Aspergillus hydrophobins - Identification, classification and characterization

Jensen, Britt Guillaume

Publication date:
2012

Document Version
Publisher's PDF, also known as Version of record

Preface

This thesis is submitted to the Technical University of Denmark and describes the results obtained during my PhD study. The work was carried out at the Center for Microbial Biotechnology (CMB) at the Department of Systems Biology from January 1st 2009 to March 1st 2012. The project was financed by the Technical University of Denmark.

First and foremost, I would like to thank my main supervisor Kristian Fog Nielsen for excellent guidance, constructive criticism and interesting discussions. My co-supervisor (previous main supervisor), Ib Søndergaard, is thanked for the initial opportunity to engage in the PhD study and very useful help and guidance. Both are highly appreciated for their endless support both in scientific and private matters. Special thanks go to my co-supervisor and former office mate, Mona Højgaard Pedersen, for endless discussions, guidance and memorable journeys to Paris and California. Thank you for having been my "hydrophobin partner in crime". I would also like to thank my co-supervisors Jens Christian Frisvad, Lars Jelsbak and Susanne Jacobsen for invaluable help, guidance and exiting discussions throughout the years.

As part of my PhD project several molecular biology techniques have been used. A big "merci beaucoup" goes to Jakob Blæsbjerg Nielsen for teaching me the ways and for your everlasting patience in the lab. Also big thanks to Anita and Olivera for being such great office mates. Thanks for the laughs and continuous supply of cookies and liquorice. Furthermore, a big thanks to the rest of CMB for providing such an inspiring environment.

My collaboration with Søren Dohn, Anja Boisen and Thomas Pedersen at Department of Micro- and Nanotechnology is also highly appreciated; Søren for unravelling the world of cantilevers and Thomas for hours in front of the SEM.

Finally, I would like to thank my family and friends for their endless support and encouragement. To my husband Peter, a big thanks for your everlasting patience and interest in my project. Thank you for always being there.

Britt Guillaume Jensen
Kgs. Lyngby, September 2012

Summary

Aspergillus species are a diverse group of fungi found ubiquitously in nature, and include species used in industry as well as human pathogens causing pulmonary infections. For immunocompromised individuals these infections are often fatal, due to lack of adequate treatment. Hydrophobins, a class of small hydrophobic proteins, are uniquely found in filamentous fungi including *Aspergillus* species. Little is known about their roles in *Aspergillus* or their possible involvement in disease. *Aspergillus* species and the bacterium *Pseudomonas aeruginosa* both commonly colonize the lungs of cystic fibrosis (CF) patients, but limited research has dealt with *Aspergillus-P. aeruginosa* interactions. Whether, hydrophobins are involved in the *Aspergillus-P. aeruginosa* interaction is not known. The overall aim of this PhD study was to provide an overview of *Aspergillus* hydrophobins and to achieve a deeper understanding of the roles of hydrophobins in *Aspergillus* species. Furthermore, *Aspergillus-P. aeruginosa* interactions were examined.

In recent years the genomes of the main *Aspergillus* species have become available providing a unique amount of data for research. Using bioinformatics, *Aspergillus* species were found to display a varying number of putative hydrophobins ranging from two in *A. oryzae* to eight in *A. niger*. Over 50 % of the *Aspergillus* hydrophobins could not be classified into the two original classes (I and II) defined by the physical properties and hydropathy patterns of hydrophobins, but displayed intermediate forms. The majority of the identified *Aspergillus* hydrophobins were either class I hydrophobins or intermediate forms. Only a single class II hydrophobin was found in *A. terreus*.

To characterize hydrophobins in *Aspergillus* species, a number of different *A. nidulans* hydrophobin deletion strains (*rodA*Δ, *dewA*Δ, AN0940Δ, AN1837Δ, AN6401Δ) were constructed. Deletion of the hydrophobins *rodA* or *dewA* resulted in reduced expression of other hydrophobins. Interestingly, deletion of both hydrophobins *rodA* and *dewA* resulted in a synthetic genetic interaction and an increased expression of the hydrophobin AN7539. The deletion strains were additionally used to test a number of previously proposed biological functions of hydrophobins. Lack of single hydrophobins had no effect on colony surface hydrophobicity or the ability of the strains to breach from an aqueous environment. Phenotypes were only apparent in *rodA*Δ strains, known to be involved in rodlet formation, and the *dewA*Δ strain, thus the role of the other hydrophobins in *A. nidulans* proved hard to

determine. If the hydrophobins AN0940, AN1837 and AN6401 are located on the conidia surface, they do not play dominant roles. These hydrophobins may only be found on the mycelium surface or secreted to the surrounding environment

An examination of the interactions between different Aspergilli and *P. aeruginosa* revealed that all tested Aspergilli were suppressed by *P. aeruginosa*. An increase in production of phenazines, a class of anti-fungal compounds produced by *P. aeruginosa*, was observed in the contact zone of the two organisms. However, *A. fumigatus* differed from the other Aspergilli by not stimulating production of phenazines. Using *P. aeruginosa* mutants, factors involved in the rpoN pathway were found to be involved in the interaction. Furthermore, common late stage CF mutations in *P. aeruginosa* seemed to alter the interaction pattern rendering the bacterium more susceptible to *A. fumigatus.* Hydrophobins did not seem to play a role in the interaction as no differences could be observed between a control strain and hydrophobin deletion strains.

Dansk resumé

Slægten *Aspergillus* er en broget gruppe af svampe, som findes overalt i naturen, og som inkluderer både arter benyttet i industrien, og sygdomsfremkaldende arter impliceret i specielt humane lungeinfektioner. Disse svampeinfektioner kan, på grund af manglende behandlingsmuligheder, være fatale for immunkompromitterede individer.
Hydrofobiner er en gruppe af små hydrofobe proteiner, som kun eksisterer i filamentøse svampe som *Aspergillus*. Der eksisterer kun begrænset viden omkring hydrofobinernes rolle i *Aspergillus* arter og deres potentielle involvering i sygdomsmekanismer. Både *Aspergillus* arter og bakterien *Pseudomonas aeruginosa* koloniserer ofte lungerne af cystisk fibrose (CF) patienter, men der er på nuværende tidspunkt begrænset viden omkring interaktionen mellem *Aspergillus* og *P. aeruginosa*. Hvorvidt, hydrofobiner spiller en rolle i *Aspergillus-P. aeruginosa* interaktioner er endnu ukendt.
Formålet med dette PhD projekt var at identificere *Aspergillus* hydrophobiner og få en dybere forståelse for hydrofobiners rolle i *Aspergillus* arter. Desuden blev *Aspergillus-P. aeruginosa* interaktioner undersøgt.

I de seneste år er de vigtigste *Aspergillus* genomer blevet tilgængelige, hvilket har resulteret i en guldmine af data. Ved brug af bioinformatik, blev et vekslende antal af hydrofobiner i *Aspergillus* arterne fundet, varierende fra to i *A. oryzae* til otte i *A. niger*. Hydrofobiner er oprindeligt blevet opdelt i to klasser (I og II) på baggrund af deres fysiske egenskaber og hydropati-plot, men over 50 % af de fundne *Aspergillus* hydrofobiner kunne ikke inddeles i de to klasser. Disse hydrofobiner synes at være intermediære former. Af de fundne *Aspergillus* hydrofobiner, var størstedelen enten klasse I eller intermediære hydrofobiner. Kun et enkelt hydrofobin i *A. terreus* blev fundet til at være et klasse II hydrofobin.

Forskellige *A. nidulans* hydrofobin deletions stammer (*rodA*Δ, *dewA*Δ, AN0940Δ, AN1837Δ, AN6401Δ) blev konstrueret for at karakterisere hydrofobiner i *Aspergillus* og benyttet til at undersøge tidligere foreslåede biologiske funktioner af hydrofobiner. Mangel på hydrofobinet *rodA* eller *dewA* resulterede i lavere gen-ekspression af andre hydrofobiner. Dog resulterede manglen på begge hydrofobiner i en syntetisk genetisk interaktion, hvorved en stigning af ekspressionen af hydrofobinet AN7539 blev observeret. Mangel på et enkelt hydrofobin havde imidlertid ingen effekt på overflade hydrofobicitet eller stammernes evne til at bryde igennem en vandoverflade. En visuel fænotype kunne kun observeres i *rodA*Δ stammer, hvor

rodA er kendt for at være involveret i dannelsen af "rodlets", og i *dewA*Δ stammen, hvorfor rollen af de resterende hydrofobiner i *A. nidulans* var svær at fastlægge. Hvis AN0940, AN1837 og AN6401 hydrofobinerne findes på spore overfladen, har disse hydrofobiner ingen dominerende rolle. Disse hydrofobiner findes muligvis kun på overfladen af mycelium eller bliver udskilt til omgivelserne.

En undersøgelse af interaktionen mellem forskellige *Aspergillus* arter og *P. aeruginosa* viste, at *P. aeruginosa* hæmmede alle undersøgte *Aspergillus* svampe. *P. aeruginosa* øgede sin produktion af de antifungale stoffer kaldet phenaziner i kontaktzonen mellem de to organismer. I modsætning til andre *Aspergillus* svampe, stimulerede *A. fumigatus* dog ikke produktionen af phenaziner. Ved at benytte forskellige *P. aeruginosa* mutanter, blev faktorer, involveret i den regulatoriske mekanisme af rpoN, fundet til at være involveret i interaktionen. Desuden synes almindelig forkomne sen-stadie CF mutationer i *P. aeruginosa* at ændre interaktionsmønstret, således at *P. aeruginosa* ikke kunne hæmme *A. fumigatus*. Der kunne ikke observeres nogen forskel i interaktionen mellem *P. aeruginosa* og henholdsvis *A. nidulans* kontrol stammer og hydrofobin deletions stammer, hvorved hydrofobiner ikke synes at spille en rolle i interaktionen.

List of original papers and other publications

Paper 1: **B.G. Jensen**, M.R. Andersen, M.H. Pedersen, J.C. Frisvad and I. Søndergaard, Hydrophobins from *Aspergillus* species cannot be clearly divided into two classes, *BMC Research Notes* (2010), **3:** 344

Paper 2: **B.G. Jensen**, L. Jelsbak, T. Pedersen, S. Damkiær, M.H. Pedersen, I. Søndergaard, J.C. Frisvad and K.F. Nielsen, Interactions between *Aspergillus fumigatus* and *Pseudomonas aeruginosa* are mediated by soluble chemical mediators involved in the RpoN pathway, manuscript in preparation for resubmitted to PLoS ONE after a favourable review

Conference proceedings:

B.G. Jensen, L. Jelsbak, I. Søndergaard, M.H. Pedersen, J.C. Frisvad and K.F. Nielsen, *Aspergillus* triggers phenazine production in *Pseudomonas aeruginosa*, Poster at 2nd European Congress on Microbial Biofilms, 6-8 july 2011, Copenhagen, Denmark

B.G. Jensen, J.B. Nielsen, M.H. Pedersen, I. Søndergaard, J.C. Frisvad and K.F. Nielsen, Characterization of *Emericella nidulans* RodA and DewA hydrophobin mutants, Poster at 26th Fungal Genetics Conference, 15–20 March 2011, Asilomar Conference Center at Pacific Grove, California, USA

B.G. Jensen, J.B. Nielsen, M.H. Pedersen, I. Søndergaard, J.C. Frisvad and K.F. Nielsen, Characterization of *Emericella nidulans* RodA and DewA hydrophobin mutants, Poster at 8th International Aspergillus Meeting, 14-15 March 2011, Asilomar Conference Center at Pacific Grove, California, USA

B.G. Jensen, L. Jelsbak, I. Søndergaard, J.C. Frisvad and K.F. Nielsen, Suppression of *Aspergillus* by *Pseudomonas aeruginosa*, Oral presentation at Biofilms in Nosocomial Fungal Infections, 31 January – 1 February 2011, Paris, France

Abbreviations

5MPCA	5-methyl-phenazinium-1-carboxylate
CF	Cystic fibrosis
CMB	Center for Microbial Biotechnology
CYA	Czapek Yeast extract Agar
DTU	Technical University of Denmark
EDTA	Ethylenediaminetetraacetic acid
FDA	U.S. Food and Drug Administration
GRAS	Generally Regarded As Safe
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
HSL	Homoserine lactone
LC	Liquid Chromatography
MIC	Minimal Inhibitory Concentration
MM	Minimal media
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
NK cells	Natural Killer cells
NMR	Nuclear Magnetic Resonance
PMS	Phenazine methosulphate
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscopy
TFA	Trifluoroactic acid
TLC	Thin Layer Chromatography
TOF	Time-of-flight
UHPLC	Ultra-High Performance Liquid Chromatography
UV	Ultraviolet

WATM Wickerhams Antibiotic Test Medium

YES Yeast Extract Sucrose agar

Thesis outline

Aspergillus species are found ubiquitously in nature including soils, decaying vegetation and dust[1,2] and many *Aspergillus* species have the ability to cause pulmonary diseases in humans ranging from benign allergies in healthy individuals to more severe and often fatal infections in immunocompromised individuals[3]. The majority of infections are caused by *Aspergillus fumigatus* being responsible for approximately 90 % of all *Aspergillus* infections[3,4]. Unfortunately, only few antifungal drugs are effective against *Aspergillus* and even these display low efficiency[4]. Due to increasing numbers of *Aspergillus* infections, high fatality rates and lack of sufficient treatment, *Aspergillus* infections have become a devastating opportunistic infection causing a significant burden on society[5].

This thesis consists of two parts. The main and first part of the thesis deals with hydrophobins, a family of small proteins found uniquely in filamentous fungi. These proteins are important for growth and survival of the fungi, but may also play a role in disease. Only few hydrophobins have been studied in *Aspergillus* species, and therefore knowledge of the roles of hydrophobins in Aspergilli is limited. The second part of the thesis examines a possible interaction between *Aspergillus* species and *Pseudomonas aeruginosa*. Both organisms are found in the lungs of cystic fibrosis patients, but little is known about their interactions. No knowledge is available of the potential role of hydrophobins in *Aspergillus-P. aeruginosa* interactions.

The overall aim of this PhD study was to achieve a deeper understanding of the roles of hydrophobins in *Aspergillus* species. The objectives were to generate an overview of putative hydrophobins from full-genome sequenced Aspergilli (Chapter 3) and create and characterize hydrophobin mutants from the model organism *Aspergillus nidulans* (Chapter 4). Furthermore an aim was to investigate the interactions between *Aspergillus* species and *P. aeruginosa* using chemical analysis of secondary metabolites (Chapter 5) and by developing a cantilever lab-on-a-chip system (Chapter 6).

To achieve this the thesis is divided into seven chapters, each with special focus on Aspergilli. The first chapter introduces hydrophobins and their biological roles. Furthermore, the chapter introduces fungal-bacterial interactions with special focus on *Aspergillus-P. aeruginosa* interactions. Chapter 2 outlines the experimental setup for the thesis. Chapters 3-6 describe

the results obtained during the PhD study, where chapters 3 and 4 focus on hydrophobins. Chapter 3 deals with the identification and classification of hydrophobins from *Aspergillus* species using a bioinformatics approach. Chapter 4 describes a current study involving the production and characterization of hydrophobin mutants from *A. nidulans*. The next two chapters focus on interactions between *Aspergillus* species and *P. aeruginosa*, where chapter 5 deals with the interactions between *P. aeruginosa* and different *Aspergillus* species with focus on *A. fumigatus* and secondary metabolite production. The involvement of hydrophobins is also examined. Chapter 6 describes results regarding the development of a cantilever lab-on-a-chip system to study interactions between Aspergilli and *P. aeruginosa*. Finally, chapter 7 is the overall discussion and conclusion.

References

1. Raper, KB, Fennell, DI, and Austwick, PKC (1965) The genus *Aspergillus*. Huntington: Robert E. Krieger Publishing Company. 686 p.

2. Dyer PS, O'Gorman CM (2012) Sexual development and cryptic sexuality in fungi: insights from *Aspergillus* species. *FEMS Microbiol Rev* **36:** 165-192.

3. Latge JP (1999) *Aspergillus fumigatus* and aspergillosis. *Clin Microbiol Rev* **12:** 310-350.

4. Denning DW (1998) Invasive aspergillosis. *Clin Infect Dis* **26:** 781-803.

5. Lin S-J, Schranz J, Teutsch SM (2001) Aspergillosis case-fatality rate: systematic review of the literature. *Clin Infect Dis* **32:** 358-366.

Table of contents

1 Introduction

Aspergillus – a filamentous fungus

Fungi are a diverse group of eukaryotes, where some species are filamentous moulds such as Ascomycetes, Basidiomycetes and Zygomycetes, while others are unicellular fungi like yeasts[1,2]. The focus of this thesis is the Ascomycete genus *Aspergillus* found ubiquitously in nature including soils, decaying vegetation and dust[3,4] (figure 1). Pier Antonio Micheli (1729) was the first to use the name *Aspergillus* after examining a mould microscopically and finding the structure of its conidiophores to resemble the aspergillum (a device used in Catholic churches to sprinkle holy water)[3]. Today more than 250 species are classified as *Aspergillus* and include beneficial species used in industry, but also species able to contaminate agricultural crops and building materials as well as human pathogens[3–5].

Figure 1: *Aspergillus* species can display a variety of different colony colours. From left: *A. oryzae*, *A. flavus*, *A. terreus*, *A. niger* and *A. nidulans.*

Many *Aspergillus* species have been full genome sequenced in the last 10 years, including *A. fumigatus, A. flavus, A. oryzae, A. niger, A. terreus, A. clavatus* and *A. nidulans*[6–10]. In this thesis the focus has been on the full genome sequenced Aspergilli and a short description of the life cycle and the different full genome sequenced Aspergilli follows below.

As a genetic model organism for other Aspergilli, *Aspergillus nidulans* is frequently used. In contrast to many Aspergilli, *A. nidulans* has a well-characterized sexual cycle, and is therefore also named by its perfect state; *Emericella nidulans*[10]. Recently, the sexual cycle of among others *Aspergillus fumigatus* (teleomorph: *Neosartorya fumigata*) and *Aspergillus flavus* (teleomorph: *Petromyces flavus*) has also been found[11–13]. Using *A. nidulans* as a model organism, the life cycle of Aspergilli can shortly be described (figure 2).

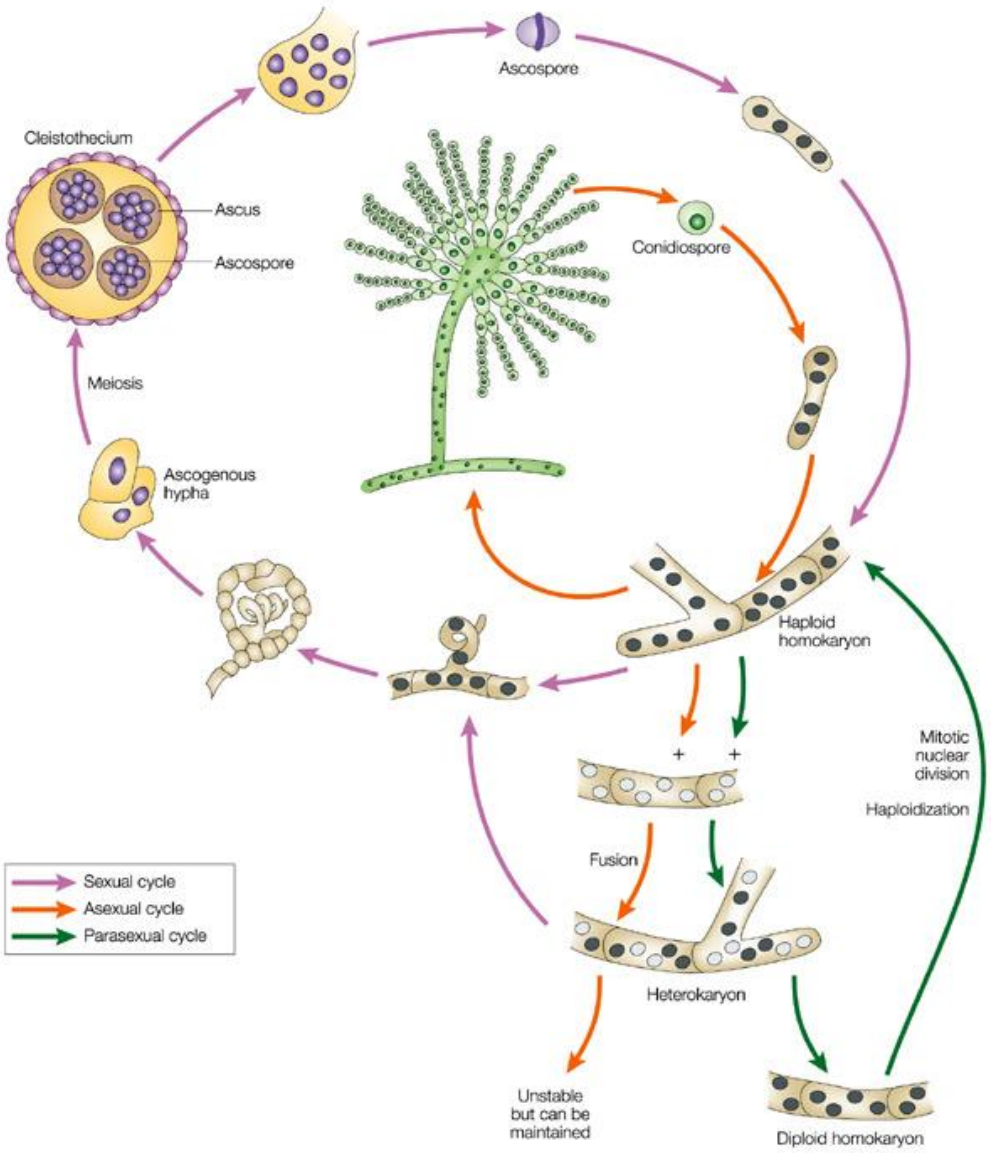


Figure 2: The life cycle of *A. nidulans*. The sexual cycle is connected with pink arrows, the asexual cycle with orange arrows and the parasexual cycle with green arrow. See text for explanation. Figure from Casselton *et al.* 2002[14].

The asexual cycle is initiated by the swelling and germination of asexual spores called conidia resulting in the formation of germ tubes. Elongation of the germ tubes leads to the formation of hyphae, which branch and form a large network of hyphae called the mycelium. Some hyphae may differentiate into conidiophores consisting of the conidiophore stalk carrying metulae, phialides and mature chains of conidia. After vegetative growth, some Aspergilli (like *A. nidulans*) can enter the sexual cycle and produce multicellular fruiting bodies called cleistothecia[1,2,14,15]. These are often surrounded by Hülle cells, which are believed to "nurse" the cleistothecia during development[4]. Upon maturation these fruiting bodies contain thousands of sexual spores termed ascospores. These ascospores are gathered in asci with

eight ascospores in each. The ascospores, conidia and pieces of mycelium may initiate a new fungal life cycle. *A. nidulans* is homothallic (it is self-fertile) and therefore has a parasexual cycle, which may be initiated by the fusion of hyphae containing genetically different nuclei and thereby form a heterokaryon. The two nuclei can then fuse within the hyphae to create a diploid homokaryon, which through haploidization and mitosis can result in a haploid homokaryon and a new fungal life cycle can be initiated[1,2,14,15].

The most prevalent airborne fungal pathogen is *Aspergillus fumigatus* causing severe and often fatal infections in immunocompromised individuals. Due to its abundant sporulation and readily airborne conidia, several hundreds of conidia are inhaled by humans each day[16]. Healthy individuals readily clear inhaled conidia, while immunocompromised individuals may acquire severe pulmonary infections[16]. Although *A. fumigatus* is responsible for app. 90% of human infections, other Aspergilli can also cause human infections including *A. flavus*, *A. niger*, *A. nidulans* and *A. terreus*[16,17].

Aspergillus flavus is the second leading cause of aspergillosis in humans, but may likewise cause diseases in humans and animals due to the consumption of contaminated food and agricultural crops spoiled by the fungus[18]. Furthermore, it produces aflatoxins the most toxic and carcinogenic biological compounds known[19,20].

Aspergillus oryzae has for many years been suspected of being a domesticated form of *A. flavus*[21]. *A. oryzae* is widely used in the Japanese fermentation industry incl. rice wine, vinegar and soy sauce production[22], and also in the production of enzymes for baking and brewing[23]. For these processes it is listed as "Generally Regarded As Safe" (GRAS) by the U.S. Food and Drug Administration (FDA), which is further supported by The World Health Organisation[22].

Aspergillus niger is likewise widely used in industry and many enzymes from *A. niger* are listed as GRAS by the FDA[24]. *A. niger* is further used as a cell factory for the production of gluconic acid and citric acid, where citric acid is used as an acidulant in, among others, the food and beverage industries[24,25]. *A. niger* can, however, produce the mycotoxins fumonisins and ochratoxins, which may be problematic on contaminated food and feedstuff, but not under industrial conditions[26,27].

Aspergillus clavatus is rarely pathogenic, but may cause allergies known as malt worker´s lung[8,28,29]. It spoils agricultural crops and has caused disease in cattle[29].

Finally, *Aspergillus terreus* is used for the production of lovastatin (an anti-cholesterolemic drug)[30] and itaconic acid[31,32].

As previously stated, many *Aspergillus* species have been full genome sequenced in recent years[6–10] providing large amounts of data for research. This data can among others be used to examine the *Aspergillus* genomes for different gene families, where one such family consists of small cysteine-rich hydrophobic proteins called hydrophobins (see below, chapter 3 and chapter 4). These proteins are the focus of this thesis.

Hydrophobins

Hydrophobins are small proteins first described in the beginning of the 1990´s by Wessels and co-workers[33,34]. The name "hydrophobins" was inspired by the hydrophobic structures found in walls of many prokaryotic and eukaryotic microorganisms[33]. Shuren *et al.*[34] had sequenced two genes, *SC3* and *SC4*, from *Schizophyllum commune* and found that these genes, together with the *SC1* gene, had similar nucleotide sequences and hydrophobicity patterns, when translated into amino acid sequences. All three hydrophobins were excreted by *S. commune* in culture medium and found in the fungal walls of aerial hyphae (SC3) and fruit body hyphae (SC1 and SC4)[33,35]. Concurrently, Stringer *et al.*[36] had discovered a gene (*rodA*) in *A. nidulans* with similarities to the *S. commune* hydrophobin genes. This gene likewise encoded a protein situated in the fungal cell wall. Following the discovery of hydrophobins, other proteins were reclassified by comparing their amino acid sequence with the available hydrophobins, leading to the identification of the hydrophobin cerato-ulmin (CU) from *Ophiostoma ulmi.*[37] Within a few years, de Vries *et al.*[38] showed that hydrophobins were found in several other filamentous fungi.

With the availability of several fungal genomes the number of hydrophobin sequences has significantly increased and it seems that hydrophobins can be found in all filamentous fungi[39]. Nevertheless, the majority of knowledge concerning hydrophobins has been based on few isolated hydrophobins from selected species (e.g. SC3), resulting in several general assumptions being made regarding the function and properties of hydrophobins across phylogenetically distinct species. Whether these assumptions are valid in *Aspergillus* species can only be assessed after isolation and characterization of the different hydrophobins.

Below hydrophobins will be described in general followed by a description of current knowledge regarding *Aspergillus* hydrophobins.

Hydrophobins in general

Hydrophobins are small hydrophobic proteins, approximately 100-150 amino acids in size[40]. These proteins appear unique to the fungal kingdom and have been found in Ascomycetes, Basidiomycetes and Zygomycetes[38,41,42]. They have been detected on fungal spores, aerial hyphae and the surface of fruiting bodies[41]. Many fungi have several genes encoding hydrophobins, thus different hydrophobins are probably expressed at different times during the life cycle of the fungi, under different environmental conditions and may serve individual functions[39]. As an example the localization of six hydrophobins from *Cladosporium fulvum* can be mentioned. Hydrophobins HCf-1, HCf-2, HCf-3 and HCf-4 were found on conidia and aerial hyphae. HCf-4 also localized on submerged hyphae, HCf-5 only appeared on early aerial hyphae and HCf-6 was secreted[43,44]. As HCf-6, many hydrophobins contain a signal sequence and thereby have the ability to be secreted into the surroundings, but may also be retained in the fungal structures[40,42,45]. Unfortunately, no method is currently available to predict whether a hydrophobin is secreted or not[45]. It is only possible to predict potential signal sequences (see chapter 3).

Based on hydropathy plots and solubility characteristics, hydrophobins were originally divided into two classes; class I and class II (figure 3+4)[46].

Figure 3: Hydrophobins display very low sequence similarity, but all display eight cysteines (highlighted in yellow) in a characteristic pattern forming four disulphide bonds. The disulphide bonds are shown by connecting lines between the cysteines. Only sequence from the first to the eighth cysteine is displayed due to high variation in the rest of the sequence. Class I hydrophobins shown are SC4 (*Schizophyllum commune*), PRI2 (*Agrocybe aegerite*), SC3 (*Schizophyllum commune*), ABH1 (*Agaricus bisporus*), EAS (*Neurospora crassa*), HCF1 (*Cladosporium fulvum*), MPG1 (*Magnaporthe grisea*) and RODA (*Aspergillus fumigatus*). Class II hydrophobins are HFBI (*Trichoderma reesei*), HFBII (*Trichoderma reesei*), SRH1 (*Trichoderma harzianum*), CU (*Ophiostoma ulmi*), CRP (*Cryphonectria parasitica*), MGP (*Magnaporthe grisea*), HCF6 (*Cladosporium fulvum*) and HYD4 (*Giberella moniliformis*). Figure from Sunde *et al*. 2008[47].

Class I hydrophobins have been found in Ascomycetes and Basidiomycetes, while class II hydrophobins only occur in Ascomycetes[39,42]. The overall sequence conservation between hydrophobins is tremendously low[40], but all hydrophobins originally classified contain eight cysteine residues in a characteristic pattern including two cysteine pairs (figure 3)[40]. This definition is however currently being broadened (see discussion). Nevertheless, the conservation of the cysteine pattern suggests that the cysteine residues are important for function and structure of hydrophobins[39,48]. In class I hydrophobins, the cysteine pairs are followed by hydrophilic amino acids, while the cysteine pairs are followed by hydrophobic amino acids in class II hydrophobins (figure 4)[40].

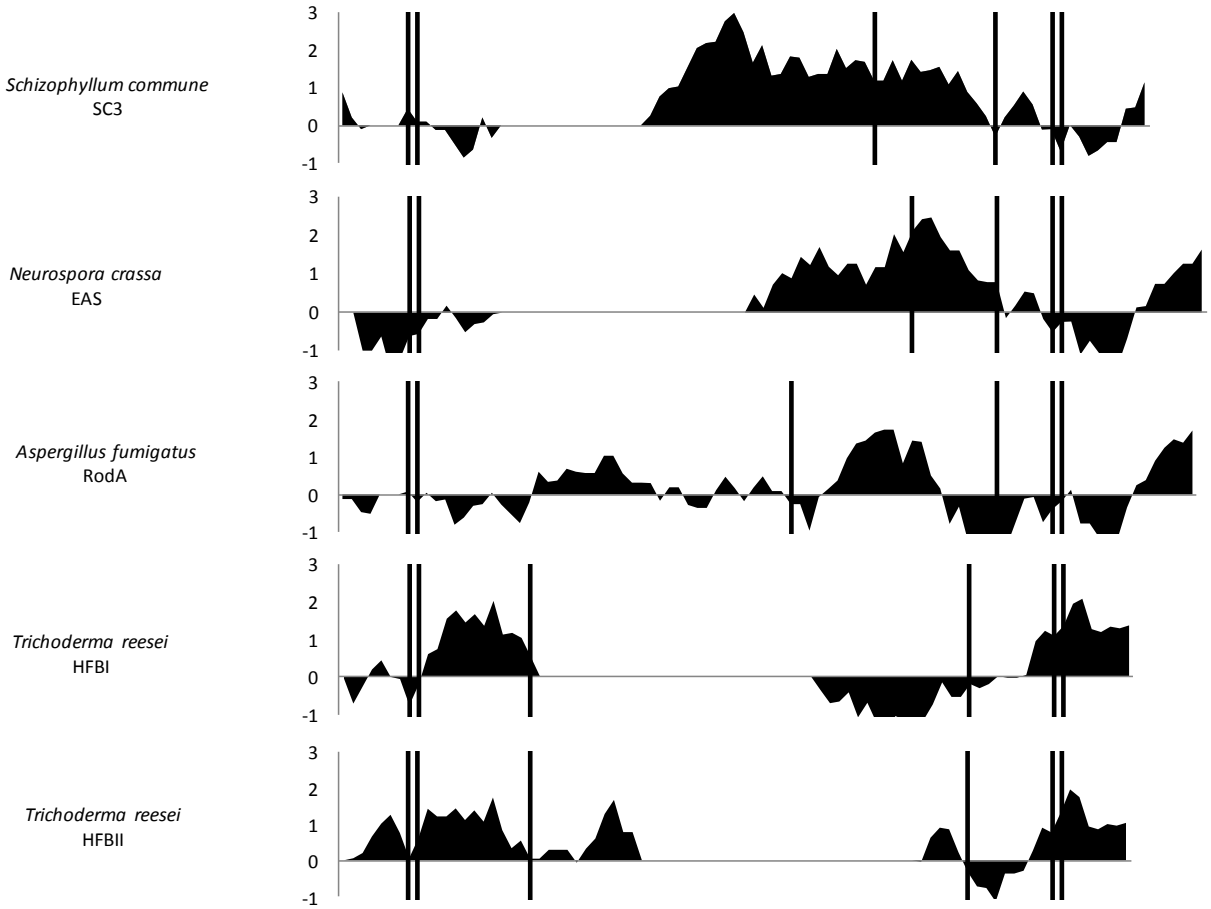


Figure 4: Hydrophobins have traditionally been divided into two classes based on their hydropathy plots and solubility characteristics. Class I hydrophobins (SC3, EAS and RodA) display hydrophilic amino acids after the cysteine pairs, while hydrophobic amino acids are found in class II hydrophobins (HFBI and HFBII). Hydrophobic amino acids are shown above the x-axis, while hydrophilic amino acids are shown below. The amino acids of the hydrophobins are shown along the x-axis, where cysteines are indicated by vertical lines. Only sequence from the first to the eighth cysteine is displayed in the hydropathy plot.

The cysteine spacing likewise varies between the two classes, where class I hydrophobins have a larger distribution of sequence lengths and higher sequence variation than class II

proteins[39]. All eight cysteines are involved in disulphide bridges[38] and form four disulphide bridges[49]. Based on the structure of class II hydrophobin HFBII from *Trichoderma reesei*[49] and class I hydrophobin EAS from *Neurospora crassa*,[50] the disulphide bonds have been found to be between Cys1-Cys6, Cys2-Cys5, Cys3-Cys4 and Cys7-Cys8 (figure 3)[50].

Few structures are available for hydrophobins and have so far included the crystallographic studies of the two class II hydrophobins HFBI and HFBII from *T. reesei* and a NMR study of the class I hydrophobin EAS from *N. crassa* (figure 5).

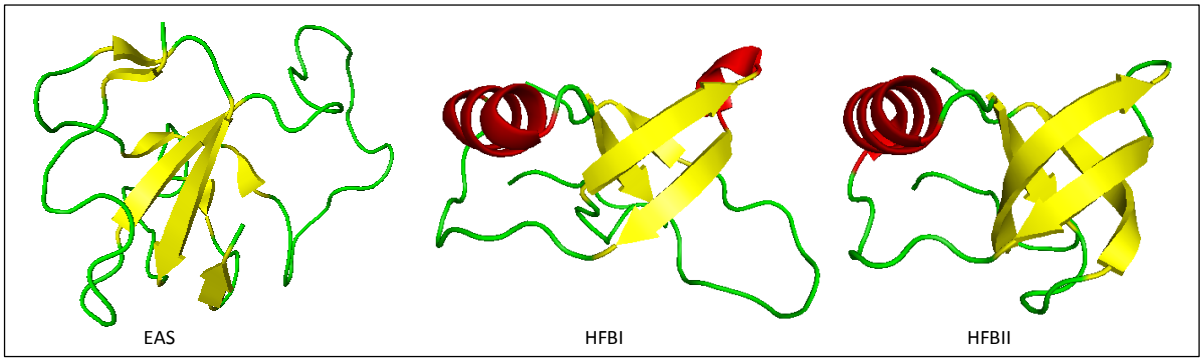


Figure 5: Three hydrophobin structures have currently been solved and deposited in the Brookhaven Protein Data Bank (www.pdb.org). All three hydrophobins contain a β-barrel of four antiparallel β-sheets. The class I hydrophobin EAS (pdb code: 2FMC) from *N. crassa* has an additional β-sheet, while class II hydrophobins HFBI (pdb code: 2FZ6) and HFBII (pdb code: 2B97) from *T. reesei* contain an α-helix. PDB files visualized using PyMOL. Loops are shown in green, sheets in yellow and helices in red.

Despite very low sequence similarity between the three hydrophobins, a similar core fold of an irregular β-barrel consisting of four antiparallel β-sheets is seen in the structures. In EAS two of the four disulphide bonds are found in the centre of the barrel, while the remaining two bonds connect the surface of the barrel with a nearby loop and an additional antiparallel β-sheet[50]. In contrast to EAS, HFBI and HFBII contain an α-helix instead of the additional structures found in EAS. Two disulphide bonds are found in the centre of the barrel, one bridge connects the β-barrel and α-helix and one bridge attaches the β-barrel and the N-terminal loop[49,51,52]. If *Aspergillus* hydrophobins have similar overall structures remains to be determined, but these hydrophobins presumably have a similar core fold consisting of an irregular β-barrel.

Aspergillus hydrophobins

With the availability of many *Aspergillus* full genome sequences, it has become apparent that these species (as with other fungal species) contain a varying amount of hydrophobin genes[6–

[10]. Table 1 provides an overview of identified hydrophobins in full genome sequenced Aspergilli. The majority of these hydrophobins have only putatively been identified and only few have been characterized in each species.

Table 1: Putative and characterized *Aspergillus* hydrophobins

Species	Genes and common names	Characterized
Aspergillus fumigatus	**Afu5g09580** (RodA)[53,54], **Afu1g17250** (RodB)[53,54], **Afu8g07060** (RodC)[53,54], Afu5g01490 (RodD)[53,54], Afu8g05890 (RodE)[53,54], **Afu5g03280** (RodF)[53,54] **Afu2g14661**[54], Afu5g09960[54], Afu7g00970[54], Afu8g01770[54]	RodA[55–57] RodB[58]
Aspergillus nidulans	**AN0940**[54,59]**, AN1837**[54,59]**, AN6401**[54,59]**, AN7539**[54,59]**, AN8006** (DewA)[54,59]**, AN8803** (RodA)[54,59], AN4845[54], AN5290[54], AN7327[54], AN6807[54]	RodA[36,57,60] DewA[60,61]
Aspergillus niger	**An01g10940** (hypB)[25,54], **An03g02360** (hypC)[25,54], **An03g02400** (hypD)[25,54], **An04g08500** (hypE)[25,54], **An07g03340** (hypF)[25,54], **An15g03800** (hypG)[25,54], **An09g05530 (**hypH)[25,54], An08g01360[54], An07g09260[54], **An08g09880**[54]	-
Aspergillus oryzae	**AO090012000143** (RolA)[54]**, AO090020000588**[54]**,** AO090012000878[54], AO090020000095[54], AO090701000512[54], AO090701000610[54]	RolA[62]
Aspergillus flavus	**AFLA_094600**[54], **AFLA_131460**[54], **AFLA_060780**[54], **AFLA_014260**[54], **AFLA_063080**, **AFLA_098380**, **AFLA_064900**[54], AFLA_059840[54], AFLA_098980[54], AFLA_101340[54]	-
Aspergillus clavatus	**ACLA_001890**[54]**, ACLA_044810**[54]**, ACLA_010960**[54]**, ACLA_072820**[54]**, ACLA_018290**[54]**, ACLA_007980**[54], ACLA_025850[54], ACLA_044070[54], ACLA_059220[54], ACLA_066600[54]	-
Aspergillus terreus	**ATEG_10285**[54]**, ATEG_08089**[54]**, ATEG_07808**[54]**, ATEG_06492**[54]**, ATEG_04730**[54]**,** ATEG_02302[54], ATEG05178[54], ATEG_06080[54], ATEG_07140[54], ATEG_10323[54]	-

Genes in bold are included in chapter 3 as these hydrophobins fulfil all original criteria[40] including having a minimum of eight cysteines, two cysteine pairs, an intact cysteine pattern, a signal sequence and have an appropriate size. For more information see chapter 3.

Two *Aspergillus* species have been and are currently being used to study hydrophobins. One species, *A. nidulans*, is used due to its role as a model organism for other Aspergilli, and the other, *A. fumigatus*, due to its pathogenicity (figure 6).

De Groot *et al*.[59] performed a genomic analysis of the *A. nidulans* cell wall genes finding six hydrophobins including RodA (AN8803), DewA (AN8006), AN0940, AN1837, AN6401 and AN7539. Hydrophobins AN1837 and RodA were predicted to be GPI (glycosylphosphatidylinositol) proteins, having the potential to attach to the cell wall carbohydrate structure and predominately exist in the plasma membrane. AN6401 had an ambiguous GPI anchor, while AN0940, AN7539 and DewA had no GPI anchor[59]. All six hydrophobins are typical hydrophobins, having eight cysteines and a signal sequence (figure 6).

In *A. fumigatus*, Beauvais *et al.*[53] described six hydrophobins (figure 6). The hydrophobins RodA (AFUA_5G09580), RodB (AFUA_1G17250), RodC (AFUA_8G07060) and RodF (AFUA_5G03280) are typical hydrophobins containing eight cysteine residues and a signal

sequence. The hydrophobin RodD (AFU5G_01490) only has seven cysteines, lacks a cysteine pair, but still has a signal sequence, while RodE (AFUA_8G05890) has no signal peptide and contains eleven cysteines. The *A. fumigatus* hydrophobins were expressed under different conditions, where RodA and RodC were not expressed under vegetative growth. RodB and RodE mRNA was found in mycelium grown under static aerial conditions and RodA and RodF mRNA was detected in mycelium grown both under static aerial and shaken submerged conditions[53]. By examining the full genome sequence of *A. fumigatus*, an additional hydrophobin is found, AFUA_2G14661, displaying eight cysteines and a signal sequence[9]. A recent article, however, states that both species contain more putative hydrophobins resulting in *A. fumigatus* having nine hydrophobins and *A. nidulans* having ten hydrophobins (see table 1)[54].

