100	37 ± 5	32 ± 6	31 ± 1	Down
An07g04430	*uncharacterized*	strong similarity to hexose transporter Ght2 - Schizosaccharomyces pombe	590 ± 273	735 ± 397	1691 ± 631	258 ± 12	253 ± 1	259 ± 16	Down
An07g04510	*uncharacterized*	hypothetical protein	96 ± 18	128 ± 46	426 ± 28	53 ± 6	61 ± 16	54 ± 5	Down
An07g05490	*uncharacterized*	strong similarity to stage V sporulation protein spoVK - Bacillus subtilis	168 ± 12	162 ± 44	102 ± 13	65 ± 5	75 ± 3	67 ± 8	Down
An07g05660	*uncharacterized*	weak similarity to hypothetical protein An14g04210 - Aspergillus niger	443 ± 195	714 ± 348	1800 ± 492	156 ± 31	116 ± 31	200 ± 51	Down
An07g06300	*uncharacterized*	strong similarity to member of the hexose transporter family of the major facilitator superfamily HXT16 - Saccharomyces cerevisiae	124 ± 4	109 ± 7	88 ± 5	44 ± 6	44 ± 8	42 ± 3	Down
An07g06480	*uncharacterized*	similarity to cytochrome 4F8 cyp4F8 - Homo sapiens	420 ± 130	586 ± 213	753 ± 142	9 ± 1	10 ± 2	9 ± 1	Down

Annotation	Gene Name*	Description- Supplemental Table 2 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An07g08250	*uncharacterized*	hypothetical protein	58 ± 15	89 ± 55	234 ± 36	36 ± 3	36 ± 3	29 ± 3	Down
An07g08940	*uncharacterized*	similarity to acetyl-esterase I of patent WO9502689-A - Aspergillus aculeatus	242 ± 13	1268 ± 407	914 ± 553	20 ± 1	31 ± 7	26 ± 0	Down
An07g08950	*eglC*	endoglucanase B eglB - Aspergillus niger	2511 ± 570	11775 ± 532	7793 ± 1370	52 ± 29	366 ± 244	422 ± 77	Down
An07g09330	*cbhA*	cellulose 1,4-beta-cellobiosidase cbhA from patent WO9906574-A1- Aspergillus niger	1157 ± 1121	7883 ± 2929	3034 ± 1406	67 ± 16	130 ± 62	170 ± 76	Down
An08g04570	*uncharacterized*	strong similarity to putative isobutene-forming enzyme and benzoate 4-hydroxylase P450rm - Rhodotorula minuta	81 ± 20	88 ± 19	61 ± 3	32 ± 3	35 ± 4	42 ± 3	Down
An08g04630	*uncharacterized*	hypothetical protein	361 ± 349	674 ± 706	305 ± 86	51 ± 1	48 ± 13	36 ± 1	Down
An08g04640	*protB*	strong similarity to putative lysosomal pepstatin insensitive protease CLN2 - Canis familiaris	98 ± 32	218 ± 127	827 ± 628	62 ± 13	58 ± 5	59 ± 2	Down
An08g05230	*uncharacterized*	strong similarity to putative endoglucanase IV - Trichoderma reesei	1152 ± 1281	2411 ± 2729	648 ± 116	55 ± 5	58 ± 11	46 ± 5	Down
An08g08370	*uncharacterized*	similarity to the alpha-1,2-mannosidase aman2 - Bacillus sp. M-90	1771 ± 843	2086 ± 211	376 ± 19	179 ± 33	78 ± 14	38 ± 4	Down
An08g09610	*agnD*	similarity to mutanase mutA - Penicillium purpurogenum	339 ± 136	389 ± 173	765 ± 330	79 ± 11	73 ± 4	73 ± 1	Down
An08g09850	*uncharacterized*	strong similarity to phosphate-repressible acid phosphatase precursor phoA - Penicillium chrysogenum	424 ± 125	551 ± 364	636 ± 431	245 ± 15	256 ± 8	132 ± 7	Down
An08g10780	*uncharacterized*	strong similarity to hypothetical protein T16K5.230 of A. thaliana	72 ± 40	140 ± 70	1109 ± 145	30 ± 8	26 ± 2	43 ± 0	Down
An08g11030	*phyB*	acid phosphatase aph, 3-phytase phyB - Aspergillus niger	436 ± 438	975 ± 1113	973 ± 889	54 ± 4	59 ± 5	44 ± 0	Down
An08g11680	*uncharacterized*	strong similarity to 2,5-dicloro-2,5-cyclohexadiene-1,4-diol dehydrogenase linC - Pseudomonas paucimobilis	320 ± 4	214 ± 41	125 ± 4	56 ± 0	68 ± 11	61 ± 1	Down
An08g11680	*uncharacterized*	strong similarity to 2,5-dicloro-2,5-cyclohexadiene-1,4-diol dehydrogenase linC - Pseudomonas paucimobilis	1198 ± 301	705 ± 255	203 ± 57	43 ± 5	27 ± 2	23 ± 0	Down
An09g00640	*uncharacterized*	similarity to transcriptional repressor RCO-1 - Neurospora crassa	77 ± 30	72 ± 3	145 ± 0	38 ± 5	40 ± 1	29 ± 2	Down
An09g00670	*gelD*	strong similarity to beta (1-3) glucanosyltransferase Gel3p - Aspergillus fumigatus	1596 ± 565	2244 ± 607	2063 ± 821	45 ± 1	39 ± 6	59 ± 10	Down
An09g00840	*uncharacterized*	weak similarity to antigenic cell wall galactomannoprotein MP1 - Aspergillus fumigatus	3153 ± 2599	10725 ± 146	3462 ± 103	29 ± 11	35 ± 22	14 ± 2	Down
An09g02160	*rgaeA*	rhamnogalacturonan acetyl esterase rgaeA - Aspergillus niger	318 ± 9	829 ± 168	409 ± 176	165 ± 41	149 ± 17	58 ± 2	Down
An09g02930	*uncharacterized*	strong similarity to high-affinity glucose transporter HGT1 - Kluyveromyces lactis	315 ± 108	405 ± 110	289 ± 126	48 ± 14	65 ± 18	35 ± 10	Down
An09g04390	*uncharacterized*	strong similarity to salicylate hydroxylase sal - Pseudomonas putida	65 ± 6	91 ± 16	32 ± 4	31 ± 3	24 ± 3	22 ± 3	Down
An09g04810	*uncharacterized*	strong similarity to high affinity glucose transporter HGT1 - Kluyveromyces lactis	1148 ± 177	951 ± 88	733 ± 427	73 ± 1	54 ± 1	47 ± 0	Down

Annotation	Gene Name*	Description- Supplemental Table 2 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An09g04830	*uncharacterized*	similarity to integral membrane protein pth11 - Magnaporthe grisea	771 ± 185	830 ± 482	849 ± 701	67 ± 12	58 ± 4	62 ± 6	Down
An09g06200	*uncharacterized*	strong similarity to PTH11 transmembrane protein - Magnaporthe grisea strain 4091-5-8	5976 ± 1928	5351 ± 2683	3632 ± 1622	442 ± 58	211 ± 45	82 ± 12	Down
An10g00310	*uncharacterized*	similarity to ferric/cupric reductase FRE2 - Saccharomyces cerevisiae	212 ± 131	279 ± 201	288 ± 18	122 ± 7	107 ± 28	73 ± 4	Down
An11g01190	*uncharacterized*	similarity to protein participating in extracellular/cell surface phenomena ECM33 - Saccharomyces cerevisiae	492 ± 82	606 ± 104	551 ± 210	223 ± 22	242 ± 18	198 ± 9	Down
An11g01660	*uncharacterized*	weak similarity to xylanase Xyl1 - Helminthosporium turcicum	1435 ± 183	1637 ± 92	5614 ± 318	278 ± 12	198 ± 22	371 ± 40	Down
An11g02100	*sm to bglR- A. nidulans*	strong similarity to furostanol glycoside 26-O-beta-glucosidase CSF26G1 - Costus speciosus	360 ± 21	1634 ± 301	808 ± 253	105 ± 38	140 ± 12	114 ± 5	Down
An11g03070	*uncharacterized*	weak similarity to the chitinase A ChiA - Emericella nidulans	87 ± 15	57 ± 3	128 ± 47	39 ± 1	34 ± 1	34 ± 0	Down
An11g03540	*uncharacterized*	strong similarity to cytochrome P450 monooxygenase TRI11 - Fusarium sporotrichioides	730 ± 642	770 ± 471	142 ± 0	30 ± 4	32 ± 1	31 ± 1	Down
An11g06080	*uncharacterized*	strong similarity to beta-glucosidase 1 bgl1 - Aspergillus aculeatus [truncated ORF]	264 ± 16	581 ± 120	449 ± 113	196 ± 2	213 ± 25	129 ± 27	Down
An11g06290	*sm to xlnR -A. ory*	strong similarity to transcriptional activator xlnR - Aspergillus niger	145 ± 30	141 ± 35	84 ± 17	54 ± 2	40 ± 4	30 ± 1	Down
An11g06350	*uncharacterized*	strong similarity to carboxypeptidase C cpy1 - Schizosaccharomyces pombe	296 ± 117	227 ± 67	91 ± 24	86 ± 0	75 ± 4	62 ± 3	Down
An11g06420	*uncharacterized*	strong similarity to sequence 253 from patent WO0100804 - Corynebacterium glutamicum	351 ± 68	343 ± 48	163 ± 56	41 ± 3	35 ± 1	38 ± 1	Down
An11g06480	*uncharacterized*	weak similarity to antigenic protein f86.aa. of patent WO9859071 - Borrelia burgdorferi	165 ± 5	154 ± 2	119 ± 6	43 ± 9	38 ± 1	43 ± 4	Down
An11g07490	*uncharacterized*	similarity to Golgi membrane mannosyltransferase complexe subunit Hoc1 - Saccharomyces cerevisiae	74 ± 4	82 ± 0	84 ± 11	48 ± 3	45 ± 5	27 ± 1	Down
An11g07940	*uncharacterized*	hypothetical protein	344 ± 132	493 ± 206	196 ± 18	131 ± 0	97 ± 5	63 ± 2	Down
An11g08020	*uncharacterized*	strong similarity to G-11 integral membrane protein PTH11 - Magnaporthe grisea	58 ± 3	76 ± 12	311 ± 8	20 ± 0	17 ± 4	20 ± 1	Down
An11g08100	*uncharacterized*	strong similarity to the hypothetical protein encoded by An12g06830 - Aspergillus niger	346 ± 3	293 ± 3	246 ± 15	58 ± 10	44 ± 3	38 ± 2	Down
An11g10260	*uncharacterized*	strong similarity to alphaN-acetylglucosamine transferase - Kluyveromyces lactis	257 ± 38	312 ± 54	712 ± 183	106 ± 18	81 ± 3	52 ± 3	Down
An12g00140	*uncharacterized*	weak similarity to mucin-like protein MUC1 - Saccharomyces cerevisiae	501 ± 162	549 ± 286	326 ± 43	162 ± 9	120 ± 27	105 ± 2	Down
An12g01320	*ppoD*	strong similarity to linoleate diol synthase - Gaeumannomyces graminis	1123 ± 612	1043 ± 345	132 ± 72	18 ± 2	20 ± 3	14 ± 2	Down
An12g04250	*uncharacterized*	strong similarity to pisatin demethylase PDAT9 - Nectria haematococca	438 ± 106	607 ± 136	345 ± 35	93 ± 18	94 ± 10	80 ± 0	Down
An12g04610	*uncharacterized*	similarity to endoglucanase IV egl4 - Trichoderma reesei	966 ± 1072	5636 ± 3072	243 ± 11	76 ± 9	108 ± 31	58 ± 5	Down

Annotation	Gene Name*	Description- Supplemental Table 2 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An12g05360	*uncharacterized*	strong similarity to cholesterol 24-hydroxylase - Mus musculus	1125 ± 155	1043 ± 11	267 ± 14	91 ± 21	88 ± 10	76 ± 5	Down
An12g05390	*uncharacterized*	weak similarity to integral membrane protein PTH11 - Magnaporthe grisea	2079 ± 127	1355 ± 14	84 ± 32	38 ± 0	31 ± 2	30 ± 3	Down
An12g05400	*uncharacterized*	similarity to cholesterol 7alpha-monooxygenase - Oryctolagus cuniculus	408 ± 39	279 ± 79	29 ± 5	26 ± 2	24 ± 0	26 ± 2	Down
An12g05960	*uncharacterized*	strong similarity to dipeptidyl peptidase II DPPII - Rattus norvegicus	105 ± 22	126 ± 40	207 ± 38	81 ± 22	81 ± 4	47 ± 7	Down
An12g09260	*uncharacterized*	weak similarity to integral membrane protein PTH11 - Magnaporthe grisea	114 ± 42	119 ± 75	254 ± 144	34 ± 2	29 ± 0	27 ± 1	Down
An12g09560	*uncharacterized*	strong similarity to cytochrome P450 monooxygenase avnA - Aspergillus parasiticus	728 ± 241	877 ± 285	194 ± 5	56 ± 6	57 ± 2	60 ± 8	Down
An12g10030	*uncharacterized*	weak similarity to membrane protein YIL140w Rev7 - Saccharomyces cerevisiae	245 ± 94	217 ± 29	198 ± 32	115 ± 21	79 ± 5	86 ± 16	Down
An12g10360	*uncharacterized*	similarity to the hypothetical protein An08g08280 - Aspergillus niger	1957 ± 80	1744 ± 229	1040 ± 87	527 ± 104	588 ± 144	585 ± 72	Down
An12g10630	*uncharacterized*	similarity to acid phosphatase aphA - Aspergillus ficuum	193 ± 67	240 ± 72	56 ± 1	38 ± 1	37 ± 0	27 ± 2	Down
An13g01520	*uncharacterized*	hypothetical protein	1397 ± 708	1305 ± 539	492 ± 121	315 ± 17	340 ± 61	208 ± 16	Down
An13g01880	*uncharacterized*	strong similarity to cephalosporin esterase - Rhodosporidium toruloides	407 ± 104	3000 ± 374	3350 ± 147	96 ± 37	194 ± 69	155 ± 2	Down
An13g01890	*uncharacterized*	similarity to hypothetical protein Rv1147 - Mycobacterium tuberculosis	106 ± 14	142 ± 14	253 ± 14	58 ± 3	65 ± 7	65 ± 2	Down
An14g01800	*uncharacterized*	strong similarity to alpha-galactosidase - Cyamopsis tetragonoloba	54 ± 4	109 ± 6	136 ± 18	27 ± 0	33 ± 3	34 ± 2	Down
An14g02150	*uncharacterized*	strong similarity to serine-type carboxypeptidase precursor cpdS - Aspergillus phoenicis	49 ± 10	57 ± 12	38 ± 7	23 ± 2	21 ± 1	23 ± 4	Down
An14g02670	*uncharacterized*	strong similarity to endoglucanase IV egl4 - Trichoderma reesei	347 ± 273	857 ± 754	737 ± 10	37 ± 3	45 ± 3	37 ± 1	Down
An14g03110	*uncharacterized*	strong similarity to cytochrome P450 monooxygenase TRI4 - Myrothecium roridum	212 ± 73	193 ± 21	70 ± 2	51 ± 3	46 ± 5	29 ± 3	Down
An14g03130	*uncharacterized*	hypothetical protein	1109 ± 560	752 ± 164	262 ± 94	49 ± 11	29 ± 4	25 ± 2	Down
An14g03850	*uncharacterized*	strong similarity to cytochrome P450 monooxygenase TRI4 - Fusarium sporotrichioides	1064 ± 231	769 ± 184	497 ± 92	78 ± 12	59 ± 3	46 ± 0	Down
An14g04200	*rhgB*	rhamnogalacturonase rhgB - Aspergillus niger	83 ± 57	177 ± 184	105 ± 34	33 ± 7	36 ± 5	34 ± 0	Down
An14g04330	*uncharacterized*	similarity to hypothetical protein An06g00600 - Aspergillus niger	158 ± 28	165 ± 4	193 ± 47	45 ± 1	41 ± 7	35 ± 3	Down
An14g05430	*uncharacterized*	similarity to meso-2,3-butanediol dehydrogenase (D-acetoin forming) budC - Klebsiella pneumoniae	113 ± 47	107 ± 44	82 ± 36	50 ± 8	40 ± 3	42 ± 2	Down
An14g05820	*uncharacterized*	strong similarity to beta-galactosidase lacA - Aspergillus niger	126 ± 37	290 ± 21	275 ± 6	60 ± 13	62 ± 12	72 ± 12	Down
An14g05850	*uncharacterized*	similarity to integral membrane protein PTH11 - Magnaporthe grisea	2042 ± 194	1706 ± 472	485 ± 477	135 ± 15	77 ± 3	41 ± 7	Down

Annotation	Gene Name*	Description- Supplemental Table 2 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An14g05960	*uncharacterized*	strong similarity to agmatinase speB - Escherichia coli	61 ± 11	92 ± 26	361 ± 161	28 ± 4	24 ± 5	46 ± 18	Down
An15g00500	*uncharacterized*	strong similarity to conserved hypothetical protein YJL055w - Saccaromyces cerevisiae	172 ± 91	153 ± 59	92 ± 32	50 ± 5	52 ± 11	45 ± 6	Down
An15g01220	*uncharacterized*	hypothetical protein	216 ± 32	206 ± 46	122 ± 25	96 ± 1	83 ± 11	61 ± 1	Down
An15g01500	*uncharacterized*	strong similarity to the fructose symporter FSY1 - Saccharomyces pastorianus	237 ± 7	230 ± 79	199 ± 139	58 ± 1	54 ± 4	62 ± 1	Down
An15g02250	*uncharacterized*	hypothetical protein	1220 ± 1202	1162 ± 905	78 ± 37	47 ± 2	49 ± 6	50 ± 9	Down
An15g03080	*uncharacterized*	similarity to BIR repeat containing ubiquitin-conjugating enzyme BRUCE - Mus musculus	694 ± 67	707 ± 22	499 ± 202	116 ± 3	85 ± 3	45 ± 4	Down
An15g04550	*xynA*	strong similarity to xylanase A xynA of patent WO200068396-A2 - Aspergillus niger	55 ± 0	384 ± 304	1176 ± 75	35 ± 4	41 ± 21	34 ± 1	Down
An15g05290	*uncharacterized*	strong similarity to hypothetical bile transporter Ybt1p - Saccharomyces cerevisiae	1216 ± 2	854 ± 42	662 ± 246	385 ± 11	323 ± 40	299 ± 15	Down
An15g05370	*pgaII*	polygalacturonase pgaII of patent EP421919-A - Aspergillus niger	224 ± 219	767 ± 877	327 ± 240	29 ± 3	25 ± 5	28 ± 5	Down
An15g05370	*pgaII*	polygalacturonase pgaII of patent EP421919-A - Aspergillus niger	106 ± 116	428 ± 512	183 ± 176	14 ± 2	16 ± 1	14 ± 1	Down
An15g07480	*uncharacterized*	weak similarity to eggshell precursor protein - Schistosoma mansoni	38 ± 7	62 ± 4	43 ± 10	20 ± 1	18 ± 1	17 ± 0	Down
An15g07580	*uncharacterized*	strong similarity to fructosyl amine oxygen oxidoreductase - Aspergillus fumigatus	193 ± 19	171 ± 7	165 ± 18	93 ± 5	85 ± 12	81 ± 13	Down
An15g07700	*protD*	strong similarity to aspergillopepsin II precursor (acid proteinase A) - Aspergillus niger	649 ± 452	5044 ± 2370	9630 ± 101	75 ± 5	76 ± 5	65 ± 2	Down
An16g00540	*uncharacterized*	similarity to putative large secreted protein - Streptomyces coelicolor [truncated ORF]	32 ± 1	89 ± 3	159 ± 14	23 ± 1	25 ± 1	26 ± 6	Down
An16g01320	*uncharacterized*	similarity to integral membrane protein PTH11 - Magnaporthe grisea	121 ± 72	107 ± 49	114 ± 60	38 ± 2	44 ± 4	46 ± 1	Down
An16g01730	*uncharacterized*	similarity to acid phosphatase precursor rPAP - Rattus norvegicus	10385 ± 1433	10580 ± 1158	7835 ± 105	3331 ± 142	2585 ± 142	4363 ± 663	Down
An16g02910	*uncharacterized*	strong similarity to hypothetical protein CC0533 - Caulobacter crescentus	865 ± 51	1033 ± 82	613 ± 238	52 ± 1	40 ± 8	42 ± 10	Down
An16g03330	*uncharacterized*	weak similarity to the endo-1,4-betaxylanase gene product CAA93120.1 - Ascochyta pisi	2688 ± 1258	2765 ± 429	7967 ± 224	562 ± 95	490 ± 128	1507 ± 46	Down
An16g03500	*uncharacterized*	weak similarity to the slow myosin heavy chain myoHC-A4 - Notothenia coriiceps	313 ± 26	338 ± 152	145 ± 114	50 ± 4	45 ± 11	53 ± 15	Down
An16g04820	*uncharacterized*	strong similarity to hypothetical protein SCD82.04c - Streptomyces coelicolor	322 ± 155	274 ± 142	177 ± 71	126 ± 8	106 ± 14	107 ± 8	Down
An16g05320	*uncharacterized*	strong similarity to trichodiene oxygenase cytochrome P450 CYP58 - Fusarium sporortrichoides	160 ± 27	118 ± 9	171 ± 7	62 ± 2	49 ± 0	45 ± 6	Down
An16g05910	*uncharacterized*	similarity to cholesterol 7alpha-hydroxylase CYP7 - Sus scrofa	902 ± 636	1646 ± 590	2001 ± 725	17 ± 3	25 ± 5	18 ± 2	Down
An16g05940	*uncharacterized*	strong similarity to cytochrome P450 monooxygenase stcS - Aspergillus nidulans	213 ± 63	382 ± 5	270 ± 28	37 ± 2	33 ± 7	38 ± 1	Down