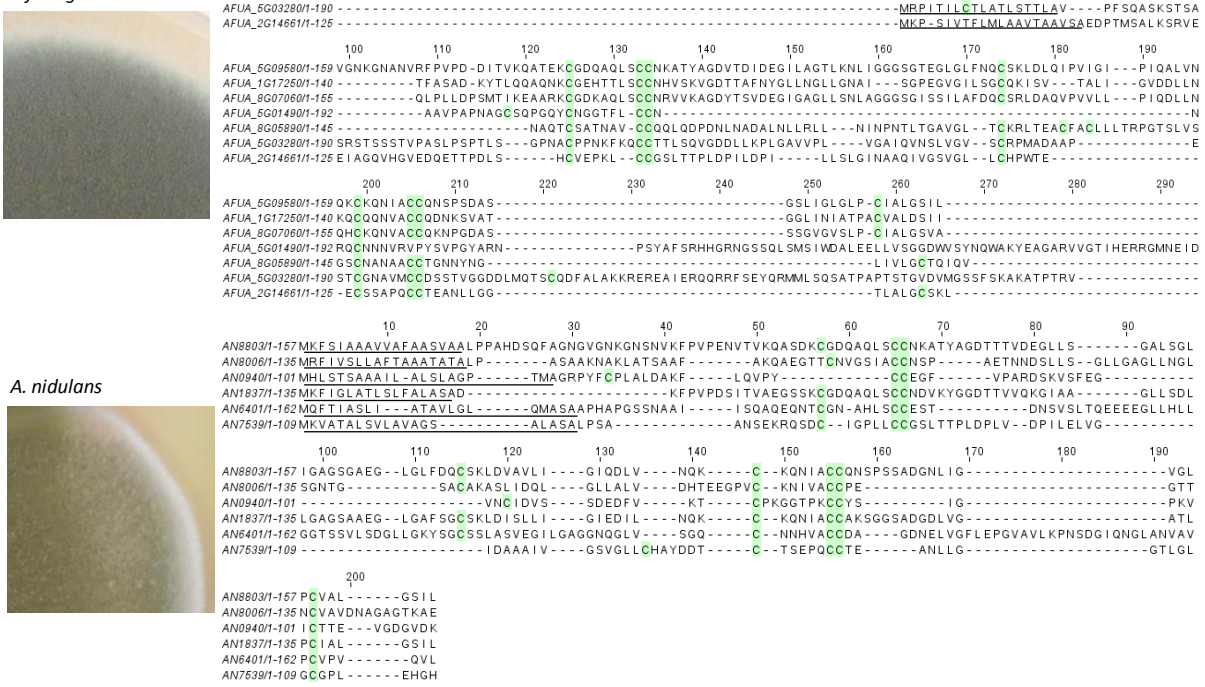


Figure 6: Hydrophobins from *Aspergillus* have primarily been studied in the model organism *A. nidulans* and the opportunistic pathogen *A. fumigatus*. Six putative hydrophobins have been identified in *A. nidulans*, while *A. fumigatus* contains seven. Signal sequences have been determined using SignalP 4.0[63] and are underlined. Cysteine residues are highlighted in green.

Despite the presence of several putative hydrophobins in both *A. nidulans* and *A. fumigatus* only two have been studied in each species. The first hydrophobin to have been discovered in Aspergilli was the RodA hydrophobin in *A. nidulans*, followed shortly after by RodA in *A. fumigatus* due to sequence homology[36,55,64]. Deletion of the *rodA* gene in *A. nidulans* revealed

wettable, darker colonies, than wild type strains, due to an abnormal accumulation of liquid on the conidiophores. In liquid the mutant conidia stuck together resulting in the formation of cylindrical spore masses[36]. Similarly, the deletion of the *rodA* gene in *A. fumigatus* leads to darker colonies. These colonies were easy wettable and the conidia stayed aggregated in water[64]. Conidia from the two *rodA* deletion strains did not easily disperse into the air, which may be attributed to the lack of rodlets (parallel rods, see next section on biological functions) on the mutant conidia[36,64]. Interestingly, the two *rodA* genes were able to complement each other, as the transformation of the *A. fumigatus rodA* gene into an *A. nidulans rodA* deletion strain resulted in the formation of a rodlet layer[55]. The phenotypes observed for the *rodA* deletion strains have likewise been seen in other fungal species. The conidia of the easily-wetted (*eas*) mutant of *N. crassa* lacks rodlets on their surface, have a smooth appearance and are not readily dispersed into the air [65]. The conidia are likewise easily wetted compared to a control strain[65], a phenotype also observed for *M. grisea* colonies lacking the Mpg1 hydrophobin[66].

An additional hydrophobin, DewA, has been studied in *A. nidulans*. This hydrophobin was, like RodA, found located on the (mature) conidia surface (figure 7). Nevertheless, no dramatic differences in morphology were observed between a *dewA* deletion strain and wild type strain and the DewA hydrophobin did not seem necessary for rodlet formation. A subtle phenotype of the *dewA* deletion strain was its increased wetting ability by mild detergent solutions (0.2 % SDS, 50 mM EDTA) leading to naming it the *detergent wettable* phenotype (*dewA*)[61]. Both the loss of *rodA* and *dewA* resulted in less hydrophobic spores. This effect was increased in a double mutant lacking both *rodA* and *dewA*[60,61].

The final hydrophobin studied in Aspergilli is the conidial hydrophobin RodB from *A. fumigatus*[58]. Colonies from a *rodB* deletion strain and a wild type strain were morphologically alike and did not wet by drops of water or mild detergent. Furthermore, the RodB hydrophobin did not seem essential for rodlet formation as rodlets were seen on the *rodB* deletion strain, but is probably involved in the building of the conidia cell wall. Despite, DewA and RodB having identical molecular masses, the *rodB* gene was unable to complement the *dewA* mutation. As in *A. nidulans*, a double mutant of *rodA* and *rodB* in *A. fumigatus* displayed a similar phenotype to the *rodA* deletion strain, having a darker colony colour, which easily wetted by water. This mutant did similarly not have any rodlet layer[58].

Figure 7: The conidial hydrophobins, RodA and DewA, render *A. nidulans* colonies highly hydrophobic. Placement of a water drop on the surface of a colony shows the hydrophobicity of the surface.

As stated previously both *A. fumigatus* and *A. nidulans* contain numerous hydrophobins, but only two conidial hydrophobins have been studied in each species. Studies of six hydrophobins in *C. fulvum* revealed differential expression and biological roles for each hydrophobin[43,44]. Therefore investigations into the biological roles of unstudied *Aspergillus* hydrophobins would be of special interest to understand their involvement in among others fungal growth and development, hydrophobicity, spore dispersal and escaping aqueous environments. In this thesis all six hydrophobins from *A. nidulans* have been examined by creating deletion strains of the hydrophobins (see chapter 4), but several other hydrophobins exist in other *Aspergillus* strains which have never been studied (see table 1).

Biological functions

Hydrophobins fulfil a large variety of functions in fungal growth and development. They are involved in coating aerial structures including conidia, hyphae and fruiting bodies rendering fungi highly hydrophobic[40]. They coat air channels in fruiting bodies allowing gas exchange and prevent collapse and water filling of the channels in humid environments[67]. Furthermore, they mediate the attachment of hyphae to hydrophobic surfaces such as plants and insects, facilitate spore dispersal in air and allow fungi to escape aqueous environments[40]. In this thesis constructed *A. nidulans* hydrophobin deletion strains have been examined for their ability to fulfil different biological functions (see chapter 4), where the main proposed functions of hydrophobins are described below. As *Aspergillus* species have many putative hydrophobins (chapter 3), these may fulfil many different biological functions. These could

include already described functions (see below), but may very well also constitute other functions not yet determined.

Rodlets

Rodlets were first observed by Sassen *et al.* and Hess *et al.* on *Penicillium* conidia using high resolution microscopy[68–70]. A few years later, rodlets were found on many *Aspergillus* species[71] and have today been seen on numerous Aspergilli[36,55,72–74]. Rodlets have also been observed on hyphae and spores of Zygomycetes and Basiodiomycets[72,75,76]. Rodlets can vary both in diameter, length and overall architecture[68,71], but are typically about 10 nm in diameter (figure 8)[40,47,76,77]. Rodlets have been detected on dormant conidia, but the rodlet layer is disrupted during germination exposing an amorphous material[78,79]. *In vitro* rodlets can be observed by drying down purified class I hydrophobin on solid surfaces[80–85], but have never been observed for class II hydrophobins[39,42]. Instead class II hydrophobins seem to display nonamyloidal and needle-like aggregates[52,86,87].

The hydrophobins of the rodlet layer have only been isolated in few fungal species, where *N. crassa* and *M. grisea* seem to have a single hydrophobin in their rodlet layer[88,89]. In contrast, two hydrophobins have been found in the conidial rodlet layer of *A. fumigatus* (RodA and RodB) and *A. nidulans* (RodA and DewA). Despite the presence of two hydrophobins, only RodA seems to be required for rodlet formation *in vivo*, as *rodB* and *dewA* deletion strains display rodlets, while *rodA* deletion strains do not (figure 8)[36,58,61,64]. Nevertheless, recombinant DewA seems to assemble into rodlets at interfaces[90]. Similarly, two hydrophobins (Hyd1 and Hyd2) have been detected in the rodlet layer of *Beauveria bassiana*. Again, distinct roles seem apparent for the two hydrophobins, where Hyd1 appears to be the major component of the rodlets, while Hyd2 may play a role in organizing the rodlets[91]. Due to the presence of several hydrophobins in fungal strains, other hydrophobins may contribute to rodlets on other fungal structures than conidia. In a *rodA* deletion strain, rodlets were observed on the stalks and vesicles of *A. nidulans* conidiophores. The appearance of these rodlets was, however, different to the conidia rodlets[36]. In this thesis effort towards examining *A. nidulans* hydrophobin deletion strains for rodlets were conducted to elucidate if different hydrophobins are essential for rodlet formation on different fungal structures (see chapter 4).

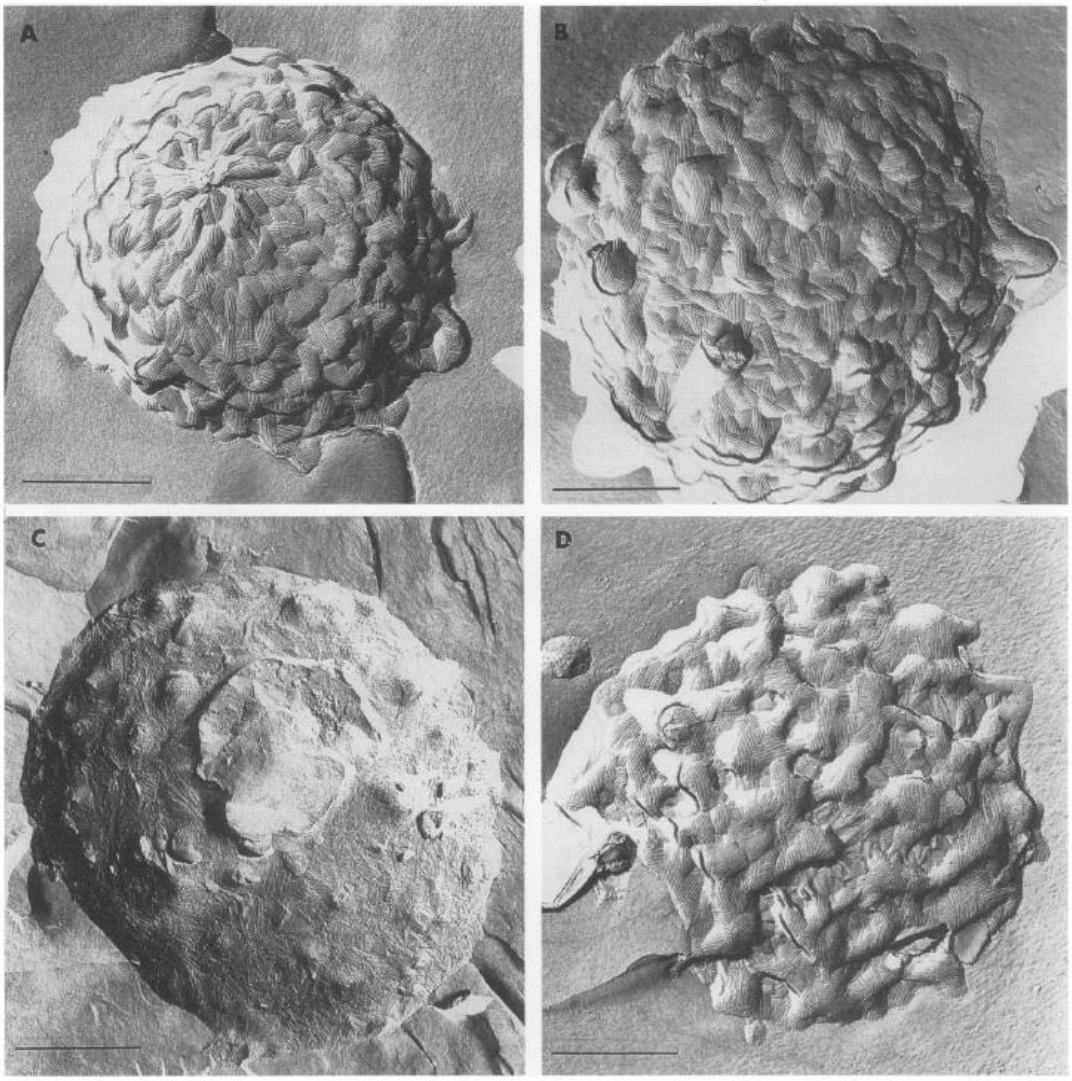


Figure 8: The hydrophobin rodlet layer has been observed on several different fungi. Both (A) *A. fumigatus* and (B) *A. nidulans* display rodlets on their conidia surface. (C) An *A. fumigatus rodA* deletion strain has no rodlets showing the involvement of RodA in rodlet formation. (D) Interestingly, the RodA hydrophobins can complement each other, as the transformation of the *A. fumigatus rodA* gene into an *A. nidulans rodA* deletion strain resulted in the formation of a rodlet layer. Figure taken from Parta *et al.* 1994[55].

Formation of amphipathic membranes

An important characteristic of hydrophobins is their ability to self-assemble into an amphipathic membrane at hydrophilic-hydrophobic interfaces found e.g. between water and air, water and oil or water and a hydrophobic surface like Teflon[46,80,81,92]. The membranes, found to be approximately 10 nm thick, can change the nature of a surface and turn a hydrophilic surface hydrophobic and vice versa[81,83,93,94]. Class I hydrophobins form highly stable aggregates, which are insoluble in water, organic solvents and 2 % SDS and can only be dissociated by formic acid or trifluoroactic acid (TFA)[33,35,38,80,95]. In contrast, aggregates

formed by class II hydrophobins readily dissociate in water, and are soluble in aqueous ethanol and 2 % SDS[40,96,97]. Some class II membranes even dissociate when cooling or pressure is applied as seen for CU (*O. ulmi*) membranes[97]. Amphipathic membranes have today been observed for several hydrophobins[67,80,84,96–98] and have been proposed to enable fungi in escaping aqueous environments as described below.

Breaching of water-air interfaces

Many filamentous fungi grow in moist substrates, e.g. soil and wood, and have to breach the water-air interface to grow into the air and form reproductive structures like spores or fruiting bodies. A proposed model for fungi to breach the water-air interface is based on the SC3 hydrophobin from *S. commune* and involves secretion of hydrophobin monomers from the tips of the submerged hyphae, which diffuse into the surrounding medium (figure 9). Upon reaching the water-air interface, the hydrophobins self-assemble into an amphipathic membrane resulting in a large drop in surface tension. This enables the hyphae to breach the interface and expand into the air. As the hyphae grow into the air, secreted hydrophobin self-assembles on the hyphae wall, exposing the hydrophobic side of the membrane to the surrounding air and the hydrophilic side towards the cell surface[99]. Upon reaching the air, the fungi can produce fruiting bodies and spores. These structures are likewise highly hydrophobic due to the presence of hydrophobins on their walls[36,84].

Hydrophobins SC1, SC3 and SC4 from *S. commune* have been found secreted into the surrounding medium by submerged hyphae[33,35,95]. Likewise, other fungi also have the ability to secrete class I hydrophobins into the surrounding medium[83,100], indicating that lowering of the water surface tension may be a common phenomena enabling aerial growth[42]. No rodlets have been observed on submerged hyphae from *N. crassa*, submerged hyphae from *S. commune* nor submerged conidia from *A. nidulans*, further supporting that the produced hydrophobins probably diffuse away into the surrounding medium[36,80,95]. Nevertheless, hydrophobins cannot always be detected in the surrounding medium indicating that all fungal species do not secrete hydrophobins[101,102]. Whether *Aspergillus* species secrete hydrophobins into the surrounding media and uses a similar mechanism as *S. commune* to escape aqueous environments remains elusive. This has been examined in the thesis using constructed *A. nidulans* hydrophobin deletion strains (see chapter 4).

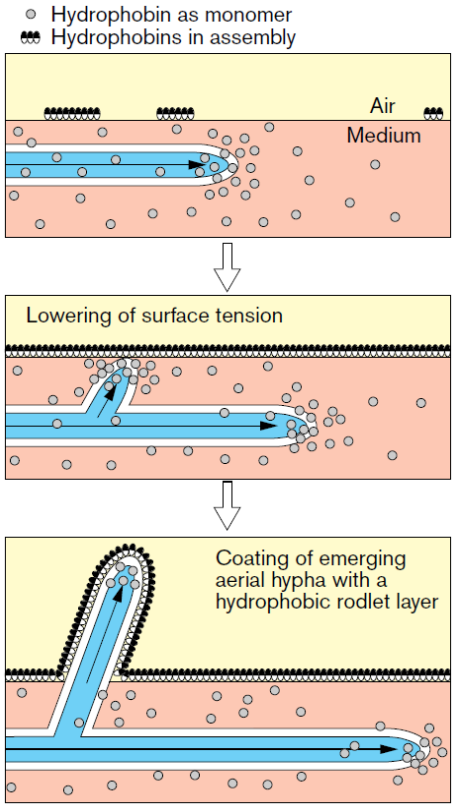


Figure 9: Proposed model for the involvement of hydrophobins in allowing fungi to breach from a moist substrate into the air. Secretion of hydrophobins into the surrounding medium lowers the water surface tension allowing hyphae to breach into the air, followed by the produced hydrophobins self-assembling on the hyphae surfaces and thereby forming a rodlet layer. Figure from Wösten *et al*. 1999[99].

Potential industrial applications of hydrophobins

Due to the many different properties of hydrophobins, hydrophobins have become increasingly interesting for both medical and technical applications. Hydrophobins seem safe for technical applications mainly due to the daily consumption of several hydrophobins by humans on eatable mushrooms like the common button mushroom (*Agaricus bisporus*)[40,84,103]. Therefore, there is interest in producing both strains lacking specific hydrophobins, but also strains over-expressing hydrophobins allowing increased production yields[104]. The technical applications for hydrophobins have not been the focus of this thesis, but an overview of different potential applications are described below to show the diversity of using hydrophobins in industry (see table 2).

Despite several potential applications for hydrophobins, only few hydrophobins have been produced in good yields (gram per litre levels)[104,105]. At CMB, the hydrophobins RodA and RodB from *A. fumigatus* have successfully been produced at 200-300 mg/l by expressing the

hydrophobins in *Pichia pastoris*[94]. In addition, the chemical company BASF have successfully cloned *A. nidulans* hydrophobins into *E. coli* and can produce hydrophobins in industrial scale[106]. If hydrophobins can be produced in significant low-cost amounts, it is certainly possible that the application of hydrophobins will gradually expand.

Table 2: Potential industrial applications for hydrophobins

Application	Result of using hydrophobins	References
Medical applications		
Prosthetic implants	Enhanced growth of fibroblasts and stem cells on surfaces	107–109
Catheters	Prevention of bacterial growth	110
Water soluble drugs	Increased bioavailability and uptake	111
Biosensors and electrodes	Stable attachments of electroactive molecules to electrodes, immobilization of enzymes and prevention of denaturation of proteins	110,112,113,114
Food and cosmetic industry		
Stabilise foam	Long lasting foams necessary in products like mousse, soft cheese and ice cream, where hydrophobins have been shown to stabilise foam for several months	115
Stabilise emulsions	Addition to hair products results in prolonged resistance to hair washing	116
Breweries	Causes economic loss as hydrophobins cause gushing of beer (the spontaneous over-foaming of beer upon opening)	45,117,118,118,119
Other applications		
Ships	Prevention of bacterial growth	110
Recycling	Stimulation of the degradation of biodegradable plastic by recruiting a polyesterase	62
Textile industry	Change the hydrophobicity of textiles	120

Fungal-bacterial interactions

Bacteria and fungi are abundantly found in many different environments and commonly co-exist. Despite the frequent co-habitation of bacteria and fungi, whether in an environmental or clinical setting, little is known about the molecular mechanisms between the species[121]. Bacteria and fungi can co-exist as planktonic cells, in biofilms or even by intra-colonization of the bacterium in e.g. the fungal hyphae (figure 10). In the mixed community the bacteria and fungi can interact in a large number of ways. This can be by the production and exchange of deleterious compounds (antibiosis), metabolites or DNA, production of signalling

molecules, modification of the surrounding environment by physiochemical changes, protein secretion or through chemotaxis (directed movement of the bacteria towards the fungus). As a result of the interaction, an effect may be observed in either species, which may consist of an effect in physiology, development or pathogenicity[121,122].

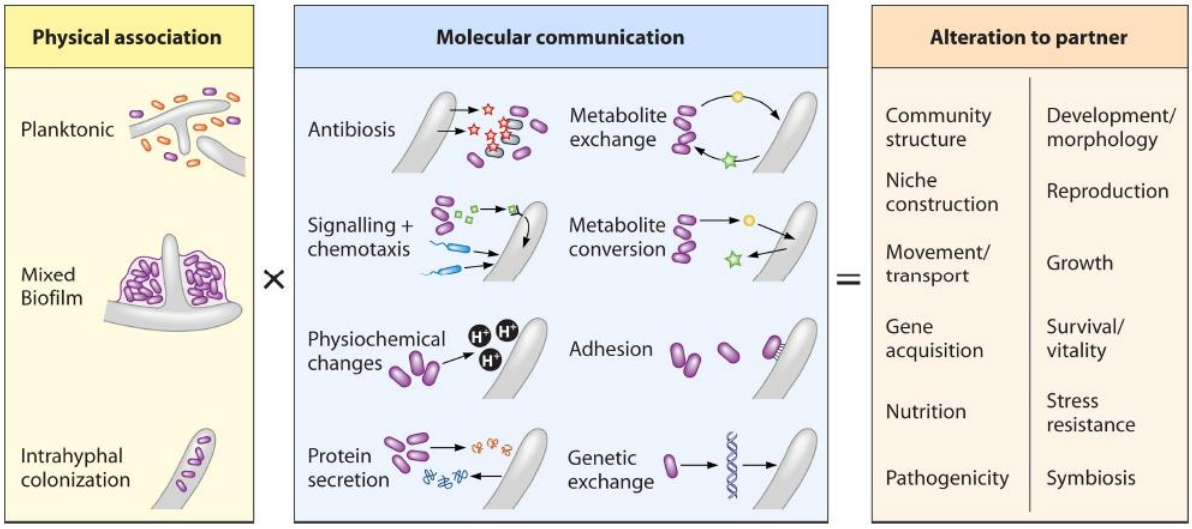


Figure 10: Fungi and bacteria can interact in many ways. Both their physical association and the interspecies communication method play a role in the outcome for each involved organism. An alteration in the fungal-bacterial interaction may further affect the surrounding environment. Figure from Frey-Klett *et al.*, 2011[122].

Several recent reviews have aimed in providing an overview of known fungal-bacterial interactions including Frey-Klett *et al.*, Peleg *et al.*, Tarkka *et al.* and Wargo and Hogan[121–124]. In a clinical perspective the main fungal species encountered are *Candida* and *Aspergillus* species. *Candida* species can infect many sites of the human body, while *Aspergillus* species are mainly lung pathogens found in the respiratory tract of patients with underlying pulmonary diseases or mechanically ventilated patients[121]. Both species commonly colonize the lungs of cystic fibrosis (CF) patients, whereas the dominant bacteria found is *Pseudomonas aeruginosa*[125]. Other bacteria found to exist in the CF lung include *Staphylococcus aureus, Haemophilus influenza* and *Burkholderia cepacia*[125,126].

Isolates of both *P. aeruginosa* and *A. fumigatus* from CF patients reveal that these organisms often coexist in the lungs of CF patients[125]. Furthermore, CF patients colonized with *Aspergillus* and *Candida albicans* or both *Aspergillus, Pseudomonas* and *C. albicans* have decreased lung functions[127]. Why a decrease in lung function is observed remains to be established, but may be caused by interactions between the organisms. A considerable amount of research has been conducted on *Pseudomonas-Candida* interactions, while the interactions between *Aspergillus* species and *Pseudomonas* are less explored. Part of this thesis deals with

the possible interaction between *Aspergillus* species and *Pseudomonas aeruginosa*. This interaction has been examined with special focus on secondary metabolite production (see chapter 5 and 6) and secondary metabolites and detection methods are described below.

Secondary metabolites

Secondary metabolites are small molecules produced by several different microorganisms including both bacteria and fungi[2]. They are often produced during the late growth phase of the producing organism, are frequently not essential for the organisms' own growth and reproduction, but may be beneficial to produce during the microorganism's different developmental stages. The formation of secondary metabolites is highly dependent upon environmental conditions and may serve a large array of functions in nature[2,128–130]. They are often not constitutively expressed[131], but are only expressed when advantageous to the organism itself[128]. In contrast to primary metabolites, secondary metabolite genes are typically clustered in the microbial genome[132,133]. Many secondary metabolite gene clusters contain a transcription factor, which regulates the expression of the genes within the cluster, but may also regulate genes outside the cluster[128,134,135]. Apart from the cluster-associated transcription factors, secondary metabolite production is also regulated by more global transcription factors allowing the regulation of genes in response to different environmental factors such as temperature, light, pH and nutrient availability[131,134,135].

Many secondary metabolites are important in modern medicine as these are used in industry due to their antibiotic and pharmaceutical properties[136]. Other secondary metabolites have been found to be involved in disease in animals and plants[135,136]. The focus of this thesis is *Aspergillus* species and *Pseudomonas*, where both groups of organisms can produce many different secondary metabolites.

Secondary metabolites from *Aspergillus* species

Aspergillus species produce a large array of secondary metabolites[135]. Examples include beneficial compounds like the cholesterol-lowering drug lovastatin, but also compounds with adverse effects like the carcinogenic compound aflatoxin and several other mycotoxins including fumonisins[19,20,30,137]. Several articles give excellent overviews of the secondary metabolites produced by *Aspergillus* species[131,137–141], where a few selected secondary metabolites are shown below (figure 11).

The secondary metabolites often have unknown functions in the fungus itself, but may be used to survive and compete with other organisms in the fungus' own natural habitat[128,134,135]. As previously mentioned, the secondary metabolite genes are typically clustered in the fungal genome[132], and Aspergilli have been found to contain between 30 – 40 clusters per species[142]. Many of the biosynthetic pathways are to a large extent similar[139], but only few secondary metabolite gene clusters are shared between species[7,8,142]. This results in that only few metabolites can be found across *Aspergillus* species[139] as can be seen by comparing metabolites from *A. niger* and *A. fumigatus*[137,138]. However, different species within a section often produce common metabolites as seen in *Aspergillus* section *Nigri* (the black Aspergilli) or *Aspergillus* section *Fumigati*[137,143]. Additionally, phylogenetically different fungi can, in few cases, have a limited number of secondary metabolites in common[144,145].

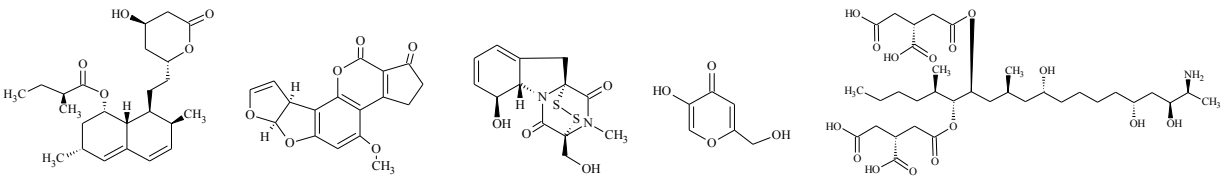


Figure 11: *Aspergillus* species produce a significant number of different secondary metabolites. A few selected metabolites exemplify the chemical diversity found in *Aspergillus*.

Secondary metabolites from *Pseudomonas*

Pseudomonas species are opportunistic pathogens found in a large variety of environments such as water, soil, plant surfaces and animals[133]. Especially *P. aeruginosa* can cause a number of infections in humans, but most commonly infect immunocompromised individuals[133]. A patient group often encountering *P. aeruginosa* infections are cystic fibrosis patients, where over 80 % are chronically infected[125].

Pseudomonas species can similarly to the *Aspergillus* species produce many different secondary metabolites, where a recent review by Gross and Loper[133] provides an excellent overview. The secondary metabolites are important for among others nutrient acquisition, virulence and competition with other organisms[133]. Of special interest in this thesis are the phenazines due to their antifungal properties and possible role in interactions between *Pseudomonas* and *Aspergillus*[133,146]. Only bacteria have been found to be a natural source of phenazines, where more than 100 different phenazine structural derivatives have been identified in nature. Many bacteria produce numerous phenazine derivatives[147]. In this thesis

the focus has been on *P. aeruginosa* (see chapter 5), which can produce at least four different phenazines including pyocyanin, phenazine-1-carboxamide, 1-hydroxyphenazine and phenazine-1-carboxylic acid (figure 12)[133,146].

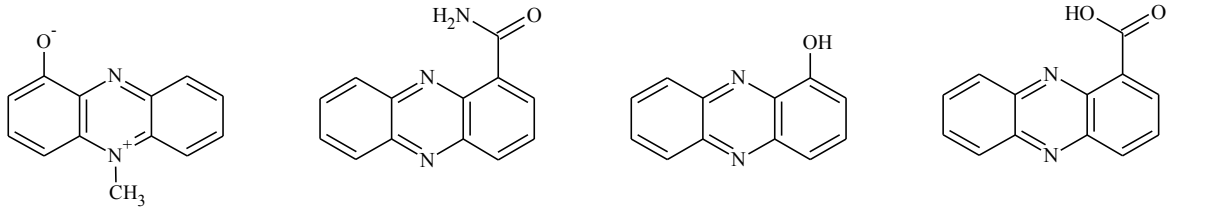


Figure 12: *P. aeruginosa* can produce several phenazines including pyocyanin, phenazine-1-carboxamide, 1-hydroxyphenazine and phenazine-1-carboxylic acid.

Chemical characterization of secondary metabolites

The chemical analysis of secondary metabolites is identical regardless of the species being a bacterium or fungus. As the focus of this thesis is primarily on fungi and the main focus area of CMB is fungi, the chemical characterization will be described in a fungal perspective, but identical methods are applicable to bacterial extracts.

A secondary metabolite profile of a given fungal species is based upon fungal extracts and consists of the extractable and detectable different compounds a fungus can produce on a specific medium[144]. The profiles of secondary metabolites are frequently highly species specific and are by mycologists used in species recognition[144,145,148]. Many fungal extracts contain compounds which have previously been characterized[149]. By dereplication, the combination of chromatographic and spectroscopic methods with database searching, a fast tentative identification of already known compounds in the extract can be achieved[148,150]. Traditionally, dereplication methodologies were based on thin layer chromatography (TLC)[151]. Developed by Filtenborg *et al.*[152,153], the fungal TLC technique involved placement of a small solvent-wetted mycelium plug on a TLC plate for a few seconds allowing efficient identification and detection of secondary metabolites. Later, this technique was followed by high-performance liquid chromatography (LC/HPLC), where dereplication was based on retention times and UV-Vis spectra[154–156]. At CMB today, the identification of individual secondary metabolite production, from a given species, is usually done by ultra-high-performance liquid chromatography (UHPLC) and often combined with mass spectrometry

(MS)[145,150]. Other modern techniques available include various combinations of LC with MS/MS, HRMS and NMR[148,157].

When examining a mass spectrum, the main objective is to assign ions to the chromatographic peaks observed (figure 13). The MS detection can be run in either positive or negative mode resulting in the formation of different ions depending on the composition of the fungal extract. Common adducts include, $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$, $[M-H]^-$ and $[M-HCOO]^-$ [148,150]. Other ions can also be present depending on the compound, the ion source parameters and solvent used.[149]

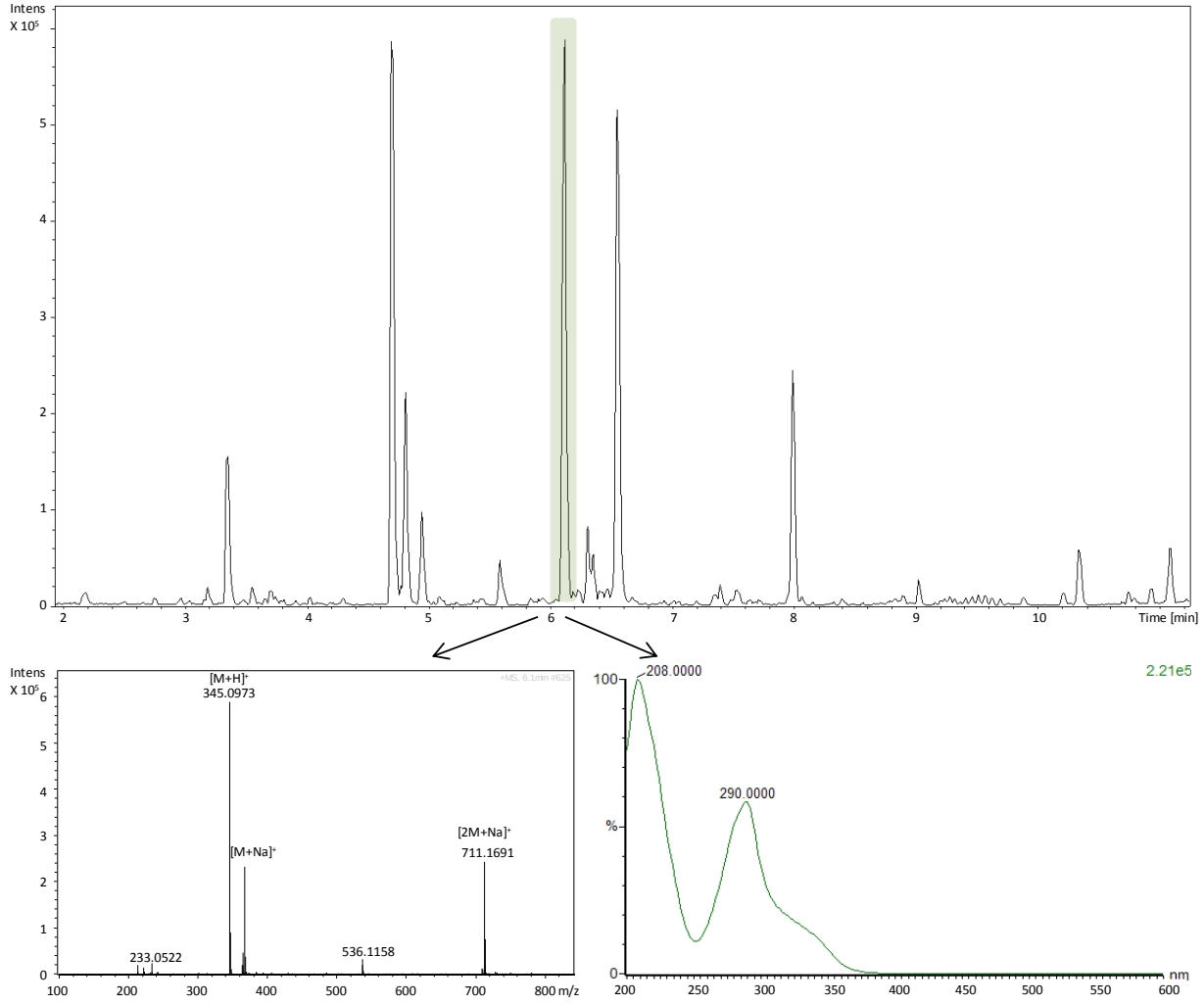


Figure 13: LC-MS analysis of a raw extract from *A. fumigatus* AF293 grown on Wickerhams Antibiotic Test Medium reveals several peaks in the chromatogram. Three ions are present in the MS spectrum of the target ion (RT = 6.1) including $[M+H]^+$, $[M+Na]^+$ and $[2M+Na]^+$. By searching the database *Antibase* for the accurate mass and combing the UV-vis spectrum and retention time of the target compound with taxonomy of the fungus, the target compound can tentatively be identified as trypacidin.

In instruments with low resolving power only the nominal mass can be acquired. The nominal mass is calculated using the mass of the most abundant isotope of each element rounded to the nearest integer value (e.g. the nominal mass for $C_{18}H_{16}O_7$ is (18x12) + (16x1) + (7x16) = 344 Da). An important benefit of newer mass spectrometers (e.g. TOF instruments) is their high resolving power enabling the acquisition of the accurate mass of a target peak[150,158], where the accurate mass is defined as a measurement of ion mass within a 5 ppm uncertainty[159]. In most cases positive mode will be sufficient to determine the accurate mass of the target compound, and negative mode is used to confirm results from positive mode[148].

After determination of the accurate mass of the target peak, database searching will provide a number of different candidates. At CMB, we use the database *Antibase* (maintained by H. Laatsh), currently containing approximately 38,000 natural compounds from microorganisms, fungi and algae[150]. As an example a raw extract from *A. fumigatus* AF293 is used displaying several peaks in the mass chromatogram (figure 13). Extraction of the MS spectrum for the peak with the retention time (RT) of 6.1 min reveals the presence of three adducts; $[M+H]^+$, $[M+Na]^+$ and $[2M+Na]^+$. If the nominal mass of 344 is used to search *Antibase*, 111 possible candidates are found. In contrast if the accurate mass is used, the number of candidates is reduced to 25 using 5 ppm mass accuracy. By using the UV-Vis spectrum from the target peak, retention time and taxonomy, several candidates can normally be eliminated, resulting in few suitable candidates[148,150]. In the above described case only two *Aspergillus* secondary metabolites are found among the candidates including trypacidin from *A. fumigatus*. By using the UV-Vis spectra, the target peak can tentatively be identified as trypacidin, while the use of NMR would be necessary for a definitive identification.

In this thesis the above described procedure has been used to examine differences in secondary metabolite production in *Aspergillus-Pseudomonas* interactions (chapter 5).

Interactions between *Aspergillus* and *Pseudomonas*

Isolates of both *P. aeruginosa* and *A. fumigatus* from CF patients reveal that these organisms often coexist in the lungs of CF patients[125]. Whether they exist in symbiosis in the CF lung, have a neutral relationship or combat each other using different chemical molecules has not yet been determined. Nevertheless, a significant decrease in lung function has been observed in CF patients colonized by both *A. fumigatus* and *P. aeruginosa* or *A. fumigatus*, *P. aeruginosa* and *C. albicans* compared to patients only colonized by a single organism[127,160,161]

and therefore studies in interactions between these organisms are of special interest. In this thesis the focus has been on *Aspergillus-P. aeruginosa* (chapter 5 and 6). Below current knowledge of *Aspergillus-P. aeruginosa* interactions is described, but examples of *Candida-P. aeruginosa* interactions are also included. As little knowledge is available regarding *Aspergillus-P. aeruginosa* interactions, it is not known if these two organisms interact at all. This has been examined in chapter 5 by looking at secondary metabolite production.

An early description of the interaction between *Aspergillus* species and *P. aeruginosa* by Mangan acknowledged an inhibitory effect of *P. aeruginosa* on *A. fumigatus* and *A. terreus* in broth culture[162]. Similarly, Blyth and Forey observed an inhibitory effect on *P. aeruginosa* cells on the hyphal growth of *A. fumigatus*, where the presence of the bacterium affected the ultrastructures of *A. fumigatus* hyphae[163,164]. Nevertheless, not all *P. aeruginosa* strains seem to inhibit *A. fumigatus* nor does *P. aeruginosa* completely inhibit *A. fumigatus*. As an example Kerr demonstrated, that *A. fumigatus* was partially inhibited by six clinical *P. aeruginosa* strains, while two other *P. aeruginosa* clinical strains showed no inhibitory effect[165]. Mowat *et al*. found that nine different *P. aeruginosa* strains, including six clinical isolates, significantly inhibited the germination of *A. fumigatus* conidia, but again did not completely abolish germination[166]. In e.g. the lungs of CF patients, *A. fumigatus* can exist as a biofilm. When a mature *A. fumigatus* biofilm was treated with *P. aeruginosa*, a minimal inhibitory effect was observed on the biofilm. Visualization of the biofilm revealed that single *P. aeruginosa* cells were distributed on the hyphae throughout the *A. fumigatus* biofilm (figure 14)[166].

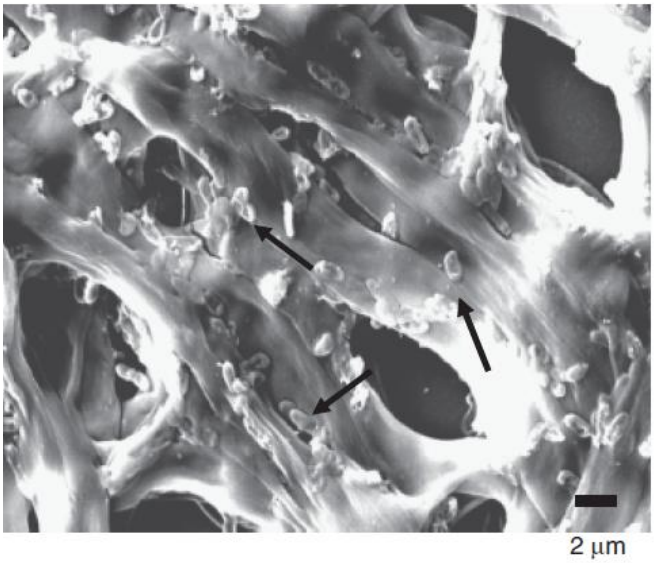


Figure 14: *P. aeruginosa* cells (arrows) adhere to the *A. fumigatus* biofilm. Picture from Mowat *et al.* 2010[166].