Annotation	Gene Name*	Description- Supplemental Table 2 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An16g05970	*uncharacterized*	similarity to UDP-glucuronosyltransferase UGT2B11 - Homo sapiens	134 ± 21	215 ± 49	355 ± 81	55 ± 12	70 ± 3	107 ± 30	Down
An16g06570	*uncharacterized*	hypothetical protein	7069 ± 619	3576 ± 3514	1162 ± 1292	62 ± 4	52 ± 8	62 ± 4	Down
An16g06800	*eglB*	strong similarity to endoglucanase eglB - Aspergillus niger	154 ± 25	226 ± 100	423 ± 232	87 ± 5	92 ± 5	77 ± 5	Down
An16g06830	*uncharacterized*	hypothetical protein	309 ± 0	274 ± 25	202 ± 27	97 ± 21	97 ± 10	110 ± 6	Down
An16g09010	*protH*	strong similarity to carboxypeptidase I protein of patent WO9814599-A1 - Aspergillus oryza	1018 ± 249	1107 ± 340	353 ± 147	312 ± 2	298 ± 3	161 ± 9	Down
An17g00300	*xarB*	strong similarity to bifunctiona xylosidase-arabinosidase xarB - Thermoanaerobacter ethanolicus	156 ± 10	534 ± 22	396 ± 19	88 ± 6	124 ± 19	144 ± 17	Down
An18g00380	*uncharacterized*	strong similarity to aminotriazole resistance protein ATR1 - Saccharomyces cerevisiae	288 ± 30	181 ± 35	76 ± 2	63 ± 3	58 ± 2	45 ± 2	Down
An18g00980	*uncharacterized*	weak similarity to integral membrane protein PTH11 - Magnaporthe grisea	7405 ± 1018	7086 ± 1306	4462 ± 275	844 ± 28	793 ± 23	560 ± 85	Down
An18g01240	*uncharacterized*	similarity to salicylate hydroxylase (nahW) - Pseuodmonas stutzeri	615 ± 42	597 ± 122	908 ± 90	426 ± 15	349 ± 16	187 ± 43	Down
An18g01480	*uncharacterized*	strong similarity to cytochrome p450 related protein eln2 - Coprinus cinereus	1238 ± 65	918 ± 208	166 ± 16	122 ± 13	99 ± 12	68 ± 6	Down
An18g05510	*uncharacterized*	similarity to mucin-like protein MUC1 - Saccharomyces cerevisiae	53 ± 5	63 ± 10	144 ± 9	36 ± 2	43 ± 3	30 ± 4	Down
An19g00340	*uncharacterized*	strong similarity to vacuolar H+/Ca2+ exchanger VCX1 - Saccharomyces cerevisiae	845 ± 108	750 ± 135	786 ± 65	237 ± 12	247 ± 24	244 ± 0	Down
An01g01450	*uncharacterized*	hypothetical protein	35 ± 3	38 ± 5	32 ± 3	147 ± 25	114 ± 40	77 ± 7	Up
An01g01540	*uncharacterized*	strong similarity to alpha,alpha-trehalase treA - Aspergillus nidulans	154 ± 10	161 ± 8	149 ± 13	568 ± 30	607 ± 43	366 ± 5	Up
An01g01630	*uncharacterized*	strong similarity to hypothetical protein An09g00510 - Aspergillus niger	69 ± 71	36 ± 26	96 ± 40	1151 ± 99	830 ± 115	802 ± 17	Up
An01g02360	*uncharacterized*	similarity to integral membrane protein PTH11 - Magnaporthe grisea	73 ± 23	67 ± 6	51 ± 8	149 ± 9	125 ± 3	237 ± 10	Up
An01g04110	*uncharacterized*	strong similarity to sulfate permease SutB - Penicillium chrysogenum [truncated ORF]	48 ± 5	70 ± 31	72 ± 16	162 ± 6	184 ± 36	529 ± 106	Up
An01g04330	*uncharacterized*	strong similarity to hypothetical protein YLR187w - Saccharomyces cerevisiae	139 ± 25	133 ± 18	75 ± 6	396 ± 13	440 ± 34	318 ± 19	Up
An01g04690	*uncharacterized*	strong similarity to peroxisomal transporter Ant1 - Saccharomyces cerevisiae	66 ± 4	65 ± 10	58 ± 16	159 ± 5	172 ± 4	131 ± 1	Up
An01g05230	*uncharacterized*	strong similarity to EST an_2899 - Aspergillus niger	4075 ± 66	5052 ± 228	5296 ± 364	10873 ± 507	10957 ± 625	7353 ± 19	Up
An01g05620	*uncharacterized*	strong similarity to lipase lipP - Pseudomonas sp.	77 ± 0	83 ± 12	70 ± 10	149 ± 28	176 ± 32	150 ± 16	Up
An01g06130	*uncharacterized*	strong similarity to probable membrane protein YLR241w - Saccharomyces cerevisiae	147 ± 6	146 ± 10	157 ± 15	338 ± 19	365 ± 53	370 ± 55	Up
An01g06280	*uncharacterized*	strong similarity to IgE-binding protein - Aspergillus fumigatus	267 ± 123	341 ± 167	136 ± 4	2010 ± 28	2634 ± 280	1002 ± 144	Up

Annotation	Gene Name*	Description- Supplemental Table 2 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An01g07000	*uncharacterized*	strong similarity to C-14 sterol reductase ERG24 - Saccharomyces cerevisiae	89 ± 14	77 ± 2	270 ± 166	498 ± 24	1009 ± 531	3138 ± 50	Up
An01g07730	*uncharacterized*	weak similarity to TcSL-2 protein precursor - Toxocara cani	118 ± 74	64 ± 3	63 ± 5	1697 ± 86	1478 ± 312	1257 ± 12	Up
An01g09050	*uncharacterized*	similarity to calcium-related protein spray - Neurospora crassa	268 ± 12	273 ± 21	271 ± 41	811 ± 32	788 ± 12	540 ± 21	Up
An01g09550	*uncharacterized*	similarity to hypothetical dihydrolipoamide dehydrogenase Rv2713 - Mycobacterium tuberculosis	36 ± 3	32 ± 2	41 ± 3	82 ± 1	101 ± 5	368 ± 103	Up
An01g10350	*sm to lacB- A. niger*	strong similarity to secreted beta-galactosidase lacA - Aspergillus niger	113 ± 5	109 ± 20	206 ± 35	469 ± 36	394 ± 38	499 ± 69	Up
An01g10860	*uncharacterized*	similarity to the hypothetical protein An04g08050 - Aspergillus niger	115 ± 10	100 ± 16	67 ± 12	205 ± 40	216 ± 58	161 ± 29	Up
An02g00330	*uncharacterized*	hypothetical protein	41 ± 8	36 ± 0	28 ± 1	122 ± 2	75 ± 3	60 ± 1	Up
An02g00740	*uncharacterized*	similarity to 6-Hydroxy-D-nicotine oxidase 6-HDNO - Arthrobacter oxidans	31 ± 1	30 ± 2	35 ± 3	61 ± 5	69 ± 11	130 ± 14	Up
An02g02660	*dfgG*	strong similarity to the protein required for filamentous growth, cell polarity, and cellular elongation Dfg5 - Saccharomyces cerevisiae	87 ± 5	110 ± 17	102 ± 21	487 ± 30	648 ± 129	532 ± 98	Up
An02g04060	*uncharacterized*	hypothetical protein	65 ± 2	70 ± 23	141 ± 13	249 ± 33	241 ± 12	300 ± 19	Up
An02g05150	*uncharacterized*	strong similarity to C-8,7 sterol isomerase - Arabidopsis thaliana	151 ± 37	165 ± 21	257 ± 6	420 ± 8	399 ± 10	502 ± 40	Up
An02g06090	*uncharacterized*	weak similarity to cellobiose dehydrogenase cdh - Trametes versicolor	153 ± 68	138 ± 21	155 ± 6	592 ± 15	384 ± 9	576 ± 42	Up
An02g06550	*uncharacterized*	similarity to ferric reductase FRE1 - Saccharomyces cerevisiae	70 ± 15	82 ± 31	62 ± 22	236 ± 31	217 ± 14	103 ± 4	Up
An02g08130	*uncharacterized*	similarity to hypothetical protein 2SCG18.24 - Streptomyces coelicolor	46 ± 3	48 ± 9	41 ± 5	2045 ± 64	2532 ± 185	3306 ± 224	Up
An02g08560	*uncharacterized*	similarity to probable dioxygenase SCOEDB - Streptomyces coelicolor	22 ± 3	23 ± 3	19 ± 0	797 ± 22	1048 ± 83	1682 ± 135	Up
An02g10940	*uncharacterized*	strong similarity to cytochrome P450 monooxygenase stcS - Aspergillus nidulans	77 ± 14	73 ± 7	72 ± 3	160 ± 13	145 ± 4	149 ± 2	Up
An02g11360	*uncharacterized*	strong similarity to the putative endo alpha-1,4 polygalactosaminidase precusor gene - Pseudomonas sp.	637 ± 2	699 ± 16	694 ± 23	1509 ± 193	1327 ± 44	1554 ± 130	Up
An02g12840	*uncharacterized*	hypothetical protein [truncated ORF]	56 ± 6	75 ± 5	95 ± 4	127 ± 14	162 ± 20	165 ± 30	Up
An02g14500	*uncharacterized*	similarity to hypothetical protein An11g03520 - Aspergillus niger	2158 ± 452	1241 ± 376	861 ± 97	6772 ± 449	7230 ± 453	3273 ± 163	Up
An03g00190	*pelB*	the pectin lyase pelB - Aspergillus niger	56 ± 4	48 ± 4	61 ± 9	338 ± 67	536 ± 67	248 ± 3	Up
An03g00460	*uncharacterized*	strong similarity to the 6-hydroxy-D-nicotine oxidase 6-HDNO - Arthrobacter oxidans	129 ± 69	90 ± 27	102 ± 7	913 ± 19	751 ± 8	198 ± 2	Up
An03g00770	*uncharacterized*	strong similarity to allergic bronchopulmonary aspergillosis allergen rAsp f 4 of patent WO9828624-A1 - Aspergillus fumigatus	93 ± 3	98 ± 34	454 ± 19	473 ± 118	437 ± 60	2858 ± 7	Up

Annotation	Gene Name*	Description- Supplemental Table 2 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An03g01770	*uncharacterized*	strong similarity to the EST an_3645 - Aspergillus niger	758 ± 657	336 ± 176	55 ± 12	10029 ± 207	8451 ± 0	1933 ± 247	Up
An03g03520	*uncharacterized*	similarity to AM-toxin synthetase AMT - Alternaria alternata	27 ± 3	26 ± 1	29 ± 1	51 ± 13	46 ± 4	138 ± 21	Up
An03g03620	*uncharacterized*	strong similarity to multidrug resistance protein atrD - Aspergillus nidulans	18 ± 1	22 ± 2	26 ± 10	83 ± 5	110 ± 1	378 ± 123	Up
An03g05210	*uncharacterized*	strong similarity to reticuline oxidase bbe1 - Eschscholzia californica	146 ± 47	180 ± 82	308 ± 23	423 ± 2	453 ± 23	767 ± 34	Up
An03g05360	*uncharacterized*	strong similarity to neutral amino acid permease mtr - Neurospora crassa	39 ± 0	36 ± 2	31 ± 2	1673 ± 148	1362 ± 88	771 ± 2	Up
An03g05740	*uncharacterized*	similarity to cellulose synthase protein of patent JP11127867-A - Acetobacter xylinum	312 ± 57	353 ± 105	380 ± 56	795 ± 44	791 ± 116	1997 ± 156	Up
An03g06220	*sm to gelD- A. nid*	strong similarity to beta (1-3) glucanosyltransferase GEL3 - Aspergillus fumigatus	42 ± 3	48 ± 11	58 ± 3	1406 ± 256	1874 ± 54	5043 ± 547	Up
An03g06310	*pmeA*	pectinesterase pmeA- Aspergillus niger	33 ± 6	36 ± 6	43 ± 5	65 ± 4	75 ± 14	179 ± 41	Up
An04g02250	*uncharacterized*	questionable ORF	250 ± 27	261 ± 0	285 ± 45	537 ± 10	657 ± 2	587 ± 68	Up
An04g03010	*uncharacterized*	similarity to cell wall antigen MP1 - Penicillium marneffei	25 ± 3	27 ± 3	28 ± 1	65 ± 2	82 ± 12	55 ± 0	Up
An04g04630	*uncharacterized*	strong similarity to capsule protein CAP59 - Cryptococcus neoformans	56 ± 15	57 ± 5	116 ± 4	123 ± 3	144 ± 1	265 ± 11	Up
An04g05530	*uncharacterized*	similarity to disintegrin and metalloproteinase ADAM19 - Homo sapiens	101 ± 20	122 ± 9	180 ± 5	270 ± 57	335 ± 51	413 ± 105	Up
An04g05840	*uncharacterized*	weak similarity to phospholipid-cholesterol acyltransferase - Aeromonas hydrophila	101 ± 35	91 ± 28	154 ± 42	329 ± 42	341 ± 36	381 ± 31	Up
An04g06400	*uncharacterized*	weak similarity to Ser/Arg-related nuclear matrix protein SRM160 - Homo sapiens	115 ± 8	138 ± 2	142 ± 13	346 ± 8	348 ± 21	322 ± 78	Up
An04g06650	*uncharacterized*	similarity to hypothetical protein SPBC19C2.15c - Schizosaccharomyces pombe	287 ± 27	249 ± 39	611 ± 9	569 ± 41	599 ± 12	1116 ± 77	Up
An04g07160	*uncharacterized*	similarity to hypothetical protein MLD14.3 - Arabidopsis thaliana	202 ± 34	215 ± 74	246 ± 38	548 ± 65	656 ± 82	666 ± 81	Up
An05g01120	*uncharacterized*	strong similarity to cytochrome P450 monooxygenase TRI11 - Fusarium sporotrichioides	29 ± 6	25 ± 3	23 ± 5	121 ± 30	82 ± 3	44 ± 1	Up
An05g01210	*uncharacterized*	strong similarity to 1-acyldihydroxyacetone-phosphate reductase Ayr1p - Saccharomyces cerevisiae	154 ± 28	108 ± 20	168 ± 51	480 ± 27	501 ± 15	728 ± 24	Up
An05g02170	*uncharacterized*	strong similarity to serine-type carboxypeptidase F CPD-II - Aspergillus niger	60 ± 17	57 ± 6	70 ± 11	109 ± 12	121 ± 19	240 ± 17	Up
An05g02250	*uncharacterized*	similarity to hypothetical serine-threonine rich protein - Schizosaccharomyces pombe	280 ± 1	363 ± 13	426 ± 6	1866 ± 39	1553 ± 125	1048 ± 6	Up
An06g00160	*uncharacterized*	weak similarity to hypothetical cell wall protein binB - Aspergillus nidulans	319 ± 29	330 ± 70	128 ± 21	3226 ± 732	2940 ± 1230	453 ± 184	Up
An06g00280	*uncharacterized*	strong similarity to hypothetical protein YBR096w - Saccharomyces cerevisiae	67 ± 12	70 ± 16	122 ± 41	209 ± 63	263 ± 1	352 ± 25	Up
An06g00720	*uncharacterized*	similarity to chloroperoxidase CPO - Caldariomyces fumago	59 ± 24	59 ± 16	40 ± 14	109 ± 12	134 ± 32	109 ± 2	Up

Annotation	Gene Name*	Description- Supplemental Table 2 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An06g01000	*uncharacterized*	strong similarity to protein related to chitinase 3 precursor - Neurospora crassa	201 ± 49	252 ± 68	397 ± 160	601 ± 188	760 ± 179	892 ± 307	Up
An06g01260	*uncharacterized*	hypothetical protein	290 ± 16	293 ± 33	160 ± 38	539 ± 88	659 ± 177	354 ± 149	Up
An07g02730	*uncharacterized*	strong similarity to SUN family protein Psu1 - Schizosaccharomyces pombe	261 ± 96	317 ± 133	1534 ± 12	2045 ± 210	2424 ± 353	3717 ± 85	Up
An07g04900	*uncharacterized*	strong similarity to mRNA sequence of cDNA clone 2589 - Aspergillus niger	1065 ± 70	929 ± 155	364 ± 239	5638 ± 245	5796 ± 687	3483 ± 369	Up
An07g05670	*uncharacterized*	strong similarity to probable membrane protein YOR389w - Saccharomyces cerevisiae	74 ± 4	80 ± 5	97 ± 19	118 ± 3	151 ± 12	266 ± 42	Up
An07g06210	*uncharacterized*	hypothetical protein	1161 ± 11	1509 ± 319	2148 ± 933	6050 ± 71	5652 ± 110	4850 ± 26	Up
An07g06210	*uncharacterized*	hypothetical protein	1037 ± 68	1302 ± 333	1693 ± 620	5175 ± 261	4936 ± 38	3936 ± 153	Up
An07g07530	*crhB*	strong similarity to cell wall protein UTR2 - Saccharomyces cerevisiae	176 ± 39	254 ± 48	1452 ± 40	1812 ± 115	1764 ± 453	2719 ± 364	Up
An07g08510	*uncharacterized*	hypothetical protein	438 ± 50	421 ± 56	653 ± 69	1186 ± 228	1438 ± 179	1441 ± 429	Up
An07g08960	*uncharacterized*	similarity to hypothetical protein SPAC15A10.09c - Schizosaccharomyces pombe	292 ± 9	490 ± 106	837 ± 174	2495 ± 1310	3211 ± 457	2662 ± 663	Up
An08g00210	*uncharacterized*	strong similarity to glycerol-3-phosphate dehydrogenase gdm1 - Mus musculus	220 ± 36	229 ± 15	262 ± 2	505 ± 24	560 ± 2	775 ± 96	Up
An08g00490	*uncharacterized*	strong similarity to lactonohydrolase - Fusarium oxysporum	160 ± 14	237 ± 17	346 ± 177	484 ± 34	516 ± 99	806 ± 14	Up
An08g01360	*uncharacterized*	weak similarity to mucin MUC5AC - Homo sapiens	98 ± 5	88 ± 12	95 ± 3	171 ± 7	187 ± 4	262 ± 4	Up
An08g03440	*uncharacterized*	similarity to hypothetical protein F49E12.10 - Caenorhabditis elegans	123 ± 4	132 ± 1	88 ± 8	235 ± 17	315 ± 101	165 ± 22	Up
An08g07350	*sm to gelB- A. nid*	strong similarity to glycophospholipid-anchored surface glycoprotein GAS1 precursor - Saccharomyces cerevisiae	226 ± 26	257 ± 8	363 ± 114	674 ± 9	839 ± 71	1001 ± 69	Up
An08g07840	*cprA*	NADPH cytochrome P450 oxidoreductase CprA - Aspergillus niger [possible sequencing error]	304 ± 38	298 ± 6	521 ± 106	668 ± 44	760 ± 61	1200 ± 0	Up
An08g08280	*uncharacterized*	weak similarity to homeobox protein slouch slou - Drosophila melanogaster	125 ± 14	176 ± 10	345 ± 4	493 ± 257	784 ± 119	630 ± 178	Up
An08g09940	*uncharacterized*	strong similarity to cytochrome P450 monooxygenase TRI11 - Fusarium sporotrichioides	163 ± 39	145 ± 44	87 ± 1	502 ± 114	496 ± 7	204 ± 12	Up
An09g00260	*aglC*	alpha-galactosidase C aglC - Aspergillus niger [truncated ORF]	31 ± 6	34 ± 4	143 ± 60	208 ± 5	216 ± 38	370 ± 31	Up
An09g00480	*uncharacterized*	weak similarity to nitrogen metabolite repression regulator NmrA - Aspergillus nidulans	65 ± 17	54 ± 4	45 ± 5	177 ± 25	180 ± 28	175 ± 30	Up
An09g00510	*uncharacterized*	strong similarity to the hypothetical protein encoded by An01g01630 - Aspergillus niger	49 ± 7	56 ± 9	118 ± 76	240 ± 14	216 ± 38	252 ± 36	Up
An09g00530	*uncharacterized*	similarity to salicylate hydroxylase nahW - Pseudomonas stutzeri	530 ± 430	231 ± 219	69 ± 13	889 ± 149	953 ± 128	1495 ± 77	Up
An09g00540	*uncharacterized*	hypothetical protein	66 ± 66	26 ± 16	17 ± 0	156 ± 2	158 ± 28	221 ± 14	Up

Annotation	Gene Name*	Description- Supplemental Table 2 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An09g01240	*uncharacterized*	strong similarity to phospholipase B - Penicillium notatum	78 ± 7	63 ± 9	104 ± 3	499 ± 142	727 ± 111	627 ± 178	Up
An09g01490	*uncharacterized*	strong similarity to the hypothetical protein encoded by An09g04690 - Aspergillus niger	39 ± 3	41 ± 7	30 ± 2	84 ± 8	115 ± 2	258 ± 18	Up
An09g01560	*uncharacterized*	weak similarity to beta-1,3-galactosyltransferase b3Gal-T8 - Homo sapiens	65 ± 5	82 ± 10	87 ± 7	226 ± 19	248 ± 43	396 ± 58	Up
An09g02150	*uncharacterized*	strong similarity to monooxygenase paxM - Penicillium paxilli	32 ± 3	34 ± 1	25 ± 0	74 ± 38	65 ± 34	61 ± 11	Up
An09g02510	*uncharacterized*	strong similarity to hypothetical membrane protein YLR251w - Saccharomyces cerevisiae	111 ± 27	94 ± 8	384 ± 124	366 ± 32	382 ± 53	712 ± 75	Up
An09g03070	*agsE*	strong similarity to alpha-glucan synthase mok1 - Schizosaccharomyces pombe	174 ± 2	142 ± 40	163 ± 50	544 ± 44	598 ± 19	458 ± 41	Up
An09g03120	*uncharacterized*	similarity to a-agglutinin core protein AGA1 - Saccharomyces cerevisiae	55 ± 15	56 ± 13	87 ± 6	145 ± 4	135 ± 15	110 ± 3	Up
An09g03450	*uncharacterized*	strong similarity to ribulose-5-phosphate-epimerase Rpe1 - Saccharomyces cerevisiae	275 ± 28	234 ± 29	458 ± 51	484 ± 4	560 ± 33	1373 ± 54	Up
An09g03650	*uncharacterized*	weak similarity to hypothetical protein Ta0309 - Thermoplasma acidophilum	84 ± 40	52 ± 1	133 ± 16	375 ± 55	488 ± 99	438 ± 1	Up
An09g04290	*uncharacterized*	hypothetical protein	100 ± 8	143 ± 19	127 ± 41	381 ± 86	415 ± 26	543 ± 5	Up
An09g04360	*uncharacterized*	weak similarity to hypothetical protein SPy1903 - Streptococcus pyogenes	35 ± 1	39 ± 8	44 ± 5	252 ± 89	239 ± 55	108 ± 9	Up
An09g05050	*uncharacterized*	questionable ORF	19 ± 4	16 ± 0	15 ± 3	2452 ± 401	2106 ± 369	5834 ± 464	Up
An09g05050	*uncharacterized*	questionable ORF	25 ± 1	24 ± 4	22 ± 3	3522 ± 1024	2872 ± 506	8559 ± 471	Up
An09g05890	*uncharacterized*	similarity to probable membrane protein YMR010w - Saccharomyces cerevisiae	196 ± 44	204 ± 82	299 ± 151	378 ± 30	444 ± 11	567 ± 6	Up
An09g06720	*uncharacterized*	weak similarity to hypothetical protein B13I18.100 - Neurospora crassa	195 ± 42	225 ± 77	202 ± 40	603 ± 45	606 ± 5	530 ± 45	Up
An10g00210	*uncharacterized*	hypothetical protein	30 ± 1	31 ± 2	27 ± 2	62 ± 0	63 ± 6	137 ± 8	Up
An10g00660	*uncharacterized*	strong similarity to cytochrome P450 monooxygenase TRI11 - Fusarium sporotrichioides	50 ± 5	43 ± 12	49 ± 5	144 ± 17	106 ± 5	79 ± 3	Up
An10g00680	*uncharacterized*	strong similarity to H+-ATPase V0 domain 17 KD subunit, vacuolar, CUP5 - Saccharomyces cerevisiae	443 ± 342	220 ± 116	119 ± 50	2783 ± 380	2761 ± 26	1102 ± 42	Up
An11g00040	*uncharacterized*	weak similarity to cDNA for 59-kDa readthrough protein RT - Sorghum chlorotic spot virus	780 ± 377	869 ± 597	1222 ± 357	3463 ± 134	3264 ± 0	2983 ± 97	Up
An11g00090	*uncharacterized*	similarity to the hypothetical protein encoded by An11g00060 - Aspergillus niger	35 ± 1	36 ± 2	40 ± 1	192 ± 31	228 ± 42	238 ± 45	Up
An11g00100	*uncharacterized*	strong similarity to triacylglycerol lipase LIP5 - Candida rugosa	71 ± 4	64 ± 3	59 ± 8	800 ± 192	866 ± 397	2265 ± 77	Up
An11g00110	*uncharacterized*	strong similarity to cutinase CUT1 - Magnaporthe grisea	29 ± 3	31 ± 11	26 ± 3	175 ± 33	107 ± 33	69 ± 16	Up
An11g00220	*uncharacterized*	similarity to ferric reductase cfl1 - Candida albicans	56 ± 0	47 ± 2	50 ± 8	88 ± 12	108 ± 9	123 ± 2	Up

Annotation	Gene Name*	Description- Supplemental Table 2 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An11g01040	*uncharacterized*	strong similarity to oligopeptide transporter OPT1 - Candida albicans	120 ± 9	127 ± 5	125 ± 23	225 ± 18	247 ± 44	308 ± 8	Up
An11g02720	*uncharacterized*	similarity to hypothetical protein C50F7.2 - Caenorhabditis elegans	35 ± 1	38 ± 6	23 ± 3	101 ± 16	58 ± 10	45 ± 7	Up
An11g04830	*uncharacterized*	hypothetical protein	67 ± 9	80 ± 2	55 ± 3	130 ± 9	158 ± 8	137 ± 4	Up
An11g05260	*uncharacterized*	strong similarity to Pichia farinosa killer toxin resistance protein PKR1 - Saccharomyces cerevisiae	198 ± 29	181 ± 33	118 ± 18	508 ± 32	551 ± 13	255 ± 2	Up
An11g07010	*uncharacterized*	strong similarity to alkane-inducible cytochrome P450 alk2 - Candida tropicalis	230 ± 125	173 ± 88	145 ± 0	494 ± 112	489 ± 42	366 ± 3	Up
An11g09170	*uncharacterized*	similarity to the secreted aspartic proteinase SAP8 - Candida albicans	48 ± 5	55 ± 11	42 ± 1	316 ± 18	441 ± 6	290 ± 41	Up
An11g10520	*uncharacterized*	similarity to conserved hypothetical protein - Rhodobacter capsulatus	658 ± 121	664 ± 101	366 ± 66	1603 ± 130	1522 ± 71	1075 ± 25	Up
An12g02410	*uncharacterized*	strong similarity to spherulin 1a precursor - Physarum polycephalum	23 ± 6	25 ± 6	23 ± 2	103 ± 72	189 ± 77	190 ± 97	Up
An12g03580	*uncharacterized*	strong similarity to microsomal glutathione S-transferase 3 MGST3 - Homo sapiens	639 ± 331	436 ± 120	557 ± 132	2284 ± 96	1268 ± 76	1093 ± 178	Up
An12g05150	*uncharacterized*	weak similarity to sequence 379 from patent WO0100842 - Corynebacterium glutamicum	31 ± 4	34 ± 1	35 ± 7	209 ± 173	208 ± 164	192 ± 41	Up
An12g07550	*uncharacterized*	weak similarity to regulatory protein CBP4 - Saccharomyces cerevisiae	189 ± 22	187 ± 5	356 ± 32	433 ± 171	526 ± 68	504 ± 90	Up
An12g07750	*uncharacterized*	similarity to serine repeat antigen BAA78500.1 - Plasmodium falciparum	53 ± 2	58 ± 3	70 ± 0	270 ± 23	270 ± 90	294 ± 35	Up
An12g10200	*uncharacterized*	weak similarity to ice nucleation gene inaX - Xanthomonas campestris	1780 ± 196	1501 ± 292	227 ± 97	5426 ± 431	7228 ± 502	1879 ± 229	Up
An12g10770	*uncharacterized*	strong similarity to aryl-alcohol oxidase precursor aao - Pleurotus pulmonarius	56 ± 20	45 ± 0	61 ± 3	172 ± 40	240 ± 0	115 ± 11	Up
An12g10790	*uncharacterized*	weak similarity to putative sterigmatocystin biosynthesis peroxidase STCC - Emericella nidulans	34 ± 0	34 ± 2	41 ± 1	198 ± 65	217 ± 44	75 ± 3	Up
An13g01340	*uncharacterized*	similarity to Rhodopsin - Alligator mississippiensis	23 ± 1	23 ± 2	44 ± 5	66 ± 2	67 ± 22	89 ± 4	Up
An14g01820	*phiA /binB*	strong similarity to hypothetical cell wall protein binB - Aspergillus nidulans	2018 ± 1048	3064 ± 430	843 ± 70	8800 ± 3676	14346 ± 1791	4066 ± 653	Up
An14g01840	*uncharacterized*	similarity to hypothetical temperature-shock induced protein TIR3 - Saccharomyces cerevisiae	227 ± 122	258 ± 66	426 ± 36	1679 ± 739	2311 ± 995	2553 ± 877	Up
An14g02010	*uncharacterized*	strong similarity to vacuolar Ca2+/H+-exchanging protein Hum1 - Saccharomyces cerevisiae	597 ± 85	608 ± 3	477 ± 52	820 ± 144	1043 ± 180	1671 ± 433	Up
An14g02470	*uncharacterized*	strong similarity to the protein PRO304 of patent WO200104311-A1 - Homo sapiens	326 ± 177	470 ± 61	384 ± 107	2772 ± 181	3044 ± 235	770 ± 49	Up
An14g02740	*uncharacterized*	strong similarity to high affinity glucose transporter HGT1 - Kluyveromyces lactis	27 ± 4	24 ± 2	17 ± 1	43 ± 3	56 ± 1	85 ± 13	Up
An14g04100	*uncharacterized*	similarity to hypothetical protein ZK632.10 - Caenorhabditis elegans	679 ± 46	600 ± 0	339 ± 44	1976 ± 153	2087 ± 115	897 ± 16	Up
An14g04210	*uncharacterized*	similarity to hypothetical protein An07g05660 - Aspergillus niger	30 ± 0	35 ± 7	31 ± 0	931 ± 174	1386 ± 104	2511 ± 5	Up

Annotation	Gene Name*	Description- Supplemental Table 2 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An14g04490	*uncharacterized*	weak similarity to hypothetical protein AF1318 - Archaeoglobus fulgidus	117 ± 34	88 ± 11	88 ± 2	393 ± 44	346 ± 10	232 ± 14	Up
An15g00620	*uncharacterized*	hypothetical protein	90 ± 16	73 ± 16	129 ± 6	257 ± 36	186 ± 56	157 ± 42	Up
An15g00630	*uncharacterized*	strong similarity to SUR1 - Saccharomyces cerevisiae	157 ± 39	162 ± 40	180 ± 50	295 ± 55	373 ± 5	490 ± 101	Up
An15g02350	*uncharacterized*	strong similarity to hypothetical spore coat protein sp96 precursor - Neurospora crassa	56 ± 7	50 ± 3	52 ± 8	194 ± 57	106 ± 15	69 ± 2	Up
An15g03550	*uncharacterized*	weak similarity to protopectinase patent WO9806832-A1 - Bacillus subtilis	57 ± 44	57 ± 45	20 ± 0	954 ± 34	1066 ± 21	330 ± 22	Up
An15g03940	*uncharacterized*	strong similarity to monosaccharide transporter Mst-1 - Amanita muscaria	244 ± 17	202 ± 47	2589 ± 838	3196 ± 21	3689 ± 943	8423 ± 210	Up
An15g06140	*uncharacterized*	strong similarity to heterokaryon incompatibility protein HET-C - Neurospora crassa	195 ± 32	177 ± 56	160 ± 96	415 ± 30	375 ± 20	372 ± 20	Up
An15g06860	*uncharacterized*	similarity to predicted protein An06g02540 - Aspergillus niger	163 ± 9	159 ± 5	117 ± 3	406 ± 180	493 ± 150	399 ± 112	Up
An16g00070	*uncharacterized*	strong similarity to EST an_2627 - Aspergillus niger	182 ± 6	151 ± 34	383 ± 188	700 ± 77	522 ± 62	364 ± 93	Up
An16g00520	*uncharacterized*	strong similarity to 6-hydroxynicotinic acid mono-oxygenase patent JP09121864-A - Pseudomonas fluorescens	65 ± 16	62 ± 14	72 ± 14	101 ± 6	297 ± 229	326 ± 116	Up
An16g01850	*uncharacterized*	similarity to blastomyces yeast phase-specific protein 1 bys1 - Ajellomyces dermatitidis	2984 ± 2759	1197 ± 780	166 ± 54	12215 ± 313	12035 ± 1137	3132 ± 648	Up
An16g01880	*lipanI*	strong similarity to lysophospholipase - Aspergillus foetidus	648 ± 245	795 ± 248	454 ± 202	10644 ± 663	10585 ± 2214	2676 ± 160	Up
An16g03550	*uncharacterized*	similarity to protein fragment SEQ ID NO: 17361 from patent EP1033405-A2 - Arabidopsis thaliana	80 ± 27	83 ± 17	114 ± 9	207 ± 8	202 ± 2	248 ± 17	Up
An16g03710	*uncharacterized*	weak similarity to cDNA clone i7e03a1.f1 - Aspergillus nidulans	277 ± 174	241 ± 101	277 ± 45	1101 ± 303	1569 ± 651	1559 ± 786	Up
An16g05730	*uncharacterized*	similarity to integral membrane protein PTH11 - Magnaporthe grisea	46 ± 15	44 ± 5	54 ± 3	184 ± 7	148 ± 1	185 ± 21	Up
An16g06270	*uncharacterized*	similarity to versicolorin reductase verA - Aspergillus nidulans	871 ± 402	672 ± 247	328 ± 99	3349 ± 361	3316 ± 149	1809 ± 369	Up
An16g07040	*sm to btgE -A. nid*	similarity to beta-1,3-glucanosyltransferase BGT1 - Aspergillus fumigatus [truncated ORF]	502 ± 224	676 ± 353	2375 ± 144	5982 ± 202	6554 ± 1346	7358 ± 380	Up
An16g08090	*dfgE*	strong similarity to hypothetical protein B2J23.120 - Neurospora crassa	114 ± 8	125 ± 5	174 ± 10	229 ± 21	280 ± 11	438 ± 28	Up
An16g08420	*uncharacterized*	similarity to hypothetical integral membrane protein SC10B7.28 - Streptomyces coelicolor	1437 ± 163	1444 ± 149	1376 ± 331	4401 ± 145	4123 ± 206	3637 ± 47	Up
An16g08870	*uncharacterized*	strong similarity to the triacylglycerol lipase I precursor lipI - Geotrichum candidum	139 ± 16	125 ± 28	84 ± 2	395 ± 28	267 ± 13	181 ± 12	Up
An17g00170	*uncharacterized*	hypothetical protein	443 ± 33	474 ± 61	642 ± 79	1527 ± 333	1952 ± 268	1452 ± 274	Up
An17g01170	*uncharacterized*	strong similarity to probable membrane protein YOR154w - Saccharomyces cerevisiae	39 ± 11	31 ± 2	63 ± 5	106 ± 4	120 ± 19	262 ± 1	Up
An18g00090	*uncharacterized*	strong similarity to short-chain alcohol dehydrogenase adhA - Aspergillus parasiticus	39 ± 4	38 ± 12	35 ± 1	83 ± 3	92 ± 8	59 ± 2	Up

Annotation	Gene Name*	Description- Supplemental Table 2 *(continued)*	wild-type zone 1	wild-type zone 3	wild-type zone 5	Δ*flbA* zone 1	Δ*flbA* zone 3	Δ*flbA* zone 5	Regulation
An18g01290	*uncharacterized*	strong similarity to predicted protein An13g01340 - Aspergillus niger	26 ± 2	24 ± 2	38 ± 10	233 ± 53	218 ± 65	405 ± 13	Up
An18g01320	*uncharacterized*	strong similarity to extracellular protease precursor BAR1 - Saccharomyces cerevisiae	148 ± 76	129 ± 56	328 ± 31	2146 ± 67	2897 ± 61	2501 ± 161	Up
An18g01570	*uncharacterized*	similarity to integral membrane protein pth11 - Magnaporthe grisea	29 ± 7	25 ± 5	33 ± 2	159 ± 53	178 ± 12	230 ± 32	Up
An18g02120	*uncharacterized*	hypothetical protein	251 ± 33	285 ± 99	287 ± 36	482 ± 60	515 ± 29	733 ± 90	Up
An18g03360	*uncharacterized*	similarity to the proteophosphoglycan ppg1 - Leishmania major	32 ± 19	46 ± 26	27 ± 5	473 ± 19	503 ± 4	242 ± 2	Up
An18g03430	*uncharacterized*	similarity to EST n1a10a1.r1 - Emericella nidulans	175 ± 36	180 ± 7	113 ± 0	487 ± 35	503 ± 58	203 ± 35	Up
An18g04070	*uncharacterized*	weak similarity to mucin 2, intestinal/tracheal - Rattus norvegicus	2317 ± 4	2561 ± 415	1675 ± 379	9398 ± 130	9665 ± 52	3639 ± 86	Up
An18g04140	*uncharacterized*	strong similarity to extracellular acid phosphatase PhoA - Penicillium chrysogenum	78 ± 2	173 ± 141	117 ± 79	427 ± 131	431 ± 88	180 ± 38	Up

Chapter 5

Deletion of *fluG* results in reduced secretome complexity and secretion heterogeneity in colonies of *Aspergillus niger*

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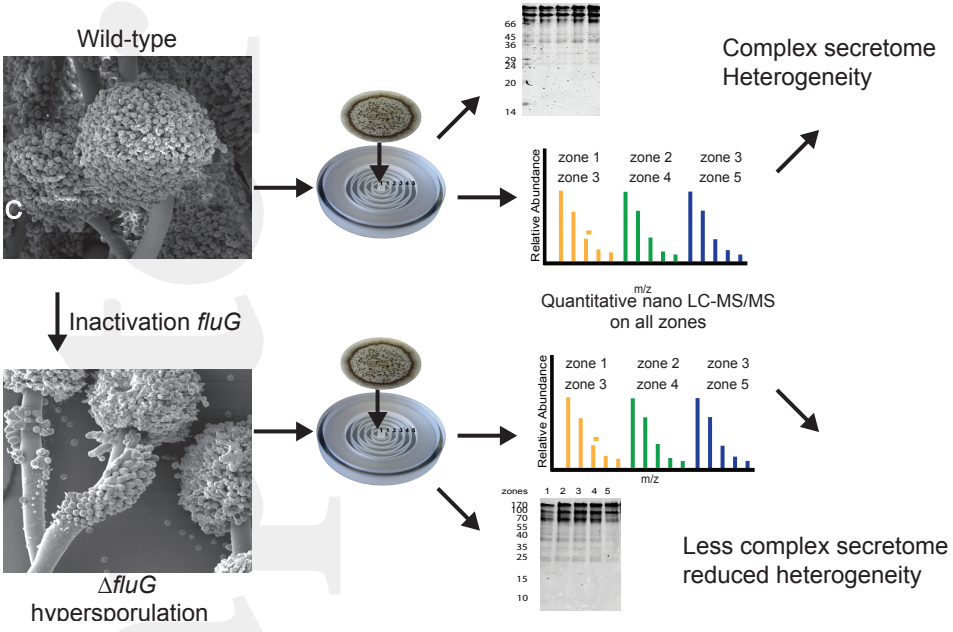
Graphical Abstract



ABSTRACT

Wild-type colonies of *Aspergillus niger* do not secrete proteins in zones that have the capacity to reproduce asexually. This indicates that sporulation inhibits secretion in the vegetative mycelium. Indeed, inactivation of the sporulation gene *flbA* results in protein secretion throughout the mycelium, which is accompanied by a more complex secretome. Here, the role of *fluG* of *A. niger* in sporulation, growth and secretion was assessed. The Δ*fluG* colonies formed fully developed conidiophores although sterigmata also developed at the stalk of these reproductive structures. Moreover, 7-days-old Δ*fluG* colonies had formed 20-60 % more biomass and growth switched from the periphery to the center when 6-days-old colonies had been transferred to fresh maltose medium for 24 h. Thus, FluG seems to repress biomass formation and impacts spatial growth in colonies under particular growth conditions. Secretion of proteins in wild-type and Δ*fluG* colonies mainly took place at the periphery. Seventy-two and 45 proteins were identified in the culture medium of wild-type and Δ*fluG* colonies, respectively. Of these proteins, 29 and 8 were differentially released into the culture medium by the different zones of the colony. These data show that inactivation of *fluG* results in reduced secreteome complexity and secretion heterogeneity. This may be mediated by a repressing activity of FluG on components of the sporulation program.

INTRODUCTION

Aspergillus species are among the most dominant fungi worldwide. They feed mainly on complex plant material.[1] To this end, they secrete a wide variety of enzymes that degrade polymers into smaller molecules that can be taken up to serve as nutrients. *Aspergilli* have a high capacity of secreting enzymes, which makes them attractive cell factories for the production of pharmaceutical proteins or enzymes that are used in the industry. This is exemplified by some strains of *Aspergillus niger* that secrete more than 30 grams per liter glucoamylase.[2]

Germination of asexual spores of aspergilli results in the formation of a mycelium that consists of an interconnected network of hyphae. Hyphae within the periphery of the colony are in contact with unexplored organic material. In contrast, the centre and intermediate zones, representing older parts of the colony, is exposed to a (partially) consumed substrate. These differences in the composition of the substrate results in zonal differences in gene expression.[3, 4, Chapter 4] However, differentiation within the vegetative mycelium has a similar impact on gene expression.[3] Differentiation within the vegetative mycelium is reflected by zonal differences in growth[3, 5, Chapter 3] secretion [5-7, Chapter 2, 3] and sporulation.[8, Chapter 3] Notably, the zone of the colony that sporulates or that has the potential to do so when an overlaying membrane is removed does not secrete proteins.[Chapter 3] This indicated that secretion by vegetative hyphae is repressed by the sporulation process.

Asexual sporulation of *aspergilli* is initiated with the formation of conidiophores. Conidiophore formation starts with the formation of a stalk. The stalk is two times wider than aerial hyphae and is formed from a footcell. The stalk stops growing when it is about 100 μm in length and then forms a vesicle at its tip that is about 10 μm wide. At the surface of this vesicle sterigmata (metulae and phialides) develop that form chains of spores.[9] Asexual development has been well studied in *Aspergillus nidulans*.[9, 10] FluG is thought to function at the start of the developmental program that leads to asexual sporulation. Overexpression of *fluG* was sufficient to induce asexual development under conditions where conidia formation is normally suppressed.[11] Vegetative and aerial hyphae were observed in strains in which the *fluG* gene was inactivated, but no further development was initiated in nutrient rich conditions.[11] However, conidiophores were observed in the Δ*fluG* strain under carbon starvation. Apparently, FluG is involved in sporulation resulting from a developmental program but not from a stress response imposed by lack of nutrients. Sporulation in the Δ*fluG* strain was also observed when it was grown next to a wild-type strain, even when these strains were separated by a dialysis membrane. From this and other data [12] it was concluded that FluG is involved in the production of an extracellular signaling molecule that initiates conidiophore formation.[13] The extracellular signaling molecule is assumed to activate a cascade of genes that ultimately leads to the activation of *brlA* encoding the central transcription factor of asexual development (Figure 1). It does so by

releasing the inhibition of SfgA on the *flbA-E* genes.[14] All *flb* mutant strains show low *brlA* expression. They form aerial hyphae but stalks are not formed.[15] FlbA is an RGS protein that regulates the signaling of the Gα-subunit FadA. GTP-bound FadA stimulates vegetative growth. FlbA converts the active FadA-GTP into the inactive FadA-GDP, thereby supressing the stimulation on vegetative growth[15, 16] (Figure 1). FlbA also (indirectly) activates *brlA* in *A. nidulans*. Inactivation of this central regulator of asexual development leads to indefinite growth of stalks. The vescicle, sterigmata and conidiospores are not formed.[10]

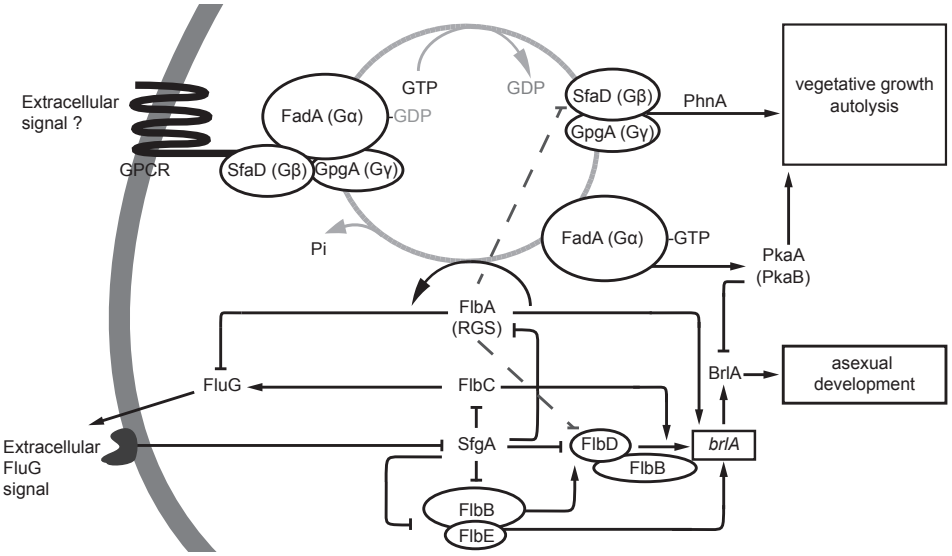


Figure 1. Model for the sporulation pathway in *A. nidulans*. FluG is involved in the synthesis and / or secretion of an extracellular factor that releases the inhibition of SfgA on Flb proteins. FlbE and FlbB form a complex that activates *brlA,* thus inducing asexual development. FlbC activates *brlA* in concert with the FlbB/FlbD transcription complex, and also activates *fluG*. In contrast, FlbA inhibits *fluG* and activates *brlA* by inactivating FadA (Adapted from [9,Chapter 1]). The sporulation pathway in *A. niger* is not identical to that in *A. nidulans.* FlbA of *A. niger* represses *sfaD* and *flbD* (stippled lines). Moreover, FluG has a different role in asexual development (this chapter).

Asexual development in *A. niger* morphologically resembles that in *A. nidulans*.[9] It was proposed that the underlying mechanism of asexual developmental of *A. nidulans* and *A. niger* are also similar. [17] Indeed, *brlA* and *flbA* of *A. niger* have a similar role in asexual reproduction as in *A. nidulans.*[8, Chapter 3] In contrast, secretion of proteins occurred throughout the mycelium of the Δ*flbA* strain of *A. niger*, while zonal secretion in the Δ*brlA* strain was similar to that in the wild-type. Apparently, inhibition of secretion is controlled early in the sporulation program. Colonies of the Δ*flbA* strain of *A. niger* also show a more complex secretome and the width of the cell wall is smaller than that observed in wild-type.[8, Chapter 3] Here, the role of *fluG* of *A. niger* in asexual development, growth

and spatial release of proteins was studied. It is shown that the Δ*fluG* strain of *A. niger* still sporulates. This is accompanied by a reduced secretome complexity and zonal secretion heterogeneity.

MATERIAL AND METHODS

Strain and Culture Conditions

A. niger N402[18] and the derived Δ*fluG* strain were grown at 30 ˚C. Colonies were grown as sandwiched cultures[5] on minimal agar medium (MM)[19] with 1.5 % agar and 25 mM maltose as a carbon source. To this end, a perforated polycarbonate (PC) membrane (0.1 µm pores, 76 mm diameter; Profiltra, Almere, Netherlands) was placed on the agar medium and topped with a 0.45 mm layer of 1.25 % agarose. A 1 mm plug of mycelium was positioned in the center of the agarose layer and covered with another 76 mm wide PC membrane. After 6 days of growth, colonies were either or not transferred for 24 h to fresh agar medium or to a ring plate.[6] The five concentric wells in the ring plate were filled with liquid MM supplemented with 25 mM maltose.

Plasmid construction

Upstream and downstream flanking regions of *fluG* (An14g03390) were amplified from chromosomal DNA of *A. niger* N402 using Taq polymerase and primer pairs 1 and 2 (Table 1). The upstream and downstream flanks were blunt end ligated in pJET1.2 (Fermentas Thermo Scientific, Waltham, USA). A *Not*I and *Xba*I/*XhoI* site had been introduced at the 5' and 3' end of the upstream flank, respectively, and a *Xho*I and *Kpn*I site at the 5' and 3' end of the downstream flank. The resulting plasmids were digested with *Not*I and *Xho*I and *Xho*I and *Kpn*I, respectively. This was followed by a three point ligation with pBluescriptII SK(+) that had been digested with *Not*I and *Kpn*I. The *Xho*I/*Xba*I fragment of pAN7-1[20] containing the hygromycine resistance cassette was inserted in the vector containing both flanks of *fluG* that had been digested with the same enzymes. This resulted in the *fluG* deletion construct BNO-*fluG*. This construct was digested with *Not*I and *Kpn*I, and the flanking sequences interspersed with the antibiotic resistance marker hygR were introduced in *A. niger* N402. Gene replacement by homologous recombination was confirmed by Southern analysis (data not shown).