Similarly, *Salmonella enterica* Typhimurium attaches to *A. niger*. Initial attachment was observed on the tip of the hyphae and resulted in a dense biofilm of *S. enterica* on the *A. niger* hyphae within few hours[167]. Interestingly, the bacteria *E. coli*, *Pantoea agglomerans* and *Pseudomonas chlororaphis* could not attach to *A. niger* hyphae. This was due to the lack of cellulose production by the strains, which was found to be necessary for initial attachment to *A. niger* hyphae and to chitin (a structural component of fungal cell walls)[167]. In contrast, *Streptomyces hygroscopicus* readily attached to *A. nidulans* hyphae[168]. *P. aeruginosa* can attach to *Candida albicans* filaments, but not to the yeast-form of *C. albicans*. As a result the filaments were killed, while the yeast-form was not[169,170]. Several other *Candida* species were likewise significantly inhibited by the presence of *P. aeruginosa*[165], as were *Torulopsis glabrata*, *Saccharomyces cerevisiae* and several *Cryptococcus* species[165,171].

To better understand the inhibitory effect of *P. aeruginosa* on *A. fumigatus*, studies into possible antifungal compounds have been conducted. Of special focus have been phenazines as these are thought to be involved in lung damage in *P. aeruginosa* infected individuals[172,173]. Kerr found that *P. aeruginosa* produced two phenazines, pyocyanin and 1-hydroxyphenazine, which were responsible for the inhibition of *A. fumigatus* and *C. albicans*[174]. MICs (Minimal Inhibitory Concentration) for *A. fumigatus* were measured to be > 100 µg/ml for pyocyanin and 50 µg/ml for 1-hydroxyphenazine, while MICs for *C. albicans* were > 100 µg/ml for pyocyanin and 25µg/ml for 1-hydroxyphenazine[174]. This finding was supported by Blyth, who showed that the germination of *A. fumigatus* conidia was inhibited by pyocyanin[163], while Mangan demonstrated that 1-hydroxyphenazine could inhibit germination of spores from *A. fumigatus*, *A. terreus*, *A. niger* and *A. flavus*[162]. In *P. aeruginosa-C. albicans* co-cultures the levels of pyocyanin were found to be increased after 48 hours compared to *P. aeruginosa* single-species cultures[175]. In addition, the accumulation of a red pigment was observed in *C. albicans* cells, when co-cultured with *P. aeruginosa* resulting in the killing of *C. albicans*. The formation of the red pigment seemed to require the presence of a phenazine and both 5-methyl-phenazinium-1-carboxylate (5MPCA) and phenazine methosulphate (PMS) induced the red pigmentation[175,176]. Similarly, the production of a red pigment (proposed to be 3-hydroxyphenazine-1-carboxylic acid) was seen in co-cultures of *Aspergillus sclerotiorum* and *Pseudomonas chlororaphis*. It seemed that both phenazine-1-carboxylic acid and phenazine-1-carboxamide could lead to the formation of the red pigment[177]. Nevertheless, the pigment had different properties than the pigment observed in *C. albicans-P. aeruginosa* cocultures[175].

Other studies showing the involvement of phenazines in fungal-bacterial interactions include interactions between different *Pseudomonas* species and *Gaeumannomyces graminis, Fusarium oxysporum, Pythium splendens* and several *Cryptococcus* species[171,178,179].

In addition to phenazines, *P. aeruginosa* can produce acetylated homoserine lactones including 3-oxo-C_{12}-HSL and C_4-HSL, which act as signalling molecules in the quorum sensing systems[180]. Interestingly, two *P. aeruginosa* quorum sensing knockout-strains displayed reduced inhibitory effect on the germination of *A. fumigatus* conidia compared to the wild type strain[166]. In *C. albicans* purified 3-oxo-C_{12}-HSL could inhibit filamentation, while C_4-HSL did not have any effect[170]. Similarly, farnesol, dodecanol and C_{12}-HSL could inhibit filamentation of *C. albicans*[170], while addition of decanol, decanoic acid and dodecanol to preformed mature *A. fumigatus* biofilm resulted in a reduction of biomass[166]. Farnesol has been identified as a quorum sensing signal in *C. albicans*,[181] and inhibited the swarming of *P. aeruginosa*[182]. Furthermore, farnesol could inhibit the growth and development of *A. nidulans* and triggered apoptosis. Apoptosis was also observed in *A. fumigatus* by addition of farnesol[183]. To add to the complexity of the mixed interactions in the CF lung, *A. fumigatus* can produce gliotoxin, which has been shown to inhibit *C. albicans* and *Cryptococcus neoforma*ns[184].

In this thesis the possible interaction between different *Aspergillus* species and *P. aeruginosa* has been examined by comparing secondary metabolite profiles of the differences species in mono and mixed cultures (chapter 5). Furthermore, the role of hydrophobins in bacterial-fungal interactions was preliminary assessed.

Hydrophobins and pathogenesis of *Aspergillus fumigatus*

The involvement of hydrophobins in bacterial-fungal infections has not previously been studied and whether hydrophobins play a role remains elusive. The involvement of hydrophobins in the interaction between *A. nidulans* and *P. aeruginosa* has slightly been touched upon in this thesis (chapter 5). However, several other studies have focused on hydrophobins and their potential role in pathogenesis caused by *Aspergillus* species showing that hydrophobins may play pivotal roles in pathogenesis.

The opportunistic fungus, *A. fumigatus*, is responsible for the vast majority of airborne fungal-infections in humans and research has primarily focused on this organism. In the lungs of healthy individuals, inhaled conidia are eliminated by innate immune mechanisms, but in

immunocompromised individuals, the ability to clear inhaled conidia is significantly impaired[16,185]. Due to the identification of several hydrophobins in *A. fumigatus* and especially the localization of RodA and RodB to the conidia cell surface, a possible role for these hydrophobins in pathogenesis has been the subject of investigation.

Aimanianda *et al.*[102] recently showed that the rodlet layer of the *A. fumigatus* conidial cell wall renders the fungus inert to innate and adaptive immunity allowing the conidia to "hide" from the immune system and await suitable germination conditions[102]. Nevertheless, a *rodA* deletion strain had similar mortality rates in mice compared to a wild type strain[64,186]. The lack of the RodA hydrophobin or rodlet layer rendered the conidia more susceptible to killing by macrophages and activated both human dendritic cells and murine alveolar macrophages *in vitro*[58,102], but not Natural Killer (NK) cells[187]. Lack of the RodB hydrophobin only had a minor effect[58].

Prolonged growth of *A. fumigatus* in the lungs of immunocompromised individuals can result in the formation of dense intertwined mycelia balls termed biofilms, which can be defined as surface attached microbial populations surrounded by an extracellular matrix[188,189]. Few reports on *A. fumigatus* biofilm formation exist showing, among others, the reduced susceptibility of the biofilms to antifungal drugs compared to single cells[190–193]. Only a single study deals with hydrophobins in biofilms showing that four genes encoding *A. fumigatus* hydrophobins are up-regulated. *rodD* was up-regulated in 24 h biofilm, while *rodB*, *rodC* and *rodE* were up-regulated in 48 h biofilm[194]. In another study examination of static, aerial grown hyphae (resembling biofilm growth) only showed expression of the *rodB* and *rodE* genes[53]. The biological role of several *A. fumigatus* hydrophobins still needs to be solved, but they may play a role in pathogenesis (as seen for RodA), biofilm formation or fungal-bacterial interactions, but more research is needed within this area.

All in all the mechanisms in fungal-bacterial interactions are diverse and whether hydrophobins play a role remains unanswered. Research into fungal-bacterial interactions has just started and as stated by Wargo and Hogan, only the "initial steps have been made in the analysis of fungal–bacterial interactions and their role in infection, but there remains a myriad of unanswered questions that will require the collaboration of mycologists, microbiologists, cell biologists and clinicians to answer in full"[123].

The present study

The focus of this thesis is *Aspergillus* species and their hydrophobins. Currently many assumptions are made regarding function and properties of hydrophobins across phylogenetically distinct fungal species, but whether these assumptions are valid in *Aspergillus* species remains to be examined. First of all it is necessary to gain an overview of putative hydrophobins in *Aspergillus* species. This can be achieved using the available genomes of several Aspergilli and bioinformatic tools such as alignments, phylogenetic analysis and hydropathy patterns (see chapter 3). Next, studies into biological roles of unstudied *Aspergillus* hydrophobins would be of special interest. This can be achieved by creating different hydrophobin mutants in the model organism *A. nidulans* and by examining the hydrophobin mutants macroscopically and microscopically, the involvement of hydrophobins in the fungal life cycle (including growth and development) can be studied. Furthermore, questions regarding the role of hydrophobins in hydrophobicity, spore dispersal and escaping aqueous environments can be examined using different assays (see chapter 4). Whether hydrophobins are involved in *Aspergillus-P. aeruginosa* infections has never been studied. By creating an assay allowing examination of interactions between different Aspergilli and *P. aeruginosa* on solid medium this can be studied. Furthermore, this assay will, by using UHPLC-DAD and UHPLC-MS, provide insight into secondary metabolites produced by the two organisms alone and in co-culture. By using different mutated *P. aeruginosa* strains commonly found in CF patients possible differences in the *A. fumigatus-P. aeruginosa* interaction pattern through the course of CF infections can be looked upon (see chapter 5). Whether these organisms can form biofilms together and to what extend hydrophobins are involved may further be studied using a cantilever lab-on-a-chip system enabling measurements of the interactions (see chapter 6).

References for chapter 1

1. Carlile, MJ, Watkinson, SC, and Gooday, GW (2001) The Fungi. London: Academic Press. 588 p.

2. Madigan, MT and Martinko, JM (2006) Brock Biology of Microorganisms. New Jersey: Pearson Prentice Hall. 1088 p.

3. Raper, KB, Fennell, DI, and Austwick, PKC (1965) The genus *Aspergillus*. Huntington: Robert E. Krieger Publishing Company. 686 p.

4. Dyer PS, O'Gorman CM (2012) Sexual development and cryptic sexuality in fungi: insights from *Aspergillus* species. *FEMS Microbiol Rev* **36:** 165-192.

5. Geiser DM, Klich MA, Frisvad JC, Peterson SW, Varga J, Samson RA (2007) The current status of species recognition and identification in *Aspergillus*. *Stud Mycol* **59:** 1-10.

6. Yu J, Payne GA, Nierman WC, Machida M, Bennett JW, Campbell BC, Robens JF, Bhatnagar D, Dean RA, Cleveland TE (2008) *Aspergillus flavus* genomics as a tool for studying the mechanism of aflatoxin formation. *Food Addit Contam Part A* **25:** 1152-1157.

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

8. Fedorova ND, Khaldi N, Joardar VS, Maiti R, Amedeo P, Anderson MJ, Crabtree J, Silva JC, Badger JH, Albarraq A, Angiuoli S, Bussey H, Bowyer P, Cotty PJ, Dyer PS, Egan A, Galens K, Fraser-Liggett CM, Haas BJ, Inman JM, Kent R, Lemieux S, Malavazi I, Orvis J, Roemer T, Ronning CM, Sundaram JP, Sutton G, Turner G, Venter JC, White OR, Whitty BR, Youngman P, Wolfe KH, Goldman GH, Wortman JR, Jiang B, Denning DW, Nierman WC (2008) Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*. *PLoS Genet* **4:** e1000046.

9. Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, Arroyo J, Berriman M, Abe K, Archer DB, Bermejo C, Bennett J, Bowyer P, Chen D, Collins M, Coulsen R, Davies R, Dyer PS, Farman M, Fedorova N, Fedorova N, Feldblyum TV, Fischer R, Fosker N, Fraser A, Garcia JL, Garcia MJ, Goble A, Goldman GH, Gomi K, Griffith-Jones S, Gwilliam R, Haas B, Haas H, Harris D, Horiuchi H, Huang J, Humphray S, Jimenez J, Keller N, Khouri H, Kitamoto K, Kobayashi T, Konzack S, Kulkarni R, Kumagai T, Lafon A, Latge JP, Li W, Lord A, Lu C, Majoros WH, May GS, Miller BL, Mohamoud Y, Molina M, Monod M, Mouyna I, Mulligan S, Murphy L, O'Neil S, Paulsen I, Penalva MA, Pertea M, Price C, Pritchard BL, Quail MA, Rabbinowitsch E, Rawlins N, Rajandream MA, Reichard U, Renauld H, Robson GD, Rodriguez de CS, Rodriguez-Pena JM, Ronning CM, Rutter S, Salzberg SL, Sanchez M, Sanchez-Ferrero JC, Saunders D, Seeger K, Squares R, Squares S, Takeuchi M, Tekaia F, Turner G, Vazquez de Aldana CR, Weidman J, White O, Woodward J, Yu JH, Fraser C, Galagan JE, Asai K, Machida M, Hall N, Barrell B, Denning DW (2005)

Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature* **438:** 1151-1156.

10. Galagan JE, Calvo SE, Cuomo C, Ma LJ, Wortman JR, Batzoglou S, Lee SI, türkmen M, Spevak CC, Clutterbuck J, Kapitonov V, Jurka J, Scazzocchio C, Farman M, Butler J, Purcell S, Harris S, Braus GH, Draht O, Busch S, D'Enfert C, Bouchier C, Goldman GH, Bell-Pedersen D, Griffiths-Jones S, Doonan JH, Yu J, Vienken K, Pain A, Freitag M, Selker EU, Archer DB, Peñalva MA, Oakley BR, Momany M, Tanaka T, Kumagai T, Asai K, Machida M, Nierman WC, Denning DW, Caddick M, Hynes M, Paoletti M, Fischer R, Miller B, Dyer P, Sachs MS, Osmani SA, Birren BW (2005) Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. *Nature* **438:** 1105-1115.

11. Samson, RA, Houbraken, J., Thrane, U., Frisvad, JC, and Andersen, B. (2010) Food and Indoor Fungi. Utrech: CBS-KNAW Fungal Biodiversity Centre Utrech. 390 p.

12. O'Gorman CM, Fuller HT, Dyer PS (2009) Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*. *Nature* **457:** 471-474.

13. Horn BW, Moore GG, Carbone I (2009) Sexual reproduction in *Aspergillus flavus*. *Mycologia* **101:** 423-429.

14. Casselton L, Zolan M (2002) The art and design of genetic screens: filamentous fungi. *Nat Rev Genet* **3:** 683-697.

15. Adams TH, Wieser JK, Yu JH (1998) Asexual sporulation in *Aspergillus nidulans*. *Microbiol Mol Biol Rev* **62:** 35-54.

16. Latge JP (1999) *Aspergillus fumigatus* and aspergillosis. *Clin Microbiol Rev* **12:** 310-350.

17. Denning DW (1998) Invasive aspergillosis. *Clin Infect Dis* **26:** 781-803.

18. Amaike S, Keller NP (2011) *Aspergillus flavus*. *Annu Rev Phytopathol* **49:** 107-133.

19. Krishnan S, Manavathu EK, Chandrasekar PH (2009) *Aspergillus flavus:* an emerging non-fumigatus *Aspergillus* species of significance. *Mycoses* **52:** 206-222.

20. Yu J, Cleveland TE, Nierman WC, Bennett JW (2005) *Aspergillus flavus* genomics: gateway to human and animal health, food safety, and crop resistance to diseases. *Rev Iberoam Micol* **22:** 194-202.

21. Rank C, Klejnstrup ML, Petersen LM, Kildgaard S, Frisvad JC, Gotfredsen CH, Larsen TO (2012) Comparative Chemistry of *Aspergillus oryzae* (RIB40) and *A. flavus* (NRRL 3357). *Metabolites* **2:** 39-56.

22. Machida M, Terabayashi Y, Sano M, Yamane N, Tamano K, Payne GA, Yu J, Cleveland TE, Nierman WC (2008) Genomics of industrial Aspergilli and comparison with toxigenic relatives. *Food Addit Contam Part A* **25:** 1147-1151.

23. Barbesgaard P, Heldthansen HP, Diderichsen B (1992) On the Safety of *Aspergillus oryzae* - A Review. *Appl Microbiol Biotechnol* **36:** 569-572.

24. Schuster E, Dunn-Coleman N, Frisvad JC, van Dijck PW (2002) On the safety of *Aspergillus niger* - a review. *Appl Microbiol Biotechnol* **59:** 426-435.

25. Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, Turner G, de Vries RP, Albang R, Albermann K, Andersen MR, Bendtsen JD, Benen JA, van den Berg M, Breestraat S, Caddick MX, Contreras R, Cornell M, Coutinho PM, Danchin EG, Debets AJ, Dekker P, van Dijck PW, van DA, Dijkhuizen L, Driessen AJ, d'Enfert C, Geysens S, Goosen C, Groot GS, de Groot PW, Guillemette T, Henrissat B, Herweijer M, van den Hombergh JP, van den Hondel CA, van der Heijden RT, van der Kaaij RM, Klis FM, Kools HJ, Kubicek CP, van Kuyk PA, Lauber J, Lu X, van der Maarel MJ, Meulenberg R, Menke H, Mortimer MA, Nielsen J, Oliver SG, Olsthoorn M, Pal K, van Peij NN, Ram AF, Rinas U, Roubos JA, Sagt CM, Schmoll M, Sun J, Ussery D, Varga J, Vervecken W, van de Vondervoort PJ, Wedler H, Wosten HA, Zeng AP, van Ooyen AJ, Visser J, Stam H (2007) Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat Biotechnol* **25:** 221-231.

26. Frisvad JC, Larsen TO, Thrane U, Meijer M, Varga J, Samson RA, Nielsen KF (2011) Fumonisin and ochratoxin production in industrial *Aspergillus niger* strains. *PLoS One* **6:** e23496.

27. Mogensen JM, Frisvad JC, Thrane U, Nielsen KF (2010) Production of Fumonisin B2 and B4 by *Aspergillus niger* on grapes and raisins. *J Agric Food Chem* **58:** 954-958.

28. Blyth W, Grant IW, Blackadder ES, Greenberg M (1977) Fungal antigens as a source of sensitization and respiratory disease in Scottish maltworkers. *Clin Allergy* **7:** 549-562.

29. Flannigan B (1986) *Aspergillus clavatus* - an allergenic, toxigenic deteriogen of cereals and cereal products. *Int Biodeterior* **22:** 79-89.

30. Manzoni M, Rollini M (2002) Biosynthesis and biotechnological production of statins by filamentous fungi and application of these cholesterol-lowering drugs. *Appl Microbiol Biotechnol* **58:** 555-564.

31. Bonnarme P, Gillet B, Sepulchre AM, Role C, Beloeil JC, Ducrocq C (1995) Itaconate biosynthesis in *Aspergillus terreus*. *J Bacteriol* **177:** 3573-3578.

32. Billington R (1969) Versatile itaconic acid and its derivatives. *Chemical Processing* **15:** 8.

33. Wessels JGH, De VO, Asgeirsdottir SA, Schuren F (1991) Hydrophobin Genes Involved in Formation of Aerial Hyphae and Fruit Bodies in *Schizophyllum*. *Plant Cell* **3:** 793-799.

34. Schuren FH, Wessels JG (1990) Two genes specifically expressed in fruiting dikaryons of *Schizophyllum commune*: homologies with a gene not regulated by mating-type genes. *Gene* **90:** 199-205.

35. Wessels JGH, de Vries OM, Asgeirsdottir SA, Springer J (1991) The thn mutation of *Schizophyllum commune*, which suppresses formation of aerial hyphae, affects expression of the Sc3 hydrophobin gene. *J Gen Microbiol* **137:** 2439-2445.

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

37. Stringer MA, Timberlake WE (1993) Cerato-ulmin, a toxin involved in Dutch elm disease, is a fungal hydrophobin. *Plant Cell* **5:** 145-146.

38. Vries OMH, Fekkes MP, Wosten HAB, Wessels JGH (1993) Insoluble hydrophobin complexes in the walls of *Schizophyllum commune* and other filamentous fungi. *Arch Microbiol* **159:** 330-335.

39. Linder MB, Szilvay GR, Nakari-Setala T, Penttila ME (2005) Hydrophobins: the protein-amphiphiles of filamentous fungi. *FEMS Microbiol Rev* **29:** 877-896.

40. Wessels JGH (1997) Hydrophobins: Proteins that change the nature of the fungal surface. *Adv Microb Physiol* **38:** 1-45.

41. Kershaw MJ, Talbot NJ (1998) Hydrophobins and repellents: proteins with fundamental roles in fungal morphogenesis. *Fungal Genet Biol* **23:** 18-33.

42. Wosten HA (2001) Hydrophobins: multipurpose proteins. *Annu Rev Microbiol* **55:** 625-646.

43. Lacroix H, Whiteford JR, Spanu PD (2008) Localization of *Cladosporium fulvum* hydrophobins reveals a role for HCf-6 in adhesion. *FEMS Microbiol Lett* **286:** 136-144.

44. Whiteford JR, Lacroix H, Talbot NJ, Spanu PD (2004) Stage-specific cellular localisation of two hydrophobins during plant infection by the pathogenic fungus *Cladosporium fulvum*. *Fungal Genet Biol* **41:** 624-634.

45. Linder MB (2009) Hydrophobins: Proteins that self assemble at interfaces. *Curr Opin Colloid In* **14:** 356-363.

46. Wessels JGH (1994) Developmental Regulation of Fungal Cell-Wall Formation. *Annu Rev Phytopathol* **32:** 413-437.

47. Sunde M, Kwan AH, Templeton MD, Beever RE, Mackay JP (2008) Structural analysis of hydrophobins. *Micron* **39:** 773-784.

48. de Vocht ML, Reviakine I, Wosten HA, Brisson A, Wessels JG, Robillard GT (2000) Structural and functional role of the disulfide bridges in the hydrophobin SC3. *J Biol Chem* **275:** 28428-28432.

49. Hakanpaa J, Linder M, Popov A, Schmidt A, Rouvinen J (2006) Hydrophobin HFBII in detail: ultrahigh-resolution structure at 0.75 A. *Acta Crystallogr D Biol Crystallogr* **62:** 356-367.

50. Kwan AH, Winefield RD, Sunde M, Matthews JM, Haverkamp RG, Templeton MD, Mackay JP (2006) Structural basis for rodlet assembly in fungal hydrophobins. *Proc Natl Acad Sci U S A* **103:** 3621-3626.

51. Hakanpaa J, Paananen A, Askolin S, Nakari-Setala T, Parkkinen T, Penttila M, Linder MB, Rouvinen J (2004) Atomic resolution structure of the HFBII hydrophobin, a self-assembling amphiphile. *J Biol Chem* **279:** 534-539.

52. Hakanpaa J, Szilvay GR, Kaljunen H, Maksimainen M, Linder M, Rouvinen J (2006) Two crystal structures of *Trichoderma reesei* hydrophobin HFBI - the structure of a protein amphiphile with and without detergent interaction. *Protein Sci* **15:** 2129-2140.

53. Beauvais A, Schmidt C, Guadagnini S, Roux P, Perret E, Henry C, Paris S, Mallet A, Prevost MC, Latge JP (2007) An extracellular matrix glues together the aerial-grown hyphae of *Aspergillus fumigatus*. *Cell Microbiol* **9:** 1588-1600.

54. Littlejohn KA, Hooley P, Cox PW (2012) Bioinformatics predicts diverse *Aspergillus* hydrophobins with novel properties. *Food Hydrocolloids* **27:** 503-516.

55. Parta M, Chang Y, Rulong S, Pinto-DaSilva P, Kwon-Chung KJ (1994) HYP1, a hydrophobin gene from *Aspergillus fumigatus*, complements the rodletless phenotype in *Aspergillus nidulans*. *Infect Immun* **62:** 4389-4395.

56. Thau N, Monod M, Crestani B, Rolland C, Tronchin G, Latge JP, Paris S (1994) Rodletless mutants of *Aspergillus fumigatus*. *Infect Immun* **62:** 4380-4388.

57. Girardin H, Paris S, Rault J, Bellon-Fontaine MN, Latge JP (1999) The role of the rodlet structure on the physicochemical properties of *Aspergillus* conidia. *Lett Appl Microbiol* **29:** 364-369.

58. Paris S, Debeaupuis JP, Crameri R, Carey M, Charles F, Prevost MC, Schmitt C, Philippe B, Latge JP (2003) Conidial hydrophobins of *Aspergillus fumigatus*. *Appl Environ Microbiol* **69:** 1581-1588.

59. de Groot PW, Brandt BW, Horiuchi H, Ram AF, de Koster CG, Klis FM (2009) Comprehensive genomic analysis of cell wall genes in *Aspergillus nidulans*. *Fungal Genet Biol* **46:** S72-S81.

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

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

62. Takahashi T, Maeda H, Yoneda S (2005) The fungal hydrophobin RolA recruits polyesterase and laterally moves on hydrophobic surfaces. *Mol Microbiol* **57:** 1780-1796.

63. Petersen TN, Brunak S, von HG, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods* **8:** 785-786.

64. Thau N, Monod M, Crestani B, Rolland C, Tronchin G, Latge JP, Paris S (1994) rodletless mutants of *Aspergillus fumigatus*. *Infect Immun* **62:** 4380-4388.

65. Beever RE, Dempsey GP (1978) Function of rodlets on the surface of fungal spores. *Nature* **272:** 608-610.

66. Talbot NJ, Ebbole DJ, Hamer JE (1993) Identification and characterization of MPG1, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. *Plant Cell* **5:** 1575-1590.

67. Lugones LG, Wosten HAB, Birkenkamp KU, Sjollema KA, Zagers J, Wessels JGH (1999) Hydrophobins line air channels in fruiting bodies of *Schizophyllum commune* and *Agaricus bisporus*. *Mycol Res* **103:** 635-640.

68. Hess WM, Sassen MM, Remsen CC (1968) Surface characteristics of *Penicillum* conidia. *Mycologia* **60:** 290-303.

69. Hess WM, Sassen MM, Remsen CC (1966) Surface structures of frozen-etched *Penicillium* conidiospores. *Naturwissenschaften* **53:** 708.

70. Sassen MM, Remsen CC, Hess WM (1967) Fine structure of *Penicillium megasporum* conidiospores. *Protoplasma* **64:** 75-88.

71. Hess WM, Stocks DL (1969) Surface characteristics of *Aspergillus* conidia. *Mycologia* **61:** 560-571.

72. Cole GT, Sekiya T, Kasai R, Yokoyama T, Nozawa Y (1979) Surface ultrastructure and chemical composition of the cell walls of conidial fungi. *Exp Mycol* **3:** 132-156.

73. van der Aa BC, Asther M, DufreÌ‚ne YF (2002) Surface properties of *Aspergillus oryzae* spores investigated by atomic force microscopy. *Colloids Surface B* **24:** 277-284.

74. Ghiorse WC, Edwards MR (1973) Ultrastructure of *Aspergillus fumigatus* conidia development and maturation. *Protoplasma* **76:** 49-59.

75. Wessels JG, Kreger DR, Marchant R, Regensburg BA, de Vries OM (1972) Chemical and morphological characterization of the hyphal wall surface of the basidiomycete *Schizophyllum commune*. *Biochim Biophys Acta* **273:** 346-358.

76. Gerin PA, Asther M, Sleytr UB, Rouxhet PG (1994) Detection of rodlets in the outer wall region of conidiospores of *Phanerochaete chrysosporium*. *Can J Microbiol* **40:** 412-416.

77. Claverie-Martin F, Diaz-Torrez MR, Geoghegan M (1986) Chemical composition and electron microscopy of the rodlet layer of *Aspergillus nidulans* conidia. *Curr Microbiol* **14:** 221-225.

78. Dague E, Alsteens D, Latge JP, Dufrene YF (2008) High-resolution cell surface dynamics of germinating *Aspergillus fumigatus* conidia. *Biophys J* **94:** 656-660.

79. Hobot JA, Gull K (1981) Changes in the organization of surface rodlets during germination of *Syncephalastrum racemosum* sporangiospores. *Protoplasma* **107:** 339-343.

80. Wosten H, De VO, Wessels J (1993) Interfacial Self-Assembly of a Fungal Hydrophobin into a Hydrophobic Rodlet Layer. *Plant Cell* **5:** 1567-1574.

81. Wosten HA, Schuren FH, Wessels JG (1994) Interfacial self-assembly of a hydrophobin into an amphipathic protein membrane mediates fungal attachment to hydrophobic surfaces. *EMBO J* **13:** 5848-5854.

82. Wosten HA, Asgeirsdottir SA, Krook JH, Drenth JH, Wessels JG (1994) The fungal hydrophobin Sc3p self-assembles at the surface of aerial hyphae as a protein membrane constituting the hydrophobic rodlet layer. *Eur J Cell Biol* **63:** 122-129.

83. Lugones LG, Wosten HA, Wessels JG (1998) A hydrophobin (ABH3) specifically secreted by vegetatively growing hyphae of *Agaricus bisporus* (common white button mushroom). *Microbiology* **144:** 2345-2353.

84. Lugones LG, Bosscher JS, Scholtmeyer K, de Vries OM, Wessels JG (1996) An abundant hydrophobin (ABH1) forms hydrophobic rodlet layers in *Agaricus bisporus* fruiting bodies. *Microbiology* **142:** 1321-1329.

85. de Vocht ML, Scholtmeijer K, van der Vegte EW, de Vries OM, Sonveaux N, Wosten HA, Ruysschaert JM, Hadziloannou G, Wessels JG, Robillard GT (1998) Structural

characterization of the hydrophobin SC3, as a monomer and after self-assembly at hydrophobic/hydrophilic interfaces. *Biophys J* **74:** 2059-2068.

86. Torkkeli M, Serimaa R, Ikkala O, Linder M (2002) Aggregation and self-assembly of hydrophobins from *Trichoderma reesei*: low-resolution structural models. *Biophys J* **83:** 2240-2247.

87. Askolin S, Linder M, Scholtmeijer K, Tenkanen M, Penttila M, de Vocht ML, Wosten HA (2006) Interaction and comparison of a class I hydrophobin from *Schizophyllum commune* and class II hydrophobins from *Trichoderma reesei*. *Biomacromolecules* **7:** 1295-1301.

88. Templeton MD, Greenwood DR, Beever RE (1995) Solubilization of *Neurospora crassa* rodlet proteins and identification of the predominant protein as the proteolytically processed *eas* (ccg-2) gene product. *Exp Mycol* **19:** 166-169.

89. Talbot NJ, Kershaw MJ, Wakley GE, De VO, Wessels J, Hamer JE (1996) MPG1 Encodes a Fungal Hydrophobin Involved in Surface Interactions during Infection-Related Development of *Magnaporthe grisea*. *Plant Cell* **8:** 985-999.

90. Morris VK, Ren Q, Macindoe I, Kwan AH, Byrne N, Sunde M (2011) Recruitment of class I hydrophobins to the air:water interface initiates a multi-step process of functional amyloid formation. *J Biol Chem* **286:** 15955-15963.

91. Zhang S, Xia YX, Kim B, Keyhani NO (2011) Two hydrophobins are involved in fungal spore coat rodlet layer assembly and each play distinct roles in surface interactions, development and pathogenesis in the entomopathogenic fungus, *Beauveria bassiana*. *Mol Microbiol* **80:** 811-826.

92. Wosten HA, de Vries OM, van der Mei HC, Busscher HJ, Wessels JG (1994) Atomic composition of the hydrophobic and hydrophilic membrane sides of self-assembled SC3p hydrophobin. *J Bacteriol* **176:** 7085-7086.

93. Zampieri F, Wosten HAB, Scholtmeijer K (2010) Creating surface properties using a palette of hydrophobins. *Materials* **3:** 4607-4625.

94. Pedersen MH, Borodina I, Moresco JL, Svendsen WE, Frisvad JC, Sondergaard I (2011) High-yield production of hydrophobins RodA and RodB from *Aspergillus fumigatus* in *Pichia pastoris*. *Appl Microbiol Biotechnol* **90:** 1923-1932.

95. Wessels JGH (1992) Gene expression during fruiting in *Schizophyllum commune*. *Mycol Res* **96:** 609-620.

96. Carpenter CE, Mueller RJ, Kazmierczak P, Zhang L, Villalon DK, Van Alfen NK (1992) Effect of a virus on accumulation of a tissue-specific cell-surface protein of the fungus *Cryphonectria (Endothia) parasitica*. *Mol Plant Microbe Interact* **5:** 55-61.

97. Russo PS, Blum FD, Ipsen JD, Abulhajj YL, Miller WG (1982) The surface-activity of the phytotoxin cerato-ulmin. *Can J Bot* **60:** 1414-1422.

98. NakariSetala T, Aro N, Kalkkinen N, Alatalo E, Penttila M (1996) Genetic and biochemical characterization of the *Trichoderma reesei* hydrophobin HFBI. *Eur J Biochem* **235:** 248-255.

99. Wosten HA, van Wetter MA, Lugones LG, van der Mei HC, Busscher HJ, Wessels JG (1999) How a fungus escapes the water to grow into the air. *Curr Biol* **9:** 85-88.

100. Asgeirsdottir SA, de Vries OM, Wessels JG (1998) Identification of three differentially expressed hydrophobins in *Pleurotus ostreatus* (oyster mushroom). *Microbiology* **144:** 2961-2969.

101. Scholtmeijer K, Wessels JG, Wosten HA (2001) Fungal hydrophobins in medical and technical applications. *Appl Microbiol Biotechnol* **56:** 1-8.

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

103. Wessels JGH (1996) Fungal hydrophobins: Proteins that function at an interface. *Trends Plant Sci* **1:** 9-15.

104. Bailey MJ, Askolin S, Horhammer N, Tenkanen M, Linder M, Penttila M, Nakari-Setala T (2002) Process technological effects of deletion and amplification of hydrophobins I and II in transformants of *Trichoderma reesei*. *Appl Microbiol Biotechnol* **58:** 721-727.

105. Askolin S, Nakari-Setala T, Tenkanen M (2001) Overproduction, purification, and characterization of the *Trichoderma reesei* hydrophobin HFBI. *Appl Microbiol Biotechnol* **57:** 124-130.

106. BASF (2012) www.basf.com, Accessed: 30-01-2012.

107. Janssen MI, van Leeuwen MB, Scholtmeijer K, van Kooten TG, Dijkhuizen L, Wosten HA (2002) Coating with genetic engineered hydrophobin promotes growth of fibroblasts on a hydrophobic solid. *Biomaterials* **23:** 4847-4854.

108. Li X, Hou S, Feng X, Yu Y, Ma J, Li L (2009) Patterning of neural stem cells on poly(lactic-co-glycolic acid) film modified by hydrophobin. *Colloids Surf B Biointerfaces* **74:** 370-374.

109. Hou S, Li X, Li X, Feng X (2009) Coating of hydrophobins on three-dimensional electrospun poly(lactic-co-glycolic acid) scaffolds for cell adhesion. *Biofabrication* **1:** 035004.

110. Hektor HJ, Scholtmeijer K (2005) Hydrophobins: proteins with potential. *Curr Opin Biotechnol* **16:** 434-439.

111. Haas Jimoh Akanbi M, Post E, Meter-Arkema A, Rink R, Robillard GT, Wang X, Wosten HAB, Scholtmeijer K (2010) Use of hydrophobins in formulation of water insoluble drugs for oral administration. *Colloid Surface B* **75:** 526-531.

112. Bilewicz R, Witomski J, Van der Heyden A, Tagu D, Palin B, Rogalska E (2001) Modification of electrodes with self-assembled hydrophobin layers. *J Phys Chem B* **105:** 9772-9777.

113. Corvis Y, Trzcinska, Rink, Bilkova, Gorecka, Bilewicz, Rogalska (2007) Electron-donor-acceptor fullerene derivative retained on electrodes using SC3 hydrophobin. *J Phys Chem C (USA)* **111:** 1176-1179.

114. Corvis Y, Rogalska E, Walcarius A, Rink R, Mrabet NT (2005) Preparing Catalytic Surfaces for Sensing Applications by Immobilizing Enzymes via Hydrophobin Layers. *Anal Chem* **77:** 1622-1630.

115. Cox AR, Aldred DL, Russell AB (2009) Exceptional stability of food foams using class II hydrophobin HFBII. *Food Hydrocolloids* **23:** 366-376.

116. Gabin, V. (2003) Cosmetic use of at least one hydrophobin for treating keratin materials and compositions used. United States patent application US 20030217419.

117. Sarlin T, Kivioja T, Kalkkinen N, Linder MB, Nakari-Setala T (2012) Identification and characterization of gushing-active hydrophobins from *Fusarium graminearum* and related species. *J Basic Microbiol* **52:** 184-194.

118. Sarlin T, Nakari-Setala T, Linder M, Penttila M, Haikara A (2005) Fungal hydrophobins as predictors of the gushing activity of malt. *J Inst Brew* **111:** 105-111.

119. Zapf MW, Theisen S, Vogel RF, Niessen L (2006) Cloning of wheat LTP1500 and two *Fusarium culmorum* hydrophobins in *Saccharomyces cerevisiae* and assessment of their gushing inducing potential in experimental wort fermentation. *J Inst Brew* **112:** 237-245.

120. Opwis K, Gutmann JS (2011) Surface modification of textile materials with hydrophobins. *Text Res J* **81:** 1594-1602.

121. Peleg AY, Hogan DA, Mylonakis E (2010) Medically important bacterial-fungal interactions. *Nat Rev Microbiol* **8:** 340-349.

122. Frey-Klett P, Burlinson P, Deveau A, Barret M, Tarkka M, Sarniguet A (2011) Bacterial-fungal interactions: hyphens between agricultural, clinical, environmental, and food microbiologists. *Microbiol Mol Biol Rev* **75:** 583-609.

123. Wargo MJ, Hogan DA (2006) Fungal-bacterial interactions: a mixed bag of mingling microbes. *Curr Opin Microbiol* **9:** 359-364.

124. Tarkka MT, Sarniguet A, Frey-Klett P (2009) Inter-kingdom encounters: recent advances in molecular bacterium-fungus interactions. *Curr Genet* **55:** 233-243.

125. Bauernfeind A, Bertele RM, Harms K, Horl G, Jungwirth R, Petermuller C, Przyklenk B, Weisslein-Pfister C (1987) Qualitative and quantitative microbiological analysis of sputa of 102 patients with cystic fibrosis. *Infection* **15:** 270-277.

126. Yang L, Jelsbak L, Molin S (2011) Microbial ecology and adaptation in cystic fibrosis airways. *Environ Microbiol* **13:** 1682-1689.

127. Chotirmall SH, O'Donoghue E, Bennett K, Gunaratnam C, O'Neill SJ, McElvaney NG (2010) Sputum *Candida albicans* presages FEV decline and hospital-treated exacerbations in cystic fibrosis. *Chest* **138:** 1186-1195.

128. Shwab EK, Keller NP (2008) Regulation of secondary metabolite production in filamentous ascomycetes. *Mycol Res* **112:** 225-230.

129. Ruiz B, Chavez A, Forero A, Garcia-Huante Y, Romero A, Sanchez M, Rocha D, Sanchez B, Rodriguez-Sanoja R, Sanchez S, Langley E (2010) Production of microbial secondary metabolites: regulation by the carbon source. *Crit Rev Microbiol* **36:** 146-167.

130. Price-Whelan A, Dietrich LE, Newman DK (2006) Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. *Nat Chem Biol* **2:** 71-78.

131. Hoffmeister D, Keller NP (2007) Natural products of filamentous fungi: enzymes, genes, and their regulation. *Nat Prod Rep* **24:** 393-416.

132. Keller NP, Hohn TM (1997) Metabolic pathway gene clusters in filamentous fungi. *Fungal Genet Biol* **21:** 17-29.

133. Gross H, Loper JE (2009) Genomics of secondary metabolite production by *Pseudomonas* spp. *Nat Prod Rep* **26:** 1408-1446.

134. Yu JH, Keller N (2005) Regulation of secondary metabolism in filamentous fungi. *Annu Rev Phytopathol* **43:** 437-458.

135. Fox EM, Howlett BJ (2008) Secondary metabolism: regulation and role in fungal biology. *Curr Opin Microbiol* **11:** 481-487.

136. Newman DJ, Cragg GM (2012) Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J Nat Prod* **75:** 311-335.

137. Nielsen KF, Mogensen JM, Johansen M, Larsen TO, Frisvad JC (2009) Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. *Anal Bioanal Chem* **395:** 1225-1242.

138. Frisvad JC, Rank C, Nielsen KF, Larsen TO (2009) Metabolomics of *Aspergillus fumigatus*. *Med Mycol* **47:** S53-S71.

139. Rank C, Larsen TO, Frisvad JC, Machida M, Gomi K (2010) Functional Systems Biology of *Aspergillus*. In: Machida M, Gomi K, editors. *Aspergillus*: Molecular Biology and Genomics. Norfolk, UK: Caister Academic Press. pp. 173-198.

140. Klejnstrup ML, Frandsen RJN, Holm DK, Nielsen MT, Mortensen UH, Larsen TO, Nielsen JB (2012) Genetics of polyketide metabolism in *Aspergillus nidulans*. *Metabolites* **2:** 100-133.

141. Sanchez R, Somoza AD, Keller NP, Wang CC (2012) Advances in *Aspergillus* secondary metabolite research in the post-genomic era. *Nat Prod Rep* **29:** 351-371.

142. Brakhage AA, Schroeckh V (2011) Fungal secondary metabolites - strategies to activate silent gene clusters. *Fungal Genet Biol* **48:** 15-22.

143. Larsen TO, Smedsgaard J, Nielsen KF, Hansen MA, Samson RA, Frisvad JC (2007) Production of mycotoxins by *Aspergillus lentulus* and other medically important and closely related species in section *Fumigati*. *Med Mycol* **45:** 225-232.

144. Frisvad JC, Andersen B, Thrane U (2008) The use of secondary metabolite profiling in chemotaxonomy of filamentous fungi. *Mycol Res* **112:** 231-240.

145. Frisvad JC, Larsen TO, de VR, Meijer M, Houbraken J, Cabanes FJ, Ehrlich K, Samson RA (2007) Secondary metabolite profiling, growth profiles and other tools for species recognition and important *Aspergillus* mycotoxins. *Stud Mycol* **59:** 31-37.

146. Mavrodi DV, Bonsall RF, Delaney SM, Soule MJ, Phillips G, Thomashow LS (2001) Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **183:** 6454-6465.

147. Pierson LS, III, Pierson EA (2010) Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and biotechnological processes. *Appl Microbiol Biotechnol* **86:** 1659-1670.

148. Larsen TO, Smedsgaard J, Nielsen KF, Hansen ME, Frisvad JC (2005) Phenotypic taxonomy and metabolite profiling in microbial drug discovery. *Nat Prod Rep* **22:** 672-695.

149. Nielsen KF, Smedsgaard J (2003) Fungal metabolite screening: database of 474 mycotoxins and fungal metabolites for dereplication by standardised liquid chromatography-UV-mass spectrometry methodology. *J Chromatogr A* **1002:** 111-136.

150. Nielsen KF, Mansson M, Rank C, Frisvad JC, Larsen TO (2011) Dereplication of microbial natural products by LC-DAD-TOFMS. *J Nat Prod* **74:** 2338-2348.

151. Hook DJ, Pack EJ, Yacobucci JJ, Guss J (1997) Approaches to automating the dereplication of bioactive natural products - The key step in high throughput screening of bioactive materials from natural sources. *J Biomol Screen* **2:** 145-152.

152. Filtenborg O, Frisvad JC, Svendsen JA (1983) Simple screening method for molds producing intracellular mycotoxins in pure cultures. *Appl Environ Microbiol* **45:** 581-585.

153. Filtenborg O, Frisvad JC (1980) A simple screening-method for toxigenic moulds in pure cultures. *Food Sci Technol-Leb* **13:** 128-130.

154. Frisvad JC, Thrane U (1987) Standardized high-performance liquid chromatography of 182 mycotoxins and other fungal metabolites based on alkylphenone retention indices and UV-VIS spectra (diode array detection). *J Chromatogr* **404:** 195-214.

155. Frisvad JC (1987) High-performance liquid chromatographic determination of profiles of mycotoxins and other secondary metabolites. *J Chromatogr* **392:** 333-347.

156. Frisvad JC, Filtenborg O, Thrane U (1989) Analysis and screening for mycotoxins and other secondary metabolites in fungal cultures by thin-layer chromatography and high-performance liquid chromatography. *Arch Environ Contam Toxicol* **18:** 331-335.

157. Wolfender JL (2009) HPLC in Natural Product Analysis: The Detection Issue. *Planta Medica* **75:** 719-734.

158. Lebedev AT (2009) Introduction to mass spectra interpretation: organic chemistry. In: Ekman R, Silberring J, Westman-Brinkmalm AM, Kraj A, editors. Mass spectrometry - Instrumentation, interpretation and applications. Hoboken: John Wiley & Sons. pp. 119-178.

159. Westman-Brinkmalm AM, Brinkmalm G (2009) Definitions and explanations. In: Ekman R, Silberring J, Westman-Brinkmalm AM, Kraj A, editors. Mass spectrometry - Instrumentation, interpretation and applications. Hoboken: John Wiley & Sons. pp. 3-13.

160. Amin R, Dupuis A, Aaron SD, Ratjen F (2010) The effect of chronic infection with *Aspergillus fumigatus* on lung function and hospitalization in patients with cystic fibrosis. *Chest* **137:** 171-176.

161. Navarro J, Rainisio M, Harms HK, Hodson ME, Koch C, Mastella G, Strandvik B, McKenzie SG (2001) Factors associated with poor pulmonary function: cross-sectional analysis of data from the ERCF. European Epidemiologic Registry of Cystic Fibrosis. *Eur Respir J* **18:** 298-305.

162. Mangan A (1969) Interactions between some aural aspergillus species and bacteria. *J Gen Microbiol* **58:** 261-266.

163. Blyth W, Forey A (1971) The influence of respiratory bacteria and their biochemical fractions on *Aspergillus fumigatus*. *Sabouraudia* **9:** 273-282.

164. Blyth W (1971) Modifications in the ultrastructure of *Aspergillus fumigatus* due to the presence of living cells of *Pseudomonas aeruginosa*. *Sabouraudia* **9:** 283-286.

165. Kerr JR (1994) Suppression of fungal growth exhibited by *Pseudomonas aeruginosa*. *J Clin Microbiol* **32:** 525-527.

166. Mowat E, Rajendran R, Williams C, McCulloch E, Jones B, Lang S, Ramage G (2010) *Pseudomonas aeruginosa* and their small diffusible extracellular molecules inhibit *Aspergillus fumigatus* biofilm formation. *FEMS Microbiol Lett* **313:** 96-102.

167. Brandl MT, Carter MQ, Parker CT, Chapman MR, Huynh S, Zhou Y (2011) *Salmonella* biofilm formation on *Aspergillus niger* involves cellulose--chitin interactions. *PLoS One* **6:** e25553.

168. Schroeckh V, Scherlach K, Nutzmann HW, Shelest E, Schmidt-Heck W, Schuemann J, Martin K, Hertweck C, Brakhage AA (2009) Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. *Proc Natl Acad Sci U S A* **106:** 14558-14563.

169. Hogan DA, Kolter R (2002) *Pseudomonas-Candida* interactions: an ecological role for virulence factors. *Science* **296:** 2229-2232.

170. Hogan DA, Vik A, Kolter R (2004) A *Pseudomonas aeruginosa* quorum-sensing molecule influences *Candida albicans* morphology. *Mol Microbiol* **54:** 1212-1223.

171. Rella A, Yang MW, Gruber J, Montagna MT, Luberto C, Zhang YM, Del PM (2012) *Pseudomonas aeruginosa* Inhibits the Growth of *Cryptococcus* Species. *Mycopathologia* **173:** 451-461.

172. Lau GW, Ran H, Kong F, Hassett DJ, Mavrodi D (2004) *Pseudomonas aeruginosa* pyocyanin is critical for lung infection in mice. *Infect Immun* **72:** 4275-4278.

173. Wilson R, Sykes DA, Watson D, Rutman A, Taylor GW, Cole PJ (1988) Measurement of *Pseudomonas aeruginosa* phenazine pigments in sputum and assessment of their contribution to sputum sol toxicity for respiratory epithelium. *Infect Immun* **56:** 2515-2517.

174. Kerr JR, Taylor GW, Rutman A, Hoiby N, Cole PJ, Wilson R (1999) *Pseudomonas aeruginosa* pyocyanin and 1-hydroxyphenazine inhibit fungal growth. *J Clin Pathol* **52:** 385-387.

175. Gibson J, Sood A, Hogan DA (2009) *Pseudomonas aeruginosa-Candida albicans* interactions: localization and fungal toxicity of a phenazine derivative. *Appl Environ Microbiol* **75:** 504-513.

176. Morales DK, Jacobs NJ, Rajamani S, Krishnamurthy M, Cubillos-Ruiz JR, Hogan DA (2010) Antifungal mechanisms by which a novel *Pseudomonas aeruginosa* phenazine toxin kills *Candida albicans* in biofilms. *Mol Microbiol* **78:** 1379-1392.

177. Hill JC, Johnson GT (1969) Microbial transformation of phenazines by *Aspergillus sclerotiorum*. *Mycologia* **61:** 452-467.

178. Chin AWT, Bloemberg GV, Mulders IH, Dekkers LC, Lugtenberg BJ (2000) Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. *Mol Plant-Microbe Interact* **13:** 1340-1345.

179. Anjaiah V, Koedam N, Nowak-Thompson B, Loper JE, Hofte M, Tambong JT, Cornelis P (1998) Involvement of phenazines and anthranilate in the antagonism of *Pseudomonas aeruginosa* PNA1 and Tn5 derivatives toward *Fusarium* spp. and *Pythium* spp. *Mol Plant-Microbe Interact* **11:** 847-854.

180. Juhas M, Eberl L, Tummler B (2005) Quorum sensing: the power of cooperation in the world of *Pseudomonas*. *Environ Microbiol* **7:** 459-471.

181. Hornby JM, Jensen EC, Lisec AD, Tasto JJ, Jahnke B, Shoemaker R, Dussault P, Nickerson KW (2001) Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl Environ Microbiol* **67:** 2982-2992.

182. McAlester G, O'Gara F, Morrissey JP (2008) Signal-mediated interactions between *Pseudomonas aeruginosa* and *Candida albicans*. *J Med Microbiol* **57:** 563-569.

183. Semighini CP, Hornby JM, Dumitru R, Nickerson KW, Harris SD (2006) Farnesol-induced apoptosis in *Aspergillus nidulans* reveals a possible mechanism for antagonistic interactions between fungi. *Mol Microbiol* **59:** 753-764.

184. Coleman JJ, Ghosh S, Okoli I, Mylonakis E (2011) Antifungal activity of microbial secondary metabolites. *PLoS One* **6:** e25321.

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

186. Shibuya K, Takaoka M, Uchida K, Wakayama M, Yamaguchi H, Takahashi K, Paris S, Latge JP, Naoe S (1999) Histopathology of experimental invasive pulmonary aspergillosis in rats: pathological comparison of pulmonary lesions induced by specific virulent factor deficient mutants. *Microb Pathog* **27:** 123-131.

187. Schmidt S, Tramsen L, Hanisch M, Latge JP, Huenecke S, Koehl U, Lehrnbecher T (2011) Human natural killer cells exhibit direct activity against *Aspergillus fumigatus* hyphae, but not against resting conidia. *J Infect Dis* **203:** 430-435.

188. Mowat E, Williams C, Jones B, McChlery S, Ramage G (2008) The characteristics of *Aspergillus fumigatus* mycetoma development: is this a biofilm? *Med Mycol* **47:** S120-S126.

189. Ramage G, Mowat E, Jones B, Williams C, Lopez-Ribot J (2009) Our current understanding of fungal biofilms. *Crit Rev Microbiol* **35:** 340-355.

190. Mowat E, Butcher J, Lang S, Williams C, Ramage G (2007) Development of a simple model for studying the effects of antifungal agents on multicellular communities of *Aspergillus fumigatus*. *J Med Microbiol* **56:** 1205-1212.

191. Mowat E, Butcher J, Williams C, Ramage G (2007) *Aspergillus fumigatus* biofilms are refractory to antifungal challenge. *Int J Androl* **29:** S147-S148.

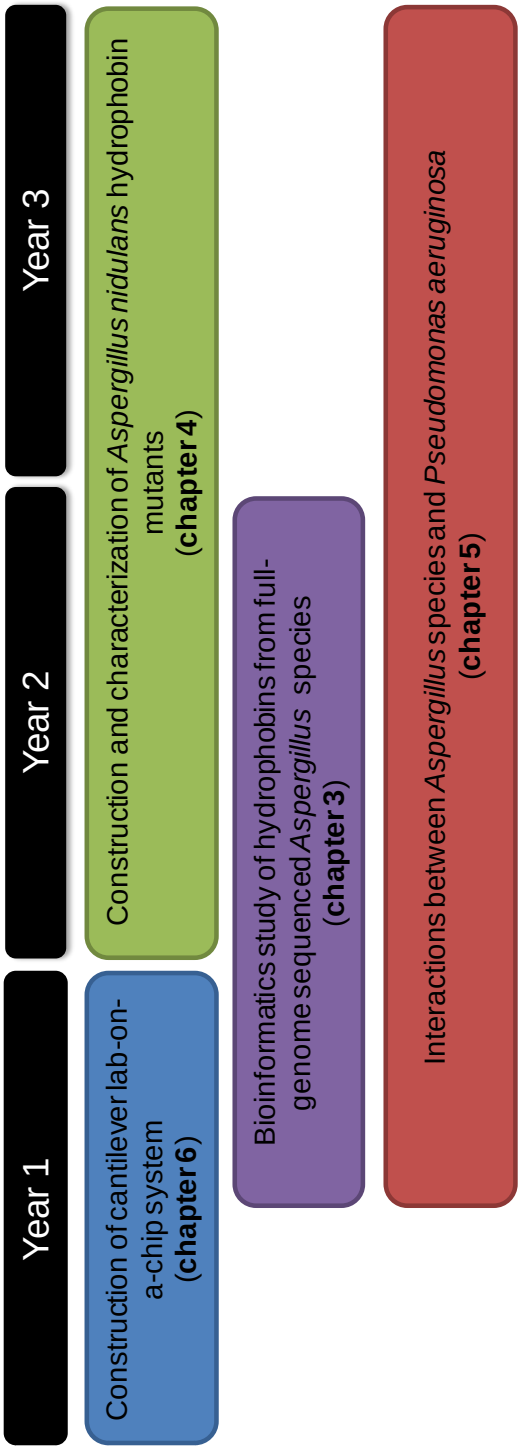
192. Mowat E, Lang S, Williams C, McCulloch E, Jones B, Ramage G (2008) Phase-dependent antifungal activity against *Aspergillus fumigatus* developing multicellular filamentous biofilms. *J Antimicrob Chemother* **62:** 1281-1284.

193. Seidler MJ, Salvenmoser S, Muller FM (2008) *Aspergillus fumigatus* forms biofilms with reduced antifungal drug susceptibility on bronchial epithelial cells. *Antimicrob Agents Chemother* **52:** 4130-4136.

194. Bruns S, Seidler M, Albrecht D, Salvenmoser S, Remme N, Hertweck C, Brakhage AA, Kniemeyer O, Muller FM (2010) Functional genomic profiling of *Aspergillus fumigatus* biofilm reveals enhanced production of the mycotoxin gliotoxin. *Proteomics* **10:** 3097-3107.

2 Overview of experimental work

This figure is intended to provide an overview of the main experiments performed during the course of this thesis. The obtained results and experimental design will be presented and discussed in the denoted chapters.



3 Identification and classification of *Aspergillus* hydrophobins

This chapter presents results from an article published in *BMC Research Notes*, 2010 3:344. For details on methods see article 1.

Introduction

Hydrophobins are a family of small hydrophobic proteins found uniquely in filamentous fungi[1]. Originally hydrophobins were characterized as proteins of approximately 100 AA with little amino acid sequence homology apart from eight conserved cysteines in a characteristic pattern (C-CC-C-C-CC-C)[2,3]. The hydrophobins contained two cysteine pairs[2,3] and the eight cysteines were found to form four disulfide bonds in the pattern Cys1-Cys6, Cys2-Cys5, Cys3-Cys4, Cys7-Cys8[4] (see figure 3 in chapter 1).

Based on their distinct hydropathy patterns (figure 1) and physical properties, hydrophobins were traditionally divided into two classes[3].

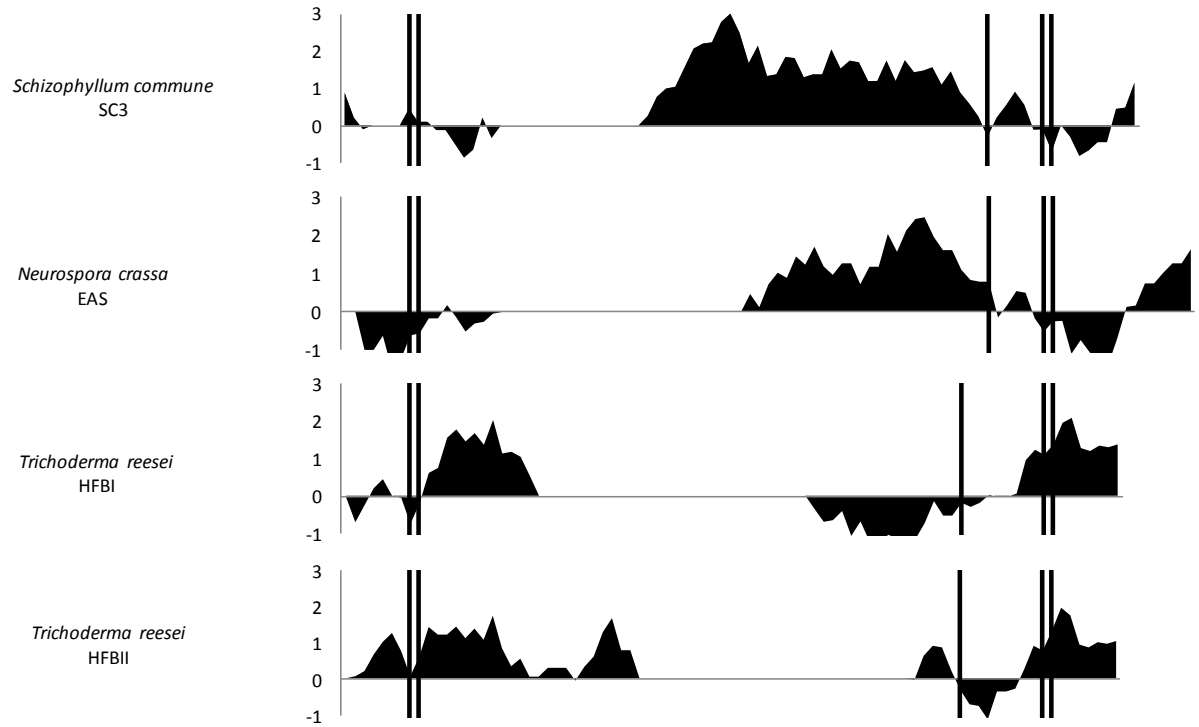


Figure 1: Hydropathy patterns of known class I hydrophobins SC3 and EAS and class II hydrophobins HFBI and HFBII. The amino acids are shown along the x-axis, where the cysteine pairs are indicated by two adjacent vertical lines. The hydrophobic amino acids are shown above the x-axis, while hydrophilic amino acids are displayed below. Only the part of the sequence from the first to the eight cysteine was used to create the hydropathy plot.

Class I hydrophobins have been identified in Ascomycetes and Basiodiomycetes, while class II hydrophobins have solely been identified in Ascomycetes[1]. Typically, a single fungal species expresses either class I or class II hydrophobins, however previous studies have shown that some species have the ability to express both classes of hydrophobins[5,6]. In class I hydrophobins the cysteine doublets are followed by hydrophilic amino acids, while hydrophobic amino acids are observed after the cysteine doublets in class II hydrophobins[2]. This can be visualized using hydropathy plots (figure 1). Furthermore, considerable variation is seen in the cysteine spacing of class I hydrophobins, while less variation is seen for class II hydrophobins[7].

In the last 10 years many *Aspergillus* species have been full genome sequenced including *A. fumigatus*, *A. niger*, *A. flavus*, *A. oryzae*, *A. nidulans*, *A. terreus* and *A. clavatus*. In this thesis the available sequences were used to examine the Aspergilli for new hydrophobins providing for the first time a complete list of putative *Aspergillus* hydrophobins. The identified hydrophobins were furthermore putatively classified as class I, class II or intermediate hydrophobins by examining the cysteine spacing and hydropathy patterns.

Results

Identification of *Aspergillus* hydrophobins

Nine full genome sequenced *Aspergillus* species were used to search for new hydrophobins including *A. fumigatus* AF293 and A1163, *A. niger* CBS 513.88 and ATCC 1015, *A. flavus* NRRL 3357, *A. oryzae* RIB40, *A. nidulans* FGSC A4, *A. terreus* NIH 2624 and *A. clavatus* NRRL 1. A total of 50 potential hydrophobins were identified (table 1) based on the criteria of two cysteine pairs, a minimum of eight cysteines, a size of app. 100 - 200 AA and an intact cysteine pattern. Proteins having up to 12 cysteines were included in the study, if additional cysteines were located outside the pattern (C-CC-C-C-CC-C) and thus did not alter the cysteine pattern.

On species level, twenty of the identified hydrophobins had not previously been mentioned in other published studies, while the number was thirty-one on strain level (table 1). The number of identified hydrophobins within the species varied from two to eight between the nine species. By using the SignalP server[17], which predicts the presence and location of signal peptide cleavage sites in amino acid sequences, all identified hydrophobins were found to contain theoretical signal sequences and therefore have the possibility of being secreted. The identified *Aspergillus* hydrophobins contained approximately 100 – 200 amino acids and were

8 – 30 kDa in size. Furthermore, they had eight to ten cysteines, where excess cysteines (above eight) were located before or after the conserved cysteine spacing pattern. By examining a multiple alignment (see figure S3 in article 1) of the putative hydrophobins very low similarity was observed between the hydrophobins.

Table 1: Identified *Aspergillus* hydrophobins.

Species	Gene	Size (Da)	n (AA)	n (cys)	Eight cysteine pattern[1]	Theoretical class	Common name[2]
A. oryzae RIB40	AO090012000143	14304	145	8	CN(8)CCN(38)CN(10)CN(5)CCN(21)C	I	RolA[a]
	AO090020000588	15231	151	8	CN(7)CCN(39)CN(17)CN(5)CCN(17)C	I	New
A. niger CBS 513.88	An03g02360[b]	12486	122	8	CN(6)CCN(32)CN(25)CN(5)CCN(4)C	I	
	An03g02400[b]	13063	131	8	CN(6)CCN(31)CN(23)CN(5)CCN(6)C	Intermediate	
	An04g08500[b]	14397	146	8	CN(7)CCN(39)CN(20)CN(5)CCN(17)C	I	
	An15g03800[b]	13225	130	8	CN(5)CCN(32)CN(6)CN(5)CCN(13)C	Intermediate	
	An01g10940[b]	10693	100	8	CN(14)CCN(17)CN(11)CN(7)CCN(8)C	Intermediate	
	An07g03340[b]	16207	162	8	CN(7)CCN(39)CN(21)CN(5)CCN(17)C	I	
	An09g05530[b]	20465	202	9	CN(8)CCN(33)CN(11)CN(5)CCN(16)C	Intermediate	
	An08g09880[b]	9169	91	9	CN(7)CCN(16)CN(6)CN(5)CCN(10)C	Intermediate	
A. niger ATCC 1015	JGI128530	10803	105	7	Fragment (similar to An07g03340)	Intermediate	(New)
	JGI35683	10693	100	8	CN(14)CCN(17)CN(11)CN(7)CCN(8)C	Intermediate	(New)
	JGI45683	13063	131	8	CN(6)CCN(31)CN(23)CN(5)CCN(6)C	Intermediate	(New)
	JGI45685	13716	132	8	CN(6)CCN(32)CN(25)CN(5)CCN(14)C	I	(New)
	JGI53462	13224	130	8	CN(5)CCN(32)CN(6)CN(5)CCN(13)C	Intermediate	(New)
	JGI194815	14397	146	8	CN(7)CCN(39)CN(20)CN(5)CCN(17)C	I	(New)
	JGI43184	20381	201	9	CN(8)CCN(33)CN(11)CN(5)CCN(16)C	Intermediate	(New)
E. nidulans FGSC A4	AN7539.2[c]	10798	109	8	CN(5)CCN(32)CN(6)CN(5)CCN(13)C	Intermediate	
	AN8803.2[c]	15625	157	8	CN(7)CCN(39)CN(18)CN(5)CCN(17)C	I	RodA[d]
	AN6401.2[c]	16131	162	8	CN(6)CCN(38)CN(22)CN(5)CCN(35)C	Intermediate	
	AN8006.2[c]	13183	135	8	CN(6)CCN(31)CN(23)CN(5)CCN(6)C	I	DewA[e]
	AN1837.2[c]	13397	135	8	CN(7)CCN(39)CN(18)CN(5)CCN(17)C	I	
	AN0940.2[c]	10594	101	8	CN(13)CCN(17)CN(12)CN(7)CCN(8)C	Intermediate	
A. fumigatus AF293	AFUA_8G07060	15996	155	8	CN(7)CCN(39)CN(21)CN(5)CCN(17)C	I	RodC[f]
	AFUA_5G09580	16153	159	8	CN(7)CCN(39)CN(21)CN(5)CCN(17)C	I	RodA[f/g]
	AFUA_2G14661	12928	125	8	CN(5)CCN(32)CN(6)CN(5)CCN(13)C	Intermediate	New
	AFUA_1G17250	14299	140	8	CN(7)CCN(36)CN(18)CN(5)CCN(18)C	I	RodB[f/h]
	AFUA_5G03280	19825	190	9	CN(7)CCN(33)CN(11)CN(5)CCN(14)C	I	RodF[f]
A. fumigatus A1163	AFUB_016640	14300	140	8	CN(7)CCN(36)CN(18)CN(5)CCN(18)C	I	(RodB New)
	AFUB_057130	16153	159	8	CN(7)CCN(39)CN(21)CN(5)CCN(17)C	I	(RodA New)
	AFUB_080740	15996	155	8	CN(7)CCN(39)CN(21)CN(5)CCN(17)C	I	(RodC New)
	AFUB_051810	19825	190	9	CN(7)CCN(33)CN(11)CN(5)CCN(14)C	Intermediate	(RodF New)
A. terreus NIH 2624	ATEG_10285	13978	129	8	CN(5)CCN(28)CN(14)CN(8)CCN(13)C	Intermediate	New
	ATEG_08089	18936	177	8	CN(8)CCN(33)CN(11)CN(5)CCN(14)C	Intermediate	New
	ATEG_07808	11677	115	8	CN(5)CCN(32)CN(6)CN(5)CCN(13)C	Intermediate	New
	ATEG_06492	17374	175	8	CN(7)CCN(40)CN(16)CN(5)CCN(17)C	I	New

	ATEG_04730	11797	121	8	CN(10)CCN(11)CN(16)CN(8)CCN(10)C	II	New
A. flavus NRRL 3357	AFLA_094600	8377	83	8	CN(7)CCN(16)CN(6)CN(5)CCN(9)C	Intermediate	New
	AFLA_131460	10867	106	8	CN(5)CCN(32)CN(6)CN(5)CCN(13)C	Intermediate	New
	AFLA_060780	27807	251	8	CN(6)CCN(30)CN(23)CN(5)CCN(4)C	I	New
	AFLA_014260	14304	145	8	CN(8)CCN(38)CN(10)CN(5)CCN(21)C	I	New
	AFLA_063080	9362	87	9	CN(5)CCN(17)CN(7)CN(7)CCN(12)C	Intermediate	New
	AFLA_098380	23415	217	10	CN(7)CCN(39)CN(17)CN(5)CCN(44)C	I	New
	AFLA_064900	9147	91	10	CN(7)CCN(15)CN(6)CN(5)CCN(8)C	Intermediate	New
A. clavatus NRRL 1	ACLA_001890	10214	100	8	CN(7)CCN(16)CN(6)CN(5)CCN(26)C	Intermediate	New
	ACLA_048810	18458	182	8	CN(7)CCN(33)CN(11)CN(5)CCN(15)C	Intermediate	New
	ACLA_010960	14671	145	8	CN(7)CCN(39)CN(21)CN(5)CCN(17)C	I	New
	ACLA_072820	16127	158	8	CN(7)CCN(39)CN(21)CN(5)CCN(17)C	I	New
	ACLA_018290	12820	126	8	CN(5)CCN(32)CN(6)CN(5)CCN(13)C	Intermediate	New
	ACLA_007980	14558	144	8	CN(7)CCN(36)CN(18)CN(5)CCN(17)C	Intermediate	New

[1]In the eight cysteine pattern, "N", signifies any other amino acid than cysteine.
[2]In the common name column, "New", signifies that the hydrophobins has not previously been mentioned in other published studies.
[a] described by Takahashi *et al.*[8], [b] mentioned by Pel *et al.*[9], [c] mentioned by de Groot *et al.*[10], [d] described by Stringer *et al.*[11], [e] described by Stringer *et al.*[12], [f] described by Beauvais *et al.*[13], [g] RodA described by Parta *et al.* and Thau *et al.*[14,15], [h] RodB described by Paris *et al.*[16]

Classification of *Aspergillus* hydrophobins

The Pfam database (pfam.sanger.ac.uk) contains a large collection of protein families characterized by unique domains in each familiy. By analyzing the identified *Aspergillus* hydrophobins for Pfam matches, forty-five of the identified proteins were found to contain domains classifying them as hydrophobins by Pfam. The remaining five hydrophobins (An01g10940, JGI35683, AN0940.2, AFLA_063080, ATEG_10285) could not be classified as hydrophobins using Pfam and do therefore not contain known domains classifying them as hydrophobins and may as a consequence not be hydrophobins. As these five proteins, however, fulfil known hydrophobin criteria (see table 1), they could constitute a novel group of hydrophobins.

To further classify the hydrophobins, hydropathy patterns were created for all identified putative hydrophobins using ProtScale[19] on the ExPASy server. Hydrophobins were classified as class I hydrophobins if the cysteine doublets were followed by hydrophilic amino acids and as class II hydrophobins if hydrophobic amino acids were observed after the cysteine doublets[2]. Twenty-three of the identified hydrophobins displayed a characteristic class I hydropathy plot (not shown) and displayed a class I cysteine pattern[7] and where therefore classified as class I hydrophobins (table 1). Twenty six of the putative hydrophobins could not be classified into the original two classes and were found to be intermediate forms due to

inconsistency between the type of cysteine spacing pattern and hydropathy pattern. Fifteen of the intermediate class hydrophobins displayed hydrophobicity patterns dissimilar to known class I and class II hydrophobins (see table 1 and additional file 2 in article 1). Only a single hydrophobin was identified as a class II hydrophobin, displayed a characteristic class II cysteine spacing pattern[7] and had a class II hydropathy pattern (table 1).

To examine the putative hydrophobins, a phylogenetic tree was constructed (figure 2) and it seemed that hydrophobins clustered according to their cysteine spacing pattern.

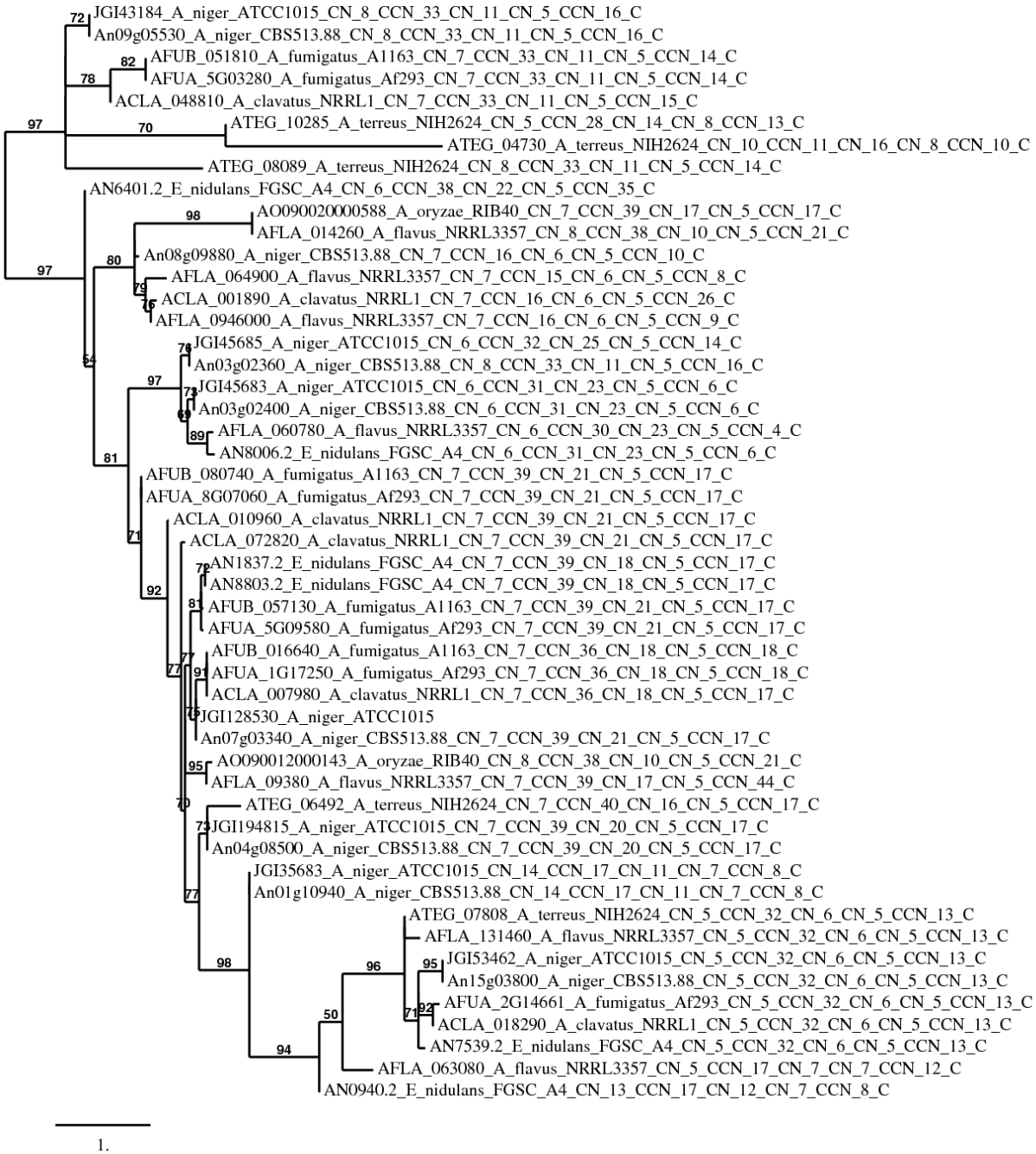


Figure 2: Phylogenetic tree of identified hydrophobins in the *Aspergillus* species. The phylogenetic tree was constructed based on a multiple alignment of identified hydrophobins using Phylogeny.fr[18]. Branches with support values less than 50 % were collapsed. "N" signifies any other amino acid than cysteine.

A common feature in 44 of the 50 hydrophobins was a conserved spacing of five amino acids between the fifth and sixth cysteines, while the remaining six hydrophobins contained either seven or eight amino acids (table 1). This spacing of five cysteines had also been observed in other known class I hydrophobins (eg. SC3, EAS and MPG1)[7] and may be a common feature in class I hydrophobins.

Four of the hydrophobins (An01g10940, JGI35683, AN0940.2, AFLA_063080) could be differentiated from the rest of the hydrophobins in displaying a distinctive cysteine pattern of CN(5-13)CCN(17)CN(7-12)CN(7)CCN(8-12)C (where "N" signifies any other amino acid than cysteine) and may constitute a new group of hydrophobins. These did, however, not have similar hydropathy patterns (figure 3) and may thus not have similar solubility characteristics and could fulfil diverse functions in the different fungi.

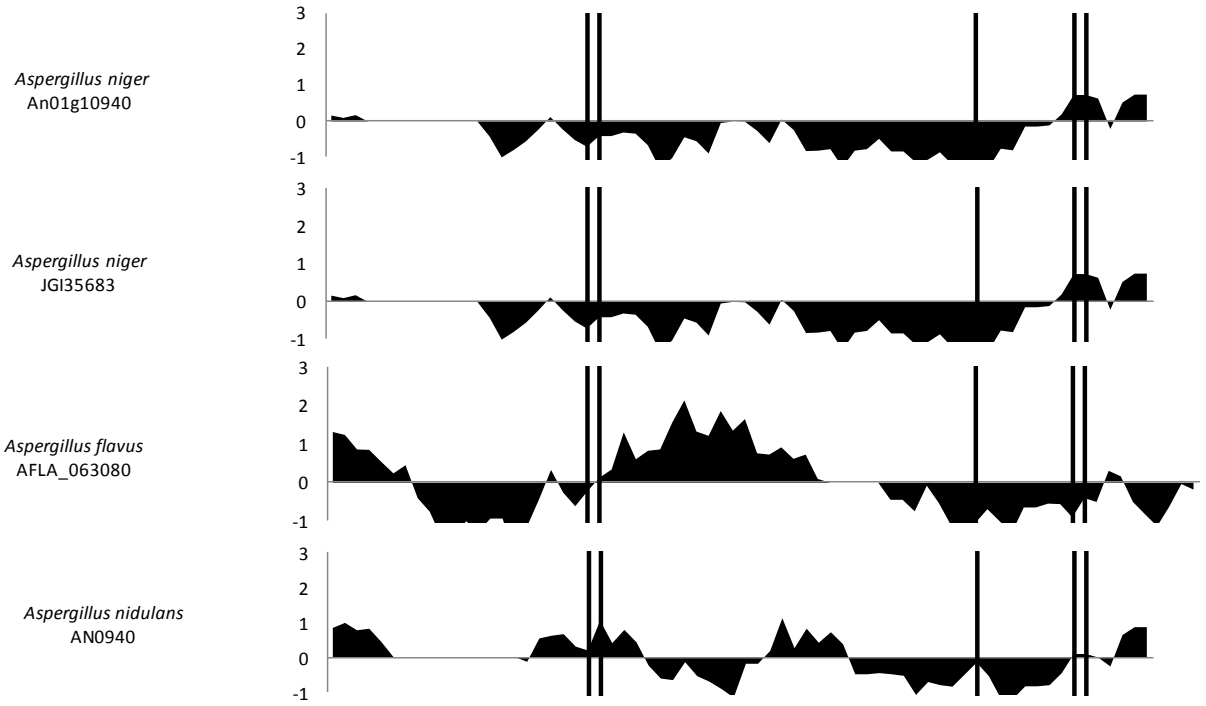


Figure 3: Hydropathy patterns of hydrophobins An01g10940, JGI35683, AFLA_063080 and AN0940. The amino acids are shown along the x-axis, where the cysteine pairs are indicated by two adjacent vertical lines. The hydrophobic amino acids are shown above the x-axis, while hydrophilic amino acids are displayed below. Only the part of the sequence from the first to the eight cysteine was used to create the hydropathy plot.

Identification of a class II hydrophobin in *Aspergillus terreus*

By examining the identified putative hydrophobins, a single hydrophobin from *A. terreus*, ATEG_04730, was found to display a characteristic class II spacing pattern (CN(10)CCN(11)CN(16)CN(8)CCN(10)C) and furthermore had a class II hydropathy pattern, when compared to known class II hydrophobins from *Trichoderma reesei* (figure 4).

Interestingly, *A. terreus* seemed to have genes for both class I and class II hydrophobins as the hydrophobin ATEG_06492 was identified as a class I hydrophobin. Comparison of ATEG_04730 to class II hydrophobins HFBI and HFBII showed 37 % and 35 % sequence identity, while comparison to class I hydrophobins RodA, SC3 and EAS showed 21 %, 16 % and 20 % sequence identity. In contrast ATEG_06492 showed 20 % and 29 % sequence identity to class II hydrophobins HFBI and HFBII, but 51 %, 21 % and 24 % to class I hydrophobins RodA, SC3 and EAS.

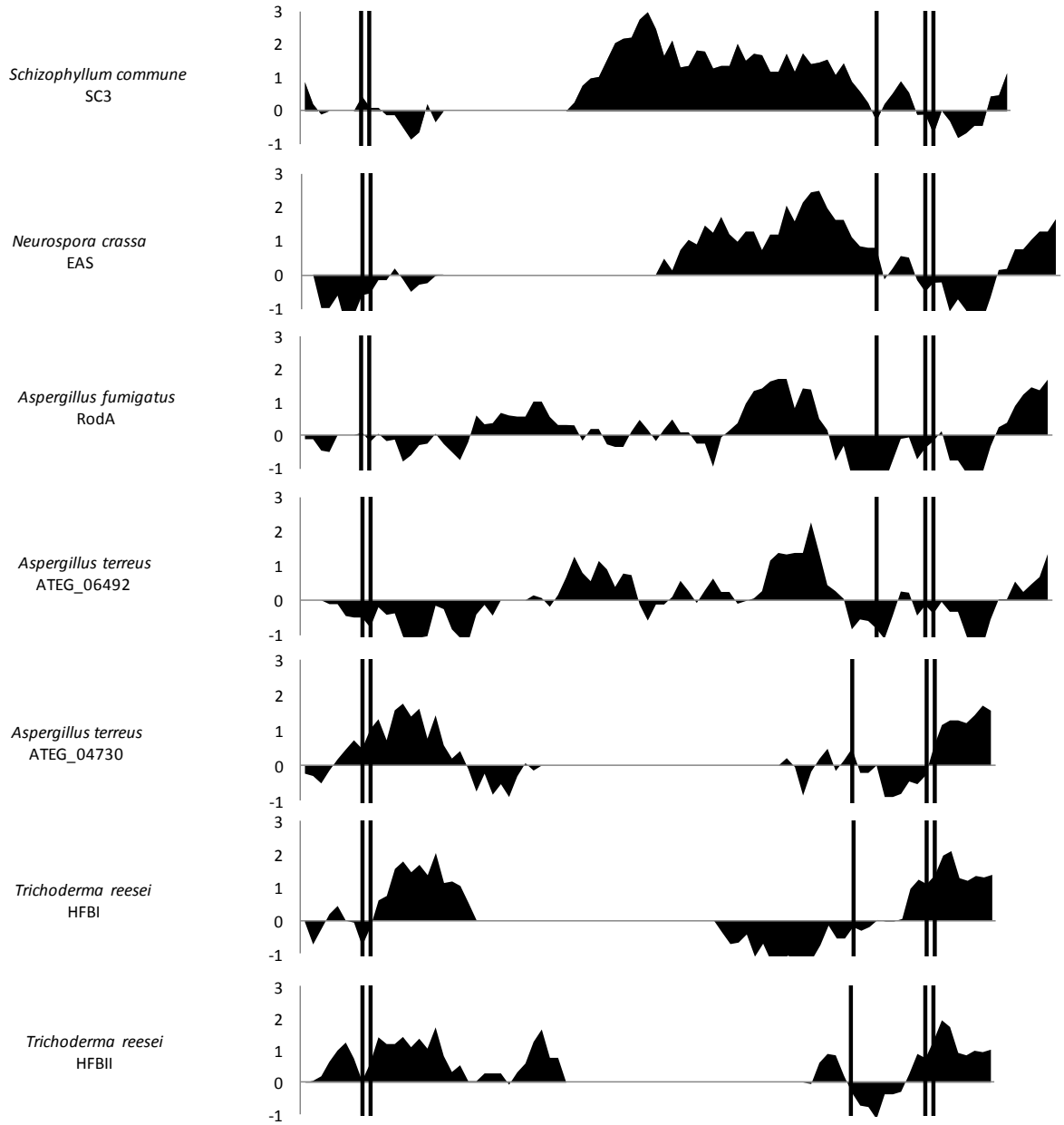


Figure 4: Comparison of hydropathy patterns of identified putative *A. terreus* hydrophobins ATEG_06492 and ATEG_04730 with hydropathy patterns of known hydrophobins SC3 from *S. commune*, EAS from *N. crassa*, RodA from *A. fumigatus* and HFBI and HFBII from *T. reesei*. The amino acids of the hydrophobins are shown along the x-axis, where cysteines are indicated by vertical lines. Hydrophobic amino acids are shown above the x-axis, while hydrophilic amino acids are shown below. Only the part of the sequence from the first to the eighth cysteine was used to create the hydropathy pattern.

Furthermore, a phylogenetic analysis (figure 5) showed that ATEG_04730 clustered with HFBI and HFBII, while ATEG_06492 clustered with RodA, EAS and SC3, strongly indicating that ATEG_04730 can indeed be classified as a class II hydrophobin, while ATEG_06492 is classified as a class I hydrophobin.