Table 1. Primers used in this study

Primers	Primer description	Sequence '5- '3
Primer 1a	5'flank *fluG* FW	ATAAGAATGCGGCCGCGTGACTTGGAGGAGAGGAGCTG
Primer 1b	5'flank *fluG* REV	CCGCTCGAGTTAATCCTAGTCTAGAGCTGATTGGTTGGCCGGTTC
Primer 2a	3'flank *fluG* FW	CCGCTCGAGACCCTGTGCATACATACATAGC
Primer 2b	3'flank *fluG* REV	CGGGGTACCCCTCGCACTGATCGCCATATC

Transformation of *Aspergillus niger*

Transformation of *A. niger* was performed using 40 mg lysing enzymes (L-1412, Sigma-Aldrich, Zwijndrecht, Netherlands) per gram wet weight of mycelium as described.[21] Transformants were isolated from MMS plates[22] containing 200 µg ml^{-1} hygromycine and 50 µg ml^{-1} caffeine.

Localization of Protein Synthesis and Protein Secretion

Protein synthesis, protein secretion, and growth were monitored as described.[3, 8] Sandwiched cultures were labelled for 4 h with 185 kBq ^{14}C-amino acids (NEC-445E amino acid mixture, L-[^{14}C(U)]- specific activity 1.94 GBq $milliatom^{-1}$ Perkin Elmer, Waltham, USA) to visualize protein synthesis. A polyvinylidene difluoride (PVDF) membrane (Immobulon-P, Millipore, Bedford, USA) was placed between the sandwiched colony and the agar medium to immobilize secreted proteins. Growth was visualized by labelling for 10 min with 185 kBq ^{14}C-N-acetylglucosamine ([glucosamine-1-14C]- (MC276), specific activity 1.665 GBq $mmol^{-1}$, Movareck Biochemicals, Brea, California). Label was absorbed to a piece of rice paper (Schleicher & Schuell, Dassel, Germany) with the size of the colony, and placed on top of the mycelium. Colonies were fixed with 4 % formaldehyde in phosphate buffered saline (PBS) for 1 h at room temperature. For the visualization of protein synthesis and secretion, sandwiched colonies and PVDF membranes were washed 3 times 60 min with 1 % casamino acids (Becton, Dickinson and Company, Le-Pont-De-Claix, France). For monitoring growth, sandwiched colonies were washed 3 times 60 min with 0.44 mM N-acetyl-glucosamine in water. After drying overnight at room temperature, colonies and PVDF membranes were exposed to Kodak Biomax XAR film (Sigma, Saint Louis, USA).

SDS-PAGE

SDS-PAGE and Coomassie Brilliant Blue staining was performed as described.[7] Gels were imaged with an Odyssey Infrared Imaging System (Licor, Bad-Homburg, Germany) using the 700 nm channel.

Sample Preparation and Dimethyl Labelling for Mass Spectrometry

Medium samples were concentrated, lys-C and trypsin-digested, and desalted and concentrated with µElution plates as described.[7] Stable isotope dimethyl labelling was performed in a total volume of 50 µl.[7, 23, 24] Proteins of zone 1 and 5 were labelled with the light dimethyl label, those of zone 2 and 4 with the intermediate dimethyl label, and those of zone 3 with heavy dimethyl label. Dimethyl labels were swapped in a biological duplicate resulting in a heavy labelled zone 1 and 5, a light labelled zone 2 and 4, and an intermediate labelled zone 3. A technical duplicate of zone 3 was labelled with an intermediate- or light dimethyl label. Labelling efficiency was ≥ 96 % as was shown by running an aliquot of the labelled

samples on a regular LC MS/MS and comparing the number of dimethyl labelled peptides versus the total number of peptides.

Nano LC-LTQ-Orbitrap-MS

Three µl aliquots of dimethyl-labelled samples were mixed and the volume was adjusted to 10 µl taking into account a final concentration of formic acid of 10 %. Samples (8 µl) were analyzed on a LTQ-Orbitrap XL (Thermo Fischer Scientific, Bremen, Germany) that was connected to an Agilent 1200 HPLC system (Agilent, Santa Clara, CA, USA).[7] Samples were loaded on a trap column (ReproSil-Pur C18-AQ, 20 mm × 100 µm inner diameter, packed in house; Dr Maisch, Ammerbuch, Germany) at 5 µL min^{-1} in 100 % solvent A (0.1 M acetic acid in water). Peptides were eluted from the trap column onto an analytical column (ReproSil-Pur C18-AQ, 40 cm × 50 µm inner diameter, packed in-house) at 100 nL min^{-1} during 120 min using a gradient from 0-28 % solvent B (0.1 M acetic acid in 8:2 v / v acetonitrile / water) in 60 min and 28-50 % solvent B in 25 min. The eluent was sprayed via distal coated emitter tips connected to the analytical column. The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS. Full-scan MS spectra (from m/z 350 to 1500) were acquired in the Orbitrap with a resolution of 60000 at m/z 400 after accumulation to a target value of 500000 in the linear ion trap. The three most intense ions were selected for collision-induced fragmentation in the linear ion trap at an normalized collision energy of 35 % after accumulation to a target value of 10000.

Protein Identification and Quantification

The database that was used to assign peptides consisted of 14166 *A. niger* CBS 513.88 sequences,[17] 14166 reversed *A. niger* sequences, 256 common contaminant sequences, and 256 reversed contaminant sequences. Thermo Proteome Discoverer 1.3.0.339 (Thermo Fischer Scientific, Bremen, Germany) was used in combination with a Mascot search engine (version 2.3.02) software platform (Matrix Science, London, UK). The following settings of Mascot were used: trypsin with 2 missed cleavages, precursor mass tolerance of 50 ppm, fragment ions mass tolerance of 0.6 Da and carbamidomethyl (C) as fixed modification. Oxidation (M), light-dimethyl (K- and N-term), intermediate-dimethyl (K- and N- term) and heavy-dimethyl (K- and N- term) were set as variable modifications. The following settings of Proteome discoverer were used: peptide mass deviation 5 ppm, peptide Mascot (ion score) > 30, maximum peptide rank = 1, peptide length: 6-35, and low peptide confidelity. Heavy/light, medium/heavy, medium/light Quan values were calculated using Thermo Proteome Discoverer 1.3.0.339. A technical control experiment was performed with zone 3 that had been labelled with two different labels (Supplemental Figure 1). The ^{2}log ratio for each of the proteins is expected to be 0. Since proteins can be identified in one zone of the

colony only, files were also loaded with the option replaced Missing Quan values, which allows quantification of 'on / off' situations. Proteins with at least one peptide in both biological replicates were included in the analysis. Protein release was considered differential when the average ^{2}log ratio value was higher than 2 or lower than -2 in one sample and at least higher than 1 or lower than -1 in the other biological replicate. The False Discovery Rate (FDR) was calculated by searching proteins against a reversed protein database. The spectra of the quantification of concentric zones of wild-type and Δ*fluG* colonies had an FDR of < 0.4 %.

Light and Scanning Electron Microscopy

Light microscopy images were taken with a Leica MZ16 FA fluorescence stereomicroscope (Leica Microsystems, Mannheim, Germany) connected to a Leica DFC420 C digital camera. Sandwiched colonies were grown in the absence of an agarose layer between PC membranes. Samples (8 x 5 mm) were cut from the periphery and the center of the colony and prepared as described.[8] Mycelium was viewed with a Field Emission Scanning Electron Microscope (FEI, Eindhoven, The Netherlands) at an acceleration voltage of 5 kV and a working distance of about 5 mm.

RESULTS

Inactivation of *fluG* results in aberrant conidiation

Gene *fluG* was deleted in *A. niger* (data not shown). The Δ*fluG* strain showed normal sporulation on agar plates when viewed by light microscopy (Figure 2ABGH). Scanning electron microscopy revealed that Δ*fluG* colonies formed fully developed conidiophores (Figure 2I-L) similar to those of the wild-type (Figure 2C-F). However, aberrant sterigmata formation was observed on the stalks of the Δ*fluG* conidiophores (Figure 2IJK). The morphology of the Δ*fluG* conidia (Figure 2L) were similar to those of the wild-type (Figure 2F).

Inactivation of Δ*fluG* affects growth and secretion

Colonies of the wild-type and the Δ*fluG* strain formed a similar biomass on maltose after 3 and 5 days of growth (Figure 3). After 7 days of growth, however, the Δ*fluG* strain showed increased biomass on maltose containing medium (Figure 3). A similar result was obtained on xylose containing medium (Supplemental Figure 2). In the next set of experiments, growth was localized in 7-days-old colonies by labeling with ^{14}C-labelled N-acetylglucosamine. Growth was most pronounced in the peripheral zone 5 of both wild-type and Δ*fluG* colonies (Figure 4AG). Growth was still observed in the peripheral zone when wild-type colonies had been transferred after 6 days to fresh maltose medium (Figure 4D). Notably, growth of transferred Δ*fluG* colonies was shown to occur mainly in the colony centre (Figure 4J). Taken together, FluG seems to repress biomass formation and impacts spatial distribution of growth under particular growth conditions.

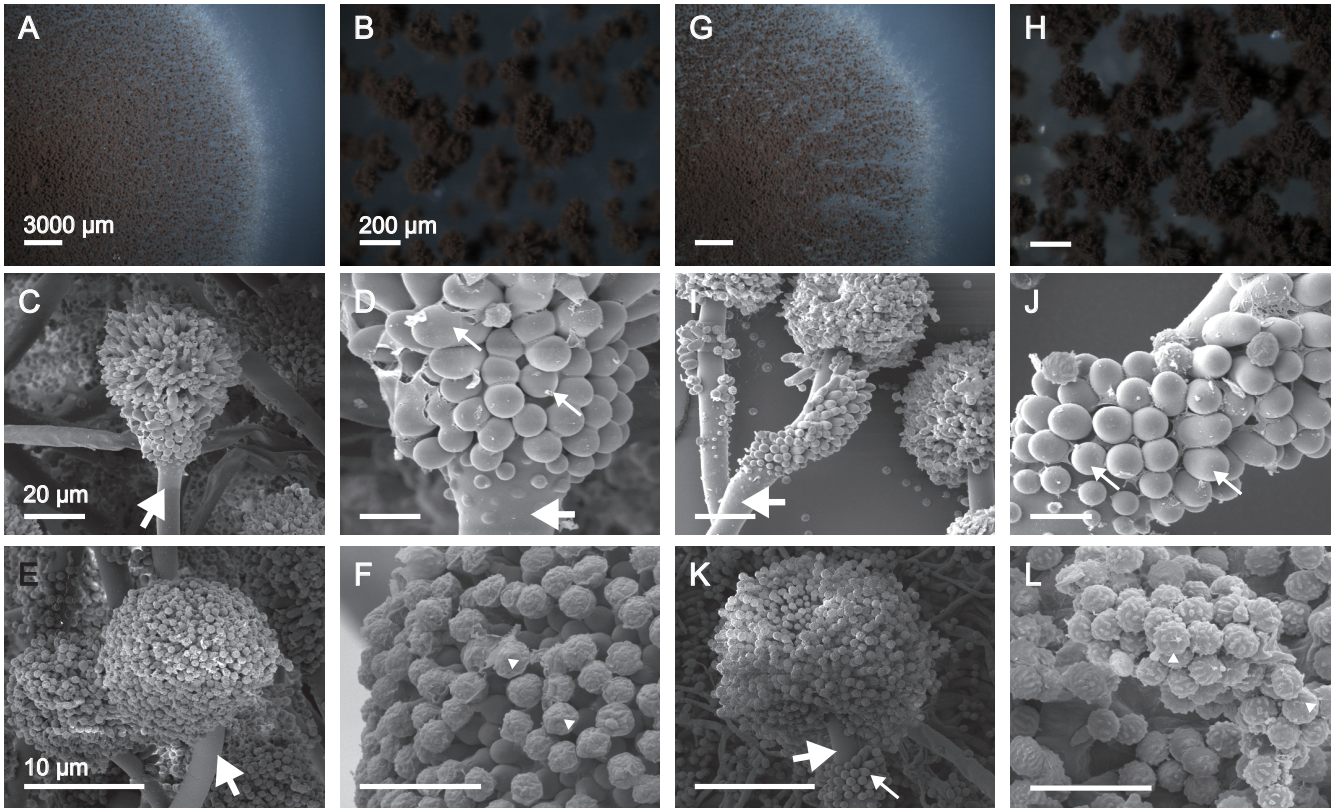


Figure 2. Light (ABGH) and scanning electron (C-F, I-L) microscopy of 6-days-old *A. niger* wild-type (N402) (A-F) and Δ*fluG* (G-L) colonies. The Δ*fluG* strain not only forms sterigmata at the surface of the vesicle of conidiophores but also on the stalks (IJK). Big arrows indicate a stalk, small arrows sterigmata, and small arrowheads conidia. Bar represent 3000 µm (AG), 200 µm (BH), 20 µm (CDIJ) and 10 µm (EFKL).

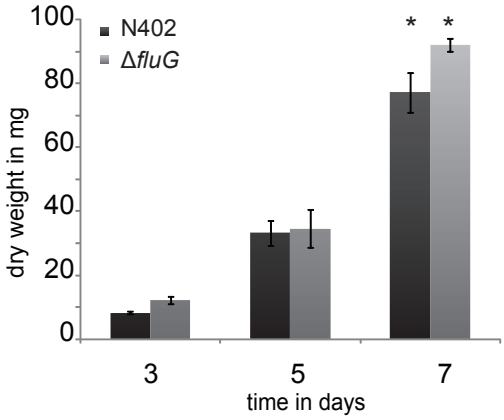


Figure 3. Dry weight of 7-days-old maltose-grown colonies of wild-type and Δ*fluG*. *indicates significant difference in biomass (*t*-test p<0.05).

Protein synthesis and secretion were monitored by labeling with ^{14}C-labelled amino acids. Protein synthesis was observed throughout the colony in non-transferred and transferred wild-type and Δ*fluG* colonies (Figure 4BEHK). Non-transferred 7-days-old wild-type and Δ*fluG* colonies secreted proteins mainly at the periphery of the colony (Figure 4CI). Secretion was still most pronounced at the periphery when colonies of both strains had been transferred to fresh medium after 6 days of growth (Figure 4FL). However, the amount of protein secreted in the intermediate and central zones had increased. This was especially the case for the Δ*fluG* colonies.

Location of growth, protein synthesis and protein secretion of wild-type and Δ*fluG* colonies was similar in non-transferred xylose-grown colonies when compared to non-transferred maltose-grown colonies (Supplemental Figure 3). Growth and protein synthesis were not affected in wild-type and Δ*fluG* colonies when they had been transferred to fresh medium after 6 days of growth. However, they secreted more proteins in the central and intermediate zones 1-3.

Quantification of the maltose grown wild-type and Δ*fluG* secretome

Proteins released by concentric zones of 7-days-old maltose-grown colonies of wild-type and *ΔfluG* were analyzed by SDS PAGE. For this, the colonies had been transferred for 24 h to a ring plate.[6, 7] The five concentric wells of the ring plate had been filled with maltose-containing minimal medium. The outermost part of the colony secreted proteins in zone 5, while the innermost zone of the colony released proteins in zone 1 of the ring plate. Proteins that had been formed during the 6-days of growth before transfer, as well as proteins that had been formed while growing on the ring plate, are part of the secretome that is visualized by Coomassie Brilliant Blue staining of the SDS-PAA gel. Proteins that had been formed before transfer had been trapped in the cell wall or had interacted with cell wall polymers, slowing down their release into the culture medium.[6, 7] The Δ*fluG* colonies showed a similar protein profile as wild-type colonies on xylose-

containing medium (Supplemental Figure 4). The protein profile of maltose-grown Δ*fluG* colonies (Figure 5B) showed some differences when compared to maltose-grown wild-type colonies (Figure 5A). For instance, Δ*fluG* colonies secreted a protein with an apparent molecular weight of 25 kDa that was not found in the culture medium of the wild-type. Cycloheximide treatment releases proteins that are trapped in the cell wall.[7] In both wild-type and Δ*fluG* colonies cycloheximide treatment resulted in higher amounts of protein in the medium when compared to untreated colonies (Figure 5). Coomassie Brilliant Blue staining did not indicate differences in the amount of proteins in the culture medium of cycloheximide-treated wild-type and Δ*fluG* colonies that had been grown on maltose (Figure 5) or xylose (Supplemental Figure 4). The 25 kDa protein band that was found in the culture medium of Δ*fluG* colonies was still absent in the wild-type secretome after cycloheximide treatment.

The secretome of maltose-grown wild-type and Δ*fluG* colonies was analysed by quantitative MS/MS. To this end, 6-days-old maltose-grown colonies had been transferred for 24 h to a ring plate. Proteins isolated from each of the wells of the ring plate were isolated, concentrated, digested with lys-C and trypsin,

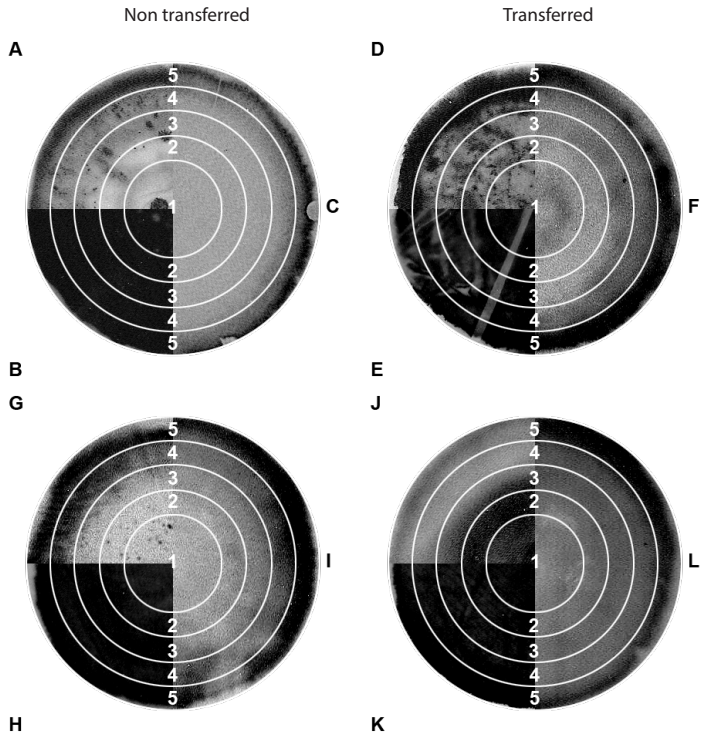


Figure 4: Growth, (ADGJ), protein synthesis (BEHK), and secretion (CFIL) in 7-days-old maltose-grown wild-type (A-F) and Δ*fluG* (G-L) colonies. Colonies were analysed before (ABC, GHI) and after transfer (DEF, JKL) to fresh maltose medium. Growth was detected with 14C-labeled N-acetylglucosamine. Protein synthesis and secretion were monitored by incorporation of ^{14}C-labeled amino acids. Secreted proteins were immobilized by a PVDF membrane that had been placed underneath the colony.

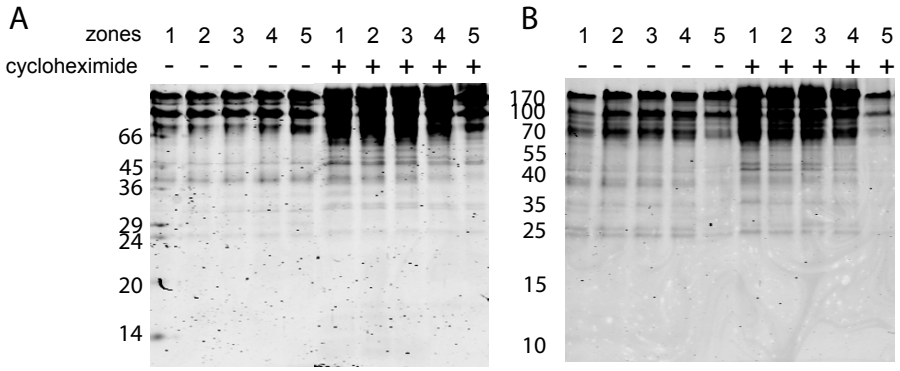


Figure 5. SDS-PAGE of the secretome of concentric zones of 7-days-old maltose-grown colonies of wild-type (A) and Δ*fluG* (B) that had either (+) or not (-) been treated with cycloheximide. Gels were stained with Coomassie Brilliant Blue. Zone 1 represents the center of the colony, whereas zone 5 represents the periphery.

and labeled with different isotopomeric dimethyl labels.[24] The reductive amination resulted in a mass shift of 28, 32, and 36 Da per primary amine of a peptide when a light-dimethyl-label, an intermediate-dimethyl-label, or a heavy-dimethyl-label was added, respectively.[24] Labeled peptides of the three innermost zones 1, 2 and 3 (containing the light, intermediate and heavy label, respectively) were mixed and analyzed in a single LC-MS/MS run. The same was done for peptides of zones 3, 4, and 5 (containing the high, intermediate and light label, respectively). By analysing the sample of zone 3 in both runs, the peptides of each of the zones could be quantitatively compared. To prevent label-specific peptide modifications, a biological duplicate was performed with a label swap on the peptides of the

Table 2. Number of representatives of protein classes identified in the culture medium of 7-days-old maltose-grown colonies of the Δ*fluG* and wild-type strain of A. *niger*. For details see Supplemental Table 2 and 4.

Protein Class	Wildype	Δ*fluG*
Cellulases	8	4
Xylanases	8	3
Mannosidase	1	0
Pectinases	7	4
Amylases	3	3
Inulinases	1	1
Proteases	16	11
Phytases	2	0
Fungal cell wall remodeling	8	7
Other enzymes	11	4
Unknown	5	5
Released via non-classical secretion (SecP)	1	1
Intracellular proteins (no SigP)	1	1
Total	**72**	**45**

different concentric zones (see Material and Methods).

A total of 72 proteins were identified in the duplicate secretome of maltose grown wild-type colonies (Supplemental Table 1). Only two of the proteins did not have a predicted signal for secretion, of which one is probably released via non-classical secretion (Table 2, Supplemental Table 1). This indicates that lysis in the colony was only minor. 28 out of the 72 identified proteins are carbohydrases (Table 2, Supplemental Table 1 and 2). Three of them are involved in starch degradation, while 24 are involved in hemicellulose (9), cellulose (8), and pectin degradation (7) (Table 2, Supplemental 1 and 2). Out of the 28 carbohydrases, 13 were found to be differentially released in the culture medium (Table 3, Supplemental Table 1, 2). Of these 13 carbohydrases, 8 were found to be released at least 4-fold lower in central zone 1, compared to the intermediate zone 3 (Table 3). Three proteins were identified in a ≥ 4-fold higher amount in the peripheral zone 5 compared to the intermediate zone 3. On the other hand, three proteins were identified to be released at least 4-fold lower in the peripheral zone 4 compared to intermediate zone 3 (Table 3, Supplemental Table 1 and 2). One of them was also identified to be released ≥ 4-fold less in the peripheral zone 5.

A total of 45 proteins identified in the wild-type secretome are involved in other processes than carbohydrate degradation. This set of proteins includes phytases, proteases, and proteins involved in cell wall synthesis / morphogenesis. Out of these 45 proteins, 16 were differentially released by the zones of the wild-type colony (Table 3, Supplemental Table 1 and 2). Seven proteins were found to be released 4-fold lower in the central zone 1 compared to the intermediate zone 3. Three of these proteins were also identified 4-fold higher in the peripheral zone 5 (Table 3). These 7 proteins include 5 (putative) proteases, of which one is released via non-classical secretion. In addition, 3 other proteins were identified to be released 4-fold higher in the culture medium compared to the intermediate zone 3. Five proteins, of which 2 proteases, were found to be released 4-fold lower in the sub-peripheral zone 4 compared to intermediate zone 3 (Table 3). One uncharacterized protein was found to be highly released in the central zones 1 and 2, compared to the intermediate zone.

AmyR is a transcription factor that regulates 14 genes encoding proteins that are involved in degradation of D-glucose or D-galactose containing oligo- and polysaccharides (including maltose).[25] For instance, AmyR regulates the genes encoding 2 α-amylases, 2 glucoamylases, 2 α-glucosidases, 2 β-glucosidases, 2 α-galactosidases, and 1 β-galactosidase.[25, 26] Of the AmyR regulated genes, 10 encode a protein with a signal sequence for secretion (Table 4). Only 4 of these proteins were identified in the secretome of maltose-grown wild-type colonies; AamA, AgdA, AglA, and GlaA.