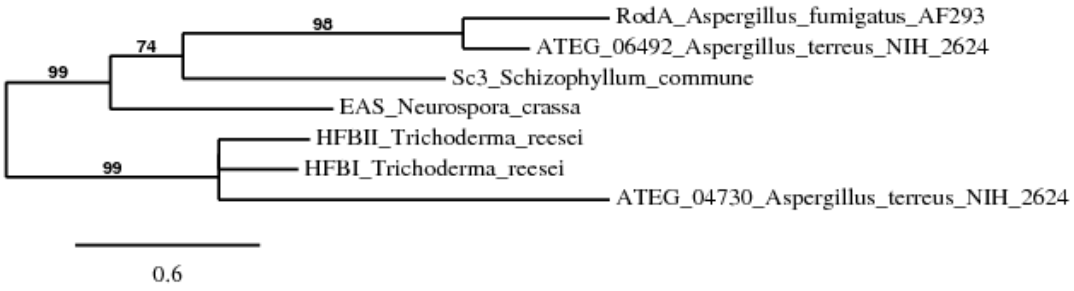


Figure 5: Phylogenetic analysis of known hydrophobins SC3 (*S. commune)*, EAS (*N. crassa*), RodA (*A. fumigatus*), HFBI and HFBII (*T. reesei*) and the putative hydrophobins ATEG_06492 and ATEG_04730 identified in *A. terreus*. The phylogenetic tree was constructed based on a multiple alignment of identified hydrophobins using Phylogeny.fr[18]. Branches with support values less than 50 % were collapsed.

Discussion

In this study fifty hydrophobins were identified in nine genome sequences from seven Aspergilli. Each species was found to display between two and eight hydrophobins, which was in agreement with Sunde *et al.*[20] who predicted that most fungal species contain between two and seven hydrophobins. The criteria used in this study for identifying the putative hydrophobins included size, a minimum of eight cysteines, two cysteine pairs and an intact characteristic spacing pattern. These criteria were based on observations by Wessels [2] and the structure of known hydrophobins[4,21–23]. Other studies[13,24] include additional potential *Aspergillus* hydrophobins, but these do not fulfil our criteria and where therefore not included in this study. If these proposed proteins are hydrophobins, the definition of hydrophobins should be expanded. This can however only be confirmed after physical isolation and characterization of the potential hydrophobins.

The cysteine spacing pattern and hydropathy plots of the identified hydrophobins were used to make a preliminary classification of the hydrophobins. Twenty-three of the identified hydrophobins could theoretically be classified as class I hydrophobins. However, the majority of the hydrophobins (twenty-six) were found to be intermediate forms and could not be classified as class I or class II hydrophobins, showing that many hydrophobins do not confer to the original classification system created by Wessels[3]. In agreement with our observations Littlejohn *et al.*[24] found several *Aspergillus* hydrophobins, which could not be classified as

class I or class II hydrophobins. Similarly, a novel set of *Trichoderma* hydrophobins have been identified which diverge and form a new subclass distinct from the original two classes [25]. This indicates that many fungal species express intermediate form hydrophobins, which may thus also exhibit solubility characteristics between the two known classes. As these intermediate forms blur the original classification, it could be speculated, whether an extension of the classical two class system would be in place as more fungal genomes become available. Nevertheless, as the majority of the identified hydrophobins have not physically been isolated and characterized, a differentiation into type of class is only provisional.

In this study a single class II hydrophobin was identified in the examined *Aspergillus* species namely ATEG_04730 from *A. terreus*. This was recently supported by Littlejohn *et al.*[24] and no other class II hydrophobin has been identified in *Aspergillus* spp. so far. In addition to the identified class II hydrophobin, *A. terreus* displayed four other hydrophobins including a potential class I hydrophobin. Many previously examined fungal species only express either class I or class II hydrophobins. Nevertheless, it seems that some species may potentially express both class I and class II hydrophobins[5,6,25]. In this study *Aspergillus* species were found to be able to express different classes of hydrophobins, where single species may express both class I hydrophobins, intermediate forms and for *A. terreus* also class II hydrophobins. The repertoire of different hydrophobins found in *Aspergillus* species may be expressed at different developmental stages of the fungal life cycle and may be required for fulfilling different biological functions in the fungus.

Concluding remarks

In this study nine genome sequences from seven Aspergilli revealed fifty hydrophobins, where each species displayed between two and eight hydrophobins. Twenty-three of the identified hydrophobins could be classified as class I hydrophobins based on their conserved cysteine spacing pattern and hydropathy pattern, but the majority seemed to be intermediate forms. A single hydrophobin, ATEG_04730, from *Aspergillus terreus* displayed a clear class II cysteine spacing and had a class II hydropathy pattern. As *Aspergillus terreus* also has the potential to express a class I hydrophobin, this is the first reported case of an *Aspergillus* species with the potential to express both class I and class II hydrophobins. The varying repertoire of hydrophobins observed in the *Aspergillus* species may be expressed at different developmental stages of the fungal life cycle. The hydrophobins may thus fulfil different biological functions and may even be able to compensate for each other.

References for chapter 3

1. Wosten HA (2001) Hydrophobins: multipurpose proteins. *Annu Rev Microbiol* **55:** 625-646.

2. Wessels JGH (1997) Hydrophobins: Proteins that change the nature of the fungal surface. *Adv Microb Physiol* **38:** 1-45.

3. Wessels JGH (1994) Developmental Regulation of Fungal Cell-Wall Formation. *Annu Rev Phytopathol* **32:** 413-437.

4. Kwan AH, Winefield RD, Sunde M, Matthews JM, Haverkamp RG, Templeton MD, Mackay JP (2006) Structural basis for rodlet assembly in fungal hydrophobins. *Proc Natl Acad Sci U S A* **103:** 3621-3626.

5. Linder MB, Szilvay GR, Nakari-Setala T, Penttila ME (2005) Hydrophobins: the protein-amphiphiles of filamentous fungi. *FEMS Microbiol Rev* **29:** 877-896.

6. Segers GC, Hamada W, Oliver RP, Spanu PD (1999) Isolation and characterisation of five different hydrophobin-encoding cDNAs from the fungal tomato pathogen *Cladosporium fulvum*. *Mol Gen Genet* **261:** 644-652.

7. Kershaw MJ, Talbot NJ (1998) Hydrophobins and repellents: proteins with fundamental roles in fungal morphogenesis. *Fungal Genet Biol* **23:** 18-33.

8. Takahashi T, Maeda H, Yoneda S (2005) The fungal hydrophobin RolA recruits polyesterase and laterally moves on hydrophobic surfaces. *Mol Microbiol* **57:** 1780-1796.

9. Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, Turner G, de Vries RP, Albang R, Albermann K, Andersen MR, Bendtsen JD, Benen JA, van den Berg M, Breestraat S, Caddick MX, Contreras R, Cornell M, Coutinho PM, Danchin EG, Debets AJ, Dekker P, van Dijck PW, van DA, Dijkhuizen L, Driessen AJ, d'Enfert C, Geysens S, Goosen C, Groot GS, de Groot PW, Guillemette T, Henrissat B, Herweijer M, van den Hombergh JP, van den Hondel CA, van der Heijden RT, van der Kaaij RM, Klis FM, Kools HJ, Kubicek CP, van Kuyk PA, Lauber J, Lu X, van der Maarel MJ, Meulenberg R, Menke H, Mortimer MA, Nielsen J, Oliver SG, Olsthoorn M, Pal K, van Peij NN, Ram AF, Rinas U, Roubos JA, Sagt CM, Schmoll M, Sun J, Ussery D, Varga J, Vervecken W, van de Vondervoort PJ, Wedler H, Wosten HA, Zeng AP, van Ooyen AJ, Visser J, Stam H (2007) Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat Biotechnol* **25:** 221-231.

10. de Groot PW, Brandt BW, Horiuchi H, Ram AF, de Koster CG, Klis FM (2009) Comprehensive genomic analysis of cell wall genes in *Aspergillus nidulans*. *Fungal Genet Biol* **46:** S72-S81.

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

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

13. Beauvais A, Schmidt C, Guadagnini S, Roux P, Perret E, Henry C, Paris S, Mallet A, Prevost MC, Latge JP (2007) An extracellular matrix glues together the aerial-grown hyphae of *Aspergillus fumigatus*. *Cell Microbiol* **9:** 1588-1600.

14. Parta M, Chang Y, Rulong S, Pinto-DaSilva P, Kwon-Chung KJ (1994) HYP1, a hydrophobin gene from *Aspergillus fumigatus*, complements the rodletless phenotype in *Aspergillus nidulans*. *Infect Immun* **62:** 4389-4395.

15. Thau N, Monod M, Crestani B, Rolland C, Tronchin G, Latge JP, Paris S (1994) rodletless mutants of Aspergillus fumigatus. *Infect Immun* **62:** 4380-4388.

16. Paris S, Debeaupuis JP, Crameri R, Carey M, Charles F, Prevost MC, Schmitt C, Philippe B, Latge JP (2003) Conidial hydrophobins of *Aspergillus fumigatus*. *Appl Environ Microbiol* **69:** 1581-1588.

17. Bendtsen JD, Nielsen H, von HG, Brunak S (2004) Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* **340:** 783-795.

18. Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard JF, Guindon S, Lefort V, Lescot M, Claverie JM, Gascuel O (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res* **36:** W465-W469.

19. Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkens MR, et al. (2005) Protein Identification and Analysis Tools on the ExPASy Server. In: Walker JM, editors. The Proteomics Protocols Handbook. Humana Press Inc. pp. 571-607.

20. Sunde M, Kwan AH, Templeton MD, Beever RE, Mackay JP (2008) Structural analysis of hydrophobins. *Micron* **39:** 773-784.

21. Hakanpaa J, Szilvay GR, Kaljunen H, Maksimainen M, Linder M, Rouvinen J (2006) Two crystal structures of *Trichoderma reesei* hydrophobin HFBI - the structure of a protein amphiphile with and without detergent interaction. *Protein Sci* **15:** 2129-2140.

22. Hakanpaa J, Linder M, Popov A, Schmidt A, Rouvinen J (2006) Hydrophobin HFBII in detail: ultrahigh-resolution structure at 0.75 A. *Acta Crystallogr D Biol Crystallogr* **62:** 356-367.

23. Hakanpaa J, Paananen A, Askolin S, Nakari-Setala T, Parkkinen T, Penttila M, Linder MB, Rouvinen J (2004) Atomic resolution structure of the HFBII hydrophobin, a self-assembling amphiphile. *J Biol Chem* **279:** 534-539.

24. Littlejohn KA, Hooley P, Cox PW (2012) Bioinformatics predicts diverse *Aspergillus* hydrophobins with novel properties. *Food Hydrocolloids* **27:** 503-516.

25. Seidl-Seiboth V, Gruber S, Sezerman U, Schwecke T, Albayrak A, Neuhof T, von DH, Baker SE, Kubicek CP (2011) Novel hydrophobins from *Trichoderma* define a new hydrophobin subclass: protein properties, evolution, regulation and processing. *J Mol Evol* **72:** 339-351.

4 Characterization of *Aspergillus nidulans* hydrophobins and their potential biological functions

This chapter presents a current study involving construction and characterizing of *Aspergillus nidulans* hydrophobins. For details on materials and methods see appendix 1.

Introduction

Hydrophobins have been proposed to be involved in a large variety of functions in fungal growth and development[1] and may be expressed at different stages of development[2]. Some hydrophobins can lower the water surface tension by assembling into stable amphipathic membranes at water-air interfaces and thereby allow hyphae to breach through the interface[3]. This is especially useful for filamentous fungi, which commonly colonize moist environments, where they sometimes have to breach the water-air interface to grow and release spores into the air. Exposed to air, hydrophobins make the fungal surfaces hydrophobic allowing spores to be dispersed in the air and capable of binding to hydrophobic surfaces[1].

Based on the full genome sequence of *Aspergillus nidulans* FGSC A4, six hydrophobins have been predicted in *A. nidulans*; namely AN0940, AN1837, AN6401, AN7539, AN8006 (DewA) and AN8803 (RodA)[4,5]. Of the six hydrophobins only two, RodA and DewA, have previously been studied[6–8]. Recently Littlejohn *et al.*[9] proposed the existence of four additional hydrophobins in *A. nidulans*. Two of the proposed hydrophobins, AN4845 and AN6807, are very large in size, contain numerous cysteines and do therefore not confer to the classical definition of hydrophobins[1,10]. The other two putative hydrophobins, AN5290 and AN7327, have nine and ten cysteines respectively, but have an intact cysteine pattern and fulfil all other hydrophobin criteria, thus these may be hydrophobins. Nevertheless, this study was initiated before the publication of the article by Littlejohn *et al.*[9] and therefore focuses on the six hydrophobins (AN0940, AN1837, AN6401, AN7539, DewA and RodA) originally identified which have eight cysteines and fulfil all original hydrophobin criteria.

The aim of this study was to gain more insight into the different roles and biological functions of hydrophobins in *A. nidulans*, by creating single deletion as well as over-expression strains of the different *A. nidulans* hydrophobins and testing them in several assays. As DewA and RodA have previously been characterized, these strains were included to verify previous

results and for comparison to the other created hydrophobin strains. This study will contribute to understanding the roles and biological functions of hydrophobins in the filamentous model fungus, *A. nidulans*.

Results

Defining the putative hydrophobins of *A. nidulans*

In the *A. nidulans* genome six genes encoding putative hydrophobins have been identified; AN0940, AN1837, AN6401, AN7539, AN8006 (*dewA*) and AN8803 (*rodA*)[4,5]. They vary in size (10 - 17 kDa), but all contain eight cysteines in the characteristic pattern for hydrophobins and have a signal sequence (figure 1).

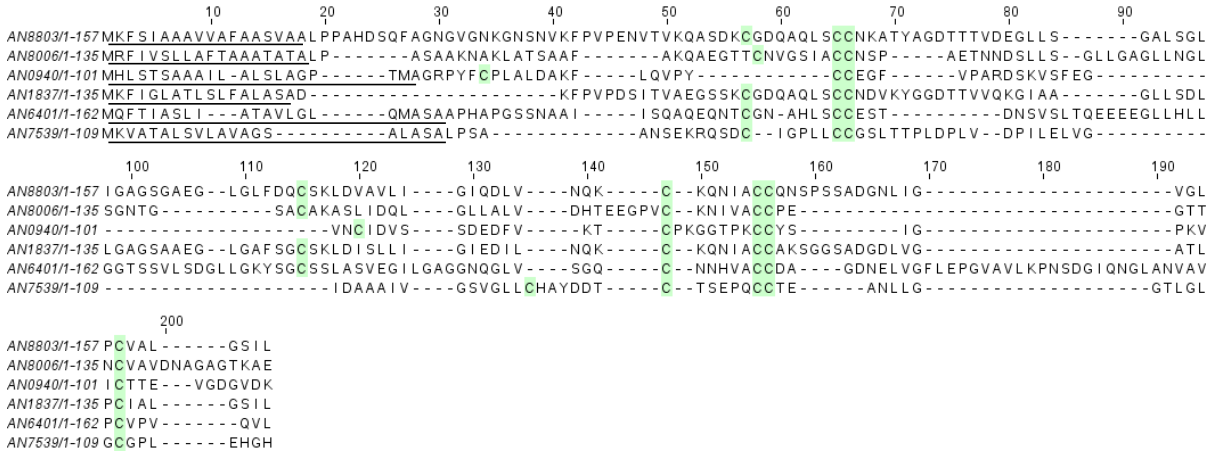


Figure 1: Alignment of *A. nidulans* hydrophobins. *A. nidulans* has six hydrophobins containing eight cysteines (highlighted in green) in the characteristic pattern for hydrophobins (C-CC-C-C-CC-C). The hydrophobins vary in size from ~ 10 – 17 kDa and contain ~ 100 – 150 amino acids. All six hydrophobins have signal sequences (underlined). Apart from the eight cysteines low sequence similarity is observed.

Five of the hydrophobins contain a stretch of five amino acids between the fifth and sixth cysteine, while AN0940 has seven amino acids between the cysteines. This is often observed in class I hydrophobins, while class II hydrophobins contain a stretch of approximately nine amino acids[11].

Based on the spacing between the cysteines and their hydropathy patterns, three hydrophobins (RodA, DewA and AN1837) can be classified as putative class I hydrophobins (figure 2). They display similar hydropathy patterns to known class I hydrophobins (SC3 and EAS) and previous work by Stringer *et al.*[6,7] has confirmed RodA and DewA as class I hydrophobins. The remaining three hydrophobins, AN0940, AN6401 and AN7539 are presumably

intermediate forms of the two classes as they do not display a characteristic class I or class II hydropathy pattern.

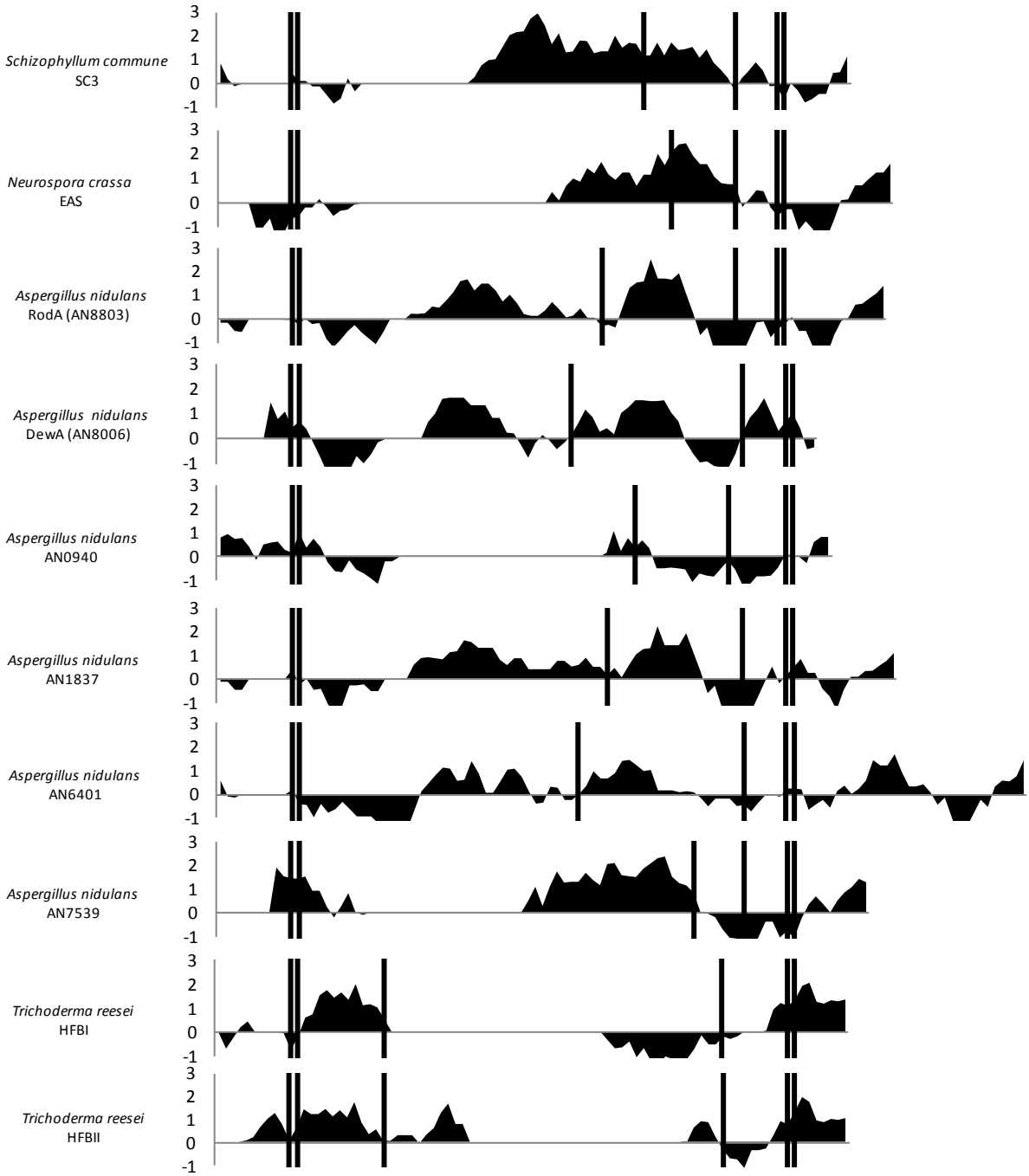


Figure 2: Hydropathy patterns of *A. nidulans* hydrophobins including hydropathy patterns of class I hydrophobins SC3 from *S. commune*, EAS from *N. crassa* and class II hydrophobins HFBI and HFBII from *T. reesei* for comparison. The amino acids of the hydrophobins are displayed along the x-axis, where cysteines are indicated by vertical lines. Hydrophobic amino acids are shown above the x-axis, while hydrophilic amino acids are shown below. Only the part of the sequence from the first to the eighth cysteine was used to create the hydropathy pattern.

Apart from the eight cysteine motif, low to moderate sequence similarity can be observed between the hydrophobins displaying from ~ 10 to 50 % identity (table 1). Highest sequence similarity is observed between RodA and AN1837, while RodA and AN0940 have the lowest sequence similarity. However, the majority display sequence similarities between ~ 10 to 25 % identity.

Table 1: Identity between *A. nidulans* hydrophobins

% identity	RodA	DewA	AN0940	AN1837	AN6401	AN7539
RodA		20.9 %	9.2 %	53.5 %	24.0 %	19.0 %
DewA	20.9 %		14.9 %	18.7 %	20.0 %	20.9 %
AN0940	9.2 %	14.6 %		10.4 %	13.4 %	14.5 %
AN1837	53.5 %	18.7 %	10.4 %		24.0 %	18.7 %
AN6401	24.0 %	20.0 %	13.4 %	14.0 %		19.5 %
AN7539	19.0 %	10.9 %	14.5 %	18.8 %	19.5 %	

Using the protein sequences of the hydrophobins, a BLAST search was conducted on the UniProt Protein Knowledgebase (www.uniprot.org) to examine the percentage of identical amino acids between the *A. nidulans* hydrophobins and other hydrophobins from full genome sequenced *Aspergillus* species (table 2).

Table 2: Comparison of *A. nidulans* hydrophobins to hydrophobins from other full genome sequenced Aspergilli

***A. nidulans* hydrophobin**	**Hydrophobins from other Aspergilli with highest percentage of identical amino acids to the *A. nidulans* hydrophobins[1]**		
RodA	75 % AFUA_5G09580 *A. fumigatus*	69 % ACLA_010960 *A. clavatus*	66 % An07g03340 *A. niger*
DewA	44 % AFLA_060780 *A. flavus*	41 % An03g02400 *A. niger*	41 % An03g02360 *A. niger*
AN0940	38 % An01g10940 *A. niger*		
AN1837	60 % AFUA_5G09580 *A. fumigatus*	57 % ACLA_010960 *A. clavatus*	55 % An07g03340 *A. niger*
AN6401	33 % ACLA_018290 *A. clavatus*	33 % An07g03340 *A. niger*	31 % AFUA_1G17250 *A. fumigatus*
AN7539	74 % ACLA_072820 *A. clavatus*	71 % AFUA_2G14661 *A. fumigatus*	56 % ATEG_07808 *A. terreus*

[1]The identified *A. nidulans* hydrophobins were compared to other full genome sequenced *Aspergillus* hydrophobins by conducting a BLAST search on the UniProt Protein Knowledgebase (www.uniprot.org). The top three matches are shown. Only a single match was found for AN0940.

If hydrophobins have high sequence similarities, these hydrophobins may have similar biological functions. Generally, RodA and AN1837 showed relatively high identity to hydrophobins annotated as RodA from other *Aspergillus* species having e.g. 75 % and 60 % identity to RodA from *A. fumigatus* (AFUA_5G09580). DewA displayed approximately 40 % identity to several hydrophobins, AN0940 and AN6401 hydrophobins had below 40 % identity to other predicted hydrophobins, while the AN7539 hydrophobin had rather high identity to both an *A. fumigatus* and an *A. clavatus* hydrophobin.

Morphological features of hydrophobin deletion strains

To investigate the function of *A. nidulans* hydrophobins, individual knockout mutants of hydrophobins RodA, DewA, AN0940, AN1837 and AN6401 were constructed as well as a double-deletion strain of *rodA*Δ*dewA*Δ. The individual knockout mutant of hydrophobin AN7539 is still under construction and will not be included in the further results and discussion part of this chapter. A *veA1* strain was isolated for use as a reference strain.
The reference and hydrophobin deletion strains were grown on both minimal media (MM[12]) and rich media; WATM (Wickerhams Antibiotic Test Medium[13]), YES (Yeast Extract Sucrose agar[14]) and CYA (Czapek Yeast extract Agar[14]) and examined for phenotypical differences both macroscopically and microscopically. No differences were observed between the strains grown on YES and CYA (not shown), while alternating phenotypes could be observed between the strains grown on MM and WATM (figure 3).

The *rodA*Δ strain displayed a darker colony colour, changing from dark green in the centre to dark brown in the periphery of the colony, compared to the green colour of the reference strain (figure 3). Sporadically white clumps of aerial mycelium were occasionally observed on the colonies, mostly situated near the centre of the colony. The conidia had a wet and sticky appearance, when observed microscopically and were found to be dispersed in clumps of chains in water. When mature colonies were held and tapped over a new empty medium plate, no or few colonies were observed on the new plate, indicating that the spores had impaired ability to be released into the air.

The *dewA*Δ strain displayed similar green coloured conidia as the reference strain (figure 3). The conidia were dry, when observed microscopically, dispersed as single chains in water and could readily be dispersed into the air. In contrast, the double mutant, *rodA*Δ*dewA*Δ,

displayed conidia with dark brown colour. Conidia were similarly to the *rodA*Δ strain dispersed clumped in chains in water and only few spores were released into the air. It seemed, however that the addition of the *dewA* deletion to the *rodA*Δ mutant enhanced the phenotype observed in the *rodA*Δ strain as the spore clumping was more pronounced with larger clumps.

The AN0940, AN1837 and AN6401 mutant strains (AN0940Δ, AN1837Δ and AN6401Δ) all had similar green conidia as the reference strain (figure 3). The conidia were dry and dispersed in single chains or as single conidia in water. When observed microscopically *dewA*Δ, AN0940Δ, AN1837Δ and AN6401Δ had normal conidiophore heads, which was in contrast to *rodA*Δ and *rodA*Δ*dewA*Δ where the heads seemed more dense and clumped. All seven strains did, however, have brown conidiophore stalks and were able to produce hülle cells and ascomata. Furthermore, no differences in radial colony growth could be seen between strains (data not shown).

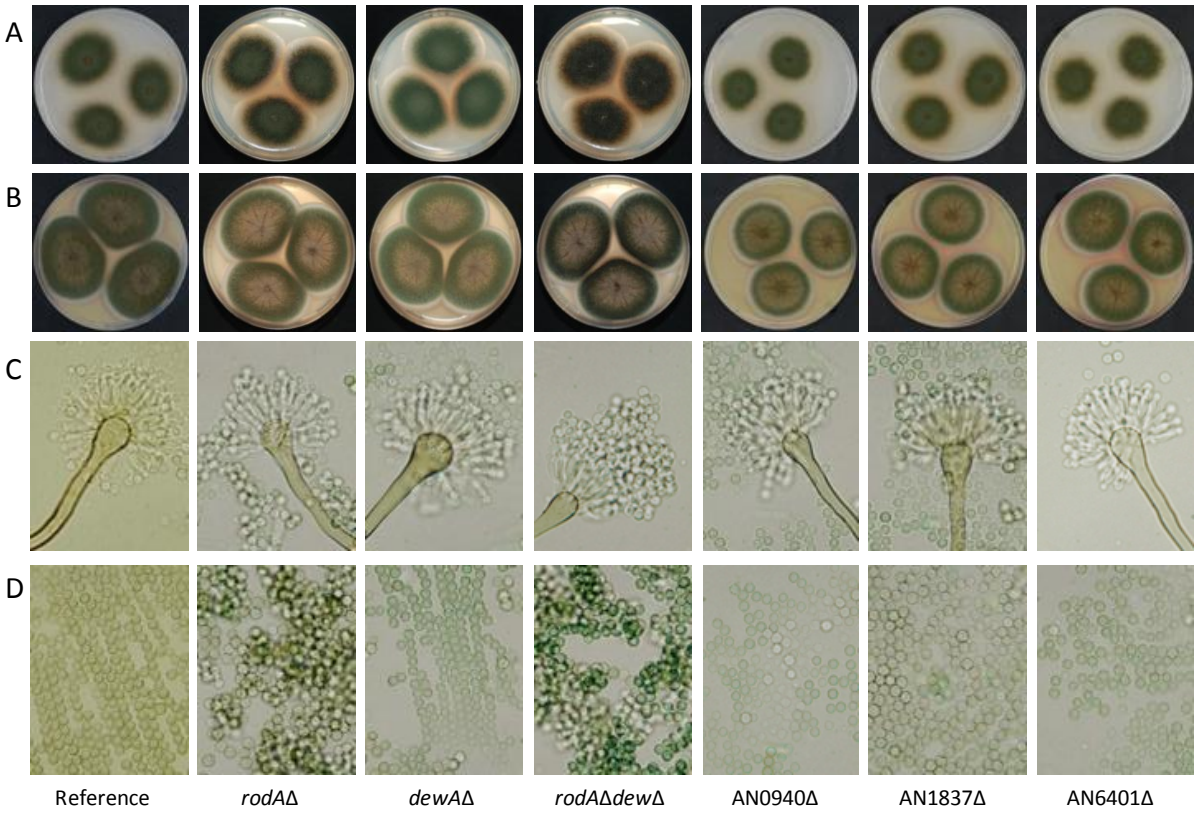


Figure 3: Morphology of hydrophobin deletion strains. *A. nidulans* hydrophobin deletion strains were grown on (A) Minimal media and (B) Wickerhams Antibiotic Test Medium in biological triplicate. The *rodA*Δ and *rodA*Δ*dewA*Δ strains displayed a darker colony colour compared to the other strains. (C) Conidial heads of *rodA*Δ and *rodA*Δ*dewA*Δ seemed more dense and clumped than the other strains and (D) spores dispersed in clumps of chains in water instead of single spores or chains. A minimum of 30 images were examined and a single representative image is shown.

Stringer *et al*.[7] had previously observed a subtle phenotype in a *dewA*Δ strain as this strain wetted rapidly by detergent[7]. To examine whether a similar phenotype could be seen in the other hydrophobin deletion strains, a drop of detergent (0.2 % SDS, 50 mM EDTA) was placed on four days old colonies. The *dewA*Δ strain readily wetted by detergent and was wetted within 30 seconds. An even faster wetting was observed for the *rodA*Δ*dewA*Δ strain, which wetted within 10 seconds, while the *rodA*Δ strain wetted within 1 minute. The remaining strains including the reference, AN0940Δ, AN1837Δ and AN6401Δ wetted within 7-10 minutes, showing that only lack of RodA and DewA give rise to this phenotype.

Rodlets

Hydrophobins RodA and DewA are found on the spore surface and *rodA* is essential for rodlet formation[6,7]. To further characterize the hydrophobin deletion strains and to examine whether the hydrophobins AN0940, AN1837 and AN6401 were involved in rodlet formation, conidia from the reference strain was initially examined by Scanning Electron Microscopy (SEM). Despite several attempts, the use of different SEM techniques and help from experts at the Center for Electron Nanoscopy at DTU visualization of the rodlets on the reference strain as well as on the wild type strain FGSC A4 were unsuccessful (figure 4).

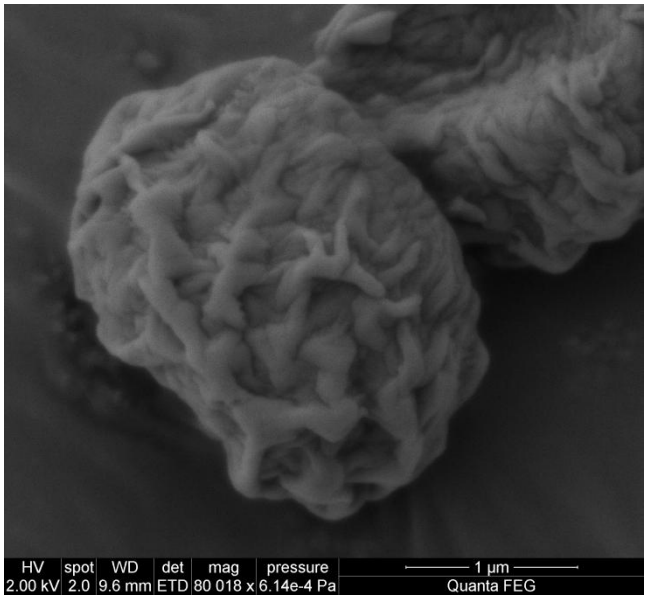


Figure 4: Scanning Electron Microscopy of the *A. nidulans* reference strain. No rodlets could be observed on the surface of the strain.

Next the *A. fumigatus* AF293 strain was included as a positive control as this specific species has previously been shown to display rodlets[15,16], but no rodlets could be found. Major

problems encountered during SEM included insufficient focus on the sample and collapse of spores, which may be caused by inadequate sample preparation. Additional sample preparation (eg. as described by Paris *et al.*[16]) or the replica plating method previously used by Parta *et al.*[15] may have proven to solve these issues. Nevertheless, due to both physical and time limitations and little prior experience with SEM on biological samples, SEM was aborted.

Morphological features of hydrophobin over expression strains

To further investigate the function of *A. nidulans* hydrophobins, strains over-expressing all six hydrophobins (RodA, DewA, AN0940, AN1837, AN6401 and AN7539) individually were constructed. The aim was to examine the phenotypic effect of over-expressing hydrophobins on the strains by examining the constructed strains both microscopically and macroscopically. However, over-expression of a single hydrophobin gene in *A. nidulans* resulted in strains displaying similar green conidia in all strains when grown on MM and WATM (not shown). By examining more than 30 samples, the conidial heads were found to resemble the reference strain. Furthermore all strains had approximately equal spore chain lengths, displayed dark ascomata and shed water instantly, due to their high degree of hydrophobicity.

Relative gene expression of hydrophobins in deletion strains

To elucidate the effect, on transcriptional level, of deleting hydrophobins on other hydrophobins, qRT-PCR was performed to determine the relative gene expression of all predicted hydrophobins in the *rodA*Δ, *dewA*Δ and *rodA*Δ*dewA*Δ strains using the reference strain as a control. Determination of the relative gene expression of all predicted hydrophobins in the AN0940Δ, AN1837Δ, AN6401Δ and AN7539Δ strains is currently ongoing and is not included in this chapter. As expected, the deleted hydrophobins were not expressed in the respective strains (figure 5). In the *rodA*Δ strain, all other hydrophobins were down-regulated compared to the reference ($P<0.025$). Fold changes ranged from ~ 0.4 - 0.7, with *dewA* and AN0940 displaying fold-change of 0.5 and 0.6 respectively. In the *dewA*Δ strain, no change in gene expression was observed for the RodA hydrophobin and a small decrease in fold change to 0.9 was observed for AN0940 ($P<0.1$). The remaining hydrophobins, AN1837, AN6401 and AN7539 were down-regulated and displayed similar

fold-changes in the *dewA*Δ strain (P<0.025) compared to the *rodA*Δ strain. In both the *rodA*Δ and *dewA*Δ strains, AN1837 displayed the lowest fold-change of ~ 0.7, AN6401 had a fold-change of 0.5 and AN7539 a fold-change of 0.4. Interestingly, when both RodA and DewA were deleted, no significant change in gene expression was observed for AN0940, AN1837 and AN6401 in the *rodA*Δ*dewA*Δ strain, when the standard deviations were taken into account. In contrast the AN7539 hydrophobin had increased expression (P<0.01) in the *rodA*Δ*dewA*Δ strain showing a fold change of 1.6.

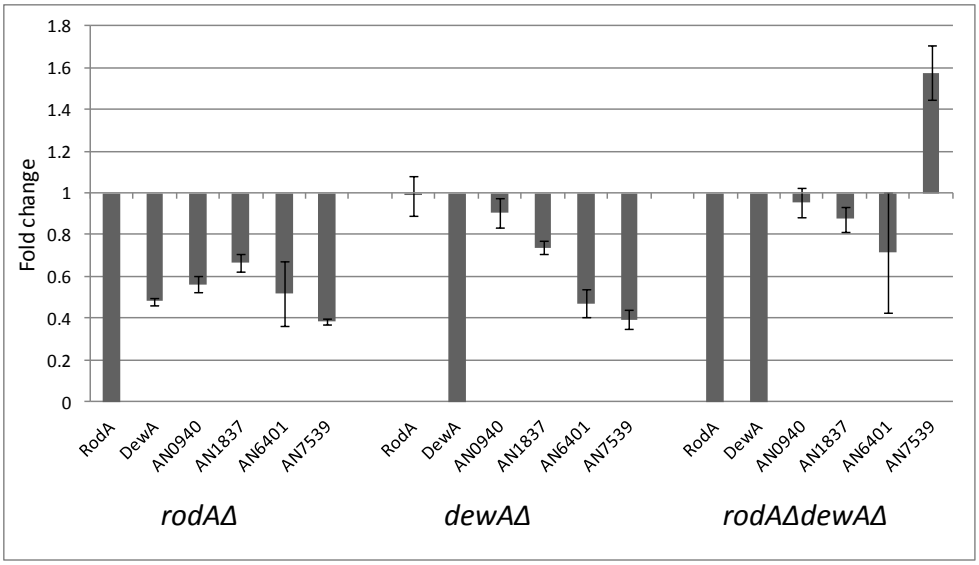


Figure 5: Relative gene expression of hydrophobin encoding genes in the *rodA*Δ, *dewA*Δ and *rodA*Δ*dewA*Δ strains using the reference as a control. The data shown are means ± standard errors from three independent experiments.

Deletion of both *rodA* and *dewA* hydrophobin genes has an effect on colony hydrophobicity

Different fungal species display varying degrees of surface hydrophobicity ranging from highly hydrophilic to very hydrophobic[17,18]. *Aspergillus* species are hydrophobic, but *rodA* deletion strains have previously demonstrated a reduced surface hydrophobicity[7,8], showing the involvement of hydrophobins in surface hydrophobicity. To examine the contribution of other *A. nidulans* hydrophobins to surface hydrophobicity, hydrophobicity of all *A. nidulans* deletion strains (except AN7539Δ) was assessed using water contact angle measurements by applying water drops on top of mature colonies and measuring the angle between the drop and colony surface. If the water contact angle is below 90° the surface is considered hydrophilic, while surfaces with water contact angles over 90° are considered hydrophobic[17].

All strains were initially found to display very high hydrophobicity having water contact angles over 140 degrees despite the lack of a hydrophobin (figure 6A). The reference strain had an initial water contact angle of 146° ± 4°. No statistical significant change of surface hydrophobicity of the *rodA*Δ, *dewA*Δ, AN0940Δ, AN1837Δ and AN6401 strains compared to our reference was measured as these strains displayed water contact angles from 142° ± 8° to 154° ± 3°. Apart from the *rodA*Δ*dewA*Δ, all strains had constant water contact angles throughout the first 60 seconds displaying below 5 degrees change even after five minutes (data not shown). Interestingly, water droplets placed on the *rodA*Δ*dewA*Δ colony soaked into the underlying agar within 3 – 5 minutes (figure 6B). The degree of surface hydrophobicity was found to decrease in average 18 degrees within the first minute and further decreased over time until no drop was visible.

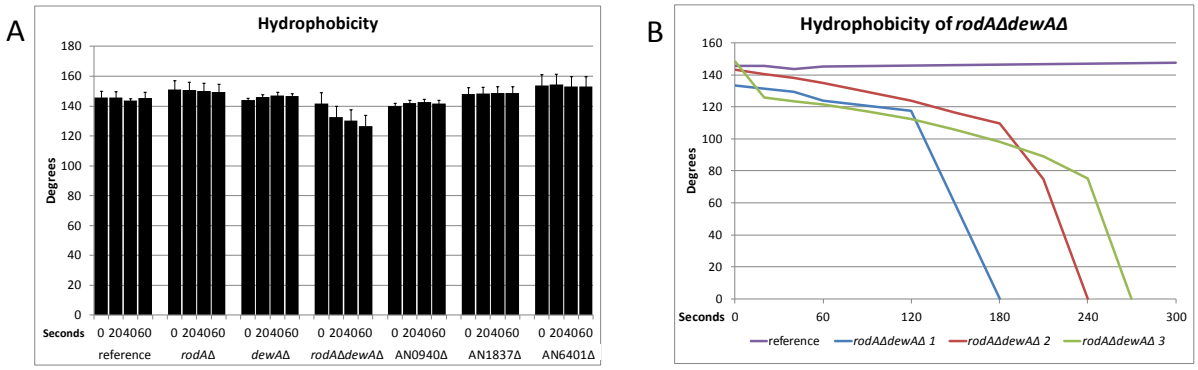


Figure 6: Hydrophobicity of *A. nidulans* deletion strains. Hydrophobicity of strains was measured using water contact angle measurements. (A) All strains (except *rodA*Δ*dewA*Δ) displayed high hydrophobicity with constant water contact angles in the first minute. The data shown are means ± standard errors from three experiments. (B) Hydrophobicity of the *rodA*Δ*dewA*Δ strain decreased over time due to the deposited water soaking into the mycelium within 3-5 min.

The ability of *A. nidulans* to breach a water-air interface is not compromised by deletion of single hydrophobin genes

Submerged hyphae from *Schizophyllum commune* secrete hydrophobins into the surrounding medium, proposing a role for hydrophobins in lowering the water surface tension, thus allowing fungi to escape aqueous environments and grow into the air[3,19]. To examine if the deletion of single hydrophobins compromises the ability of *A. nidulans* in escaping aqueous environments and examine if a similar mechanism may be used by *A. nidulans*, all hydrophobin deletion strains were inoculated on solid media and subsequently submerged in water. Initial experiments using wild type strains of *A. fumigatus, A. oryzae* and *A. niger* verified that *Aspergillus* species are able to breach from an aqueous environment into the air.

Using the hydrophobin deletion strains, our results indicate that lack of a single hydrophobin or two (*rodA*Δ*dewA*Δ) does not affect the ability of *A. nidulans* to breach the water air interface. After 4-5 days of growth at 20 °C below water level, all strains had breached through the water forming a colony of vegetative mycelium and conidiophores on top of the water (figure 7). The colony was connected to the solid submerged media by easily visible mycelia allowing the floating colony to acquire nutrients.

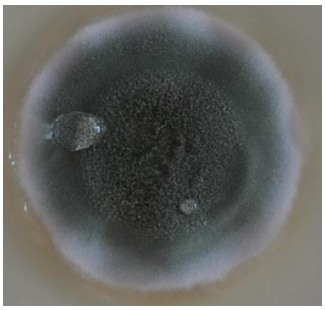


Figure 7: All *Aspergillus* hydrophobin deletion species were able to breach through water into the air forming a mature colony atop the water after 4-5 days of growth at 20 °C. All experiments were conducted in triplicate. The reference strain is shown as an example.

Discussion

Hydrophobins have been proposed to fulfil many different biological functions in fungi including hydrophobicity, facilitation of spore dispersal and allowing the fungus to escape aqueous environments[1] and may therefore be expressed at different stages of fungal development[2]. *Aspergillus* species display a varying number of hydrophobins[4,9] and six putative hydrophobins have been identified in *A. nidulans* (AN0940, AN1837, AN6401, AN7539, AN8006 and AN8803)[4,5]. Recently, Littlejohn *et al*.[9] proposed the existence of an additional four hydrophobins in *A. nidulans*. These four hydrophobins do, however, not confer to the accepted standards for hydrophobins originally presented by Wessels[1,10] and may therefore not be able to maintain a globular overall fold similar to other hydrophobins[20–23]. In this study the focus was on the six hydrophobins (AN0940, AN1837, AN6401, AN7539, RodA and DewA) fulfilling all criteria to classify them as hydrophobins including size, eight cysteines, two cysteine pairs and an intact characteristic cysteine pattern. As with other hydrophobins[1], the *A. nidulans* hydrophobins display low to moderate sequence similarity towards each other and varying sequence similarity to hydrophobins from other full genome sequenced Aspergilli. Three hydrophobins (RodA, DewA and AN1837) were

putatively classified as class I hydrophobins based on their cysteine pattern and hydropathy plots, while AN0940, AN6401 and AN7539 probably are intermediate forms. Many other *Aspergillus* species likewise display different classes of hydrophobins[4,9], but the classes of hydrophobins can only been confirmed after isolation and characterization of the hydrophobins.

To examine the function of *A. nidulans* hydrophobins, deletion strains of the *A. nidulans* hydrophobins were constructed. Of the six *A. nidulans* hydrophobins, one hydrophobin (AN7539) was not included in the experimental part of the present study and will not be discussed as a knockout mutant of the strain is still under construction.

Previous studies have shown that deletion of single *Aspergillus* hydrophobins resulted in altered phenotypes compared to control strains[6,7,24], and therefore the constructed deletion strains were examined both macroscopically and microscopically. However, only the *rodA*Δ and *rodA*Δ*dewA*Δ strains had different phenotypes compared to the reference strain, where the *rodA*Δ*dewA*Δ strain displayed a more severe phenotype than the *rodA*Δ strain. The darkened, wet and sticky appearance of the conidia in growing colonies was in agreement with previous results by Stringer *et al.*[6]. This phenotype had also been observed in a *rodA* knockout mutant of *A. fumigatus*[25] and an *eas* mutant from *Neurospora crassa*[26], showing that this phenotype often is associated with the lack of the conidia rodlet layer. The remaining strains (*dewA*Δ, AN0940Δ, AN1837Δ, AN6401Δ and reference) displayed dry spores, were not easily wetted and resisted suspension in water. This indicates that these hydrophobins, as previously shown for DewA[7], may not be essential for rodlet formation, but could still be located on the conidia cell wall. To examine if the hydrophobins AN0940, AN1837 and AN6401 are involved in rodlet formation, a visualization of the conidium surface was attempted using SEM. Unfortunately this was not successful and therefore the involvement of AN0940, AN1837 and AN6401 in rodlet formation still remains elusive.

Another phenotype linked to the loss of conidial hydrophobins in *A. nidulans* is the detergent wettable phenotype in *dewA*Δ strains[7] and therefore the other hydrophobin deletion strains were examined for this phenotype. The *dewA*Δ, *rodA*Δ*dewA*Δ and *rodA*Δ strains displayed rapid uptake of drops of detergent (0.2 % SDS, 50 mM EDTA) in the colonies from instantly to 1 min. The AN0940Δ, AN1837Δ and AN6401Δ strains did not display a detergent wettable phenotype, indicating that these hydrophobins may not have dominant roles on conidia if

present at all. These hydrophobins could only be present in the mycelium cell wall or may be complemented by other hydrophobins. This was examined using qRT-PCR.

Beauvais *et al.*[27] had previously examined the expression of *A. fumigatus* hydrophobins under different conditions finding that *rodA* and *rodC* were expressed during vegetative growth, *rodB* and *rodE* in mycelium grown under static aerial conditions, while *rodD* and *rodF* were expressed in both mycelium grown under static aerial conditions and shaken submerged conditions. Similarly, the hydrophobins from *Cladosporium fulvum* have been found to be differentially expressed under different culturing conditions[28–30]. These previous expression studies reveal overall regulation following different stages of development. To examine if hydrophobins are coupled in expression or are able to complement each other, expression levels of the six *A. nidulans* hydrophobins were examined in the *rodA* and *dewA* deletion strains by qRT-PCR. In the *rodA*Δ strain, deletion of *rodA* resulted in all other hydrophobins having a reduced gene expression including the other known conidial hydrophobin DewA. In contrast the expression level of *rodA* was not changed in the *dewA*Δ strain. This interestingly shows that changes in *rodA* affect *dewA* expression, but not vice versa. This may be due to RodA being the major outer wall hydrophobin on the conidia, while DewA has been proposed to be a part of the inner layer[7]. It seems that the presence of *dewA* is not a prerequisite for the expression of *rodA*, which is confirmed by the observation of rodlets in *dewA*Δ strains[7], but the decreased expression of *rodA* and thereby lack of rodlet layer appears to affect the expression of all other hydrophobins.

The deletion of *dewA*, did not result in any significant change in the expression levels of AN0940. However, AN1837, AN6401 and AN7539 had reduced expression similar to the *rodA*Δ strain. It seems that these three hydrophobins display comparable reduced expression levels irrespectively of which conidial hydrophobin (*rodA* or *dewA*) has been deleted. In the *rodA*Δ*dewA*Δ strain, the hydrophobins AN0940, AN1837 and AN6401 only had a minimal change in expression and were only slightly down-regulated showing that the deletion of the two hydrophobins does not reduce the expression of the other hydrophobins to the same extent compared to the single deletion strains. These hydrophobins may become more important as more hydrophobins are missing. Interestingly, the deletion of both *rodA* and *dewA*, resulted in an increased expression of AN7539, which was in contrast to the reduced expression observed in the single deletion strains. This hydrophobin may thus fulfil some of the functions of the hydrophobins RodA and DewA, when both hydrophobins have been

deleted. Future expression studies on the AN7539Δ strain will reveal if RodA and DewA are affected by the deletion of the AN7539 hydrophobin, but as RodA is the major conidial hydrophobin this is not likely.

The colony surface hydrophobicity of the deletion strains was measured using water contact angle measurements, where all strains displayed high surface hydrophobicity. Different fungal species display varying degrees of surface hydrophobicity[17,18], but *A. nidulans* generally has high hydrophobicity[8] . In agreement with our results, Dynesen *et al.*[8] did not observe any difference in surface hydrophobicity between the *dewA*Δ and reference strain, but did in contrast to our results see a decrease in surface hydrophobicity in the *rodA*Δ strain. Our *rodA*Δ*dewA*Δ strain allowed passage of water to the underlying agar, a phenotype previously described in an *A. fumigatus rodA*Δ strain[25]. This may be due to the lower hydrophobicity of the *rodA*Δ*dewA*Δ strain, previously confirmed by Stringer *et al.*[7]. The minor difference in surface hydrophobicity of AN0940Δ, AN1837Δ and AN6401Δ from the reference strain indicated that these hydrophobins are not essential for colony hydrophobicity, and that RodA plays a more pronounced role in making the colony hydrophobic. Water contact angle measurements were done on a sporulating colony containing both conidia and mycelium, which may explain why no major differences could be observed between the strains. Further experiments on pure conidia or mycelium, could provide insight into the hydrophobicity of single fungal structures. This could be achieved using the "microsphere adhesion assay" previously described by Beauvais *et al.*[27]. In this assay the binding of fluorescent polystyrene latex beads to fungal structures indicate the hydrophobicity of the fungus.

Hydrophobins have been suggested to actively help fungi to escape aqueous environments by lowering the water surface tension[3]. Previously, Wessels *et al.* have shown that hydrophobins from *S. commune* are secreted into the surrounding medium by submerged hyphae, thereby lowering the water surface tension and allowing the fungus to breach into the air[19,31,32]. If a similar strategy is used in Aspergilli then at least a single hydrophobin should be secreted into the liquid medium. To test this hypothesis, we submerged immature colonies of our mutant strains in water. After 4 -5 days of growth at 20 °C all strains had breached through the water and had formed mature colonies on the water. This implies that the lack of a single *A. nidulans* hydrophobin or both the RodA and DewA hydrophobins does not impair the ability to escape an aqueous environment. This may be a result of the hydrophobins complementing each other allowing differential secretion into the surrounding medium or an indication that a

different strategy is used by *Aspergillus* compared to *S. commune* in allowing the fungus to breach into the air.

Concluding remarks

Hydrophobins have been proposed to be involved in many different functions of fungal growth and development. They are involved in hydrophobicity, facilitate spore dispersal and allow fungi to escape aqueous environments[1]. The aim of this study was to examine putative hydrophobins from *A. nidulans* by creating single deletion and over-expression strains of six hydrophobins and testing them in several assays. Previous results by Stringer *et al.*[6,7] were confirmed for the RodA and DewA hydrophobins. As no phenotype was apparent for the AN0940, AN1837 and AN6401 deletion strains, roles of these hydrophobins remains to be determined. It seems that hydrophobins in *A. nidulans* have different roles in the fungus, where some are necessary for the fungus to survive in nature, while the roles of others are still elusive. Further studies may reveal, yet undiscovered, biological functions for hydrophobins.

References for chapter 4

1. Wessels JGH (1997) Hydrophobins: Proteins that change the nature of the fungal surface. *Adv Microb Physiol* **38:** 1-45.

2. Wosten HA (2001) Hydrophobins: multipurpose proteins. *Annu Rev Microbiol* **55:** 625-646.

3. Wosten HA, van Wetter MA, Lugones LG, van der Mei HC, Busscher HJ, Wessels JG (1999) How a fungus escapes the water to grow into the air. *Curr Biol* **9:** 85-88.

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

5. de Groot PW, Brandt BW, Horiuchi H, Ram AF, de Koster CG, Klis FM (2009) Comprehensive genomic analysis of cell wall genes in *Aspergillus nidulans*. *Fungal Genet Biol* **46:** S72-S81.

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

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

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

9. Littlejohn KA, Hooley P, Cox PW (2012) Bioinformatics predicts diverse *Aspergillus* hydrophobins with novel properties. *Food Hydrocolloids* **27:** 503-516.

10. Wessels JGH (1994) Developmental Regulation of Fungal Cell-Wall Formation. *Annu Rev Phytopathol* **32:** 413-437.

11. Kershaw MJ, Talbot NJ (1998) Hydrophobins and repellents: proteins with fundamental roles in fungal morphogenesis. *Fungal Genet Biol* **23:** 18-33.

12. Nielsen JB, Nielsen ML, Mortensen UH (2008) Transient disruption of non-homologous end-joining facilitates targeted genome manipulations in the filamentous fungus *Aspergillus nidulans*. *Fungal Genet Biol* **45:** 165-170.

13. Raper, K. B. and Thom, C. (1949) A manual of the penicillia. Baltimore: Williams & Wilkens Co. 920 p.

14. Frisvad JC, Samson RA (2004) *Penicillium* subgenus *Penicillium* - A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. *Stud Mycol* **49:** 1-173.

15. Parta M, Chang Y, Rulong S, Pinto-DaSilva P, Kwon-Chung KJ (1994) HYP1, a hydrophobin gene from *Aspergillus fumigatus*, complements the rodletless phenotype in *Aspergillus nidulans*. *Infect Immun* **62:** 4389-4395.

16. Paris S, Debeaupuis JP, Crameri R, Carey M, Charles F, Prevost MC, Schmitt C, Philippe B, Latge JP (2003) Conidial hydrophobins of *Aspergillus fumigatus*. *Appl Environ Microbiol* **69:** 1581-1588.

17. Chau HW, Si BC, Goh YK, Vujanovic V (2009) A novel method for identifying hydrophobicity on fungal surfaces. *Mycol Res* **113:** 1046-1052.

18. Smits TH, Wick LY, Harms H, Keel C (2003) Characterization of the surface hydrophobicity of filamentous fungi. *Environ Microbiol* **5:** 85-91.

19. Wessels JGH, De VO, Asgeirsdottir SA, Schuren F (1991) Hydrophobin Genes Involved in Formation of Aerial Hyphae and Fruit Bodies in *Schizophyllum*. *Plant Cell* **3:** 793-799.

20. Hakanpaa J, Paananen A, Askolin S, Nakari-Setala T, Parkkinen T, Penttila M, Linder MB, Rouvinen J (2004) Atomic resolution structure of the HFBII hydrophobin, a self-assembling amphiphile. *J Biol Chem* **279:** 534-539.

21. Kwan AH, Winefield RD, Sunde M, Matthews JM, Haverkamp RG, Templeton MD, Mackay JP (2006) Structural basis for rodlet assembly in fungal hydrophobins. *Proc Natl Acad Sci U S A* **103:** 3621-3626.

22. Hakanpaa J, Linder M, Popov A, Schmidt A, Rouvinen J (2006) Hydrophobin HFBII in detail: ultrahigh-resolution structure at 0.75 A. *Acta Crystallogr D Biol Crystallogr* **62:** 356-367.

23. Hakanpaa J, Szilvay GR, Kaljunen H, Maksimainen M, Linder M, Rouvinen J (2006) Two crystal structures of *Trichoderma reesei* hydrophobin HFBI - the structure of a protein amphiphile with and without detergent interaction. *Protein Sci* **15:** 2129-2140.

24. Thau N, Monod M, Crestani B, Rolland C, Tronchin G, Latge JP, Paris S (1994) Rodletless mutants of *Aspergillus fumigatus*. *Infect Immun* **62:** 4380-4388.

25. Thau N, Monod M, Crestani B, Rolland C, Tronchin G, Latge JP, Paris S (1994) rodletless mutants of *Aspergillus fumigatus*. *Infect Immun* **62:** 4380-4388.

26. Beever RE, Dempsey GP (1978) Function of rodlets on the surface of fungal spores. *Nature* **272:** 608-610.

27. Beauvais A, Schmidt C, Guadagnini S, Roux P, Perret E, Henry C, Paris S, Mallet A, Prevost MC, Latge JP (2007) An extracellular matrix glues together the aerial-grown hyphae of *Aspergillus fumigatus*. *Cell Microbiol* **9:** 1588-1600.

28. Nielsen PS, Clark AJ, Oliver RP, Huber M, Spanu PD (2001) HCf-6, a novel class II hydrophobin from *Cladosporium fulvum*. *Microbiol Res* **156:** 59-63.

29. Whiteford JR, Spanu PD (2001) The hydrophobin HCf-1 of *Cladosporium fulvum* is required for efficient water-mediated dispersal of conidia. *Fungal Genet Biol* **32:** 159-168.

30. Whiteford JR, Lacroix H, Talbot NJ, Spanu PD (2004) Stage-specific cellular localisation of two hydrophobins during plant infection by the pathogenic fungus *Cladosporium fulvum*. *Fungal Genet Biol* **41:** 624-634.

31. Wessels JGH, de Vries OM, Asgeirsdottir SA, Springer J (1991) The thn mutation of *Schizophyllum commune*, which suppresses formation of aerial hyphae, affects expression of the Sc3 hydrophobin gene. *J Gen Microbiol* **137:** 2439-2445.

32. Wessels JGH (1992) Gene expression during fruiting in *Schizophyllum commune*. *Mycol Res* **96:** 609-620.

5 Examination of potential *Aspergillus-Pseudomonas* interactions

This chapter deals with interactions between *Aspergillus* species and *Pseudomonas aeruginosa.* The majority of results are included in article 2. Methods can be seen in article 2 and appendix 2.

Introduction

A part of this PhD study has focused on the potential interaction between different *Aspergillus* species and the bacterium *Pseudomonas aeruginosa*, which both commonly colonize the lungs of cystic fibrosis (CF) patients[1,2]. Cystic fibrosis is a chronic inherited genetic disorder characterized by recurrent infections of the lower respiratory tract due to an impaired mucociliary clearance and thickened bronchial mucus providing a favourable growth environment for bacteria and fungi. Over time the recurrent infections and inflammation lead to a severely impaired lung function[3].

Pseudomonas aeruginosa is the most dominant bacterium found in CF lungs colonizing approximately 80 % of CF patients[1,2]. As CF patients reach early childhood, most CF patients eventually become chronically infected by *P. aeruginosa* strains[4]. *P. aeruginosa* changes genetically during the course of chronic CF lung infections resulting in loss-of-function mutations and changes in phenotypes[5,6]. Common mutations include loss of motility[7] and inactivation of the anti-sigma factor MucA resulting in a mucoid phenotype[8]. Furthermore, the quorum sensing regulator LasR is commonly affected[5,9] resulting in a decrease in expression of many virulence factors[10–13]. Frequent mutations in the alternative sigma factor, RpoN, similarly affect many virulence factors including synthesis of pili, flagella, pyocyanin and rhamnolipids[14–17].

Aspergillus spp. can cause disease in immunocompromised hosts and individuals with underlying pulmonary diseases, where *Aspergillus fumigatus* is responsible for approximately 90% of human infections with *Aspergillus* species[18]. In CF patients, *A. fumigatus* is the most common isolated filamentous fungus occurring in between 6 – 58 % of CF patients[1,2,19–23] and is the most persistent organism to colonize CF patients next to *P. aeruginosa*[1]. Many CF patients harbour several *A. fumigatus* genotypes in their lungs[24–26], with some strains being chronically present, while others are found more transiently[26]. Bakare *et al.* found that 64% of *A. fumigatus* positive patients also had *P. aeruginosa* infections[2], and patients with *A. fumigatus* more frequently had *P. aeruginosa*[20,27]. Other *Aspergillus* species found in cystic fibrosis patients are *A. flavus, A. niger, A. nidulans, A.*

versicolor and *A. terreus*[19,20,28,29]. Most are possibly only found transiently, but *A. terreus* seems to be able to chronically colonize the airways of CF patients[28].

Both *Aspergillus* species and *Pseudomonas aeruginosa* are frequently isolated from CF patients[2], but few studies have focused on a possible interaction between the species in mixed populations[30–34]. During the writing of this chapter Moree *et al.*[35], however, published a study dealing with *A. fumigatus* - *P. aeruginosa* interactions. In this chapter the potential interaction between *Pseudomonas aeruginosa* and different *Aspergillus* species was examined. If the two organisms interact, this may lead to changes in behaviour of one of the organisms, changes in secondary metabolite profiles, increased inflammatory response and worsening of underlying pulmonary disease. As a result of genetic adaption to the CF environment mutational changes in *P. aeruginosa* may alter the interactions between *A. fumigatus* and *P. aeruginosa* during the course of a CF infection. This has been examined using different *P. aeruginosa* mutants. Finally the involvement of hydrophobins in the interaction has briefly been touched upon.

Results

Development of an assay to investigate *Aspergillus-Pseudomonas* interactions

In order to examine *Aspergillus-Pseudomonas* interactions it was pivotal to find a medium allowing balanced growth between the two organisms and develop an assay allowing visualization of the interaction. The fungal nutritional rich media Wickerhams Antibiotic Test Medium (WATM)[36] provided appropriate and balanced growth of both organisms and was chosen as standard medium. Other media tested included Luria broth (LB)[37] and Yeast Extract Sucrose agar (YES)[38], but neither provided balanced growth between the two organisms.

Next an appropriate model for streaking the two organisms was examined. The aim was to find a model system enabling the examination of the two organisms close together, but also containing areas with each organism alone further from the other organism for comparison. Several different models for streaking out the two organisms were tested (figure 1). The first model (A) was found to suppress the fungal growth too much compared to the bacterium. In this model the fungus was completely surrounded by the bacterium, whereby fungal areas further from the bacterium were unavailable. The second model (B) was found to under-

suppress the fungus, as it over time covered the whole plate except the bacterium streak. Hereby the direction of the fungus could not be controlled and no bacterium area without adjacent fungus was available. The third model (C) consisted of a single fungal streak on the left side of the plate and four bacterial streaks perpendicular to the fungal streak. Initially the bacterium was streaked at varying distances, but was subsequently modified to be streaked at equal distances. This model provided both zones in the bacterium and the fungus adjacent to the other organism, but also further from the other organism.

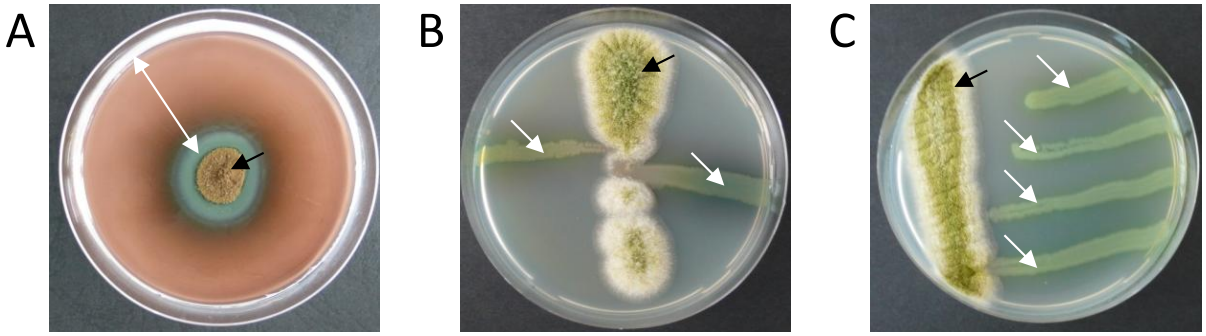


Figure 1: Several different models for streaking out the *Aspergillus* species and *P. aeruginosa* was tried out using 9 cm media plates. Models are shown using *A. flavus*. Model A: *A. flavus* inoculated in the middle, *P. aeruginosa* streaked around the fungus covering the rest of the plate. Model B: *A. flavus* streaked in the middle of the plate, *P. aeruginosa* streaked from each side perpendicular to the fungus. Model C: *A. flavus* streaked on the left side of the plate. *P. aeruginosa* streaked four times perpendicular to the fungus at varying distance from the fungus. (black arrow: *A. flavus*, white arrow: *P. aeruginosa*)

The developed assay thus consisted of WATM plates, where the *Aspergillus* species was streaked on the left side of the plate, while *P. aeruginosa* was streaked on the right allowing the organisms to initially colonize separately (figure 2).

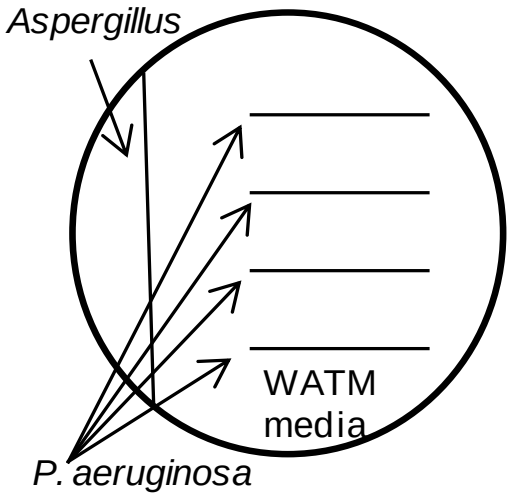


Figure 2: The standard plating method was used throughout this study. *Aspergillus* was streaked on the left side of the 9 cm plate, while *P. aeruginosa* was streaked four times perpendicular to the fungal streak on the right side. The initial distance between the fungal and bacterial streaks was 2 cm.

Aspergillus species are suppressed by *P. aeruginosa*

Several *Aspergillus* species may infect CF patients[19,20,28,29]. Previous studies in *Aspergillus-Pseudomonas* interactions have primarily focused on *A. fumigatus*[30–32], but in this study *A. niger*, *A. oryzae* and *A. flavus* were also included.

By using the developed plating assay *P. aeruginosa* PAO1[39] was found to suppress all tested Aspergilli including; *A. fumigatus, A. niger, A. oryzae* and *A. flavus* (figure 3). For *A. oryzae* and *A. flavus*, *P. aeruginosa* produced a greenish compound in the contact zone to the Aspergilli. This greenish compound is presumably pyocyanin, known to have a green/blue colour and an increase in pyocyanin production by *P. aeruginosa* was confirmed by LC-MS in the contact zone of *A. oryzae* and *A. flavus.* No coloration was observed for *P. aeruginosa* in the contact zone of *A. fumigatus* or *A. niger*, nor was the production of pyocyanin increased. *P. aeruginosa* seemed growth restricted in the contact zone with *A. niger*. This may be a result of citric acid produced by *A. niger*, previously shown to inhibit *P. aeruginosa*[40]. No inhibition was observed when 1 M HCl was applied to bacterium, thus the observed inhibitory effect is specific to the produced acid by *A. niger* and not a pH effect. This shows differential interaction patterns, despite all tested *Aspergillus* being suppressed and was later examined in more detail (see below).

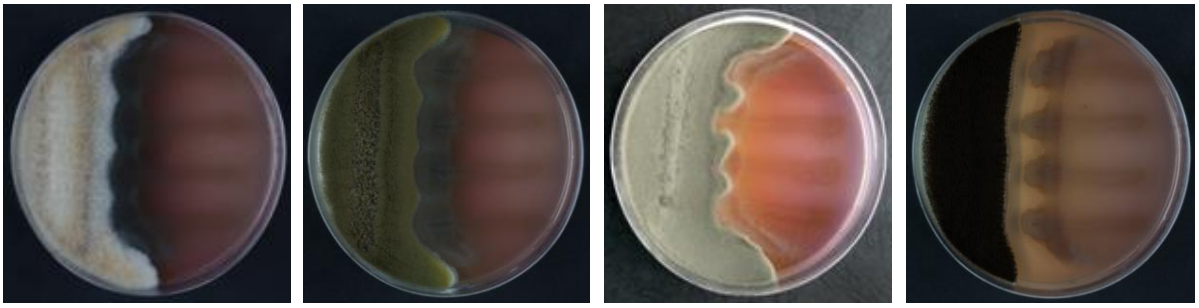


Figure 3: *P. aeruginosa* suppressed growth of *A. oryzae*, *A. flavus*, *A. fumigatus* and *A. niger* on WATM plates. The pictures were taken after five days of growth at 37 °C.

Both clinical and environmental *A. fumigatus* strains are suppressed by *P. aeruginosa* PAO1

As *A. fumigatus* is the major *Aspergillus* species isolated from CF patients[18], it was interesting to examine if the suppression of *A. fumigatus* by *P. aeruginosa* was a general phenomena for *A. fumigatus* strains. Therefore ten different *A. fumigatus* strains were selected, including six clinical and four environmental strains, and tested against *P.*

aeruginosa PAO1 (table 1). This could also provide insight to differences in the interaction between *P. aeruginosa* and *A. fumigatus* strains with different origin.

Table 1: Interaction between *A. fumigatus* strains and *P. aeruginosa* PAO1 and the production of secondary metabolites by *A. fumigatus* strains.

		WATM medium											LB medium[1]
***A. fumigatus* strain**	**Type of strain**	**Color of *P. aeruginosa* in contact zone**	**Dusty (*)**	**Fumigaclavins**	**Pseurotins**	**Tryptoquivalins**	**Fumiquinazolins**	**Trypacidins**	**Fumagillins**	**Helvolic acid**	**Pyripyropenes**	**Fumitremorgins**	**Gliotoxin production (ng/cm^2)**
AF293	Clinical	-	+	+	+	+	+	+	+	+	+	-	40
AF41	Clinical	Green	-	+	-	+	+	+	+	+	+	+	ND
A37941	Clinical	Green	-	-	-	-	+	-	+	+	-	-	ND
CBS 144.89	Clinical	Green	-	+	+	+	+	-	+	+	+	+	180
ATCC 201531	Clinical	-	+	+	+	+	+	+	+	+	+	+	190
AF250	Clinical	Yellow	-	+	+	+	+	+	+	+	+	+	40
NRRL1979	Environmental	Green	-	+	-	+	+	+	+	+	+	+	ND
TUBF-32	Environmental	-	+	+	+	+	+	-	+	+	+	-	50
AFIR974	Environmental	-	+	+	-	+	+	+	+	+	+	+	ND
TUBF-440	Environmental	-	+/-	+	+	+	+	+	+	-	+	+	650

[1]Gliotoxin is mainly produced on low carbohydrate containing media[41] and therefore LB medium was used.
ND: < 20 ng/cm^2

All *A. fumigatus* strains were inhibited by *P. aeruginosa* (figure 4), showing that inhibition of *A. fumigatus* by *P. aeruginosa* is a common phenomenon independent of the fungal strain. This is in agreement with previous studies showing that *A. fumigatus* strains (with few exceptions) are partly or completely suppressed by *P. aeruginosa*[30–32]. Five strains (AF293, ATCC 201531, TUBF-32, AFIR 974 and TUBF-440) had a dusty appearance (2-3 mm spore layer almost floating on top of the culture), while the remaining strains were less dusty. The strains AF41, A37941, CBS 144.89 and NRRL1979 unexpectedly triggered the production of a green compound by *P. aeruginosa* in the contact zone (figure 4), while AF250 triggered the production of a yellow compound. The remaining strains did not trigger any visual response in *P. aeruginosa* as previously observed for the AF293 strain. It seemed that the less dusty strains triggered the production of the green compound in *P. aeruginosa*, while the very dusty strains did not. This variation in the interaction may explain why some *A. fumigatus* strains chronically infect CF patients while other *A. fumigatus* strains are found more transiently[26].

To further examine the background for this altering interaction pattern, the ten strains were analysed by HPLC to elucidate if the colour reaction from *P. aeruginosa* was correlated to the production of one or several secondary metabolites by *A. fumigatus.* As expected, based on previously published results by Frisvad *et al.*[41], the *A. fumigatus* strains were found to produce different classes of secondary metabolites (table 1). Of special interest was gliotoxin, previously found in the lungs of CF patients and able to damage human respiratory epithelium[42,43]. Nevertheless, no correlation could be found between secondary metabolites from *A. fumigatus* and the green colour observed in *P. aeruginosa*, so other factors not involving secondary metabolites or other undetectable secondary metabolites may be involved. Even so all *A. fumigatus* strains were suppressed by *P. aeruginosa*.

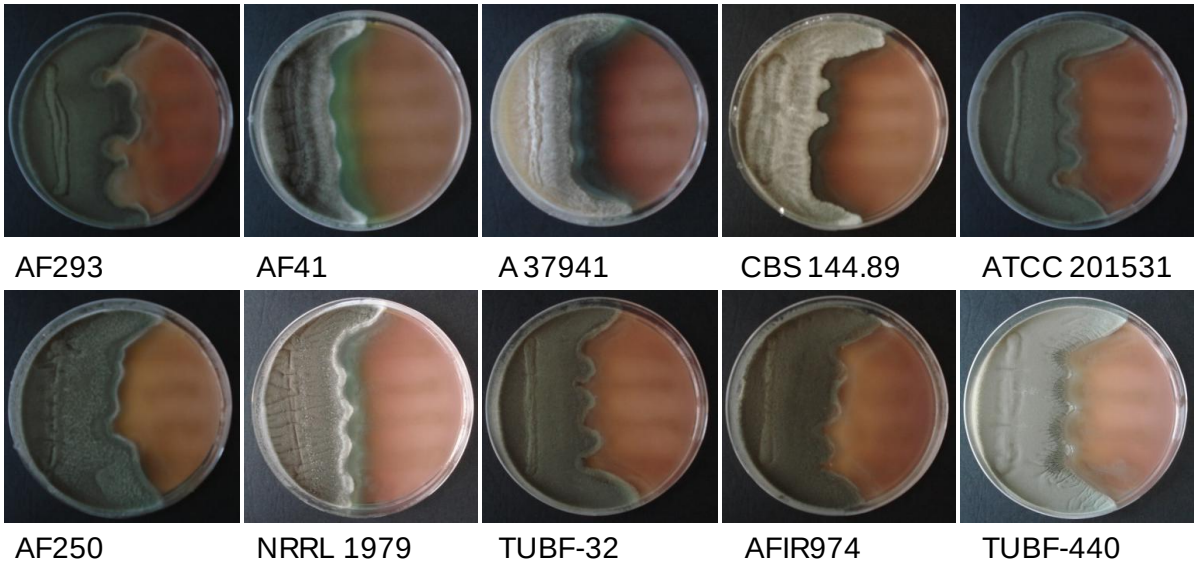


Figure 4: Ten different *A. fumigatus* strains were suppressed by *P. aeruginosa* PAO1. Six strains were clinical isolates (AF293, AF41, A37941, CBS 144.89, ATCC 201531 and AF250), while four strains were environmental strains (NRRL 1979, TUBF-32, AFIR974 and TUBF-440).

Common mutations in *P. aeruginosa* changes the interaction between *A. fumigatus* and *P. aeruginosa*

Next several different *P. aeruginosa* mutants were included in the study. These strains contained mutations frequently found in genetically CF adapted *P. aeruginosa* strains[5,6] (table 2) and could potentially provide additional insight into the *A. fumigatus*-*P. aeruginosa* interaction. This study is, to my knowledge, the first to include several different *P. aeruginosa* mutant strains to elucidate the role of different genes in mixed cultures.

Table 2: *P. aeruginosa* mutant strains used

***P. aeruginosa* strains**		
P. aeruginosa fliM	Motility mutant	Mutant lacks the polar flagellum[44]
P. aeruginosa pilA	Motility mutant	Mutant lacks type IV pili[44]
P. aeruginosa rhlA	Rhamnolipid mutant	Rhamnolipids are involved in surface motility, immune modulation and virulence[45,46]
P. aeruginosa pvdA	Pyoverdin mutant	Pyoverdins are siderophores used to acquire Fe^{3+} ions from the environment[47]
P. aeruginosa lasR	Quorum sensing mutant	Mutations in LasR results in a decrease in expression of many virulence factors including pyocyanin[10–13]
P. aeruginosa rpoN	Regulatory mutant	Mutations in the alternative sigma factor, RpoN, affect many virulence factors including synthesis of pili, flagella, pyocyanin and rhamnolipids[14–17].
P. aeruginosa mucA	Mucoid mutant	Inactivation of the anti-sigma factor MucA results in a mucoid phenotype due to increased production of alginate[8]

First the regulatory strains were examined. Both the *lasR* and *mucA* mutant strains were initially found to suppress growth of *A. fumigatus* AF293 (figure 5). The mucoid *mucA* strain was slightly overgrown by *A. fumigatus* in the interaction zone after five days incubation, increasing a little over time, while the *lasR* mutant was able to grow in between the *P. aeruginosa* streaks.

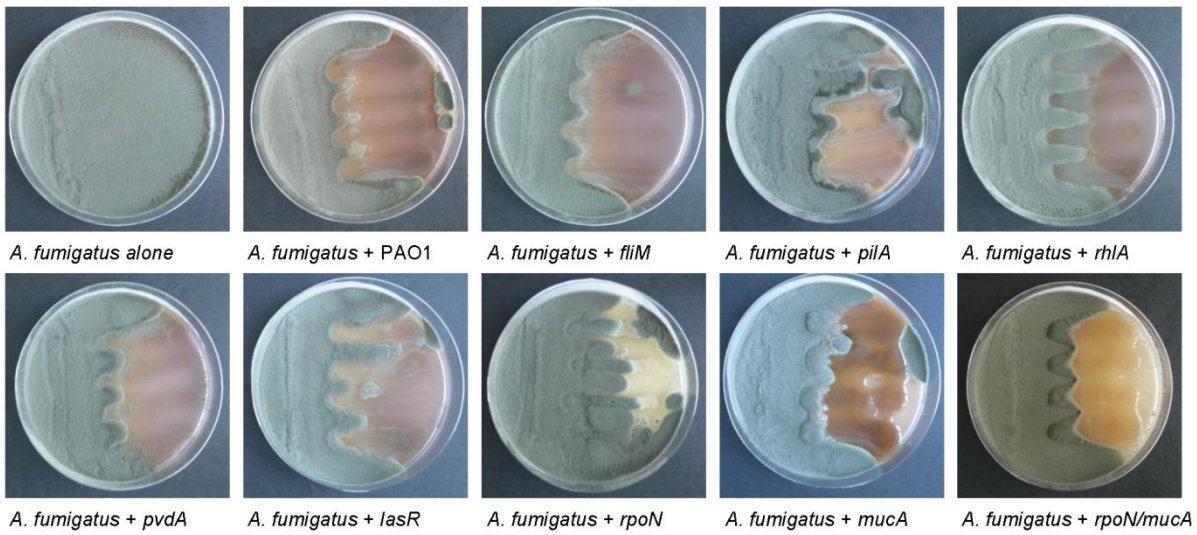


Figure 5: Eight different *P. aeruginosa* mutants were tested against *A. fumigatus* AF293. Six mutants suppressed *A. fumigatus*, while the *rpoN*, *mucA* and *rpoN/mucA* mutants were unsuccessful. All pictures were taken in a LAF bench (after five days incubation at 37 °C) due to safety reasons, wherefore the colours of *A. fumigatus* AF293 vary due to insufficient photo setup.

Next a mutant defective in the alternative sigma factor RpoN was included. This strain initially suppressed growth of *A. fumigatus* AF293, but began to be overgrown by the fungus after 4-6 days of growth until completely overgrown. It seemed that *A. fumigatus* preferred growing on top of the *rpoN* mutant rather than in between the streaks as seen in other

mutants tested (figure 5). Similarly all other *A. fumigatus* strains (both clinical and environmental strains) were not suppressed by the *rpoN* mutant and grew over the mutant.

As both the *rpoN* and *mucA* mutants were unable to suppress *A. fumigatus* AF293, a double mutant (*rpoN/mucA*) was introduced next. Commonly CF isolated *P. aeruginosa* strains contain multiple mutations including the combination of *mucA*, *lasR* and *rpoN* mutations[6,48]. As expected this mutant was completely overgrown by *A. fumigatus* after seven days of incubation resulting in the mucoid *P. aeruginosa* forming the base of a lawn of *A. fumigatus* (figure 6). This combination of mutations in *P. aeruginosa* seemed to provide the most ideal environment for *A. fumigatus* growth.

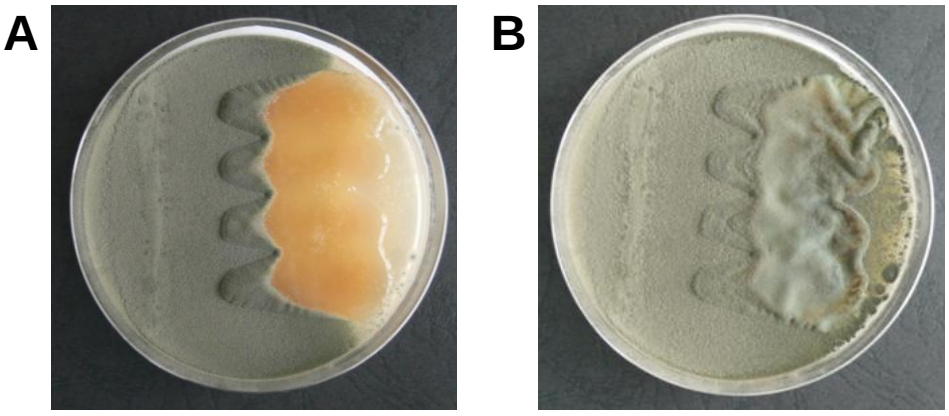


Figure 6: *A. fumigatus* AF293 was able to grow over the *P. aeruginosa rpoN/mucA* mutant. Initially the bacterium was able to partly suppress the fungus (A), but after seven days of incubation at 37 °C *A. fumigatus* AF293 completely overgrew the mutant resulting in the mucoid *P. aeruginosa* forming the base of a lawn of *A. fumigatus* (B).

The alternative sigma factor RpoN controls production of multiple virulence factors[14–17] and therefore individual contribution of different factors to the growth inhibition of *A. fumigatus* was examined to provide insight into which factors may be involved in the observed suppression. Both lack of motility, rhamnolipid and pyoverdin production did not seem to change the interaction as these mutants were able to suppress *A. fumigatus* AF293 (figure 5). Long-term incubation (13 days) resulted in *A. fumigatus* growing in between the *P. aeruginosa pilA* and *rhlA* mutant streaks, while no change was observed on the *fliM* mutant plates over time. It seems that all *P. aeruginosa* mutants initially suppress *A. fumigatus*, but differences in the ability of *P. aeruginosa* strains were observed over time (table 3). Whether a similar picture could be observed in other *Aspergillus* species was examined next.

Table 3: Suppression of *P. aeruginosa* strains

***P. aeruginosa* strains**		**Suppression of *A. fumigatus***	**Growth of *A. fumigatus* between *P. aeruginosa* streaks**
P. aeruginosa PAO1	Control strain	+	-
P. aeruginosa fliM	Motility mutant	+	-
P. aeruginosa pilA	Motility mutant	+	+
P. aeruginosa rhlA	Rhamnolipid mutant	+	+
P. aeruginosa pvdA	Pyoverdin mutant	+	-
P. aeruginosa lasR	Quorum sensing mutant	+	+
P. aeruginosa rpoN	Regulatory mutant	-	+
P. aeruginosa mucA	Mucoid mutant	-	-

All *P. aeruginosa* mutants can suppress *A.* oryzae, *A. flavus* and *A. niger*

To further examine the effect of frequently occurring regulatory mutations on the *P. aeruginosa–Aspergillus* interaction, *A. niger*, *A. oryzae* and *A. flavus* was included.

As previously mentioned a mutant defective in the alternative sigma factor RpoN could not suppress the growth of *A. fumigatus*, but was overgrown by the fungus. Unexpectedly, neither *A. niger, A. oryzae* nor *A. flavus* could overgrow the *rpoN* mutant (figure 7).

The *lasR* mutant strain was able to suppress growth of *A. fumigatus* and similarly suppressed growth of *A. oryzae, A. niger* and *A. flavus* (figure 7). In the contact zone of the *P. aeruginosa lasR* mutant and *A. oryzae/A. flavus* a large accumulation of pyocyanin was observed. This had similarly been observed in the contact zone of *P. aeruginosa* PAO1 and *A. flavus/A. oryzae*, as described above, however the *lasR* mutant seemed to produce larger amounts pyocyanin. In general both *A. flavus* and *A. oryzae* triggered the production of pyocyanin in all *P. aeruginosa* strains except the *rpoN* strain.

Finally, we tested the *mucA* mutant (figure 7) and the remaining mutants (*fliM, pilA, pvdA* and *rhlA*). All mutants could initially suppress growth of *A. oryzae*, *A. niger* and *A. flavus* as previously observed in *A. fumigatus* AF293, but could in contrast to *A. fumigatus* also suppress after prolonged incubation.