The secretome of maltose-grown Δ*fluG* colonies appears to be less complex than that of the wild-type. Only 45 proteins were identified (Supplemental Tables 3 and 4). One of them did not have a singal sequence for secretion,

Table 3. Secreted proteins with a ^{2}log fold change ≥ 2 in at least one of the concentric zones of 7-days-old maltose-grown wild-type colonies. Protein release was considered differential when the average ^{2}log ratio value was higher than 2 or lower than -2 in one sample and at least higher than 1 or lower than -1 in the other biological replicate. Green and red shading indicate increased and decreased abundance in the secretome compared to zone 3, respectively. * In this case the average ^{2}log value was below 2, or higher than -2, but the value was at least (-)2 or (-)1 in both biological replicates. ** In this case the average ^{2}log value was higher than 2 or lower than -2, but not at least higher than 1, or lower than -1 in both biological replicates.

Annotation	Protein name	Description - Table 3	average ^{2}log ratio			
		cellulases	zone 1/3	zone 2/3	zone 4/3	zone 5/3
An07g09330	CbhA	1,4-beta-D-glucan cellobiohydrolase A precursor cbhA - Aspergillus niger	-3.0	-1.3	0.2	1.0
An16g06800	EglB	strong similarity to endoglucanase eglB - Aspergillus niger	-3.4	-1.8	-1.1	0.7
		xylanolytic enzymes				
An04g09700		strong similarity to endo-xylogalacturonan hydrolase xghA - Aspergillus tubingensis	-0.8	-0.1	1.7	2.0*
An12g05010	AxeA	acetyl xylan esterase axeA - Aspergillus niger	0.0	-0.4	1.3	2.0*
An03g00960	AxhA	1,4-beta-D-arabinoxylan arabinofuranohydrolase axhA - Aspergillus niger	-3.1	-1.5	0.6	1.3
An02g11150	AglB	alpha-galactosidase aglB - Aspergillus niger	-5.4	-3.3	-0.4	0.9
		pectinolytic enzymes				
An02g04900	PgaB	endo-polygalacturonases B pgaB - Aspergillus niger	0.0	0.0	-4.3	-1.0**
An15g05370	PgaII	polygalacturonase pgaII - Aspergillus niger	0.0	0.0	-4.2	-2.0
An02g10550	AbnC	endo-alpha-1,5-arabinanase abnC - Aspergillus niger	-0.4	0.1	3.0	2.0
		amylolitic enzymes				
An04g06920	AgdA	extracellular alpha-glucosidase agdA- Aspergillus niger	-2.8	-1.1	-0.9	-0.2
An03g06550	GlaA	glucan 1,4-alpha-glucosidase glaA - Aspergillus niger	-2.0*	-0.8	-2.0	-0.2
An11g03340	AamA	acid alpha-amylase aamA - Aspergillus niger	-2.0*	-0.6	-0.3	0.5
		inulinolytic enzymes				
An12g08280	InuE	exo-inulinase inu1 - Aspergillus niger	-4.1	-2.1	0.1	1.4
		(putative) proteases				
An07g08030	PepF	serine carboxypeptidase pepF - Aspergillus niger	-2.0	-0.8	1.6	2.0*
An02g13750		strong similarity to glutaminase A gtaA - Aspergillus oryzae	-2.1	-1.1	0.7	1.2
An07g01320	Anafp	strong similarity to antifungal protein precursor paf - Penicillium chrysogenum	-1.4	-0.5	-2.9	-1.1
An12g03300	ProtG	strong similarity to aspartic protease pr1 - Phaffia rhodozyma	-2.2	-0.6	-0.2	0.3
An06g00190		strong similarity to lysosomal pepstatin insensitive protease CLN2 - Homo sapiens	0.0	0.0	-4.5	-2.7
An14g02150		strong similarity to serine-type carboxypeptidase precursor cpdS - Aspergillus phoenicis	-2.2	-0.2	0.6	1.0
An02g04690		strong similarity to serine-type carboxypeptidase I cdpS - Aspergillus saitoi	-2.2	-0.2	0.9	0.9
		(putative) cell wall synthesis/ morphogenesis enzyme				
An14g01820	PhiA	cell wall protein binB - Aspergillus nidulans	-2.8	-1.6	-0.2	1.4
An06g00160		strong similarity to hypothetical protein AN1813.2 - Aspergillus nidulans	-1.1	-0.1	2.0	2.0*
		related to other enzymes				
An01g14940		similarity to nonhemolytic phospholipase C PC-PLC - Burkholderia pseudomallei	-2.0*	-0.3	2.3	2.0
An03g00500		strong similarity to diglycosidase related protein SEQ ID NO:10 from patent WO200018931-A1 - Aspergillus fumigatus	0.0	0.0	-2.6	0.2
An08g04630		strong similarity to hypothetical protein Afu2g15420 - Aspergillus fumigatus	0.0	0.0	0.8	2.0*

Annotation	Protein name	Description - Table 3 *(continued)*	average ^{2}log ratio			
			zone 1/3	zone 2/3	zone 4/3	zone 5/3
An14g02660		strong similarity to hypothetical necrosis and ethylene inducing protein BH0395 - Bacillus halodurans	0.0	-0.3	-2.2	-0.3
An14g02470		strong similarity to protein PRO304 from patent WO200104311-A1 - Homo sapiens	0.0	0.0	-4.2	-0.1
		related to unknown				
An16g00670		similarity to hypothetical protein AAO51454.1 - Dictyostelium discoideum	2.0	2.0	0.0	0.0

whereas another protein was predicted to be released via non-classical secretion (Table 2). This indicates that lysis is minimal in Δ*fluG* colonies. 15 out of the 44 identified proteins with a signal sequence for secretion are carbohydrases that are involved in the degradation of cellulose (4), xylan (3), pectin (4), and starch (4) (Supplemental Table 3). Twelve of these proteins were also identified in the wild-type secretome. Only 2 pectinases and 1 amylase were not found in the wild-type secretome (Supplemental Table 4). Five out of the 15 carbohydrases (i.e. EglB, AbfB, PgaE, AamA, and InuE) were found to be differentially released by zones of the colony (Table 5). The amylolytic protein AamA and the inulinase InuE were found to be ≥ 4-fold lower released in the central zones 1 and 2 compared to zone 3. The hemicellulase AbfB and the pectinase PgaE were released ≥ 4-fold higher in the peripheral zone 5 compared to zone 3. On the other hand, the cellulase EglB was found to be released ≥ 4-fold lower in the sub-peripheral zone 4 compared to intermediate zone 3. Of the 10 proteins with a signal sequence for secretion

Table 4. MS/MS detection of AmyR regulated proteins in concentric zones of 7-days-old maltose-grown wild-type and Δ*fluG A. niger* colonies.

Annotation	Protein name	Description - Table 4	SigP	Wild-type	ΔfluG
An04g06920	AgdA	extracellular alpha-glucosidase aglU - Aspergillus niger	y	**y**	(y)
An01g10930	AgdB	Putative alpha-glucosidase	y	n	n
An11g03340	AamA	acid alpha-amylase aamA - Aspergillus niger	y	**y**	**y**
An03g06550	GlaA	glucan 1,4-alpha-glucosidase glaA - Aspergillus niger	y	**y**	**y**
An12g03070	GlaB	Putative glucoamylase	n	n	n
An04g06930	AmyC	Alpha-amylase	y	n	n
An05g02100	AmyA/ AmyB	extracellular alpha-amylase amyA/amyB - Aspergillus niger	y	(y)	**y**
An06g00170	AglA	alpha-galactosidase aglA - Aspergillus niger	y	**y**	n
An06g00290	LacC	Beta-galactosidase lacC	y	n	n
An14g01770	(Bgl)	strong similarity to beta-glucosidase bgln - Candida molischiana	y	n	n
An09g00260	AglC	alpha-galactosidase C aglC - Aspergillus niger [truncated ORF]	y	n	n
An09g00270	AglC	alpha-galactosidase C aglC - Aspergillus niger [truncated ORF]	nc	n	n
An04g02700	(Agl)	hypothetical protein	n	n	n
An06g02040	(Bgl)	strong similarity to beta-glucosidase Cbg1 - Agrobacterium tumefaciens	n	n	n

that are encode by a AmyR-regulated gene, only 3 (AamA, AgdA and GlaA) were found to be released in the culture medium of Δ*fluG* colonies (Table 4).

A total of 30 other proteins were identified in the Δ*fluG* secretome. This set comprises 11 proteases, 1 catalase, and 7 cell wall synthesis / morphogenesis enzymes. Of these proteins only three proteases were found to be differentially released in the medium (Table 5). Taken together, these data indicate that heterogeneity in the colony is reduced upon deletion of *fluG*.

Table 5. Proteins with a ^{2}log fold change ≥2 in at least one of the concentric zones of 7-days-old-maltose-grown colonies of the *ΔfluG* strain of *A. niger*. Protein release was considered differential when the average ^{2}log ratio value was higher than 2 or lower than -2 in one sample and at least higher than 1 or lower than -1 in the other biological replicate. Green and red shading indicate increased and decreased abundance in the secretome compared to zone 3, respectively.

Annotation	Protein name	Description - Table 5	average ^{2}log ratio			
		(putative) cellulases	zone 1/3	zone 2/3	zone 4/3	zone 5/3
An16g06800	EglB	strong similarity to endoglucanase eglB - Aspergillus niger	0.4	0.9	-2.1	-1.0
		xylanolytic enzymes				
An15g02300	AbfB	arabinofuranosidase B abfB - Aspergillus niger	-0.1	0.3	2.5	2.2
		pectinolytic enzymes				
An01g14670	PgaE	polygalacturonase E precursor pgaE - Aspergillus niger	-0.1	0.1	2.0	1.5
		amylolitic enzymes				
An11g03340	AamA	acid alpha-amylase aamA - Aspergillus niger	-2.0	-2.1	-1.1	-0.1
		inulinolytic enzymes				
An12g08280	InuE	exo-inulinase inu1 - Aspergillus niger	-2.7	-2.2	1.1	1.6
		(putative) proteases				
An02g13750		strong similarity to glutaminase A gtaA - Aspergillus oryzae	-2.4	-2.4	0.0	1.1
An14g04710	PepA	aspartic proteinase aspergillopepsin I pepA - Aspergillus niger	0.0	0.0	0.0	-3.0
An01g01750		similarity to lysosomal protease CLN2 - Rattus norvegicus	0.0	0.0	-2.5	-0.2

Quantification of the zonal release of proteins in Δ*fluG* compared to wild-type

Proteins in the medium of zone 1, 3, and 5 of wild-type and Δ*fluG* colonies were quantitatively analysed to assess whether Δ*fluG* colonies have a higher secretion capacity. To this end, proteins of zone 1 of wild-type and Δ*fluG* colonies were mixed and analysed in a LC-MS/MS run. This was also done for zones 3 and 5. A total of 65 proteins could be used for the zonal quantification (Supplemental Table 5 and 6). Of these, 25 were found to be differentially released between Δ*fluG* and wild-type zones (≥ 4-fold difference) (Table 6). These proteins did not contain amylolytic proteins. Twenty-two proteins were found to be released 4-fold higher in at least one zone of the wild-type colony, while only 3 proteins were found to be higher released by at least one zone of the Δ*fluG* colonies (Table 6). These 3 proteins consist of the arabinofuranosidase AbfB, pectinase PgaE, and inulinase InuE.

Table 6. Quantitative difference between the secretome of colonies of the wild-type and the Δ*fluG* strain of *A. niger*. Proteins released in zones 1, 3, and 5 of Δ*fluG* were compared to zones 1, 3, and 5 of the wild-type. Heat map indicates the ^{2}log fold change ≥2 of a protein identified in the Δ*fluG* secretome compared to that of wild-type. Protein release was considered differential when the average ^{2}log ratio value was higher than 2 or lower than -2 in one sample and at least higher than 1 or lower than -1 in the other biological replicate. Green and red shading indicate increased and decreased abundance in the secretome compared to zone 3, respectively. * In this case the average ^{2}log value was below 2, or higher than -2, but the value was at least (-)2 or (-)1 in both biological replicates. ** In this case the average ^{2}log value was higher than 2 or lower than -2, but not at least higher than 1, or lower than -1 in both biological replicates.

Annotation	Protein name	Description - Table 6	average ^{2}log ratio Δ*flug/wt*		
		cellulases	zone 1/1	zone 3/3	zone 5/5
An16g06800	EglB	strong similarity to endoglucanase eglB - Aspergillus niger	0.1	0.6	-4.3
		xylanolytic enzymes			
An01g00780	XlnB	endo-1,4-xylanase xlnB - Aspergillus niger	-2.4	0.0	-2.4
An01g14600		strong similarity to endo-1,4-beta-xylanase B xynB from patent WO9414965 - Aspergillus tubingensis	-3.0	-2.9	0.0
An15g02300	AbfB	arabinofuranosidase B abfB - Aspergillus niger	2.2	3.6	0.0
An02g11150	AglB	alpha-galactosidase aglB - Aspergillus niger	0.0	0.0	-4.2
An04g09700		strong similarity to endo-xylogalacturonan hydrolase xghA - Aspergillus tubingensis	0.0	-4.6	0.0
		inulinolytic enzymes			
An12g08280	InuE	exo-inulinase inu1 - Aspergillus niger	0.0	2.0*	1.0
		pectinolytic enzymes			
An16g06990	PgaA	endo-polygalacturonase A pgaA - Aspergillus niger [putative frameshift]	0.0	-4.3	-1.1
An01g14670	PgaE	polygalacturonase E precursor pgaE - Aspergillus niger	0.0	3.0	0.0
An03g06310	PmeA	pectinesterase pmeA - Aspergillus niger	0.0	-4.3	-2.0*
		(putative) cell wall synthesis/ morphogenesis enzyme			
An03g05290	BgtB	glucan endo-1,3-beta-glucosidase eglC precursor - Aspergillus niger	-2.1	-1.5	-0.5
An01g12450	ExsG	strong similarity to hypothetical glucan beta-1,3 exoglucanase exgS - Aspergillus phoenicis	-2.0*	-1.2	-1.0
An06g00160		strong similarity to hypothetical protein AN1813.2 - Aspergillus nidulans	0.0	-2.3	0.0
An14g01820	PhiA	strong similarity to hypothetical cell wall protein binB - Aspergillus nidulans	-3.5	-5.6	0.0
		phospholypase			
An01g14940		similarity to nonhemolytic phospholipase C PC-PLC - Burkholderia pseudomallei	0.0	-4.8	0.0
		proteases			
An03g05200	ProtF	strong similarity to carboxypeptidase S1 - Penicillium janthinellum	-3.3	-2.9	-2.0*
An02g13750		strong similarity to glutaminase A gtaA - Aspergillus oryzae	-2.6	-2.1	-1.4
An14g04710	PepA	aspartic proteinase aspergillopepsin I pepA - Aspergillus niger	0.0	0.0	-5.0
An07g08030	PepF	serine carboxypeptidase pepF - Aspergillus niger	0.0	-2.0	0.0
An02g04690		strong similarity to serine-type carboxypeptidase I cdpS - Aspergillus saitoi	-1.1	-3.0	0.0
An08g04640	ProtB	strong similarity to hypothetical lysosomal pepstatin insensitive protease CLN2 - Canis lupus	-2.2	-1.2	-0.3
An14g02150		strong similarity to serine-type carboxypeptidase precursor cpdS - Aspergillus phoenicis	0.0	-3.7	0.0
		related to other enzymes			
An07g01320	Anafp	strong similarity to antifungal protein precursor paf - Penicillium chrysogenum	0.0	-2.3	0.0
An08g10780		strong similarity to hypothetical protein T16K5.230 - Arabidopsis thaliana	0.0	-2.5	0.0

Annotation	Protein name	Description - Table 6 *(continued)*	average ^{2}log ratio Δ*flug/wt*		
			zone 1/1	zone 3/3	zone 5/5
An10g00800		strong similarity to purine nucleoside permease NUP - Candida albicans	0.0	0.0	-2.4
		related to unknown proteins			
An02g13650		strong similarity to hypothetical protein Afu4g00380 - Aspergillus fumigatus	0.0	0.0	-4.8

DISCUSSION

FluG of *A. nidulans* has been proposed to be involved in the production of an extracellular signaling molecule that is involved in the initiation of conidiophore formation.[13] Here, the role of *fluG* of *A. niger* in sporulation, growth and secretion was studied. Inactivation of *fluG* of *A. niger* did not affect formation of conidia. This phenotype resembles that of Δ*fluG* of *A. fumigatus*[27] but is different from that of *A. nidulans*[15,12] and *A. oryzae*.[28] Scanning electron microscopy revealed that sterigmata were not only formed at the vesicle of the conidiophores of the Δ*fluG* strain of *A. niger* but also at the stalk. Scanning electron microscopy should reveal whether this is also the case in *A. fumigatus*. From these data we propose that FluG of *A. niger* is responsible for proper spatial and / or temporal expression of component(s) of the sporulation pathway, thereby ensuring proper development of the conidiophore. So far, gene expression analysis in *A. nidulans, A. oryzae* and *A. fumigatus* has been performed on whole colonies. As a consequence, spatial differences in expression of genes involved in asexual development will not be revealed and interactions between genes may be easily missed. Zonal expression of genes involved in conidiophore formation in the Δ*fluG* strain will be subject of future studies. This should reveal a better understanding of the interaction of *fluG* with other genes. It is expected that it is more complex than that shown in Figure 1.

Growth was shown to occur at the periphery of 7-days-old maltose-grown colonies of the wild-type and the Δ*fluG* strain. As expected,[3] spatial distribution of growth did not change in the wild-type when 6-days-old maltose-grown colonies had been transferred to fresh medium. In contrast, growth mainly occurred in the center of transferred Δ*fluG* colonies, a phenomenon not found when xylose was used as carbon source. Growth in the center of the colony was also observed after transfer of xylose-grown[8, Chapter 3] and maltose-grown (unpublished data) Δ*flbA* colonies. In this case, growth was even observed throughout the colony. The impact of FluG on growth is also illustrated by the fact that Δ*fluG* colonies produced 20 % and 60 % more biomass than the wild-type after 7-days of growth on medium with maltose and xylose, respectively. These data indicate that not only FlbA but also FluG is a repressor of growth, at least in particular zones of the colony.

Protein secretion in the wild-type and the Δ*fluG* strain was most

pronounced at the periphery. However, the center of wild-type and Δ*fluG* colonies secreted more proteins after transfer to fresh medium. This effect was most pronounced in the case of the Δ*fluG* strain. Increased protein secretion in central and intermediate zones of the Δ*fluG* strain compared to the wild-type was not observed by quantitative mass spectrometry. Twenty-two proteins were more abundantly released in at least one zone of the wild-type colony, while this was only the case for 3 proteins in the Δ*fluG* strain (one of which may be the 25 kDa protein that was exclusively found in the secretome of the Δ*fluG* strain as shown by SDS PAGE). This discrepancy can be explained by the fact that localization of protein secretion was performed by pulse labeling with radioactive amino acids, while quantitative mass spectrometry was performed on these newly formed proteins as well as on proteins that had been formed before transfer. The latter proteins that represent the major part of the secretome[6] are slowly released into the culture medium because they had been trapped in the cell wall or had been associated with cell wall polymers. Such proteins will not be labeled with radioactive amino acids in a pulse labelling. These data imply that FluG has a positive impact on release of proteins into the culture medium. As mentioned above, we propose that FluG of *A. niger* is responsible for proper spatial and / or temporal expression of component(s) of the sporulation pathway, thereby ensuring proper development of the conidiophore. FluG may have local repressing activity on genes involved in asexual development. This repression may stimulate secretion. Such a mechanism would be in line with the phenomenon of sporulation inhibited secretion.[8, Chapter 3]

We have identified 72 and 45 proteins in the secretome of maltose grown wild-type and Δ*fluG* colonies, respecively. Of these 45 proteins, 39 were also found in the secretome of the wild-type. This shows that the secretome of Δ*fluG* is less complex than that of the wild-type and far less complex than that of Δ*flbA* that consisted of 138 proteins with a signal sequence for secretion.[8] 68 % and 59 % of the proteins of the secretome of the wild-type were also found in the secretome of xylose-grown colonies of the wild-type and Δ*flbA*, respectively. This was 67 % and 55 % for the Δ*fluG* strain. These data show that there is a considerable overlap between the secretomes of cultures that have been grown on different carbon sources. This can be explained by the fact that the carbon source is depleted in the central and intermediate zones of colonies that are continuously grown on the same agar medium.[3] Most of the proteins that have been detected in our quantitative MS/MS have been formed during the time that the colony was exposed to the depleted agar medium (see above). AmyR is a transcription factor that activates 10 genes encoding secreted enzymes that degrade D-glucose or D-galactose containing oligo- and polysaccharides such as maltose.[25] Of these, GlaA, AgdA, AamA, AglA were found in the secretome of maltose grown colonies, while GlaA, AamA, and AmyA/AmyB were found in the secretome of Δ*fluG* colonies. The medium of xylose-grown colonies contains 3 enzymes that are encoded by AmyR-regulated genes.[7] Cycloheximide treatment released another 3

AmyR regulated enzymes from the cell walls of these colonies.[7] It may well be that maltose-grown colonies also have AmyR regulated proteins in their cell wall that can only be released upon cycloheximide treatment. It would be of interest to assess whether such enzymes are indeed found in the cell wall and whether they are instrumental in degradation of D-glucose or D-galactose containing oligo- and polysaccharides.

Taken together, we have shown that FluG is involved in proper development of conidiophores, that it represses growth and that it has a positive impact on secretion heterogeneity and the complexity of the secretome. The latter may be due to a repressing effect of FluG on components of the sporulation pathway. As such, FluG has an opposite role when compared to FlbA. This protein reduces the complexity of the secretome and stimulates sporulation. Like FluG, FlbA represses growth.

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ASSOCIATED CONTENT

Supplemental Table 1. Quantitative secretome analysis of 7-days-old maltose-grown wild-type colonies of *A. niger*. Heat map indicates the ^{2}log fold change of a protein compared to the intermediate zone 3. Protein release was considered differential when the average ^{2}log ratio value was higher than 2 or lower than -2 in one sample and at least higher than 1 or lower than -1 in the other biological replicate. Green and red shading indicate increased and decreased abundance of proteins when compared to zone 3, respectively. * In this case the average ^{2}log value was below 2, or higher than -2, but the value was at least (-)2 or (-)1 in each of the biological replicates. ** In this case the average ^{2}log value was higher than 2 or lower than -2, but not at least higher than 1, or lower than -1 in both biological replicates.Y: yes; nc: non-classical; n: no.

Annotation	Protein name	Description - Supplemental Table 1	average ^{2}log ratio				sigP
		cellulases	zone 1/3	zone 2/3	zone 4/3	zone 5/3	
An07g09330	CbhA	1,4-beta-D-glucan cellobiohydrolase A precursor cbhA - Aspergillus niger	-3.0	-1.3	0.2	1.0	y
An14g02760	EglA	endoglucanase A eglA - Aspergillus niger	-0.3	0.1	1.3	1.7	y
An16g06800	EglB	strong similarity to endoglucanase eglB - Aspergillus niger	-3.4	-1.8	-1.1	0.7	y
An18g03570	BglA	Probable beta-glucosidase A precursor bglA - Aspergillus niger	0.0	0.0	-0.3	1.0	y
An08g05230		strong similarity to hypothetical endoglucanase IV - Trichoderma reesei	0.0	0.0	-0.8	1.2	y
An14g02670		strong similarity to endoglucanase IV egl4 - Trichoderma reesei	0.0	0.0	0.7	0.8	y
An03g05530		strong similarity to endo-beta-1,4-glucanase EGIII-like from patent WO9931255-A2 - Emericella desertoru	-0.3	0.3	0.8	0.8	y
An03g04190		similarity to cellulase #2 from patent US2003036176-A1 - Xanthomonas campestris	0.4	-0.1	0.0	0.5	y
		xylanolytic enzymes					
An01g00780	XlnB	endo-1,4-xylanase xlnB - Aspergillus niger	-1.0	-0.4	0.2	1.2	y
An01g14600		strong similarity to endo-1,4-beta-xylanase B xynB from patent WO9414965 - Aspergillus tubingensis	-0.1	0.3	1.6	1.9	y
An04g09700		strong similarity to endo-xylogalacturonan hydrolase xghA - Aspergillus tubingensis	-0.8	-0.1	1.7	2.0*	y
An12g05010	AxeA	acetyl xylan esterase axeA - Aspergillus niger	0.0	-0.4	1.3	2.0*	y
An03g00960	AxhA	1,4-beta-D-arabinoxylan arabinofuranohydrolase axhA - Aspergillus niger	-3.1	-1.5	0.6	1.3	y
An06g00170	AglA	alpha-galactosidase aglA - Aspergillus niger	0.0	0.0	0.0	0.5	y
An02g11150	AglB	alpha-galactosidase aglB - Aspergillus niger	-5.4	-3.3	-0.4	0.9	y
An15g02300	AbfB	arabinofuranosidase B abfB - Aspergillus niger	1.3	1.3	0.0	0.0	y
		pectinolytic enzymes					
An03g06310	PmeA	pectinesterase pmeA - Aspergillus niger	0.1	0.4	0.1	0.3	y
An16g06990	PgaA	endo-polygalacturonase A pgaA - Aspergillus niger [putative frameshift]	-1.2	-0.4	0.7	0.9	y
An09g03260	PgaD	endo-polygalacturonase D pgaD - Aspergillus niger	-0.2	-0.1	1.0	0.9	y
An02g04900	PgaB	endo-polygalacturonases B pgaB - Aspergillus niger	0.0	0.0	-4.3	-1.0**	y
An15g05370	PgaII	polygalacturonase pgaII - Aspergillus niger	0.0	0.0	-4.2	-2.0	y
An02g10550	AbnC	endo-alpha-1,5-arabinanase abnC - Aspergillus niger	-0.4	0.1	3.0	2.0	y
An18g05940	GalA	arabinogalactan endo-1,4-beta-galactosidase galA - Aspergillus aculeatus	-0.9	0.0	0.0	1.2	y
		amylolitic enzymes					
An04g06920	AgdA	extracellular alpha-glucosidase agdA- Aspergillus niger	-2.8	-1.1	-0.9	-0.2	y
An03g06550	GlaA	glucan 1,4-alpha-glucosidase glaA - Aspergillus niger	-2.0*	-0.8	-2.0	-0.2	y
An11g03340	AamA	acid alpha-amylase aamA - Aspergillus niger	-2.0*	-0.6	-0.3	0.5	y

Annotation	Protein name	Description - Supplemental Table 1 *(continued)*	average ^{2}log ratio				sigP
			zone 1/3	zone 2/3	zone 4/3	zone 5/3	
		inulinolytic enzymes					
An12g08280	InuE	exo-inulinase inu1 - Aspergillus niger	-4.1	-2.1	0.1	1.4	y
		mannosidases					
An01g12550	MsdS	strong similarity to mannosyl-oligosaccharide 1,2-alpha-mannosidase msdS - Aspergillus saitoi	0.0	-1.0	0.3	1.0	y
		(putative) proteases					
An14g04710	PepA	aspartic proteinase aspergillopepsin I pepA - Aspergillus niger	-1.4	-0.4	-0.8	-0.1	y
An07g08030	PepF	serine carboxypeptidase pepF - Aspergillus niger	-2.0	-0.8	1.6	2.0*	y
An15g06280	PepAa	strong similarity to aspartic proteinase aspergillopepsin I pepA - Aspergillus niger [truncated ORF]	-0.9	-0.1	1.0	1.2	y
An02g01550		strong similarity to secreted serine protease 19 kDa CS antigen CS-Ag - Coccidioides immitis	-0.2	0.4	1.7	1.6	y
An02g13750		strong similarity to glutaminase A gtaA - Aspergillus oryzae	-2.1	-1.1	0.7	1.2	y
An01g01750		similarity to lysosomal protease CLN2 - Rattus norvegicus	-0.1	0.0	-1.6	0.0	y
An04g01440		strong similarity to precursor of pepsin A3 - Homo sapiens	-1.0	0.0	1.4	1.1	y
An07g01320	Anafp	strong similarity to antifungal protein precursor paf - Penicillium chrysogenum	-1.4	-0.5	-2.9	-1.1	y
An08g04490	ProtA	Endoprotease Endo-Pro- precursor Epr - Aspergillus niger	0.1	0.3	0.6	0.8	y
An03g05200	ProtF	strong similarity to carboxypeptidase S1 - Penicillium janthinellum	-0.2	0.1	0.4	0.5	y
An12g03300	ProtG	strong similarity to aspartic protease pr1 - Phaffia rhodozyma	-2.2	-0.6	-0.2	0.3	y
An06g00190		strong similarity to lysosomal pepstatin insensitive protease CLN2 - Homo sapiens	0.0	0.0	-4.5	-2.7	y
An18g01320		strong similarity to extracellular protease precursor Bar1 - Saccharomyces cerevisiae	-0.1	0.3	-0.4	0.2	y
An08g04640	ProtB	strong similarity to hypothetical lysosomal pepstatin insensitive protease CLN2 - Canis lupus	0.2	0.3	1.3	1.4	y
An14g02150		strong similarity to serine-type carboxypeptidase precursor cpdS - Aspergillus phoenicis	-2.2	-0.2	0.6	1.0	y
An02g04690		strong similarity to serine-type carboxypeptidase I cdpS - Aspergillus saitoi	-2.2	-0.2	0.9	0.9	y
		(putative) phytases					
An12g01910	PhyA3	strong similarity to phytase phyA3 - Aspergillus fumigatus	0.0	0.0	-1.8	0.2	y
An09g00670	GelD	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanosyltransferase gel3 - Aspergillus fumigatus	-0.3	0.3	0.1	0.8	y
		(putative) cell wall synthesis/ morphogenesis enzyme					
An14g01820	PhiA	strong similarity to hypothetical cell wall protein binB - Aspergillus nidulans	-2.8	-1.6	-0.2	1.4	y
An03g05290	BgtB	glucan endo-1,3-beta-glucosidase eglC precursor - Aspergillus niger	0.2	0.5	0.7	1.0	y
An01g11010	CrhD	strong similarity to cell wall protein Crh1 - Saccharomyces cerevisiae	0.1	0.4	0.6	0.6	y
An01g12450	ExsG	strong similarity to hypothetical glucan beta-1,3 exoglucanase exgS - Aspergillus phoenicis	0.6	0.5	1.5	1.4	y
An07g07530	CrhB	strong similarity to cell wall protein Utr2 - Saccharomyces cerevisiae	0.0	0.0	-1.4	-0.3	y
An06g00160		strong similarity to hypothetical protein AN1813.2 - Aspergillus nidulans	-1.1	-0.1	2.0	2.0*	y
An02g00850		strong similarity to hypothetical mixed-linked glucanase precursor related protein MLG1 - Neurospora crassa	-0.7	0.0	0.0	0.0	y
An04g01230	EcmA	strong similarity to hypothetical ECM33 homolog SPCC1223.12c - Schizosaccharomyces pombe	-0.5	0.0	0.6	0.8	y
		related to other enzymes					
An08g07350		strong similarity to precursor of glycophospholipid-anchored surface glycoprotein Gas1 - Saccharomyces cerevisiae	-0.6	-0.4	-0.9	0.1	y
An01g14940		similarity to nonhemolytic phospholipase C PC-PLC - Burkholderia pseudomallei	-2.0*	-0.3	2.3	2.0	y

Annotation	Protein name	Description - Supplemental Table 1 *(continued)*	average ^{2}log ratio				sigP
			zone 1/3	zone 2/3	zone 4/3	zone 5/3	
An03g00500		strong similarity to diglycosidase related protein SEQ ID NO:10 from patent WO200018931-A1 - Aspergillus fumigatus	0.0	0.0	-2.6	0.2	y
An08g04630		strong similarity to hypothetical protein Afu2g15420 - Aspergillus fumigatus	0.0	0.0	0.8	2.0*	y
An10g00800		strong similarity to purine nucleoside permease NUP - Candida albicans	-0.5	0.1	-0.5	0.1	y
An11g00040		weak similarity to cDNA for 59-kDa readthrough protein RT - Sorghum chlorotic spot virus	-1.4	-0.2	0.5	0.9	y
An14g01685		strong similarity to hypothetical acetyltransferase Afu4g03600 - Aspergillus fumigatus	0.0	0.4	0.0	0.0	y
An14g02660		strong similarity to hypothetical necrosis and ethylene inducing protein BH0395 - Bacillus halodurans	0.0	-0.3	-2.2	-0.3	y
An14g02470		strong similarity to protein PRO304 from patent WO200104311-A1 - Homo sapiens	0.0	0.0	-4.2	-0.1	y
An01g10580		strong similarity to ribonuclease T2 precursor rntB - Aspergillus oryzae	-0.8	0.0	1.2	1.4	y
An02g13650		strong similarity to hypothetical protein Afu4g00380 - Aspergillus fumigatus	-1.5	-0.5	0.8	1.1	y
		related to unknown					
An15g02250		hypothetical protein	-1.3	-0.5	-1.5	-0.4	y
An14g00800		similarity to hypothetical protein CAC38347.1 - Schizosaccharomyces pombe	-1.1	-0.4	0.7	0.7	y
An15g07520		similarity to hypothetical protein mlr2143 - Mesorhizobium loti	-0.8	0.0	-0.3	0.3	y
An16g00670		similarity to hypothetical protein AAO51454.1 - Dictyostelium discoideum	2.0*	2.0*	0.0	0.0	y
An08g10780		strong similarity to hypothetical protein T16K5.230 - Arabidopsis thaliana	-0.6	0.1	0.7	1.0	y
		released via non-classical secretion (secP)					
An01g00370	PepAb	strong similarity to aspergillopepsin apnS - Aspergillus phoenicis	-0.2	-0.8	2.5	2.0	nc
		related to intracellular proteins (no SigP)					
An07g08490		similarity to ATP-dependent helicase pcrA - Bacillus stearothermophilus	0.0	0.0	0.0	0.0	n

Supplemental Table 2. Quantitative analysis of the secretome of concentric zones of 7-days-old maltose-grown wild-type colonies of *A. niger*. This material is available free of charge via the internet at http://goo.gl/BH1Kd

Supplemental Table 3. Quantitative secretome analysis of 7-days-old maltose-grown colonies of the Δ*fluG* strain of *A. niger*. Heat map indicates the ^{2}log fold change of a protein identified in the Δ*fluG* secretome compared to that of wild-type. Protein release was considered differential when the average ^{2}log ratio value was higher than 2 or lower than -2 in one sample and at least higher than 1 or lower than -1 in the other biological replicate. Green and red shading indicate increased and decreased abundance of proteins when compared to zone 3, respectively. Y: yes; nc: non-classical; n: no.

Annotation	Protein name	Description - Supplemental Table 3	average ^{2}log ratio				sigP
		(putative) cellulases	zone 1/3	zone 2/3	zone 4/3	zone 5/3	
An14g02760	EglA	endoglucanase A eglA - Aspergillus niger	-0.5	0.0	1.7	1.2	y
An16g06800	EglB	strong similarity to endoglucanase eglB - Aspergillus niger	0.4	0.9	-2.1	-1.0	y
An03g04190		similarity to cellulase #2 from patent US2003036176-A1 - Xanthomonas campestris	0.0	0.0	0.0	0.0	y
An03g05530		strong similarity to endo-beta-1,4-glucanase EGIII-like from patent WO9931255-A2 - Emericella desertoru	0.3	0.3	0.4	0.4	y
		xylanolytic enzymes					
An01g00780	XlnB	endo-1,4-xylanase xlnB - Aspergillus niger	-1.4	-1.5	0.7	0.6	y
An01g14600		strong similarity to endo-1,4-beta-xylanase B xynB from patent WO9414965 - Aspergillus tubingensis	-0.5	-1.0	0.0	0.0	y
An15g02300	AbfB	arabinofuranosidase B abfB - Aspergillus niger	-0.1	0.3	2.5	2.2	y
		pectinolytic enzymes					
An14g04370	PelA	pectin lyase pelA - Aspergillus niger	0.0	0.0	0.0	-1.3	y
An16g06990	PgaA	endo-polygalacturonase A pgaA - Aspergillus niger [putative frameshift]	0.0	0.0	-2.3	-1.2	y
An01g14670	PgaE	polygalacturonase E precursor pgaE - Aspergillus niger	-0.1	0.1	2.0	1.5	y
An02g10550	AbnC	endo-alpha-1,5-arabinanase abnC - Aspergillus niger	0.4	0.4	1.7	1.2	y
		amylolitic enzymes					
An03g06550	GlaA	glucan 1,4-alpha-glucosidase glaA - Aspergillus niger	-0.8	-0.9	-0.4	0.3	y
An05g02100		extracellular alpha-amylase amyA/amyB - Aspergillus niger	0.0	0.0	0.0	0.0	y
An11g03340	AamA	acid alpha-amylase aamA - Aspergillus niger	-2.0	-2.1	-1.1	-0.1	y
		inulinolytic enzymes					
An12g08280	InuE	exo-inulinase inu1 - Aspergillus niger	-2.7	-2.2	1.1	1.6	y
		(putative) proteases					
An02g13750		strong similarity to glutaminase A gtaA - Aspergillus oryzae	-2.4	-2.4	0.0	1.1	y
An14g04710	PepA	aspartic proteinase aspergillopepsin I pepA - Aspergillus niger	0.0	0.0	0.0	-3.0	y
An03g05200	ProtF	strong similarity to carboxypeptidase S1 - Penicillium janthinellum	-0.4	-0.7	-0.2	0.3	y
An01g01750		similarity to lysosomal protease CLN2 - Rattus norvegicus	0.0	0.0	-2.5	-0.2	y
An08g04490	ProtA	Endoprotease Endo-Pro- precursor Epr - Aspergillus niger	-0.3	-0.1	0.5	0.4	y
An14g02150		strong similarity to serine-type carboxypeptidase precursor cpdS - Aspergillus phoenicis	0.0	0.0	-1.2	-0.7	y
An08g04640	ProtB	strong similarity to hypothetical lysosomal pepstatin insensitive protease CLN2 - Canis lupus	-0.6	-0.4	0.3	0.5	y
An12g03300	ProtG	strong similarity to aspartic protease pr1 - Phaffia rhodozyma	-1.5	-0.1	0.0	0.0	y
An02g01550		strong similarity to secreted serine protease 19 kDa CS antigen CS-Ag - Coccidioides immitis	-0.6	0.0	1.0	1.3	y
An02g04690		strong similarity to serine-type carboxypeptidase I cdpS - Aspergillus saitoi	0.0	0.0	-1.0	-0.5	y
An18g01320		strong similarity to extracellular protease precursor Bar1 - Saccharomyces cerevisiae	-0.2	0.1	-0.3	0.1	y

Annotation	Protein name	Description - Supplemental Table 3 *(continued)*	average ^{2}log ratio				sigP
			zone 1/3	zone 2/3	zone 4/3	zone 5/3	
		superoxide dismutase					
An01g01820	CatR	catalase R catR - Aspergillus niger	0.0	0.0	0.0	0.0	y
		(putative) cell wall synthesising/ morphogenesis enzymes					
An14g01820	PhiA	strong similarity to hypothetical cell wall protein binB - Aspergillus nidulans	-0.5	0.0	-0.1	0.4	y
An03g05290	BgtB	glucan endo-1,3-beta-glucosidase eglC precursor - Aspergillus niger	-0.2	0.0	-0.2	0.0	y
An01g11010	CrhD	strong similarity to cell wall protein Crh1 - Saccharomyces cerevisiae	0.2	0.4	0.8	0.4	y
An07g01160	CrhC	strong similarity to cell wall protein Utr2 - Saccharomyces cerevisiae	0.0	0.0	0.3	1.2	y
An01g12450	ExsG	strong similarity to hypothetical glucan beta-1,3 exoglucanase exgS - Aspergillus phoenicis	0.0	0.2	1.5	1.6	y
An04g01230	EcmA	strong similarity to hypothetical ECM33 homolog SPCC1223.12c - Schizosaccharomyces pombe	-0.3	0.0	1.0	0.8	y
An09g00670	GelD	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanosyltransferase gel3 - Aspergillus fumigatus	-0.3	0.0	0.4	0.5	y
		related to other enzymes					
An11g00040		weak similarity to cDNA for 59-kDa readthrough protein RT - Sorghum chlorotic spot virus	-0.4	-0.1	0.2	0.5	y
An08g10780		strong similarity to hypothetical protein T16K5.230 - Arabidopsis thaliana	0.0	0.0	1.3	0.0	y
An01g10580		strong similarity to ribonuclease T2 precursor rntB - Aspergillus oryzae	-0.4	0.2	0.8	0.8	y
An10g00800		strong similarity to purine nucleoside permease NUP - Candida albicans	0.0	0.0	0.0	0.0	y
		related to unknown					
An14g00800		similarity to hypothetical protein CAC38347.1 - Schizosaccharomyces pombe	-0.4	0.4	0.8	0.8	y
An15g07520		similarity to hypothetical protein mlr2143 - Mesorhizobium loti	0.0	0.0	-0.2	0.4	y
An06g00160		strong similarity to hypothetical protein AN1813.2 - Aspergillus nidulans	0.0	0.0	0.0	0.0	y
An16g00670		similarity to hypothetical protein AAO51454.1 - Dictyostelium discoideum	1.3	1.1	0.0	0.0	y
An02g13650		strong similarity to hypothetical protein Afu4g00380 - Aspergillus fumigatus	0.0	0.0	0.0	-1.4	y
		released via non-classical secretion (secP)	0.0	0.0	0.0	0.0	
		released via non-classical secretion (secP)					
An14g01685		strong similarity to hypothetical acetyltransferase Afu4g03600 - Aspergillus fumigatus	1.2	1.2	0.0	0.0	nc
		related to intracellular (no sigP)					
An13g01830		weak similarity to SAK-b serine-threonine kinase from patent CA2150789-A - Mus musculus	-0.2	-0.3	0.0	0.0	n

Supplemental Table 4. Quantitative analysis of the secretome of concentric zones of maltose-grown colonies of the Δ*fluG* strain of *A. niger*. This material is available free of charge via the internet at http://goo.gl/BH1Kd

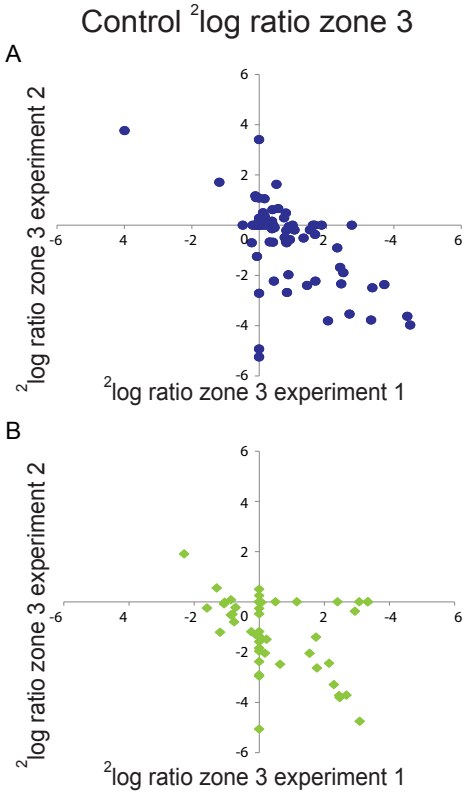
Supplemental Table 5. Quantitative difference between the secretome of Δ*fluG* and wild-type *A. niger* colonies. Proteins released in zones 1, 3 and 5 of Δ*fluG* were compared to zones 1,3 and 5 of the wild-type, respectively. Heat map indicates the ^{2}log fold change of a protein identified in the Δ*fluG* secretome compared to that of wild-type. Protein release was considered differential when the average ^{2}log ratio value was higher than 2 or lower than -2 in one sample and at least higher than 1 or lower than -1 in the other biological replicate. Green and red shading indicate increased and decreased abundance of proteins when compared to zone 3, respectively. * In this case the average ^{2}log value was below 2, or higher than -2, but the value was at least (-)2 or (-)1 in both biological replicates. ** In this case the average ^{2}log value was higher than 2 or lower than -2, but not at least higher than 1, or lower than -1 in both biological replicates.Y: yes; nc: non-classical; n: no.