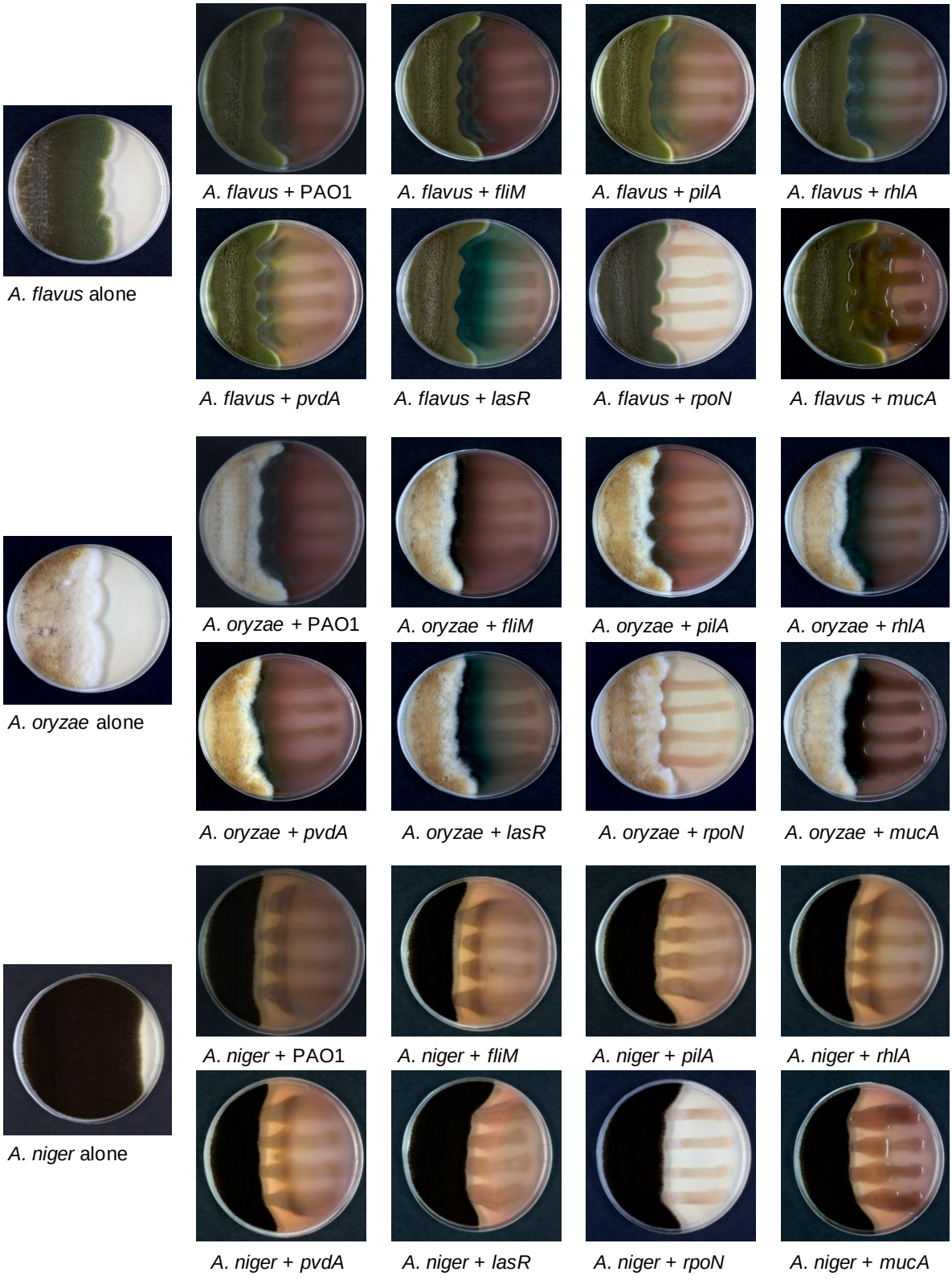


Figure 7: All *P. aeruginosa* mutants could suppress *A. oryzae*, *A. flavus* and *A. niger*. All *P. aeruginosa* mutants, except *rpoN*, produced pyocyanin in the contact zone of *A. oryzae* and *A. flavus*. Especially the *lasR* mutant produced visually large amounts of this compound. *P. aeruginosa* was likewise slightly inhibited by *A. niger*, probably due to the production of citric acid. Plates had been incubated at 37 °C for five days.

Chemical investigation of the interaction between *P. aeruginosa* and *Aspergillus* species

Another approach used to examine the interaction between the *Aspergillus* species and *P. aeruginosa* included HPLC analysis of predefined zones on the plates (figure 8). This could potentially explain the differentiable interaction pattern observed between *A. fumigatus* and the other *Aspergillus* strains.

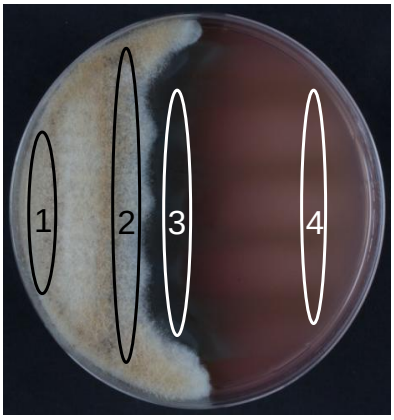


Figure 8: To examine the interaction between the *Aspergillus* species and *P. aeruginosa* four zones were defined on each plate, two in the fungus and two in the bacterium.

Each plate was divided into four zones (figure 8). Two in the fungus (zone 1 and 2) and two in the bacterium (zone 3 and 4). By comparing levels of extractable and detectable secondary metabolites from the fungus and bacterium in each zone an indication of potential chemical response could be investigated for the four *Aspergillus* species (*A. fumigatus*, *A. niger*, *A. oryzae* and *A. flavus*).

P. aeruginosa increased the production of phenazine-1-carboxamide and phenazine-1-carboxylic acid in the contact zone (zone 3, figure 9) of *A. oryzae* and *A. flavus* compared to zone 4. Only phenazine-1-carboxylic acid was increased in response to *A. niger*, while *A. fumigatus* AF293 did not seem trigger the production of phenazines by *P. aeruginosa* (figure 9). Similarly no other clinical or environmental *A. fumigatus* strain triggered the production of phenazine-1-carboxamide or phenazine-1-carboxylic acid. *P. aeruginosa* PAO1 is known to produce at least four different phenazines; pyocyanin, 1-hydroxyphenazine, phenazine-1-carboxylic acid and phenazine-1-carboxamide[49] and phenazines have previously been demonstrated to have antifungal activity[50–53]. Pyocyanin could be detected in zone 3 on *A. flavus/A.oryzae* with *P. aeruginosa* plates (correlating with the production of the green compound), but was undetected in zone 3 on *P. aeruginosa* alone plates. 1-

hydroxyphenazine was not detected.

These findings show that *P. aeruginosa* responds to the presence of some Aspergilli by increasing the production of phenazine compounds. Nevertheless, it does not appear that the growth inhibitory effect of *P. aeruginosa* requires an increased production of phenazines, as growth of *A. fumigatus* was inhibited in a similar way as other Aspergilli. Interestingly, no differences in secondary metabolite profiles were seen between the two zones in the fungi (with or without *P. aeruginosa*) indicating that the *Aspergillus* species do not alter their secondary metabolism to the presence of the bacterium by increasing or reducing expression of secondary metabolites.

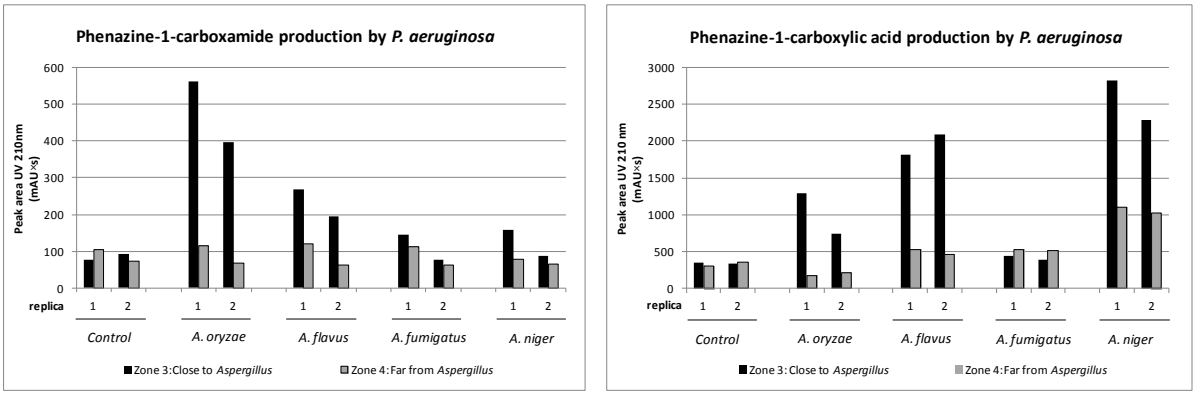


Figure 9: Production of two phenazines (phenazine-1-carboxamide and phenazine-1-carboxylic acid) by *P. aeruginosa* was increased in the presence of *A. oryzae* and *A. flavus*, while phenazine-1-carboxylic acid was increased in the presence of *A. niger*. No increase was measured in response to *A. fumigatus*. Phenazines were measured in zone 3 and 4 by HPLC after five days of incubation and results from two independent biological replica experiments are shown. Control: *P. aeruginosa* only**.**

Next the interaction between the *Aspergillus* species and the different *P. aeruginosa* mutants was similarly examined using HPLC. Initially the production of secondary metabolites by the different *P. aeruginosa* mutants was compared to the PAO1 strain (table 4).

Table 4: Comparison of the secondary metabolite production by the *P. aeruginosa* PAO1 strain and the *P. aeruginosa* mutants. Strains grown on WATM plates at 37 °C without *Aspergillus* present.

Difference in secondary metabolite profile between mutant and PAO1		
P. aeruginosa fliM	Motility mutant	No difference in secondary metabolite profile (see paper 2)
P. aeruginosa pilA	Motility mutant	No difference in secondary metabolite profile (see paper 2)
P. aeruginosa rhlA	Rhamnolipid mutant	No rhamnolipid detected in mutant
P. aeruginosa pvdA	Pyoverdin mutant	No difference in secondary metabolite profile (see paper 2)
P. aeruginosa lasR	Quorum sensing mutant	Phenazine-1-carboxamide and phenazine-1-carboxylic acid reduced in mutant
P. aeruginosa rpoN	Regulatory mutant	Reduced expression of several phenazines and quinolones in mutant
P. aeruginosa mucA	Mucoid mutant	No major difference in secondary metabolite profile

Of special interest were the three regulatory mutants; *mucA*, *lasR* and *rpoN* (figure 10). No major difference in secondary metabolite profile could be observed between the PAO1 strain and the *mucA* strain showing that the overgrowth of the mutant by *A. fumigatus* was not due to reduced expression of any detectable secondary metabolites, but may be due to the increased production of alginate (not detectable by the methods used) and possible reduced expression of virulence factors[6].

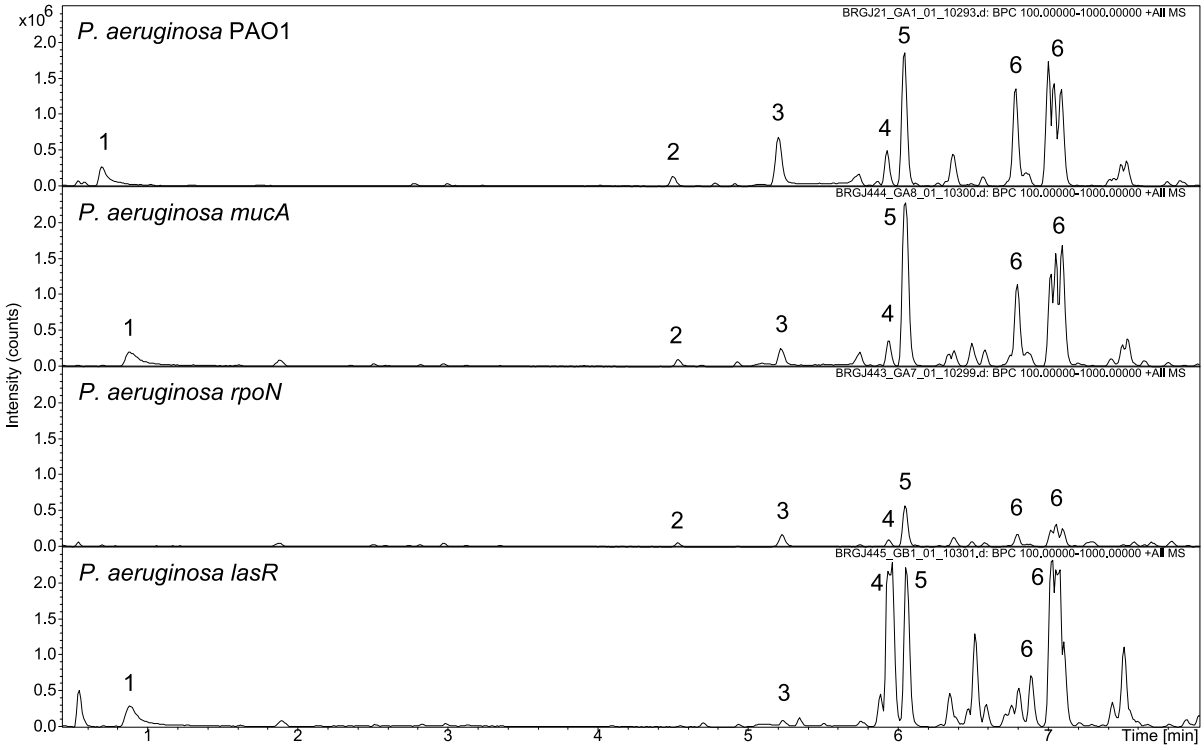


Figure 10: Comparison of secondary metabolite production of PAO1, *mucA*, *lasR* and *rpoN* mutant measured by HPLC-MS. Base peak chromatogram of extracts from *P. aeruginosa* PAO1, *mucA*, *lasR* and the *rpoN* mutant. Peaks are; 1: pyocyanin, 2: phenazine-1-carboxamide, 3: phenazine-1-carboxylic acid, 4: 2-n-Heptyl-4-oxy-quinoline/2-n-Heptyl-(1H)-quinolin-4-one, 5: 2-Heptyl-3-hydroxy-4(3H)-quinolinone, 6: unidentified quinolones with same elemental composition. All mutants had grown on WATM media for five days.

Both the *lasR* and *rpoN* mutant are known to have reduced expression of several virulence factors[6]. In the *lasR* mutant phenazine-1-carboxamide and phenazine-1-carboxylic acid were reduced, while the *rpoN* mutant had a reduced expression of several secondary metabolites including the phenazines as well as quinolones. Surprisingly (in contrast to *A. fumigatus*) several phenazines and quinolones were increased in the *rpoN* mutant in response to the three other Aspergilli (figure 11). These included among others phenazine-1-carboxamide, phenazine-1-carboxylic acid and 2-Heptyl-3-hydroxy-4(3H)-quinolinone. As no increase in phenazine production was detected against *A. fumigatus*, these data suggest that *P. aeruginosa* responds differentially toward different Aspergilli, and that the increased

phenazine production observed for co-culture with *A. niger*, *A. oryzae* or *A. flavus* is independent of RpoN.

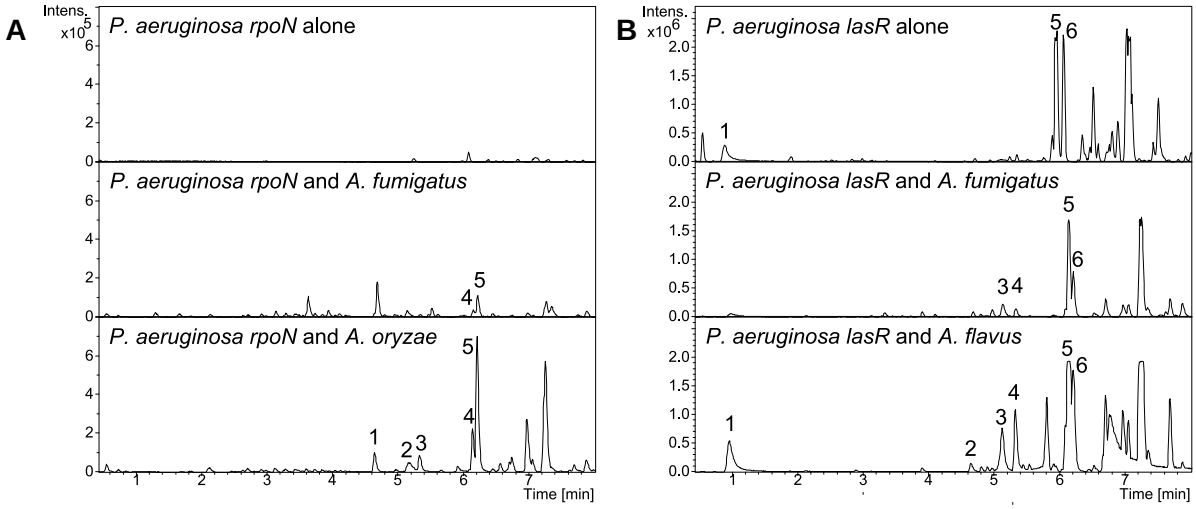


Figure 11: (A) Comparison of the secondary metabolite profile of the *P. aeruginosa rpoN* mutant alone and together with *A. oryzae* in zone 3 revealed an increase in phenazines (1: phenazine-1-carboxamide, 2: 1-hydroxyphenazine and 3: phenazine-1-carboxylic acid) and several quinolones (4: 2-n-Heptyl-4-oxy-quinoline/2-n-Heptyl-(1H)-quinolin-4-one, 5: 2-Heptyl-3-hydroxy-4(3H)-quinolinone) by *P. aeruginosa* in response to *A. oryzae*. No phenazines could be detected in response to *A. fumigatus*, while quinolones were detected in lower amounts. All experiments were done in duplicate. (B) Comparison of the secondary metabolite profile of the *P. aeruginosa lasR* mutant alone and together with *A. flavus* in zone 3 revealed an increase in all four phenazines (1: pyocyanin, 2: phenazine-1-carboxamide, 3: 1-hydroxyphenazine and 4: phenazine-1-carboxylic acid) by *P. aeruginosa*. Phenazines could similar be detected in response to *A. fumigatus*, but at lower levels. Quinolones (5: 2-n-Heptyl-4-oxy-quinoline/2-n-Heptyl-(1H)-quinolin-4-one and 6: 2-Heptyl-3-hydroxy-4(3H)-quinolinone) were detected in all three cases. All experiments were done in duplicate and measured by HPLC-MS.

In the *lasR* mutant strain chemical analysis revealed an increase in production of all four phenazines, including pyocyanin, 1-hydroxyphenazine, phenazine-1-carboxamide and phenazine-1-carboxylic acid (figure 11) by *P. aeruginosa lasR* towards *A. oryzae* and *A. flavus*. For *A. niger* an increase in phenazine-1-carboxylic acid was observed (not shown), while a minimal increase in phenazine-1-carboxylic acid was observed in response to *A. fumigatus.* No differences in quinolone production were observed. Again a differentiable interaction pattern was observed between *P. aeruginosa* and the different *Aspergillus* strains.

The involvement of hydrophobins in *A.nidulans-P.aeruginosa* interactions

The main focus of this thesis is hydrophobins and previous studies have shown a potential involvement in hydrophobins in disease[54–57]. After observing the potential of the developed plating assay and having constructed several *A. nidulans* hydrophobins deletion strains (chapter 4), it was intriguing to examine if hydrophobins could be involved in or altered the interaction pattern between *A. nidulans* and *P. aeruginosa*. In addition to the hydrophobin

deletion strains, an *A. nidulans* wild type strain (FGSC A4) was included as a control strain. *P. aeruginosa* was found to suppress all tested *Aspergillus* strains. *P. aeruginosa* produced pyocyanin in the contact zone of all tested Aspergilli and the strain was interestingly more mucoid in this zone (figure 12). The mucoidy was especially apparent upon plug-extraction as the hole borer was covered by sticky bacteria in this zone.

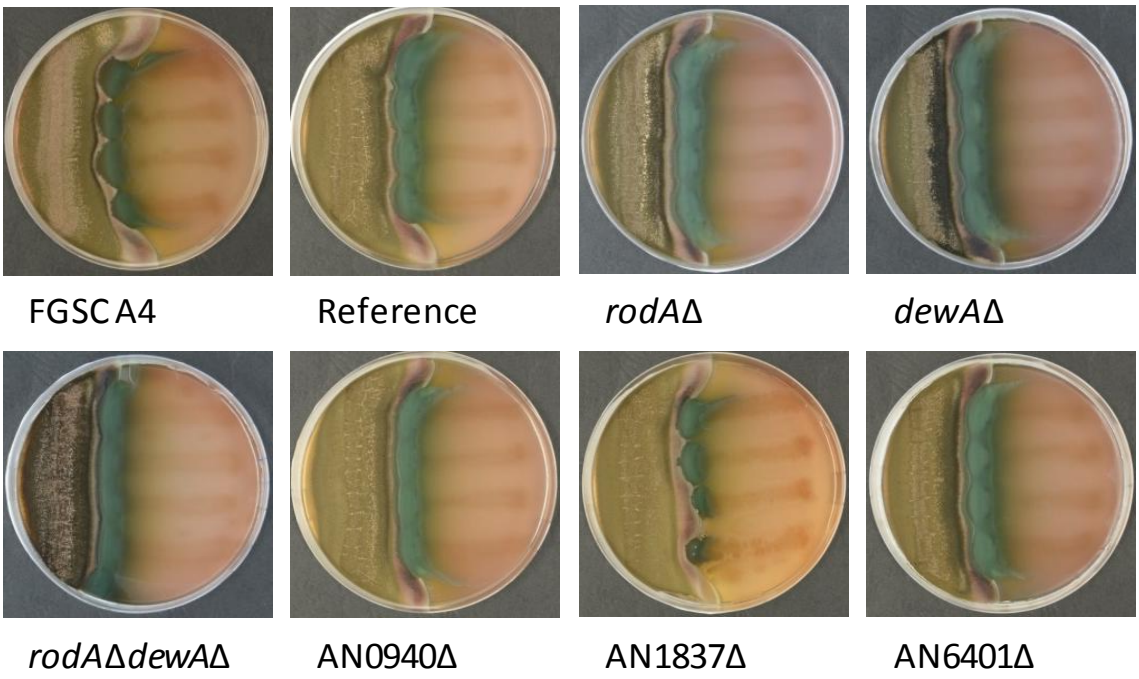


Figure 12: All *A. nidulans* strains were suppressed by *P. aeruginosa* PAO1 on WATM agar plates. Pictures were taken after five days of incubation at 37 °C. The Aspergilli turned red in the contact zone to *P. aeruginosa*, while the bacterium became mucoid and produced pyocyanin in the contact zone to the fungus. All experiments were done in duplicate.

Further from the fungus, no coloration of *P. aeruginosa* was observed and the strain was non-mucoid (minimal bacteria sticking to the hole borer). In the *Aspergillus* colony the colour changed to red in the contact zone with *P. aeruginosa*. This red area consisted of pure mycelium and had a padded appearance. In contrast the rest of the fungus had a normal appearance. *A. nidulans* was the only *Aspergillus* species to have been visually affected by the presence of *P. aeruginosa* compared to the previously four tested *Aspergillus* species (*A. fumigatus*, *A. niger*, *A. flavus* and *A. oryzae*).

Next HPLC analysis on the four previously defined zones was used to examine the interaction between *P. aeruginosa* PAO1 and the *Aspergillus* species. HPLC analysis, however, showed no change in detectable fungal secondary metabolites in the red zone compared to the rest of the (green) fungus, showing that secondary metabolite production in the fungus was not affected in the red zone, but only the appearance of the fungus.

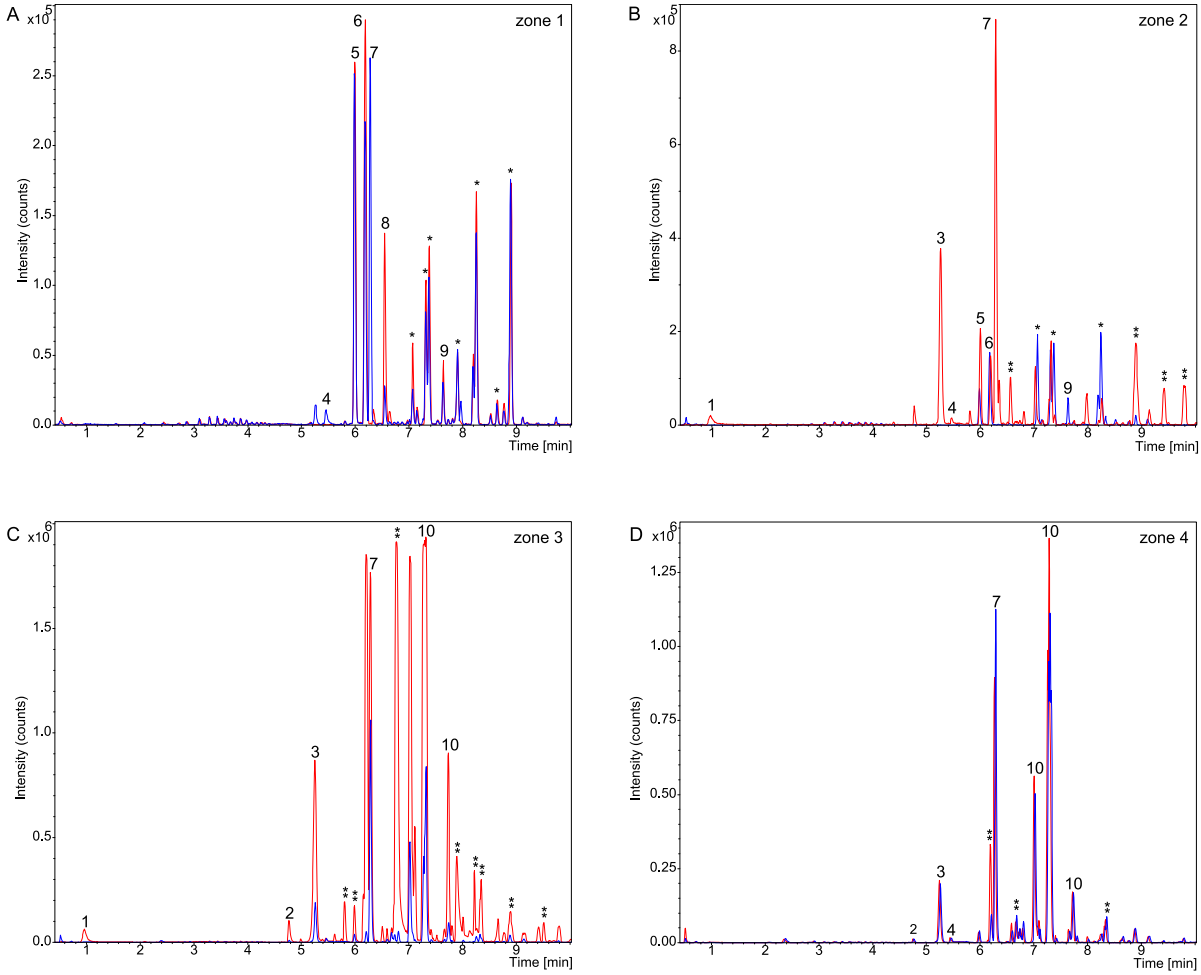


Figure 13: Comparison of secondary metabolite profiles of control plates and interaction plates. Base peak chromatograms from control plates are blue, while chromatograms from interaction plates are red. For zone 1 and zone 2 plates with *Aspergillus* alone were used as control plates, while plates with *Pseudomonas* alone were used as control plates for zone 3 and 4. Chromatograms of the reference strain is shown as similar results were obtained for all strains. (A) zone 1, (B) zone 2, (C) zone 3 and (D) zone 4. Peak 1: pyocyanin. Peak 2: phenazine-1-carboxamide. Peak 3: 1-hydroxyphenazine. Peak 4: phenazine-1-carboxylic acid. Peak 5: austinol. Peak 6: dehydroaustinol. Peak 7: 2-heptyl-3-hydroxy-4(3H)-quinolone. Peak 8: sterigmatocystin. Peak 9: emericellamide A. Peak 10: unidentified quinolones. (*): unidentified *A. nidulans* metabolite. (**): unidentified *P. aeruginosa* metabolite. *P. aeruginosa* secondary metabolites could be detected in all four zones, while *A. nidulans* secondary metabolites could only be detected in zone 1 and zone 2.

Next the predefined zones were compared using mixed culture plates and mono cultured plates. In zone 1 and 2 (figure 13A and 13B) all *A. nidulans* strains (*rodA*Δ, *dewA*Δ, AN0940Δ, AN1837Δ, AN6401Δ and reference) were found to produce similar repertoires of secondary metabolites. No differences in secondary metabolite profile could be seen between the *Aspergillus* strains with or without *P. aeruginosa*, indicating that similar chemical interaction patterns are observed between the hydrophobin deletion strains and *Pseudomonas* (figure 13A and 13B). In zone 1, however, sterigmatocystin was slightly increased on the mixed culture plates, while several *Pseudomonas* metabolites diffused into zone 2 on the mixed culture plate.

In zone 3 phenazines and quinolones were increased in production towards all *A. nidulans* hydrophobin mutants (figure 13C), while no difference could be seen in zone 4 between bacterial plates with or without *Aspergillus* (figure 13D). This had previously been observed for other *Aspergillus* strains. As no differences could be found between the hydrophobin deletion strains and control strains, it does not seem that hydrophobins alter the interaction pattern between the bacterium and fungus and may thus not play any significant role in the interaction.

Discussion

The aim of this part of the thesis was to examine the potential interaction between *Aspergillus* species and *P. aeruginosa.* The interaction between *P. aeruginosa* and several *Aspergillus* species was tested and showed that growth of all tested Aspergilli (*A. fumigatus, A. niger, A. oryzae, A. flavus* and *A. nidulans*) was suppressed by *P. aeruginosa.* Previous studies have tested the interaction of *P. aeruginosa* and Aspergilli showing both inhibitory and non inhibitory effects[30–32,35], which may be due to different culture conditions and the type of assay used (media plates or liquid culture). *P. aeruginosa* has likewise been shown to inhibit other fungal species including *Candida* species[31,50,58–60]. Despite the inhibitory affect observed on all tested *Aspergillus* species in this study, the two organisms grew as close as possible to each other. In agreement with our observations, Blyth[33] likewise found a close spatial relationship between *A. fumigatus* and *P. aeruginosa*, but the presence of *P. aeruginosa* affected ultrastructures of *A. fumigatus* hyphae[33]. In mature *A. fumigatus* biofilm, *P. aeruginosa* cells have been found distributed throughout the filamentous network with a minimal effect on *A. fumigatus* biomass[61]. It seems that *P. aeruginosa* and *A. fumigatus* can have a close spatial relationship, when coming in contact, but preferably occupy their own area. Similarly, it seems that other Aspergilli can grow close to *P. aeruginosa*, but preferably occupy their own area.

Genetic adaption and evolution of *P. aeruginosa* is common during the course of chronic CF lung infections and several genes have been found to be inactivated by mutations in clinical *P. aeruginosa* isolates[5,6,8]. Common mutations include the *lasR*, *rpoN* and *mucA* genes which all encode proteins with regulatory functions[6]. To further examine the interaction between *Aspergillus* species and *P. aeruginosa*, several *P. aeruginosa* knock-out mutants were

included.

A. fumigatus AF293 was initially suppressed by the *mucA* strain, but could after prolonged co-culturing partially grow over the mucoid *P. aeruginosa* strain, while *A. flavus, A. oryzae* and *A. niger* were suppressed. The *mucA* strain is known to have reduced virulence[6], including a reduced pulmonary clearance in animal models and increased alginate production[62]. These factors may enable *A. fumigatus* to partially grow over the bacterium. Nevertheless, the fungus is still suppressed indicating that genetic changes in the *P. aeruginosa* strains are not severe enough to completely abolish the inhibitory effect of the *P. aeruginosa* strain.

rpoN, an alternative sigma factor, is required for initial promoter recognition and consequently for transcription of a subset of genes by *P. aeruginosa* RNA polymerase[14,15]. Several virulence factors are affected in a *rpoN* mutant including the synthesis of pili, flagella, pyocyanin and rhamnolipids[14–17]. *rpoN* mutants are also less virulent[63] and form poorer biofilms[16,60] compared to wildtype strains. Interestingly, *A. fumigatus* AF293 completely grew over a *P. aeruginosa rpoN* mutant, while *A. flavus, A. oryzae* and *A. niger* were suppressed. Therefore we examined the differential interaction between the *rpoN* mutant and the different Aspergilli by chemical analysis. Our data showed that the *rpoN* mutant in mono-culture was defective in production of several quinolones and phenazines compared to PAO1. This reduced secondary metabolite production may remove the inhibition of *A. fumigatus* and allow it to grow over the *P. aeruginosa rpoN* mutant. Interestingly, the production of several phenazines and quinolones was increased in the *rpoN* mutant in response to *A. flavus, A. oryzae* and *A. niger*, but not *A. fumigatus*. This finding suggests that the increased phenazine production observed for co-culture with *A. niger, A. oryzae* or *A. flavus* may be independent of RpoN and that regulation of phenazine production most likely is multifactorial.

In contrast to the *mucA* and *rpoN* deletion strains, a *lasR* mutant suppressed growth of all tested Aspergilli including *A. fumigatus*. This supports findings by Mowat *et al.*[61], who previously demonstrated that two quorum sensing knockout strains (PAO1:Δ*LasR* and PAO1:Δ*LasI*) inhibited *A. fumigatus* biofilm formation. When comparing interactions between the *lasR* mutant and the Aspergilli, we observed that phenazines were increased in response to the presence of *A. oryzae, A. flavus* and *A. niger*, while no response was seen to the presence of *A. fumigatus.* In the contact zone of the *lasR* mutant and *A. oryzae/A. flavus* a

large accumulation of pyocyanin was observed. This accumulation was higher in the *lasR* mutant compared to PAO1. Cugini *et al.*[64] similarly observed a large enhancement of pyocyanin by a *lasR* mutant, when inoculated onto a *C. albicans* lawn indicating that some fungal species may trigger the production of pyocyanin.
Finally individual contribution of motility, rhamnolipid and pyoverdin to the suppression of the Aspergilli was examined, but did not individually seem to play a role as all Aspergilli were suppressed by these *P. aeruginosa* mutants.

To further examine the interaction, secondary metabolite production was examined in both the bacterium and fungal species. An increase in phenazine production (including phenazine-1-carboxamide and phenazine-1-carboxylic acid) by *P. aeruginosa* was observed in the contact zone of some *Aspergillus* species. These metabolites readily diffused and could be found throughout the plates. This was similarly observed by Moree *et al.*[35]. Both phenazine-1-carboxylic acid and phenazine-1-carboxamide are known antifungal compounds[51], and the increase of the phenazines may thus be a response from *P. aeruginosa* to the presence of the Aspergilli. The increase did, however, not prevent the Aspergilli from growing as close to the *Pseudomonas* as possible, indicating that the phenazines may both be a signal to the Aspergilli of the existence of the *Pseudomonas* and an attempt to inhibit the fungi. Despite the Aspergilli being suppressed by *P. aeruginosa*, no changes in *Aspergillus* secondary metabolite profile could surprisingly be detected in any of the Aspergilli. In contrast Moree *et al.*[35] found several *A. fumigatus* metabolites suppressed by *P. aeruginosa* PA14. This may be due to the use of different *P. aeruginosa* strains, type of medium or instrument sensitivity.
Several studies indicate that phenazines seem to play a role in antagonistic interactions among different fungal species[50,65–67]. Gibson *et al.*[53] described the accumulation of a red pigment in *Candida albicans* cells and subsequently killing of the cells, when co-cultured with *P. aeruginosa.* The formation of red pigment required 5-methyl-phenazinium-1-carboxylate (5MPCA) produced by *P. aeruginosa*. Morales *et al.*[68] demonstrated that phenazine methosulphate likewise killed *C. albicans* and induced accumulation of red pigment. No red pigments were observed in this study, nor were the fungi killed indicating that different mechanisms apply in varying bacterial-fungal interactions.

Recently, Moree *et al.*[35] showed that *A. fumigatus* can convert phenazine-1-carboxylic acid and pyocyanin into other phenazines, when co-cultured with *P. aeruginosa.* Interestingly, in

our study *A. fumigatus* strains did not trigger any increase of phenazines by *P. aeruginosa*, nor were any differences in *A. fumigatus* secondary metabolites observed, resulting in no detectable secondary metabolic effect on either organism. Even though it did not seem that the two organisms affected each other visually or chemically, it has previously been shown that there is a significant decrease in lung function in CF patients colonized with both organisms compared to patients colonized with only *A. fumigatus* or *P. aeruginosa*, respectively[27,69]. Similarly, it has been observed that CF patients colonized with both *C. albicans* and *Aspergillus* or *Pseudomonas*, *C. albicans* and *Aspergillus* have decreased lung functions and body mass index[70]. This may "just" be a consequence of the heavily affected lungs of CF patients, but could potentially also be due to interactions between the colonizing organisms. In contrast the reduced lung function may provide favourable conditions for co-colonization. Further studies are required in this area to elucidate the role of interactions in human health.

As a smaller experiment the involvement of hydrophobins in the interaction between *A. nidulans* and *P. aeruginosa* was examined. Hydrophobins may play a role in human pathogenicity[54], but their role in fungal-bacterial interactions has to my knowledge not previously been examined. *A. nidulans* was (as previously seen for other Aspergilli) suppressed by *P. aeruginosa*. The hydrophobin deletion strains did not alter this inhibition, indicating that hydrophobins are not involved in the interaction between *A. nidulans* and *P. aeruginosa*. No differences in secondary metabolite production were observed between the hydrophobin deletion strains and control strains and all strains triggered the production of phenazines in *P. aeruginosa*. However, hydrophobins vary between *Aspergillus* species (see chapter 4 and introduction) and other hydrophobins in e.g. *A. fumigatus* may display a different pattern. This remains to be examined.

Concluding remarks

The interaction between *P. aeruginosa* and different *Aspergillus* species proved diverse and *P. aeruginosa* was found to interact differently towards *A. fumigatus* than *A. niger, A. oryzae* and *A. flavus*. These species, also counting *A. nidulans*, triggered the production of anti-fungal phenazines, while *A. fumigatus* did not. Furthermore, frequently observed regulatory mutations in *P. aeruginosa* during long-term CF infections changed the bacterial-fungal

interactions as measured in defined culture conditions. Both *mucA* and *rpoN* mutants were unable to suppress *A. fumigatus*, but could suppress the other examined *Aspergillus* species showing the diversity in *Aspergillus-Pseudomonas* interactions. Further studies are required to investigate if such genetically adapted *P. aeruginosa* strains are less competitive towards *A. fumigatus* in the CF airways (e.g. using mouse models), and if there are clinical effects associated with particular mutations in *P. aeruginosa*. Of special interest would be the use of phenazine deficient mutants to further elucidate the role of phenazines in the interaction. Finally, significant more research is needed within this area before all questions have been answered. I feel that only the surface has been lifted upon the enormous mechanism constituting *Aspergillus-Pseudomonas* interactions.

References for chapter 5

1. Bauernfeind A, Bertele RM, Harms K, Horl G, Jungwirth R, Petermuller C, Przyklenk B, Weisslein-Pfister C (1987) Qualitative and quantitative microbiological analysis of sputa of 102 patients with cystic fibrosis. *Infection* **15:** 270-277.

2. Bakare N, Rickerts V, Bargon J, Just-Nubling G (2003) Prevalence of *Aspergillus fumigatus* and other fungal species in the sputum of adult patients with cystic fibrosis. *Mycoses* **46:** 19-23.

3. Horre R, Symoens F, Delhaes L, Bouchara JP (2010) Fungal respiratory infections in cystic fibrosis: a growing problem. *Med Mycol* **48 Suppl 1:** S1-S3.

4. Govan JR, Deretic V (1996) Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev* **60:** 539-574.

5. Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, Miller SI, Ramsey BW, Speert DP, Moskowitz SM, Burns JL, Kaul R, Olson MV (2006) Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc Natl Acad Sci U S A* **103:** 8487-8492.

6. Yang L, Jelsbak L, Marvig RL, Damkiaer S, Workman CT, Rau MH, Hansen SK, Folkesson A, Johansen HK, Ciofu O, Hoiby N, Sommer MO, Molin S (2011) Evolutionary dynamics of bacteria in a human host environment. *Proc Natl Acad Sci U S A* **108:** 7481-7486.

7. Mahenthiralingam E, Campbell ME, Speert DP (1994) Nonmotility and phagocytic resistance of *Pseudomonas aeruginosa* isolates from chronically colonized patients with cystic fibrosis. *Infect Immun* **62:** 596-605.

8. Hoiby N (1975) Prevalence of mucoid strains of *Pseudomonas aeruginosa* in bacteriological specimens from patients with cystic fibrosis and patients with other diseases. *Acta Pathol Microbiol Scand Suppl* **83:** 549-552.

9. Hoffman LR, Kulasekara HD, Emerson J, Houston LS, Burns JL, Ramsey BW, Miller SI (2009) *Pseudomonas aeruginosa* lasR mutants are associated with cystic fibrosis lung

disease progression. *J Cyst Fibros* **8:** 66-70.

10. Pesci EC, Milbank JB, Pearson JP, McKnight S, Kende AS, Greenberg EP, Iglewski BH (1999) Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **96:** 11229-11234.

11. Gambello MJ, Iglewski BH (1991) Cloning and characterization of the *Pseudomonas aeruginosa* lasR gene, a transcriptional activator of elastase expression. *J Bacteriol* **173:** 3000-3009.

12. Juhas M, Eberl L, Tummler B (2005) Quorum sensing: the power of cooperation in the world of *Pseudomonas*. *Environ Microbiol* **7:** 459-471.

13. D'Argenio DA, Wu M, Hoffman LR, Kulasekara HD, Deziel E, Smith EE, Nguyen H, Ernst RK, Larson Freeman TJ, Spencer DH, Brittnacher M, Hayden HS, Selgrade S, Klausen M, Goodlett DR, Burns JL, Ramsey BW, Miller SI (2007) Growth phenotypes of *Pseudomonas aeruginosa* lasR mutants adapted to the airways of cystic fibrosis patients. *Mol Microbiol* **64:** 512-533.

14. Totten PA, Lara JC, Lory S (1990) The rpoN gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. *J Bacteriol* **172:** 389-396.