Annotation	Protein name	Description - Supplemental Table 4	average ^{2}log ratio Δflug/ wt			SigP
		cellulases	zone 1/1	zone 3/3	zone 5/5	
An14g02760	EglA	endoglucanase A eglA - Aspergillus niger	-0.7	-0.4	-0.9	y
An16g06800	EglB	strong similarity to endoglucanase eglB - Aspergillus niger	0.1	0.6	-4.3	y
An07g09330	CbhA	1,4-beta-D-glucan cellobiohydrolase A precursor cbhA - Aspergillus niger	0.0	0.0	0.0	y
An01g03340		Probable xyloglucan-specific endo-beta-1,4-glucanase A precursor xgeA - Aspergillus niger	0.0	0.3	0.0	y
An14g02670		strong similarity to endoglucanase IV egl4 - Trichoderma reesei	0.0	0.0	0.0	y
An03g04190		similarity to cellulase #2 from patent US2003036176-A1 - Xanthomonas campestris	0.0	0.0	0.0	y
		xylanolytic enzymes				
An01g00780	XlnB	endo-1,4-xylanase xlnB - Aspergillus niger	-2.4	0.0	-2.4	y
An01g14600		strong similarity to endo-1,4-beta-xylanase B xynB from patent WO9414965 - Aspergillus tubingensis	-3.0	-2.9	0.0	y
An12g05010	AxeA	acetyl xylan esterase axeA - Aspergillus niger	0.0	0.0	0.0	y
An03g00960	AxhA	1,4-beta-D-arabinoxylan arabinofuranohydrolase axhA - Aspergillus niger	0.0	0.0	0.0	y
An15g02300	AbfB	arabinofuranosidase B abfB - Aspergillus niger	2.2	3.6	0.0	**y**
An02g11150	AglB	alpha-galactosidase aglB - Aspergillus niger	0.0	0.0	-4.2	y
An04g09700		strong similarity to endo-xylogalacturonan hydrolase xghA - Aspergillus tubingensis	0.0	-4.6	0.0	y
		amylolitic enzymes				
An03g06550	GlaA	glucan 1,4-alpha-glucosidase glaA - Aspergillus niger	0.9	0.5	-0.4	y
An11g03340	AamA	acid alpha-amylase aamA - Aspergillus niger	-0.4	0.1	0.9	y
An05g02100		extracellular alpha-amylase amyA/amyB - Aspergillus niger	0.0	0.0	0.0	y
An04g06920	AgdA	extracellular alpha-glucosidase agldA- Aspergillus niger	0.0	0.0	0.0	y
		inulinolytic enzymes				
An12g08280	InuE	exo-inulinase inu1 - Aspergillus niger	0.0	2.0*	1.0	**y**
		pectinolytic enzymes				
An16g06990	PgaA	endo-polygalacturonase A pgaA - Aspergillus niger [putative frameshift]	0.0	-4.3	-1.1	y
An01g14670	PgaE	polygalacturonase E precursor pgaE - Aspergillus niger	0.0	3.0	0.0	**y**
An14g04200	RhgB	rhamnogalacturonase rhgB - Aspergillus niger	0.0	0.0	0.0	y
An14g04370	PelA	pectin lyase pelA - Aspergillus niger	0.0	0.0	-0.8	y
An02g10550	abnC	endo-alpha-1,5-arabinanase abnC - Aspergillus niger	-0.7	-1.3	-0.6	y
An03g06310	PmeA	pectinesterase pmeA - Aspergillus niger	0.0	-4.3	-2.0*	y
		mannosidase				
An01g12550	MsdS	strong similarity to mannosyl-oligosaccharide 1,2-alpha-mannosidase msdS - Aspergillus saitoi	0.0	0.0	0.0	y
		(putative) cell wall synthesis/ morphogenesis enzyme				

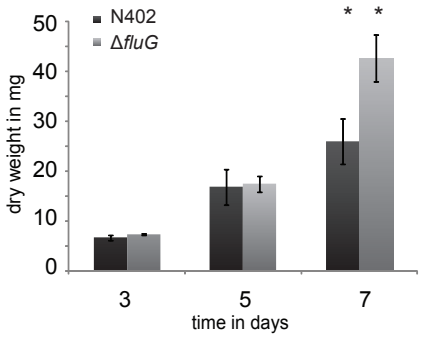
Annotation	Protein name	Description - Supplemental Table 4 *(continued)*	average ^{2}log ratio Δflug/wt			SigP
			zone 1/1	zone 3/3	zone 5/5	
An03g05290	BgtB	glucan endo-1,3-beta-glucosidase eglC precursor - Aspergillus niger	-2.1	-1.5	-0.5	y
An01g12450	ExsG	strong similarity to hypothetical glucan beta-1,3 exoglucanase exgS - Aspergillus phoenicis	-2.0*	-1.2	-1.0	y
An01g11010	CrhD	strong similarity to cell wall protein Crh1 - Saccharomyces cerevisiae	0.7	0.6	0.6	y
An04g01230	EcmA	strong similarity to hypothetical ECM33 homolog SPCC1223.12c - Schizosaccharomyces pombe	0.5	0.3	0.1	y
An03g05530		strong similarity to endo-beta-1,4-glucanase EGIII-like from patent WO9931255-A2 - Emericella desertoru	0.8	0.3	0.6	y
An06g00160		strong similarity to hypothetical protein AN1813.2 - Aspergillus nidulans	0.0	-2.3	0.0	y
An07g07530	CrhB	strong similarity to cell wall protein Utr2 - Saccharomyces cerevisiae	0.0	0.0	0.0	y
An14g01820	PhiA	strong similarity to hypothetical cell wall protein binB - Aspergillus nidulans	-3.5	-5.6	0.0	y
		phospholypase				
An01g14940		similarity to nonhemolytic phospholipase C PC-PLC - Burkholderia pseudomallei	0.0	-4.8	0.0	y
		Superoxide dismutases				
An01g01820	CatR	catalase R catR - Aspergillus niger	0.0	0.0	0.6	y
		(putative) proteases				
An03g05200	ProtF	strong similarity to carboxypeptidase S1 - Penicillium janthinellum	-3.3	-2.9	-2.0*	y
An02g13750		strong similarity to glutaminase A gtaA - Aspergillus oryzae	-2.6	-2.1	-1.4	y
An15g06280	PepAa	strong similarity to aspartic proteinase aspergillopepsin I pepA - Aspergillus niger [truncated ORF]	0.0	0.0	0.0	y
An14g04710	PepA	aspartic proteinase aspergillopepsin I pepA - Aspergillus niger	0.0	0.0	-5.0	y
An07g08030	PepF	serine carboxypeptidase pepF - Aspergillus niger	0.0	-2.0	0.0	y
An06g00190		strong similarity to lysosomal pepstatin insensitive protease CLN2 - Homo sapiens	0.0	0.0	-0.8	y
An01g01750		similarity to lysosomal protease CLN2 - Rattus norvegicus	0.0	-1.0	0.0	y
An04g01440		strong similarity to precursor of pepsin A3 - Homo sapiens	0.0	0.0	0.0	y
An02g01550		strong similarity to secreted serine protease 19 kDa CS antigen CS-Ag - Coccidioides immitis	-0.1	-0.2	0.3	y
An02g04690		strong similarity to serine-type carboxypeptidase I cdpS - Aspergillus saitoi	-1.1	-3.0	0.0	y
An08g04490	ProtA	Endoprotease Endo-Pro- precursor Epr - Aspergillus niger	-1.3	-1.6	-0.9	y
An08g04640	ProtB	strong similarity to hypothetical lysosomal pepstatin insensitive protease CLN2 - Canis lupus	-2.2	-1.2	-0.3	y
An18g01320		strong similarity to extracellular protease precursor Bar1 - Saccharomyces cerevisiae	0.0	-0.9	-1.4	y
An01g00370	PepAb	strong similarity to aspergillopepsin apnS - Aspergillus phoenicis	0.0	0.0	0.0	n
An14g02150		strong similarity to serine-type carboxypeptidase precursor cpdS - Aspergillus phoenicis	0.0	-3.7	0.0	y
An08g05230		strong similarity to hypothetical endoglucanase IV - Trichoderma reesei	0.0	0.0	0.0	y
		related to other				
An07g01320	Anafp	strong similarity to antifungal protein precursor paf - Penicillium chrysogenum	0.0	-2.3	0.0	y
An12g03300	ProtG	strong similarity to aspartic protease pr1 - Phaffia rhodozyma	0.0	0.5	-0.3	y
An08g10780		strong similarity to hypothetical protein T16K5.230 - Arabidopsis thaliana	0.0	-2.5	0.0	y
An09g00670	GelD	strong similarity to glycosylphosphatidylinositol-anchored beta(1-3)glucanosyltransferase gel3 - Aspergillus fumigatus	1.4	1.4	0.0	y
An10g00800		strong similarity to purine nucleoside permease NUP - Candida albicans	0.0	0.0	-2.4	y
An11g00040		weak similarity to cDNA for 59-kDa readthrough protein RT - Sorghum chlorotic spot virus	-0.4	-1.3	-1.2	y
An14g00800		similarity to hypothetical protein CAC38347.1 - Schizosaccharomyces pombe	0.0	0.1	0.0	y

Annotation	Protein name	Description - Supplemental Table 4 *(continued)*	average ^{2}log ratio Δflug/wt			SigP
		related to unknown proteins	zone 1/1	zone 3/3	zone 5/5	
An02g13650		strong similarity to hypothetical protein Afu4g00380 - Aspergillus fumigatus	0.0	0.0	-4.8	y
An08g04630		strong similarity to hypothetical protein Afu2g15420 - Aspergillus fumigatus	0.0	0.0	0.0	y
An01g10580		strong similarity to ribonuclease T2 precursor rntB - Aspergillus oryzae	-1.1	-1.6	-1.2	y
An16g00670		similarity to hypothetical protein AAO51454.1 - Dictyostelium discoideum	0.1	0.0	0.0	y
An15g02250		hypothetical protein	0.0	0.0	0.0	y
		released via non-classical secretion (secP)				
An13g01830		weak similarity to SAK-b serine-threonine kinase from patent CA2150789-A - Mus musculus	0.0	1.4	0.0	nc
		related to intracellular proteins (no sigP)				
An14g01685		strong similarity to hypothetical acetyltransferase Afu4g03600 - Aspergillus fumigatus	**0.6**	**0.0**	**0.0**	n

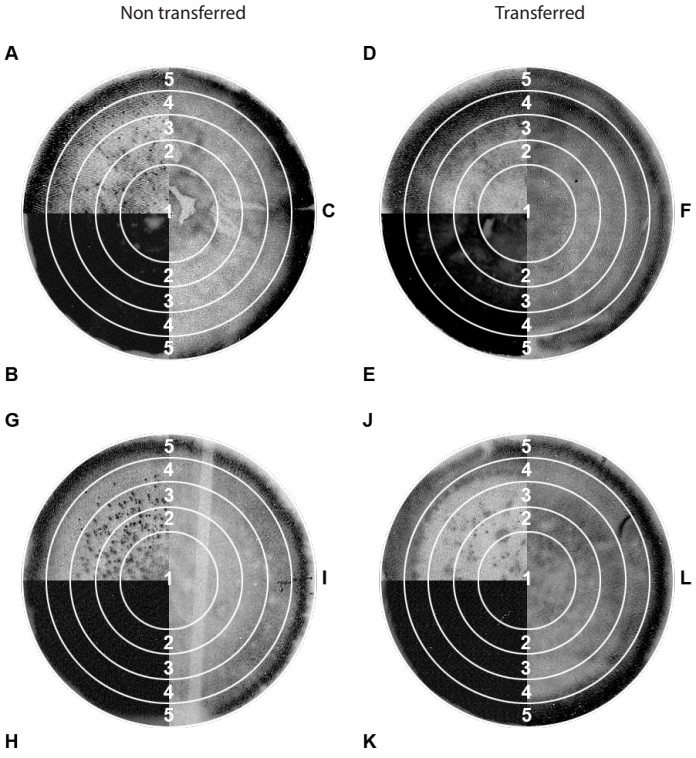
Supplemental Table 6. Quantitative analysis of the secretome of concentric zones of maltose-grown colonies of the Δ*fluG* strain of *A. niger* compared to the wild-type. This material is available free of charge via the internet at http://goo.gl/BH1Kd



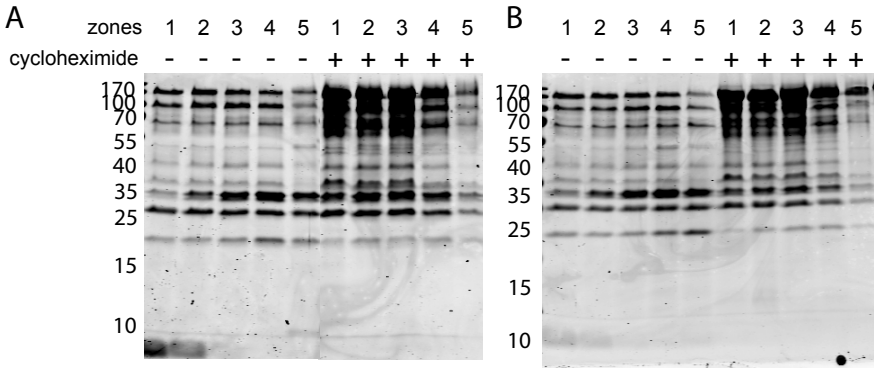
Supplemental Figure 1: 2log ratios of the technical duplicate of individual proteins of zone 3 of wild-type (A) and Δ*fluG* (B) as was obtained by labeling the peptides with two different dimethyl labels. The data of the biological duplicates are shown.



Supplemental Figure 2. Dry weight of 7-days-old xylose-grown colonies of wild-type and Δ*fluG*. *indicates significant difference in biomass (*t*-test p<0.05).



Supplemental Figure 3: Growth, (ADGJ), protein synthesis (BEHK), and secretion (CFIL) in 7-days-old xylose-grown wild-type (A-F) and Δ*fluG* (G-L) colonies. Colonies were analysed before (ABC, GHI) and after transfer (DEF, JKL) to fresh xylose medium. Growth was detected with 14C-labeled N-acetylglucosamine. Protein synthesis and secretion were monitored by incorporation of ^{14}C-labeled amino acids. Secreted proteins were immobilized by a PVDF membrane that had been placed underneath the colony.



Supplemental Figure 4: SDS-PAGE of the secretome of concentric zones of 7-days-old xylose-grown colonies of wild-type (A) and Δ*fluG* (B) that had either (+) or not (-) been treated with cycloheximide. Gels were stained with Coommassie Brilliant Blue. Zone 1 represents the central zone, whereas the peripheral zone is represented by zone 5.

Chapter 6

Summary and General Discussion

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Chapter 6

Summary and General Discussion

INTRODUCTION

Aspergillus species are among the most abundant fungi in the world. The *Aspergillus* genus comprises up to 837 species[1-3] that contribute to element recycling in nature by degrading dead organic material. They particularly degrade complex plant material.[4] As such, aspergilli can cause spoilage of food and feed. Some *Aspergillus* species are pathogenic for plants, animals and humans, and can affect the quality of the indoor environment. Yet, they are also beneficial for mankind by functioning as cell factories. The capacity of aspergilli to secrete proteins is enormous. For instance, some strains of *Aspergillus niger* secrete more than 30 grams per liter glucoamylase.[5] The fact that aspergilli secrete a large variety of enzymes that degrade for instance plant material makes these fungi also of interest as cell factories. Many of these enzymes can be used in the food and feed industry, in the production of bioethanol, or can be used as pharmaceutical proteins.

An *Aspergillus* colony consists of a network of interconnected hyphae, also known as mycelium.[6] Colonies can grow as centimeter-scale macro-colonies on agar medium, or (sub-) millimeter-scale micro-colonies during submerged growth in liquid medium. The center of these colonies represents the oldest part of the mycelium, whereas the youngest part of the mycelium is found at the colony periphery. Hyphae at the outer part of the colony are exposed to unexplored substrate, whereas hyphae in intermediate and central zones are confronted with a (partly) utilized medium. As a consequence, zones of colonies of *A. oryzae*[7] and *A. niger*[8-10] are heterogeneous with respect to gene-expression. Notably, the differences in medium composition only explain 50% of the zonal differences in RNA composition.[9] The other half of the variation is caused by differentiation processes in the vegetative mycelium. Zones of colonies are also heterogeneous with respect to growth and secretion.[9, 11] For instance, colonies that have been grown for 5-7 days on an agar medium with maltose or xylose as a carbon source only grow and secrete at the outer part of the colony.

Aspergilli initiate sexual or asexual development after a period of vegetative growth. The sexual cycle has been identified in a number of aspergilli (i.e. in *A. nidulans,*[12] *A. flavus*,[13] *A. parasiticus*,[14] *A. fumigatus,*[15]and *A. nomius*[16]). Genes underlying sexual development have been identified in *A. niger*,[17] but until now only asexual development has been observed. The morphological process of asexual development in *A. niger* resembles that of *A. nidulans* (Chapter 1).[6] The first step of asexual development is the formation of a stalk. This aerial hypha develops from a foot cell in the vegetative mycelium. A vesicle is formed at the tip of the stalk after it has reached a length of about 100 μm. Metulae are formed at the surface of the vesicle by budding and they in turn give rise to phialides. The phialides finally bud into chains of (mainly) uninucleate conidia (Chapter 1).[6] Genes underlying the asexual developmental pathway have been well studied in *A. nidulans*.[6] Gene *fluG* is assumed to act at the start of the sporulation pathway.[18]

It has been proposed to be involved in the production of an extracellular factor that binds to an unknown receptor, thus activating the sporulation program.[18] Formation of asexual reproductive structures is abolished in the Δ*fluG* strain of *A. nidulans*.[19] FluG of *A. nidulans* releases the repression of the SfgA protein on the *flbA-E* genes.[20, 21] The phenotype of either of the Δ*flb* colonies of *A. nidulans* is similar to that of Δ*fluG*. They only form vegetative and aerial hyphae.[19] Gene *flbA* encodes a Regulator of G-protein signaling. It hydrolyses the intrinsic activity of the active GTP-bound Gα-subunit FadA into its inactive GDP-bound conformation. The inactive Gα-submit is part of a heterotrimeric complex together with the Gβ- and Gγ- subunits that are encoded by *sfaD* and *gpgA*, respectively.[22-25] When GDP is exchanged for GTP on the Gα-subunit, it dissociates from the β- and γ-subunit. Both the activated Gα-subunit and the Gβγ-dimer promote vegetative growth and inhibit asexual development. Like FlbB-E, FlbA stimulates expression of the central regulatory gene of asexual development *brlA*.[19, 22] A Δ*brlA* colony of *A. nidulans* forms vegetative and aerial hyphae and also initiates stalk formation. However, these stalks do not stop their growth when they reach a length of 100 μm and do not form vesicles.[26] This gives the colony a *bristle* phenotype.

The aim of this Thesis was to study spatial secretion patterns in macro-colonies of the cell factory *A. niger* and to identify genes that are involved in zonal secretion. It is shown that FlbA and FluG but not BrlA impact zonal secretion. The role of FlbA on spatial protein secretion is explained, at least in part, by its impact on gene expression and the thickness of the cell wall.

ASEXUAL DEVELOPMENT IN *A. NIGER*

Asexual development in *A. oryzae*[27] is identical to that in *A. nidulans*[19, 28] with respect to the functions of *flbA, brlA,* and *fluG.* In contrast, the functions of these genes of *A. fumigatus* differ. Like *A. nidulans, flbA* of *A. fumigatus* activates asexual development via induction of *brlA* but the Δ*flbA* strain of *A. fumigatus* does not show hyphal disintegration.[29] Moreover, the Δ*fluG* strain of *A. fumigatus* is not affected in conidiophore formation[29], while its *brlA* gene functions earlier in the developmental program as compared to *A. nidulans*. The latter was concluded from the fact that both conidiophore and stalk formation were abolished in the *ΔbrlA* mutant.[29] In my Thesis, I studied the role of *fluG* (Chapter 5), *flbA* (Chapter 3 and 4) *and brlA* (Chapter 3) in asexual development of *A. niger.* Inactivation of *brlA* of *A. niger* resulted in a bristle phenotype like that in *A. nidulans*[19, 28] and *A. oryzae*.[27] The Δ*brlA* colonies of *A. niger* formed stalks that did not stop their growth. They reached a length of up to 1000 μm, which is 20-30 times longer than that of wild-type stalks. Inactivation of *flbA* of *A. niger* also showed a phenotype similar to that observed in *A. nidulans*[19, 28] and *A. oryzae*.[27] The Δ*flbA* strain of *A. niger* neither formed conidiophores nor stalks. Instead, masses of aerial hyphae were observed. Inactivation of *flbA* did not have a major impact on the expression of genes involved in asexual development (Chapter 4). 13 out of 68 genes implicated

in reproduction were differentially expressed when compared to the wild-type. Genes *flbD* and *sfaD* were among the 6 genes that were up-regulated ≥ 2-fold in the Δ*flbA* strain. In contrast, expression of these genes was not increased in the Δ*flbA* strains of *A. nidulans* and *A. oryzae*.[27, 30] Transcripts of *brlA* were absent in wild type *A. niger* colonies, while some *brlA* expression was observed in the center of Δ*flbA* colonies. This indicates that FlbA is a repressor of *brlA* expression in part of the colony. So far, FlbA has only been reported as a (in)direct stimulator of *brlA* expression in *A. nidulans*,[19] *A. oryzae*,[27] and *A. fumigatus*.[29]

Inactivation of *fluG* in *A. niger* resulted in fully developed conidiophores. Notably, sterigmata were also formed at the stalks (Chapter 5). I propose that FluG of *A. niger* is responsible for proper spatial and or temporal expression of component(s) of the sporulation pathway, thereby ensuring proper development of the conidiophore.It cannot be excluded that formation of sterigmata on stalks also takes place in the Δ*fluG* strain of *A. fumigatus.* This phenoptype is only observed by scanning electron microscopy, a method that was not used to study the deletion strain of *A. fumigatus*. Taken together, it can be concluded that the sporulation pathway of *A. niger* is similar but not identical to that of *A. nidulans, A. oryzae,* and *A. fumigatus.*

Sporulation-inhibited secretion in *A. niger*

Until recently it was believed that only growing hyphae secrete proteins. This was based on the finding that both protein secretion and growth are mainly observed at the periphery of sandwiched colonies and that proteins are released at tips of growing hyphae only.[11] Levin et al.[9] and I (Chapter 2 and 5), however, showed that protein secretion can also occur in non-growing zones of a sandwiched colony. Growth was still localized at the periphery when 7-days-old xylose- or maltose-grown colonies were transferred for 24 hours to fresh medium, but proteins were now also secreted in the center (Chapter 2 and 5). Only a sub-peripheral zone (zone 4) still did not release newly synthesized proteins in the medium, as was shown by labeling with radioactive amino acids. In the next set of experiments the composition of the secretome of 5 concentric zones of transferred xylose-grown colonies (zone 1 representing the inner center and zone 5 the outer periphery) was determined by quantitative mass spectrometry (Chapter 2). It had been shown that a large part of secreted proteins is associated with or trapped in the cell wall of *A. niger*.[10] I used the protein synthesis inhibitor cycloheximide to distinguish between proteins that had been synthesized and secreted into the culture medium immediately after transfer to fresh medium and proteins that had been formed before transfer and that are slowly released in the medium. Thus, newly secreted proteins in the secretome were expected to be identified by subtracting the secretome of cyclohemixide treated colonies from non-treated cultures. As expected, less proteins were released at the periphery of cycloheximide-treated colonies. However, the treated colonies released more secreted proteins in

intermediate and central zones when compared to untreated colonies. How can this phenomenon be explained? Cycloheximide treatment resulted in thinner cell walls in these inner zones but not at the colony periphery. Fluffy material surrounding the hyphae in these zones, as observed by transmission electron microscopy, indicated that the cell wall had been partially degraded during cycloheximide treatment. The degradation of the cell wall would release proteins that are trapped in the cell wall or that interacted with cell wall polymers in a faster way than would occur in non-treated colonies.

As mentioned above, a major part of the proteins are associated with the cell walls of *A. niger.* This is also reflected by the fact that 124 and 59 proteins were detected in the culture medium of wild-type colonies that had or had not been treated with cycloheximide (Chapter 2). In addition, 70 and 65 proteins were ≥ 4-fold more abundant in the central zone 1 and the intermediate zone 3, respectively, in cycloheximide treated colonies when compared to untreated colonies. From these data it was concluded that cycloheximide can be used to obtain a near complete qualitative and quantitative secretome of *A. niger.* The fact that 19 proteins have been detected in this study that had not been identified in the secretome of *A. niger* before[31-34] is of interest from a biotechnological point of view. The protein mixture resulting from cycloheximide treatment may show improved degradation of plant material, which may be of interest for the bioethanol industry.

The non-growing sub-peripheral zone 4 still did not secrete proteins when colonies were transferred to fresh medium (Chapter 2). This zone started to sporulate when the upper membrane of the sandwiched colony was removed (Chapter 3). This indicated that sporulation represses secretion in the vegetative mycelium. To test this hypothesis, zonal secretion of the *ΔfluG, ΔflbA, and ΔbrlA* strain was localized (Chapter 3, 4, and 5). Zonal growth and secretion were not affected in the *ΔbrlA* strain (Chapter 3). From this it was concluded that sporulation inhibited secretion acts upstream of BrlA. Indeed, growth and secretion patterns had changed in the case of the *ΔfluG* and *ΔflbA* colonies (Chapter 3 and 5). Growth in 7-days-old maltose- and xylose-grown colonies of the Δ*fluG* strain occurred at the periphery, as was shown in the wild-type. Transfer of 6-days-old Δ*fluG* colonies to fresh xylose medium for 24 h did not change the growth pattern. This was also observed for the wild-type that had been grown on xylose or maltose.[9] In contrast, growth mainly occurred in the center of transferred maltose-grown Δ*fluG* colonies. Growth in the center of the colony was also observed after transfer of xylose- and maltose-grown (unpublished data) Δ*flbA* colonies (Chapter 3). In fact, in this case growth was shown to occur throughout the colony. The impact of FluG on growth was also illustrated by the fact that Δ*fluG* colonies produced 20-60 % more biomass than the wild-type after 7-days of growth (Chapter 5). In contrast, the biomass of Δ*flbA* colonies was similar or even less than the wild-type (Chapter 3). This may be explained by accelerated autolysis of the mycelium

(see below). Taken together, these data indicate that both FlbA and FluG act as repressors of vegetative growth, at least in intermediate (FlbA) and central (FlbA, FluG) zones of the colony. Only 10 and 2 genes out of 102 genes predicted to be involved in cell wall synthesis[17] were up- and down-regulated in 7-days-old non-transferred colonies of the Δ*flbA* strain, respectively (Chapter 4). These genes may be involved in the changed spatial distribution of growing hyphae in the colony. The expression of the *chsF* chitin synthase gene is most interesting. This gene is periphery specific in wild-type colonies. Expression levels are similar in the central zones of the Δ*flbA* strain when compared to the periphery of the wild-type strain, and are even higher at the periphery of the Δ*flbA* strain.

Proteins were most abundantly secreted at the periphery of non-transferred maltose and xylose grown Δ*fluG* and wild-type colonies (Chapter 5), while both the periphery and the colony center were active in secretion in non-transferred Δ*flbA* colonies (Chapter 3). Notably, the colony center of transferred xylose-grown wild-type colonies showed a similar secretion pattern as non-transferred Δ*flbA* colonies, while secretion occurred throughout the mycelium when Δ*flbA* colonies had been transferred to fresh xylose-containing medium. Transferred maltose-grown Δ*fluG* and wild-type colonies also secreted more protein in the colony center but protein secretion was still most abundant at the colony periphery (Chapter 5). 71 and 44 proteins with a signal sequence for secretion were identified in the secretome of maltose-grown wild-type and Δ*fluG* colonies, respectively. Moreover, 1 protein in each case was identified with a motif for non-classical secretion. 39 out of the 45 proteins of the Δ*fluG* secretome were also found in the secretome of the wild-type (Chapter 5). This shows that the secretome of Δ*fluG* is less complex than that of the wild-type and far less complex than that of Δ*flbA*. In the latter case even 138 proteins with a signal sequence for secretion were identified, as well as 15 proteins with a motif for non-classical secretion (Chapter 3). Quantitative MS/MS analysis showed that 22 proteins were more abundantly released in the wild-type colony when compared to the Δ*fluG* strain, while only 3 proteins were secreted to a lower extent (Chapter 5). These data imply that FluG has a positive impact on protein secretion into the culture medium. In contrast, FlbA has a negative effect on protein secretion as judged from the secretome complexity and the spatial distribution of protein secretion of the Δ*flbA* strain (Chapter 3).

Quantitative mass spectrometry analysis showed that zonal secretion heterogeneity was less pronounced in the Δ*flbA* colonies when compared to the wild-type colonies (Chapter 3). For instance, only 8 and 4 out of the 138 proteins were differentially released in the central zone 1 and the sub-peripheral zone 4 compared to the intermediate zone 3, respectively (Chapter 3). These numbers were 8 and 16 out of the 59 secreted proteins in the case of the wild-type (Chapter 2). Reduced secretion heterogeneity in the Δ*flbA* colonies coincided with a more complex secretome when compared to the wild-type. As mentioned above, the

secretome of Δ*flbA* colonies consisted of 138 proteins with a signal sequence and 15 proteins with a motif for non-classical secretion. Of these, 18 and 12 had not been reported in any secretome study[31-33, 35, 36] (Chapter 3). Moreover, 101 and 70 proteins had not been identified in the secretome of 7-days-old xylose-grown wild-type colonies that had not or had been treated with cycloheximide (Chapter 2, 3). 23 out of the 70 proteins were differentially expressed in 7-days-old non-transferred xylose-grown colonies, of which 22 were up-regulated in the Δ*flbA* strain (Chapter 4). Thus, part of the differences in the secretome between the wild-type strain and the Δ*flbA* strain is caused by transcriptional control. Another part of the differences may be explained by induction of genes after transfer to fresh medium and by (post)-translational regulation. Moreover, part of the secretome of the wild-type may still be trapped in or associated with the cell wall after cycloheximide treatment. This may be different in the Δ*flbA* strain. This is based on the finding that the width of the cell wall of hyphae at the periphery of colonies of the Δ*flbA* strain was 85 % of that of peripheral hyphae of the wild-type, while it was only 58 % in the colony center. Moreover, unlike the wild-type (Chapter 2) cycloheximide treatment did not affect the width of the cell wall in the centre of Δ*flbA* colonies (Chapter 3). A role of FlbA in cell wall thickness may be linked to its activity as a repressor of vegetative growth. Indeed, the G-protein complex stimulating vegetative growth has a role in cell wall synthesis in *A. nidulans*.[37] Inactivation of the Gβ subunit *sfaD* resulted in increased chitin content of the cell wall. As a consequence, porosity of the cell wall was reduced. It is tempting to speculate that overexpression of *sfaD* would result in reduced chitin levels, improved porosity and thinner cell walls. This would be in line with the finding that *sfaD* expression was 2-fold up-regulated in the Δ*flbA* strain of *A. niger* (Chapter 4). The role of FlbA in cell wall synthesis may be mediated via the differential expression of 12 genes involved in this process (Chapter 4). These genes encode proteins with synthesizing activity, cell wall processing activity (i.e. crosslinking), as well as degradation activity (*An04g04670*), all of them may function in architecture and thickness of the cell wall.

55 proteins were identified in the wild-type secretome that were absent in the culture medium of the Δ*flbA* strain (Chapter 3). 35 out of the 55 genes encoding these proteins were differentially expressed in non-transferred xylose-grown colonies, of which 33 were down-regulated in the Δ*flbA* strain (Chapter 4). From this it is concluded that transcriptional control has a major impact on genes encoding proteins that are released by the wild-type strain but not by the Δ*flbA* strain.

Hyphae of the Δ*flbA* strain of *A. nidulans*,[19, 27, 28, 38] but not those of *A. fumigatus*[29] go in autolysis when colonies get older. So far, it has not been established whether autolysis takes place in sandwiched cultures of the *A. niger* Δ*flbA* strain. The secretome of the *A. niger* Δ*flbA* strain only contained 15 proteins without a signal sequence or a motif for non-classical secretion. Moreover, a semi-

tryptic Mascot search assigned only twelve extra proteins (i.e. 8.5 %) in the culture medium. This indicates that proteinases are not highly active in the culture medium of the Δ*flbA* strain. The strongest indication for autolysis in the *A. niger* Δ*flbA* strain is the presence of chitinase *chiB* (*An02g07020*) in the culture medium. This enzyme has been related to autolysis of the Δ*flbA* mycelium of *A. nidulans*.[39] It would be of interest to assess whether inactivation of *chiB* also reduces release of intracellular proteins in the Δ*flbA* strain of *A. niger*, as was shown for *A. nidulans*.

CONCLUDING REMARKS

In this Thesis I have shown that asexual development in *A. niger* is not identical to that of *A. nidulans.* FluG of *A. niger* is involved in proper development of conidiophores, whereas this protein initiates sporulation in *A. nidulans.* FluG also represses growth and has a positive impact on secretion heterogeneity and the complexity of the secretome. The latter may be due to a local repressing effect of FluG on components of the sporulation pathway that also results in proper development of the conidiophores. Such a mechanism would be in line with the

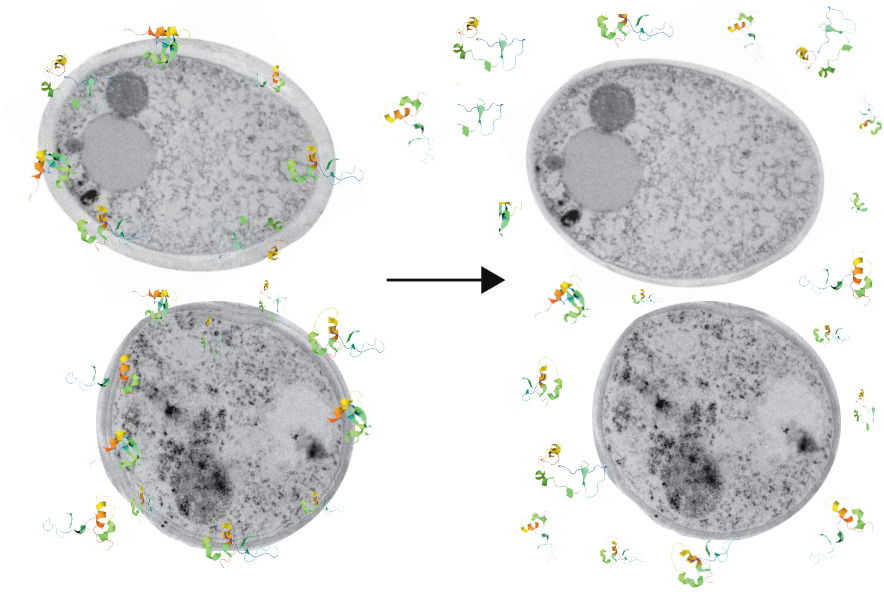

Figure 1. FlbA has an effect on expression of genes encoding transcriptional regulators and genes involved in cell wall synthesis. Modulation of expression of such genes may result in thinner cell walls. Secreted proteins will be more easily trapped in a thicker cell wall or interact with the cell wall components (left) when compared to a thinner cell wall (right). Therefore, strains with a thinner cell wall are of interest for the industry that uses fungi as a cell factory.

phenomenon of sporulation inhibited secretion. Like FluG, FlbA increases zonal secretion heterogeneity and represses growth. However, FlbA has an opposite role when compared to FluG by reducing the complexity of the secretome and

stimulating sporulation. It was also shown that FlbA is involved in cell wall thickness, thereby reducing release of proteins into the culture medium. FlbA may impact zonal heterogeneity in growth and secretion, secretome complexity and cell wall thickness via different transcription factors (Chapter 4). 38 transcriptional regulators were found to be differentially regulated in the Δ*flbA* strain. Most of these regulators have not been characterized. This should be subject of future studies because modulation of expression of one or more of these transcription factors may improve *A. niger* as a cell factory. For instance, strains can be obtained with a thinner cell wall that allows more efficient release of proteins in the medium (Figure 1).

Part of the genes involved in reproduction are differentially expressed in colonies of *A. niger.* Genes *ppoA* and *ganB* were up-regulated in the colony center (Chapter 4). Moreover, *brlA* and *dewA* are repressed by FlbA in the colony centre. So far, pathways involved in asexual development have been based on gene expression analysis of whole colonies on *A. nidulans, A. oryzae* and *A. fumigatus*. The average expression levels within the colony are probably not representative for zones that have or do not have the potential to sporulate. Thus, the model of asexual development is more complex. This is illustrated by the fact that so far FlbA is only considered to be a (in)direct activator of *brlA* (see for instance Figure 1 of Chapter 4), while I have shown that it represses this gene in the colony center. Such spatial and temporal effects should also be considered for genes involved in growth, secretion and cell wall architecture.

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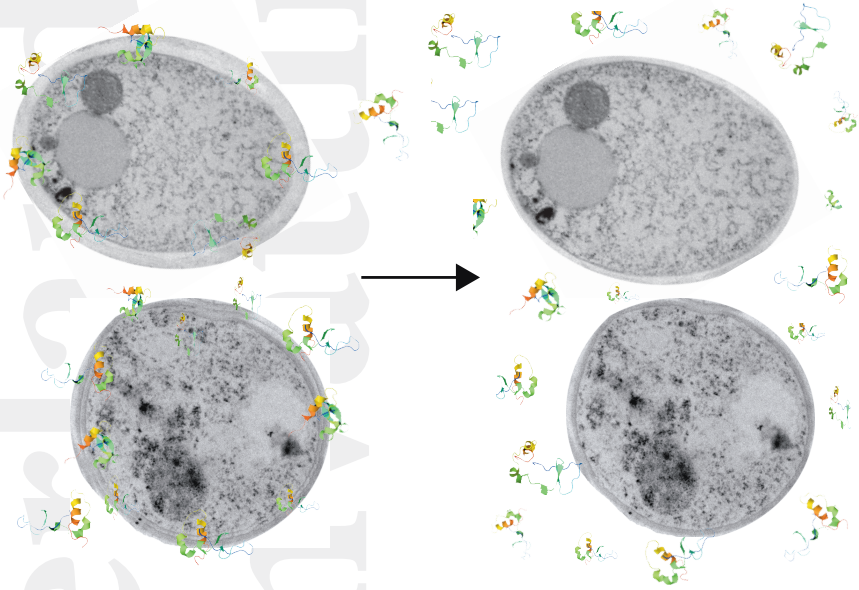
Appendix

Nederlandse Samenvatting



Bacteriën en schimmels hebben zich aan vrijwel elke denkbare situatie aangepast, met een ontelbaar aantal micro-organismen als gevolg. Alleen het schimmel-geslacht *Aspergillus* kent al 837 soorten. Aspergilli zijn filamenteuze schimmels die vooral op dood plantenmateriaal leven. Dit type schimmel groeit door middel van hyfen (draden) die aan hun top groeien en die meer naar achteren toe vertakken, hierdoor ontstaat een netwerk van duizenden hyfen die samen het mycelium of de kolonie van de schimmel vormen. Met uitgescheiden enzymen breken *Aspergillus*-soorten plantenmateriaal af tot kleine moleculen die ze opnemen als voedingsstoffen. Zo scheiden zij bijvoorbeeld amylases uit die zetmeel omzetten in glucose. Met uitgescheiden xylanases en pectinases breekt de schimmel respectievelijk xylan en pectinase af. Aspergilli kunnen grote hoeveelheden enzymen uitscheiden. Verschillende aspergilli, zoals *Aspergillus niger* en *Aspergillus oryzae,* zijn dankzij deze eigenschap uitstekende productiesystemen voor het produceren van eiwitten die gebruikt worden als medicijn of in de industrie. Zo kunnen bepaalde stammen van *A. niger* tot wel 30 gram per liter glucoamylase uitscheiden, wat men gebruikt voor de grootschalige productie van glucosestroop uit zetmeel.

Wild-type kolonies van *A. niger*, gevormd op agarmedium, groeien en scheiden enzymen uit aan de buitenkant van het mycelium. De hyfen in deze zone worden geconfronteerd met onaangetast organisch materiaal, terwijl hyfen in het midden en het centrum van de kolonie juist in contact staan met (deels) verbruikt substraat. De compositie van het substraat verklaart 50% van de variatie in genexpressie in de kolonie. Differentiatie (ontwikkelings-) processen in de kolonie veroorzaken de andere helft van de variatie. Vanaf het moment dat A. niger zich aseksueel voort kan planten, vormt de schimmel zogenaamde conidioforenDe vorming van conidioforen begint met het maken van een 'stalk', een soort stam die ontstaat uit een verdikte hyfe, ook wel de voetcel genoemd. De stalk is groter en dikker dan gewone luchthyfen en stopt met groeien wanneer het ~100-1000 µm lang is. Aan het uiteinde van de stalk vormt zich een vesikel, waarop zich metulae vormden door knopvorming. Uit deze metulae ontstaan phialides, die door deling een ketting van conidiosporen vormen. Dit differentiatieproces is goed bestudeerd in *A. nidulans*. Veel genen zijn betrokken bij deze sporulatieroute. Het gen *fluG* is waarschijnlijk het eerste gen dat betrokken is bij de initiatie van aseksuele differentiatie. Hoogstwaarschijnlijk is dit gen verantwoordelijk voor de aanmaak van een extracellulair signaalmolecuul dat betrokken is bij de start van conidiofoor-vorming. Deze moleculen zouden de remming weghalen van SfgA op verschillende *flb*-genen die het *brlA*-gen activeren. Dit laatstgenoemde gen codeert voor de centrale transcriptiefactor van sporulatie. Na het uitschakelen van het *brlA*-gen in *A. nidulans* vormt zich een kolonie met stalks die ongeremd door blijven groeien en zich niet verder ontwikkelen tot conidioforen. Met uitgeschakelde *fluG*- of *flb*-genen worden pluizige kolonies gevormd met een lage *brlA* expressie. Deze stammen vormen wel luchthyfen, terwijl stalkontwikkeling

afwezig blijft. Het gen *flbA* is een regulator van het actieve GTP-gebonden Gα-subunit en stimuleert de omzetting naar het inactieve en GDP-gebonden α-subunit. De stimulatie van vegetatieve groei die door het actieve Gα-subunit FadA wordt bewerkstelligd, vervalt hierdoor.

Groei vindt nog steeds plaats aan de buitenkant van de kolonie wanneer een 7-dagen-oude *A. niger* culture overgeplaatst wordt naar vers medium. De secretie vindt dan echter niet alleen plaats in de buitenste (nieuwste) zone 5 van de kolonie, maar ook in de binnenste (oudste) zones 1, 2 en 3. De zone (4) doet niet mee in het uitscheiden van eiwitten. Wij lieten zien dat deze zone sporuleert wanneer we het bovenste membraan weghalen (Hoofdstuk 3). Daaruit ontstond de vraag of sporulatie de secretie remt. Verschillende genen, betrokken bij de sporulatie-route, werden uitgeschakeld en geanalyseerd op onder andere hun secretiecapaciteit. Deletie van het *fluG*-gen in *A. niger* resulteert in een kolonie die nog steeds aseksueel ontwikkelt. Alleen ontwikkelen sterigmata (metulae en phialides) zich ook op de stalk (Hoofdstuk 5). Dit fenotype lijkt anders te zijn dan het niet-sporulerende Δ*fluG* fenotype dat gevonden is in *A. nidulans*. Echter, het lijkt wel op het Δ*fluG* fenotype van A. fumigatus, dat ook nog steeds in staat is om conidioforen te maken. De biomassa van de Δ*fluG*-kolonies was 20-60 % hoger dan dat van wild-type *A. niger* kolonies. Groei van de Δ*fluG*-kolonies op xylose vindt net als in wild-type kolonies voornamelijk plaats aan de periferie van de kolonie, onafhankelijk van het feit of het medium ververst is. Echter, wanneer we de kolonie op vers maltosemedium plaatsen, zien we de groei voornamelijk in het centrum plaatsvinden, terwijl vóór het verplaatsen de groei aan de periferie plaats vond. Dit impliceert dat *fluG* de groei normaalgesproken remt.

De 56 eiwitten, uitgescheiden door het wild-type dat is gegroeid op xylose (Hoofdstuk 2), verschillen per zone in de kolonie. Zo waren er op xylose acht eiwitten die tenminste vier keer minder uitgescheiden werden in het centrum vergeleken met de middelste zone van de kolonie. Zestien andere eiwitten werden meer dan vier keer meer (6) of minder (10) uitgescheiden door de periferie in vergelijking met de middelste zone (Hoofdstuk 2). Dit verschijnsel namen we ook waar wanneer het wild-type groeide met maltose als koolstofbron (hoofdstuk 5). In dit geval werden er 72 eiwitten gevonden, waarvan negen en elf eiwitten in verschillende hoeveelheden uitgescheiden werden door respectievelijk de oudste zone (1,2) en jongste zone (4,5) van de kolonies in vergelijking tot de middelste zone (zone 3). De deletie van *fluG* resulteerde in een minder complex secretoom vergeleken met wild-type kolonies (Hoofdstuk 5). Daarbij was er ook minder heterogeniteit tussen de zones in uitgescheiden eiwitten. Dit duidt erop dat het gen *fluG* naast remming van groei, ook betrokken is bij stimulatie van plaatselijke (zonale) secretie van eiwitten.

Inactivatie van de sporulatiegenen *brlA* en *flbA* in *A. niger* resulteerde respectievelijk in kolonies met oneindige groei van stalks of in kolonies die alleen luchthyfen vormden. Dit fenotype lijkt op de waargenomen fenotypes van de

overeenkomende deletiemutanten in *A. nidulans*. De deletie van *brlA* in *A. niger* resulteerde niet in een verschil in groei of secretie in de kolonie. Het lijkt erop dat dit gen te ver in de sporulatie-route zit om nog invloed te hebben op deze processen. Echter, de *flbA*-deletie zorgde wel voor een verandering in het groei- en secretiepatroon in de kolonie. Deze Δ*flbA*-kolonies scheidden eiwitten niet alleen uit aan de buitenkant van de kolonie, maar ook in het centrum (Hoofdstuk 3). Bovendien zorgde de *flbA*-deletie ervoor dat de kolonies, ten opzichte van wild-type kolonies (Hoofdstuk 2), meer eiwitten (138) in het medium uitscheidden (Hoofdstuk 3). Het *flbA*-gen lijkt dus betrokken te zijn bij de remming van secretie. Daarbij hadden de hyfen van Δ*flbA* kolonies een dunnere celwand (Hoofdstuk 3). Hieruit kunnen we concluderen dat *flbA* ook betrokken is bij celwandvorming. De resultaten uit mijn proefschrift duiden erop dat een groot deel van de eiwitten normaal gesproken gevangen worden in de celwand van de schimmel voordat zij langzaam vrijkomen in de omgeving. Een deletie van het *flbA*-gen resulteert in een dunnere celwand, waardoor eiwitten sneller in het medium terecht kunnen komen. Inderdaad bleek dat eiwitten ook sneller in de omgeving terechtkwamen indien we een wild-type kolonie behandelden met cycloheximide. Deze stof breekt de celwand van *A. niger* gedeeltelijk af (Hoofdstuk 2). We hebben dus voor het eerst laten zien dat toevoeging van cycloheximide, een remmer van de eiwitsynthese, voor een hogere afgifte van eiwitten zorgt. Dit is interessant voor de industrie die gebruik maakt van schimmels voor de enzymproductie.

De deletie van *flbA* zorgt ervoor dat 1152 genen hun expressie tweevoudig veranderen ten opzichte van het wild-type (Hoofdstuk 4). Hierbij horen twaalf genen die betrokken zijn bij celwandsynthese, dertien genen die betrokken zijn in aseksuele ontwikkeling, en 345 genen die een signaalsequentie hebben om uitgescheiden te worden. Daarnaast zijn er 38 transcriptiefactorgenen waarvan de expressie tenminste twee keer veranderde. Deze transcriptiefactoren zijn dus mogelijk betrokken bij aseksuele ontwikkeling, bij secretie, en bij groei en celwandvorming. Mogelijk kan door het veranderen van de expressie van één of meerdere van deze genen een *A. niger*-stam worden gemaakt die normaal groeit, een dunnere celwand maakt en zo dus meer eiwit uitscheidt. Ook kan het leiden tot een stam die een grotere variatie aan eiwitten uitscheidt waardoor plantenmateriaal makkelijker afgebroken wordt. Dit is bijvoorbeeld van belang voor de productie van enzymcocktails die men bij bio-ethanolproductie gebruikt.

Curriculum vitae

Curriculum vitae

Pauline Krijgsheld was born on March 30th, 1984 in Voorburg, The Netherlands. She followed her secondary education at the Oosterlicht College Nieuwegein, The Netherlands, and Graduated in 2002 with a Gymnasium-level diploma without classic languages. In September the same year she began her Bachelor Biology at Utrecht University followed by the Master Biomolecular Sciences. She did an internship within the Cellular Architecture and Dynamics group of the Department of Biology at Utrecht University under the supervision of Dr. David-Lutje Hulsik, Dr. Hendrik Adams, and Prof. dr. Theo Verrips. This was followed by a second internship in the Molecular Microbiology group of the Department of Biology at Utrecht University, The Netherlands under the supervision of Dr. Wieke Teertstra and Prof. dr. Han Wösten and in the Terrestrial Microbiology group of the Max Planck Institute in Marburg, Germany under the supervision of Prof. dr. Jörg Kämper. Pauline obtained her diploma in December 2007. In April 2008 she started her PhD within the Molecular Microbiology group of the Department of Biology at Utrecht University under the supervision of Prof. dr. Han Wösten. The research on sporulation inhibited protein secretion in the filamentous fungus *Aspergillus niger* was financially supported by the Kluyver Center for Genomics of Industrial Fermentation, and the Netherlands Proteomic Center, both part of the Netherlands Genomics Initiative. During her PhD track she coordinated the master course Introduction in Fungal Biology of the Environmental Biology Masters (2011, 2012), and a practical microbiology course of the University College Utrecht program (2010, 2012, 2013). In April 2013 she started working as a post-doc in collaboration between Utrecht University and DSM, Delft, The Netherlands. This project is financially supported by a KIT grant from the Kluyver Center for Genomics of Industrial Fermentation.

List of Publications

Krijgsheld P, Wösten HAB (2013) Transcriptome analysis of zones of colonies of the Δ*flbA* strain of *Aspergillus niger*. *Fungal Gen. Biol.* Submitted

Wösten HAB, van Veluw GJ, de Bekker C, Krijgsheld P (2013) Heterogeneity in the mycelium; implications for the use of fungi as cell factories. *Biotechnol. Lett.* 2013 Apr 17. *[Epub ahead of print]*

Krijgsheld P, Nitsche BM, Post H, Levin AM, Müller WH. Ram AFJ, Heck AJR, Altelaar AFM, Wösten HAB (2013) Deletion of *flbA* results in increased secretome complexity and reduced secretion heterogeneity in colonies of *Aspergillus niger*. *J. Proteome Res. Journal of Proteome Research* 12: 1808-1819.

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

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

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

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

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Teertstra WR, Krijgsheld P, Wösten HAB (2011) Absence of repellents in *Ustilago maydis* induces genes encoding small secreted proteins, *Antonie Leeuwenhoek*. **100**: 219–229.

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