15. Ishimoto KS, Lory S (1989) Formation of pilin in *Pseudomonas aeruginosa* requires the alternative sigma factor (RpoN) of RNA polymerase. *Proc Natl Acad Sci U S A* **86:** 1954-1957.

16. Thompson LS, Webb JS, Rice SA, Kjelleberg S (2003) The alternative sigma factor RpoN regulates the quorum sensing gene rhlI in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **220:** 187-195.

17. Heurlier K, Denervaud V, Pessi G, Reimmann C, Haas D (2003) Negative control of quorum sensing by RpoN (sigma54) in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **185:** 2227-2235.

18. Latge JP (1999) *Aspergillus fumigatus* and aspergillosis. *Clin Microbiol Rev* **12:** 310-350.

19. Mortensen KL, Jensen RH, Johansen HK, Skov M, Pressler T, Howard SJ, Leatherbarrow H, Mellado E, Arendrup MC (2011) *Aspergillus* species and other molds in respiratory samples from patients with cystic fibrosis: a laboratory-based study with focus on *Aspergillus fumigatus* azole resistance. *J Clin Microbiol* **49:** 2243-2251.

20. Paugam A, Baixench MT, Demazes-Dufeu N, Burgel PR, Sauter E, Kanaan R, Dusser D, Dupouy-Camet J, Hubert D (2010) Characteristics and consequences of airway colonization by filamentous fungi in 201 adult patients with cystic fibrosis in France. *Med Mycol* **48:** S32-S36.

21. Valenza G, Tappe D, Turnwald D, Frosch M, Konig C, Hebestreit H, Abele-Horn M (2008) Prevalence and antimicrobial susceptibility of microorganisms isolated from sputa of patients with cystic fibrosis. *J Cyst Fibros* **7:** 123-127.

22. Mroueh S, Spock A (1994) Allergic bronchopulmonary aspergillosis in patients with cystic fibrosis. *Chest* **105:** 32-36.

23. Sudfeld CR, Dasenbrook EC, Merz WG, Carroll KC, Boyle MP (2010) Prevalence and risk factors for recovery of filamentous fungi in individuals with cystic fibrosis. *J Cyst Fibros* **9:** 110-116.

24. Neuveglise C, Sarfati J, Debeaupuis JP, Vu TH, Just J, Tournier G, Latge JP (1997) Longitudinal study of *Aspergillus fumigatus* strains isolated from cystic fibrosis patients. *Eur J Clin Microbiol Infect Dis* **16:** 747-750.

25. de Valk HA, Klaassen CH, Yntema JB, Hebestreit A, Seidler M, Haase G, Muller FM, Meis JF (2009) Molecular typing and colonization patterns of *Aspergillus fumigatus* in patients with cystic fibrosis. *J Cyst Fibros* **8:** 110-114.

26. Vanhee LM, Symoens F, Bouchara JP, Nelis HJ, Coenye T (2008) High-resolution genotyping of *Aspergillus fumigatus* isolates recovered from chronically colonised patients with cystic fibrosis. *Eur J Clin Microbiol Infect Dis* **27:** 1005-1007.

27. Amin R, Dupuis A, Aaron SD, Ratjen F (2010) The effect of chronic infection with *Aspergillus fumigatus* on lung function and hospitalization in patients with cystic fibrosis. *Chest* **137:** 171-176.

28. Pihet M, Carrere J, Cimon B, Chabasse D, Delhaes L, Symoens F, Bouchara JP (2009) Occurrence and relevance of filamentous fungi in respiratory secretions of patients with cystic fibrosis--a review. *Med Mycol* **47:** 387-397.

29. Nelson LA, Callerame ML, Schwartz RH (1979) Aspergillosis and atopy in cystic fibrosis. *Am Rev Respir Dis* **120:** 863-873.

30. Blyth W, Forey A (1971) The influence of respiratory bacteria and their biochemical fractions on *Aspergillus fumigatus*. *Sabouraudia* **9:** 273-282.

31. Kerr JR (1994) Suppression of fungal growth exhibited by *Pseudomonas aeruginosa*. *J Clin Microbiol* **32:** 525-527.

32. Mangan A (1969) Interactions between some aural aspergillus species and bacteria. *J Gen Microbiol* **58:** 261-266.

33. Blyth W (1971) Modifications in the ultrastructure of *Aspergillus fumigatus* due to the presence of living cells of *Pseudomonas aeruginosa*. *Sabouraudia* **9:** 283-286.

34. Leclair LW, Hogan DA (2010) Mixed bacterial-fungal infections in the CF respiratory tract. *Med Mycol* **48 Suppl 1:** S125-S132.

35. Moree WJ, Phelan VV, Wu CH, Bandeira N, Cornett DS, Duggan BM, Dorrestein PC (2012) Interkingdom metabolic transformations captured by microbial imaging mass spectrometry. *Proc Natl Acad Sci U S A* **109:** 13811-13816.

36. Raper, K. B. and Thom, C. (1949) A manual of the penicillia. Baltimore: Williams & Wilkens Co. 920 p.

37. Bertani G (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol* **62:** 293-300.

38. Frisvad JC, Samson RA (2004) *Penicillium* subgenus *Penicillium* - A guide to identification

of food and air-borne terverticillate Penicillia and their mycotoxins. *Stud Mycol* **49:** 1-173.

39. Holloway BW, Morgan AF (1986) Genome organization in *Pseudomonas*. *Annu Rev Microbiol* **40:** 79-105.

40. Nagoba BS, Deshmukh SR, Wadher BJ, Mahabaleshwar L, Gandhi RC, Kulkarni PB, Mane VA, Deshmukh JS (1998) Treatment of superficial pseudomonal infections with citric acid: an effective and economical approach. *J Hosp Infect* **40:** 155-157.

41. Frisvad JC, Rank C, Nielsen KF, Larsen TO (2009) Metabolomics of *Aspergillus fumigatus*. *Med Mycol* **47:** S53-S71.

42. Amitani R, Taylor G, Elezis EN, Llewellyn-Jones C, Mitchell J, Kuze F, Cole PJ, Wilson R (1995) Purification and characterization of factors produced by *Aspergillus fumigatus* which affect human ciliated respiratory epithelium. *Infect Immun* **63:** 3266-3271.

43. Lewis RE, Wiederhold NP, Chi J, Han XY, Komanduri KV, Kontoyiannis DP, Prince RA (2005) Detection of gliotoxin in experimental and human aspergillosis. *Infect Immun* **73:** 635-637.

44. Klausen M, Heydorn A, Ragas P, Lambertsen L, Aaes-Jorgensen A, Molin S, Tolker-Nielsen T (2003) Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Mol Microbiol* **48:** 1511-1524.

45. Abdel-Mawgoud AM, Lepine F, Deziel E (2010) Rhamnolipids: diversity of structures, microbial origins and roles. *Appl Microbiol Biotechnol* **86:** 1323-1336.

46. Soberon-Chavez G, Aguirre-Ramirez M, Sanchez R (2005) The *Pseudomonas aeruginosa* RhlA enzyme is involved in rhamnolipid and polyhydroxyalkanoate production. *J Ind Microbiol Biotechnol* **32:** 675-677.

47. Visca P, Ciervo A, Orsi N (1994) Cloning and nucleotide sequence of the pvdA gene encoding the pyoverdin biosynthetic enzyme L-ornithine N5-oxygenase in *Pseudomonas aeruginosa*. *J Bacteriol* **176:** 1128-1140.

48. Yang L, Jelsbak L, Molin S (2011) Microbial ecology and adaptation in cystic fibrosis airways. *Environ Microbiol* **13:** 1682-1689.

49. Mavrodi DV, Bonsall RF, Delaney SM, Soule MJ, Phillips G, Thomashow LS (2001) Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **183:** 6454-6465.

50. Rella A, Yang MW, Gruber J, Montagna MT, Luberto C, Zhang YM, Del PM (2012) *Pseudomonas aeruginosa* Inhibits the Growth of *Cryptococcus* Species. *Mycopathologia* **173:** 451-461.

51. Gross H, Loper JE (2009) Genomics of secondary metabolite production by *Pseudomonas* spp. *Nat Prod Rep* **26:** 1408-1446.

52. Kerr JR, Taylor GW, Rutman A, Hoiby N, Cole PJ, Wilson R (1999) *Pseudomonas aeruginosa* pyocyanin and 1-hydroxyphenazine inhibit fungal growth. *J Clin Pathol* **52:** 385-387.

53. Gibson J, Sood A, Hogan DA (2009) *Pseudomonas aeruginosa-Candida albicans* interactions: localization and fungal toxicity of a phenazine derivative. *Appl Environ Microbiol* **75:** 504-513.

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

55. Shibuya K, Takaoka M, Uchida K, Wakayama M, Yamaguchi H, Takahashi K, Paris S, Latge JP, Naoe S (1999) Histopathology of experimental invasive pulmonary aspergillosis in rats: pathological comparison of pulmonary lesions induced by specific virulent factor deficient mutants. *Microb Pathog* **27:** 123-131.

56. Thau N, Monod M, Crestani B, Rolland C, Tronchin G, Latge JP, Paris S (1994) rodletless mutants of *Aspergillus fumigatus*. *Infect Immun* **62:** 4380-4388.

57. Schmidt S, Tramsen L, Hanisch M, Latge JP, Huenecke S, Koehl U, Lehrnbecher T (2011) Human natural killer cells exhibit direct activity against *Aspergillus fumigatus* hyphae, but not against resting conidia. *J Infect Dis* **203:** 430-435.

58. Treat J, James WD, Nachamkin I, Seykora JT (2007) Growth inhibition of *Trichophyton* species by *Pseudomonas aeruginosa*. *Arch Dermatol* **143:** 61-64.

59. Bandara HM, Yau JY, Watt RM, Jin LJ, Samaranayake LP (2010) *Pseudomonas aeruginosa* inhibits in-vitro *Candida* biofilm development. *BMC Microbiol* **10:** 125.

60. Hogan DA, Kolter R (2002) *Pseudomonas-Candida* interactions: an ecological role for virulence factors. *Science* **296:** 2229-2232.

61. Mowat E, Rajendran R, Williams C, McCulloch E, Jones B, Lang S, Ramage G (2010) *Pseudomonas aeruginosa* and their small diffusible extracellular molecules inhibit *Aspergillus fumigatus* biofilm formation. *FEMS Microbiol Lett* **313:** 96-102.

62. Boucher JC, Yu H, Mudd MH, Deretic V (1997) Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: characterization of muc mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection. *Infect Immun* **65:** 3838-3846.

63. Hendrickson EL, Plotnikova J, Mahajan-Miklos S, Rahme LG, Ausubel FM (2001) Differential roles of the *Pseudomonas aeruginosa* PA14 rpoN gene in pathogenicity in plants, nematodes, insects, and mice. *J Bacteriol* **183:** 7126-7134.

64. Cugini C, Morales DK, Hogan DA (2010) *Candida albicans*-produced farnesol stimulates *Pseudomonas* quinolone signal production in LasR-defective *Pseudomonas aeruginosa* strains. *Microbiology* **156:** 3096-3107.

65. Thomashow LS, Weller DM (1988) Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. *J Bacteriol* **170:** 3499-3508.

66. Chin AWT, Bloemberg GV, Mulders IH, Dekkers LC, Lugtenberg BJ (2000) Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. *Mol Plant-Microbe Interact* **13:** 1340-1345.

67. Anjaiah V, Koedam N, Nowak-Thompson B, Loper JE, Hofte M, Tambong JT, Cornelis P (1998) Involvement of phenazines and anthranilate in the antagonism of *Pseudomonas aeruginosa* PNA1 and Tn5 derivatives toward *Fusarium* spp. and *Pythium* spp. *Mol Plant-Microbe Interact* **11:** 847-854.

68. Morales DK, Jacobs NJ, Rajamani S, Krishnamurthy M, Cubillos-Ruiz JR, Hogan DA (2010) Antifungal mechanisms by which a novel *Pseudomonas aeruginosa* phenazine toxin kills *Candida albicans* in biofilms. *Mol Microbiol* **78:** 1379-1392.

69. Navarro J, Rainisio M, Harms HK, Hodson ME, Koch C, Mastella G, Strandvik B, McKenzie SG (2001) Factors associated with poor pulmonary function: cross-sectional analysis of data from the ERCF. European Epidemiologic Registry of Cystic Fibrosis. *Eur Respir J* **18:** 298-305.

70. Chotirmall SH, O'Donoghue E, Bennett K, Gunaratnam C, O'Neill SJ, McElvaney NG (2010) Sputum *Candida albicans* presages FEV decline and hospital-treated exacerbations in cystic fibrosis. *Chest* **138:** 1186-1195.

6 Construction of a microfluidic cantilever lab-on-a-chip system for studying interactions between *Aspergillus* and *Pseudomonas aeruginosa*

This chapter presents efforts towards constructing a microfluidic cantilever lab-on-a-chip system.

Introduction

In recent years micro-cantilevers have shown great potential for biosensing applications. They can be used in food quality control, environmental control, *in vitro* diagnostics and in the genomic and proteomic field. Cantilevers are especially useful due to their small size and quick response. Furthermore, cantilevers display high sensitivity and can be integrated into "lab-on-a-chip" devices[1–3]. A complete cantilever chip consists of a chip body for handling and a number of separate cantilevers (figure 1).

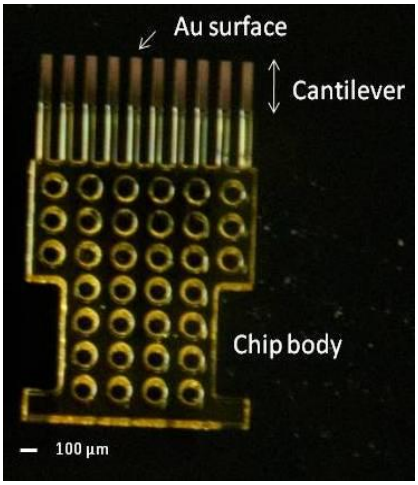


Figure 1: A complete cantilever chip consisting of a chip body for handling the cantilever and ten separate cantilevers. The cantilevers can be functionalized by a layer sensitive to a specific compound. In this case a gold surface has been applied.

One surface of the cantilever can be functionalized with a layer sensitive to a specific compound, thereby allowing the detection of specific chemical or biological compounds as well as biochemical interactions[1,4]. Furthermore, micro-cantilevers do not require labelling of the compounds. This saves both time and effort, but also limits variability and uncertainty in the results due to differentiable binding of the label to the compound[5,6].

Cantilever sensors can be operated in two main modes, either static mode or dynamic mode. In static mode the degree of bending of the cantilever is measured. When molecules bind to the functionalised cantilever surface, they generate surface stress, which results in the cantilever bending. In dynamic mode the resonance frequency of the cantilever is monitored.

As molecules bind to the surface of the cantilever, the overall mass increases, resulting in the resonance frequency decreasing. In both modes the deflection of the cantilevers can be monitored by using an external optical readout system, where a laser beam is focused onto the cantilever and the reflection monitored by a position sensitive diode[4,6,7].

Several groups have used cantilevers as detectors in both static and dynamic modes showing the ability to detect DNA[8–11], RNA[12] and proteins[11,13–15]. Furthermore, cantilevers have been used to detect *Staphylococcus* enterotoxin B[16], Bovine capillary endothelial cells[17], virus[18], bacteria[19–21] and fungi[22,23]. Most bio-chemical reactions are performed in static mode[6] and was also the method of choice in this study.

Aspergillus species are widely used in the fermentation and food industry, but are also known to be involved in pathogenesis. They can produce a large number of extrolites including secondary metabolites and hydrophobins[24–29]. *Pseudomonas aeruginosa* is the most common isolated pathogen in cystic fibrosis (CF) patients. However, *Aspergillus fumigatus* and other Aspergilli are also commonly found in the respiratory tract secretions from CF patients[30,31].

In this study an attempt to create a microfluidic cantilever lab-on-a-chip system to study possible interactions between *Aspergillus* and *P. aeruginosa* was conducted. Due to safety reasons, *Aspergillus oryzae* was used as a model organism for *A. fumigatus.* By covering the cantilever surface with *Aspergillus* conidia, an examination of whether *P. aeruginosa* can adhere to and affect germination of *Aspergillus* conidia or biofilm formation would be possible. Furthermore, covering the cantilever surface with *P. aeruginosa* would provide insight into whether *Aspergillus* can adhere to and germinate on *P. aeruginosa* cells. In addition, by using previously constructed *Aspergillus nidulans* hydrophobin mutants (see chapter 4), a possible role of hydrophobins in the interaction with *P. aeruginosa* could be examined. To my knowledge no one has yet examined interactions between two organisms in a microfluidic cantilever lab-on-a-chip system.

Materials and methods

Design of lab-on-a-chip system

Three main parts were fabricated to complete the microfluidic system; a bottom plate, a sealing and a top plate (figure 2). All items were designed using Synrad WinMark Pro and cut out using a 48-5s Duo-Lase carbon dioxide laser marking system, Synrad Inc.

The bottom plate was cut out from a 5 mm thick poly (methyl methacrylate) plate (PMMA) and had the dimensions 39 x 39 x 5 mm. It contained a channel (2.8 x 3.6 x 0.9 mm) for placing the chip and another channel for flow of media with the dimensions (5.9 x 0.76 x 0.77 mm). Inlet and outlet holes were marked at each end of the flow channel and holes (4.7 mm in diameter) were cut in each corner of the plate for later assembly. The bottom plate was annealed for 30 min at 85 °C to reduce stress caused by the laser cutting. Inlet and outlet holes (4 mm deep) were drilled using a 3.2 mm drill and thread was cut in the holes.

The top plate was cut from a 1.5 mm thick PMMA plate and had the dimensions 39 x 39 x 1.5 mm. A square (9.6 x 9.0 x 1.5 mm) was cut in the middle of the plate and holes (4.7 mm in diameter) were likewise cut in each corner of the plate. The top plate was annealed for 30 min at 85 °C to reduce stress caused by the laser cutting.

The sealing was cut from 1 mm thick polydimethylsiloxane (PDMS) and had the dimensions 11.9 x 11.9 x 1 mm. A hole (6.9 x 0.6/1.1 mm) was cut in the sealing to prevent clocking the flow channel with PDMS after setup assembly.

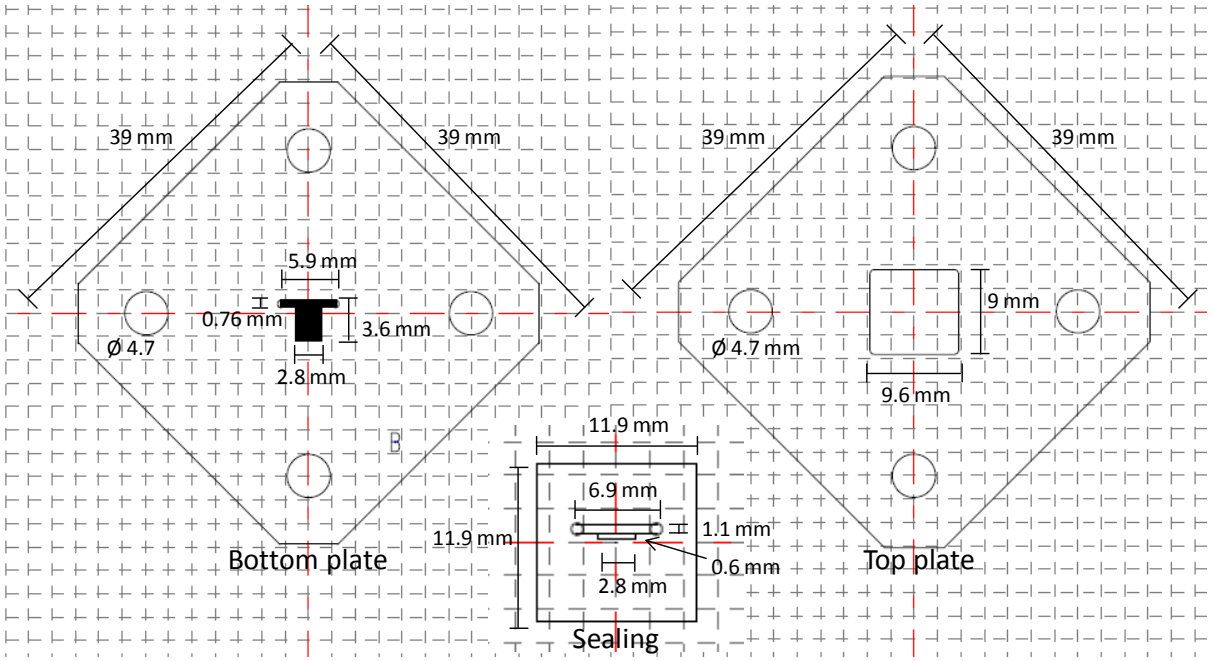


Figure 2: Dimensions of the bottom plate, top plate and sealing.

Cantilever fabrication

The complete cantilever chip, consisting of a chip body for handling and ten separate cantilevers, was constructed in the photo-sensitive polymer SU8 by UV lithography as described by Nordstrom *et al.*[6] Each cantilever was 100 µm wide, 500 µm long and 5.5 µm

thick. To achieve sufficient reflection for optical readout and sensitize the cantilever surface to molecule binding, a gold layer was applied to the top surface of the cantilevers.

Bending of cantilevers

Distilled water, 0.9 % sodium chloride and CYA broth[32] were used to examine the bending of cantilevers in different media. Pictures were taken of each cantilever using a Carl Zeiss "Axio Scope.A1" Vario reflected-light microscope fitted with an "Infinity X" digital camera and the degree of bending was calculated before and after 20 hours incubation in media.

Germination of *Aspergillus oryzae*

Timeframes for conventional fungal growing phases were determined by inoculating *A. oryzae* spores in liquid CYA broth and incubating under shaking conditions at room temperature. Each hour samples were taken and examined under microscope.

Compatibility of microfluidic lab-on-a-chip components and *Aspergillus*

The components going to be in contact with *Aspergillus* during experiments were tested for compatibility. *A. oryzae* RIB40 was used as a model for the opportunistic human pathogen *A. fumigatus*. Each component (bottom plate, sealing, glass slide and chip) was placed on a Czapek Yeast Autolysate agar (CYA) media plate[32] inoculated with *A. oryzae*. The plates were incubated at 25 °C for 8 days.

To further examine the ability of *Aspergillus* spores to adhere to the cantilevers a spore suspension of *A. oryzae* was prepared from a 14 day old CYA media plate. The chips were dipped in the *A. oryzae* spore suspension, briefly left to air dry and examined under microscope. To examine the ability of *A. oryzae* spores to adhere to and germinate on the cantilevers under flow, a Watson Marlow multi-channel pump, with a flowrate of 30 µl/min, was connected to the microfluidic setup. CYA broth was used as media. The setup was inoculated with *A. oryzae* spores and left for 1 hour before the flow was started. After 17 and 21 hours the cantilevers were examined.

Results and discussion

Construction of the lab-on-a-chip system

The complete lab-on-a-chip system consisted of five parts; a PMMA bottom plate, a PDMS sealing, a glass cover, a PMMA top plate and a SU-8 cantilever chip. The system was assembled on a purpose built holder as shown on figure 3A.

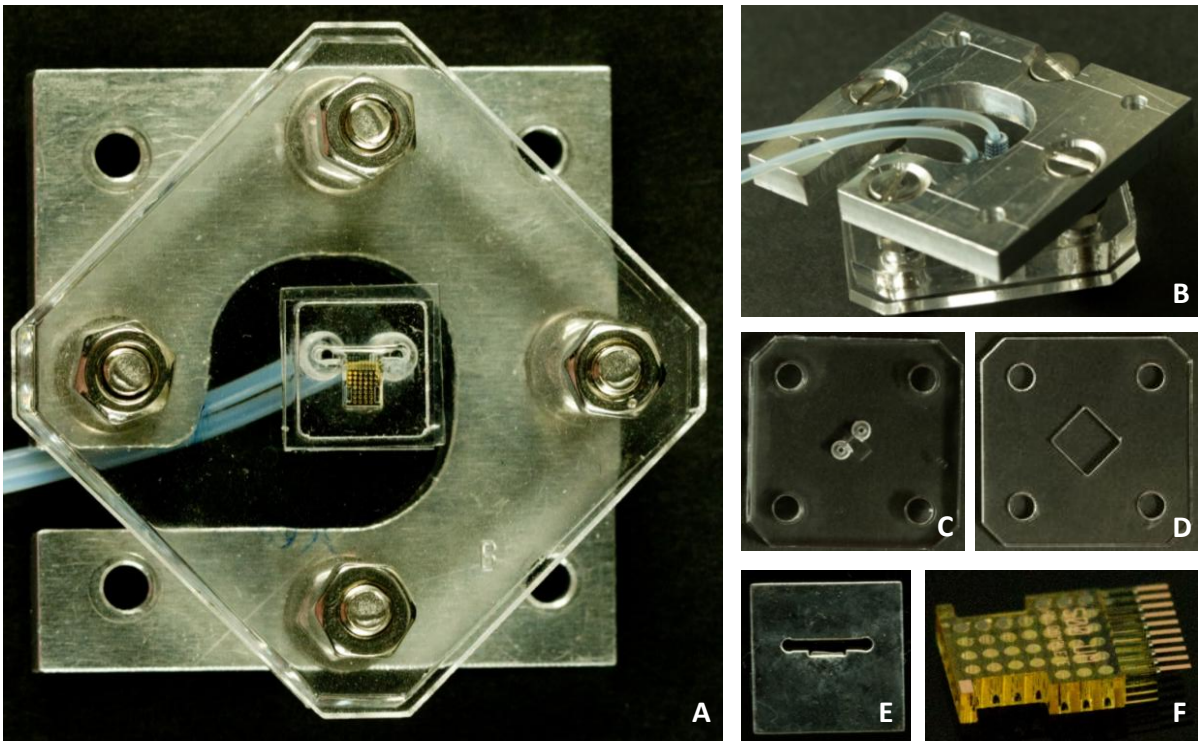


Figure 3: The assembled lab-on-a-chip system. (A) The system was assembled on a purpose built holder. (B) Tubes fitted to the bottom of the PMMA bottom plate allowed filling of the system with media. The lab-on-a-chip system consisted of (C) a PMMA bottom plate, (D) a PMMA top plate, (E) a PDMS sealing, a glass cover (not shown) and (F) a SU-8 cantilever chip.

To prevent leaking, size 0.8 O-rings were inserted into the inlet and outlet holes in the bottom plate and two screw connectors fitted with tubes were fixed to the holes allowing filling of the microfluidic system with media (figure 3B). Next the bottom plate was fitted on the holder and the chip was placed in the channel. The sealing was then placed over the chip and channel to prevent leakage, followed by a glass cover and finally the top plate. The assembled lab-on-a-chip system was held in place by four screws. Prior to the setup shown in figure 3 several efforts were made to construct a leak free microfluidic system, but unfortunately this was not accomplished. The final system setup did however prove to have limited leakage and could be used for preliminary experiments.

Bending of cantilevers

Initial bending of cantilevers may pose a problem, when using very sensitive polymer-based cantilevers to examine biochemical interactions. Functionalization of individual cantilevers will further change their bending, and aligning several different functionalized cantilevers may pose a problem due to differences in bending. Bending of up to several micrometers may occur and are often beyond the detection limit for sensitive optical detection systems[33]. To minimize initial bending, the effect of different media on cantilever bending was investigated by incubating the SU-8 cantilevers in distilled water, CYA broth and 0.9 % NaCl. After incubation in all three media, the degree of cantilever bending was reduced (figure 4). Incubation in CYA broth resulted in the lowest reduction in bending, while both distilled water and 0.9% NaCl showed a higher reduction in bending. Based on the results, it was decided to pre-incubate all cantilevers in distilled water prior to use in further experiments to limit initial bending of the cantilevers.

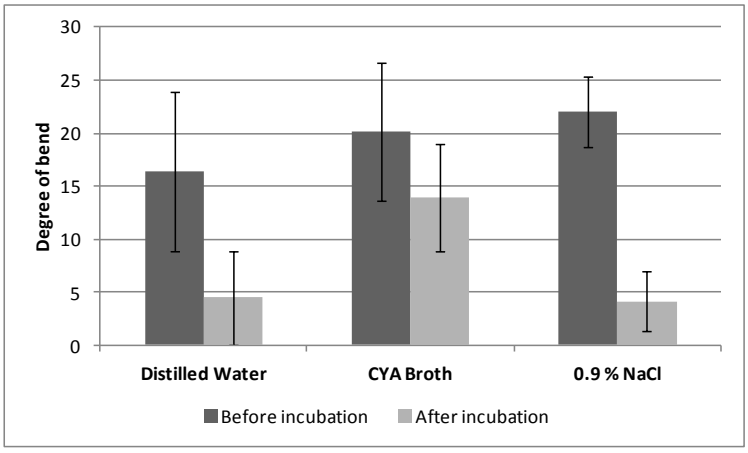


Figure 4: Degree of cantilever bending before and after 20 h incubation in media. Measurements were conducted on 10 separate cantilever tips and an average of the degree of bend was calculated.

Compatibility of microfluidic lab-on-a-chip components and *Aspergillus*

Several parts of the microfluidic system had the possibility of coming in contact with the *Aspergillus* during experimental setups. Therefore the compatibility of the *Aspergillus* with the components of the microfluidic system was examined. As can be seen on figure 5 none of the parts showed any inhibitory effect on *A. oryzae* demonstrating the ability of the system to be used in further experiments. *A. oryzae* grew as close to both PMMA and the glass cover as possible, while PDMS and the SU-8 cantilever chips were nearly completely overgrown.

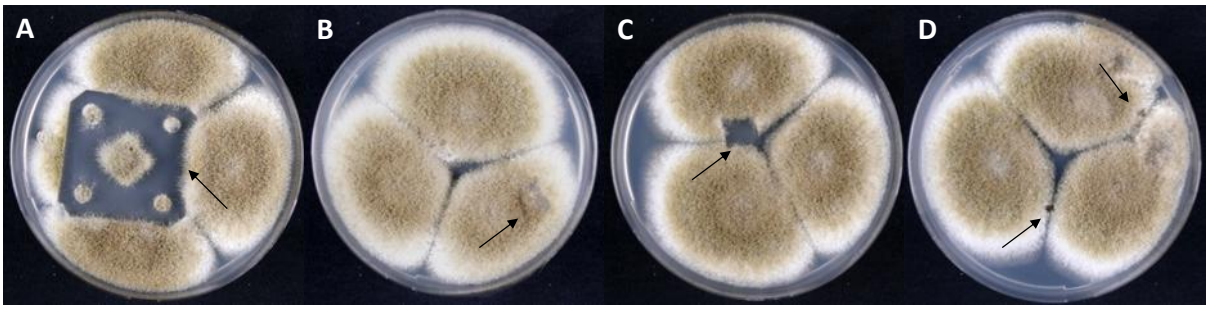


Figure 5: Examination of inhibitory effect of microfluidic lab-on-a-chip components on *Aspergillus oryzae* RIB40. (A) PMMA bottom plate, (B) PDMS sealing, (C) glass cover and (D) SU-8 chips. The different parts are indicated by arrows.

Immobilization of *Aspergillus* on cantilevers

The immobilization of *A. oryzae* spores on the cantilevers was an essential part in the experiments. *Penicillium roqueforti* spores had successfully been immobilized on cantilevers (Mona H. Pedersen, unpublished results), so the cantilevers were initially dipped in an *A. oryzae* spore suspension to examine binding capabilities. The cantilevers had a functionalized top gold surface to achieve sufficient reflection for optical readout and sensitize the cantilever surface to molecule binding, while the bottom side consisted of SU-8.

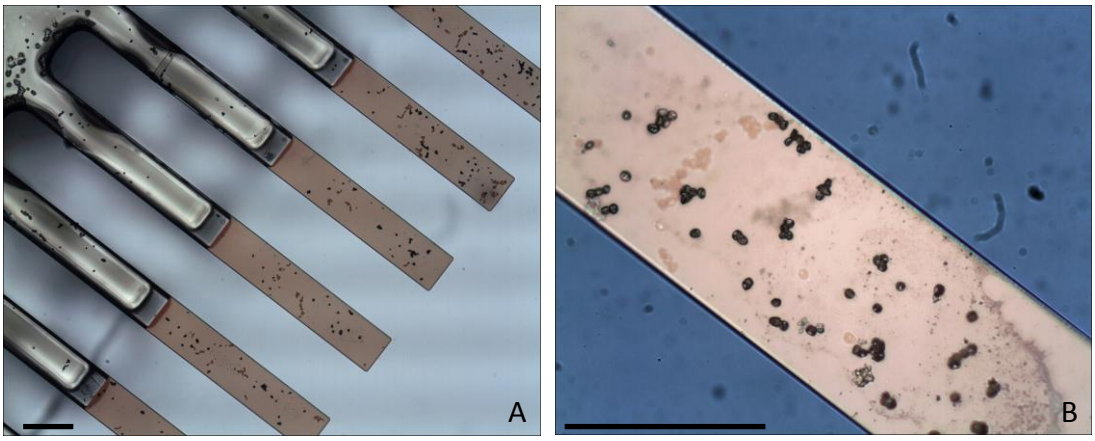


Figure 6: (A) Immobilization of *A. oryzae* spores on cantilevers. (B) Spores bound both to the top (dark brown spores) and bottom of the cantilever (light brown spores). Bar: 100 µm

Aspergillus oryzae spores bound both to the top (dark brown spores) and the bottom of the cantilevers (light brown spores) (figure 6). However, this unspecific binding may cause an increase in total mass on the cantilever leading to a larger degree in bending. Nugaeva *et al.*[22] had previously used anti-*Aspergillus niger* polyclonal antibodies to immobilize *A. niger* spores on cantilevers, while Blagoi *et al.*[34] had functionalized SU-8 surfaces with IgG proteins. Therefore, by functionalizing the top of the cantilever with anti-*A. oryzae* polyclonal

antibodies, a higher degree of specific binding could be achieved, reducing the effect of unspecific binding on the total mass. Nugaeva *et al.*[23] had previously demonstrated the ability to differentiate between dormant spores and active *A. niger* spores on cantilevers by observing a shift in resonance frequency as the spores started to germinate. Our setup did not allow simultaneous measurements of bending and visualization of the spores on the cantilevers. Therefore to determine timeframes for all fungal growing phases, the germination of *A. oryzae* spores was followed over time (figure 7).

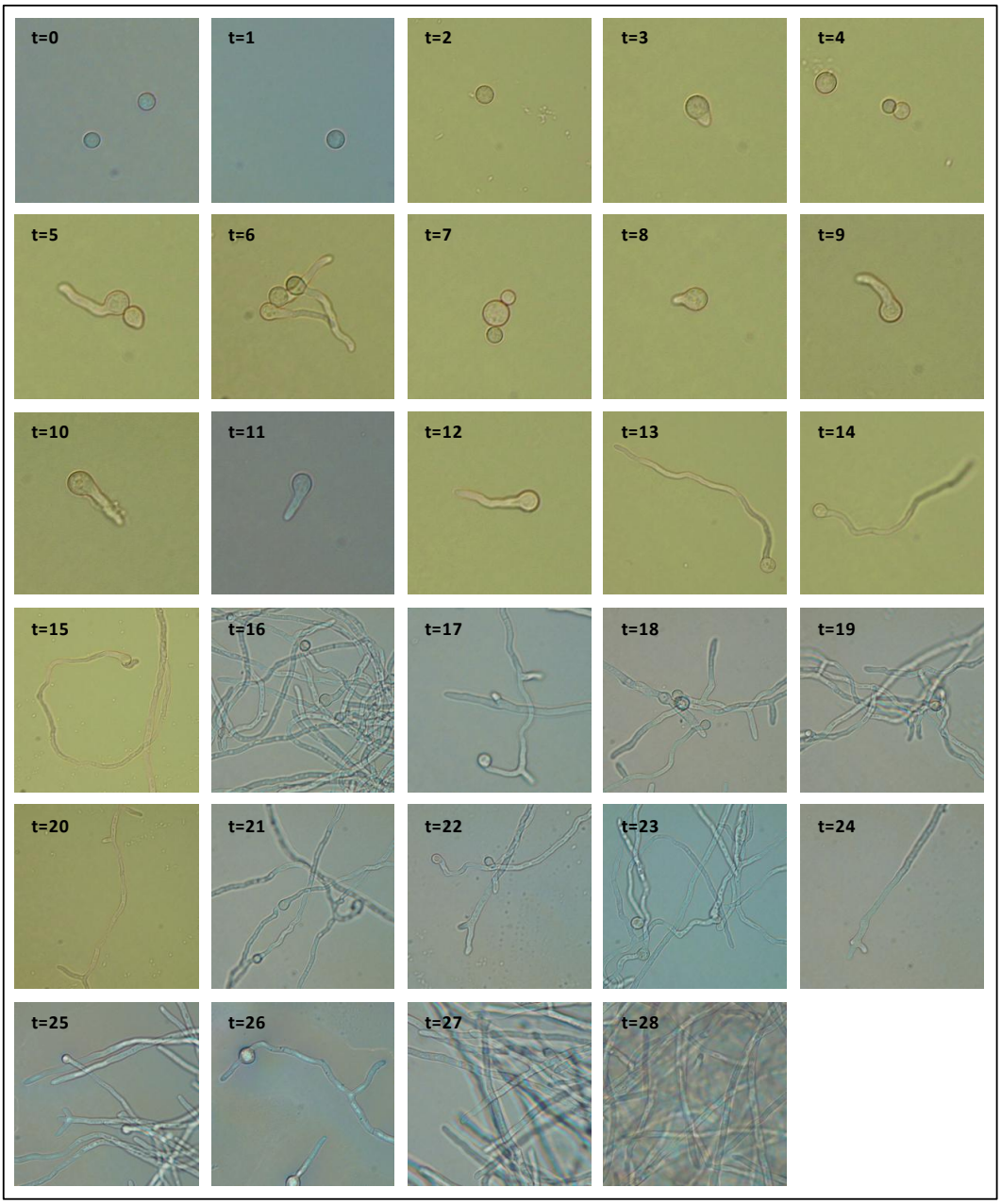


Figure 7: Germination of *A. oryzae* spores in CYA broth. All fungal growing phases could be observed including swelling (1-4 h), emergence of germ tubes (3-8 h), elongation and branching of hyphae (8-20 h) and formation of mycelium (20 h).

Initially, the spores started to swell (1-4 hours) followed by the emergence of germ tubes (3-8 hours). Then an elongation of the hyphae followed, resulting in initial branching after app. 15 hours of incubation. After 20 hours, extensive branching was observed and a dense mycelium had been formed.

Next the immobilization of *A. oryzae* spores was examined under flow conditions. Dormant *A. oryzae* spores were able to adhere to the cantilevers under flow conditions without further functionalizing the cantilever surface apart from the gold layer. After respectively 17 and 21 hours the spores had germinated and mycelium had formed (figure 8) showing the ability to detect *A. oryzae* growth on cantilevers under flow conditions. Unfortunately, germination caused the spores to detach resulting in drift of germinating spores and mycelium in the system, and thereby a homogenous picture could not be achieved. This may be overcome by using a lower initial spore concentration and by functionalizing the surface with anti-*A. oryzae* polyclonal antibody, as previously described. Nugaeva *et al.*[22] have shown the ability to detect all the conventional fungal growing phases by comparing the resonance frequency shift with a conventional fungal growth curve. This shows the potential of examining the effect of bacteria (*Pseudomonas aeruginosa*) on different stages of fungal germination or biofilm formation. Several groups have successfully detected bacteria using cantilevers[19–21], but to my knowledge no efforts have been made to examine mixed biofilms using cantilevers.

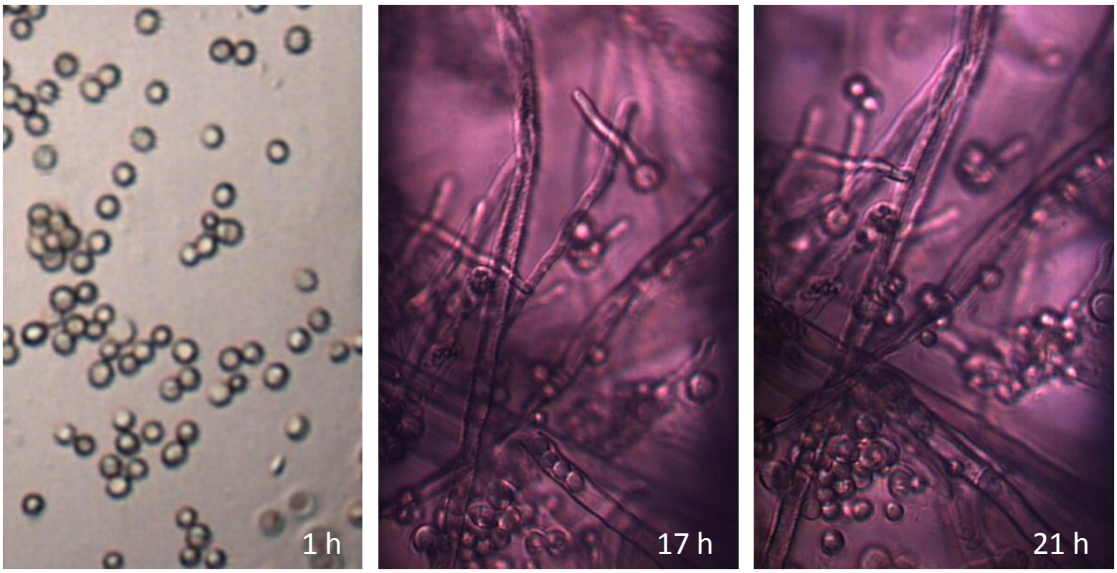


Figure 8: Adhesion of *A. oryzae* spores on cantilevers under flow conditions. Pictures taken with 200X magnification after 1 hour, 17 hours and 21 hours.

Concluding remarks

Cantilevers are being used in many different fields and have been used to detect both fungi and bacteria[16,19,21–23]. The aim of this study was to construct a microfluidic cantilever lab-on-a-chip system to study the effect of bacteria (*P. aeruginosa*) on the germination of *Aspergillus* conidia and examine possible mixed biofilms. Furthermore, using previously constructed hydrophobin deletion strains, the role of hydrophobins in mixed biofilms could be studied. Nugaeva *et al.*[22] had previously detected all conventional fungal growing phases on a cantilever tip. Yet, those experiments were conducted under either dry or humid conditions. In this study, we attempted to conduct similar measurements under flow conditions. Several efforts were made to construct a microfluidic cantilever lab-on-a-chip system enabling these studies, but due to extensive leakage problems and lack of sufficient measuring equipment, this was not achieved. However, our results do show a potential for studying interactions between fungi and bacteria using cantilevers, as the ability to immobilize *A. oryzae* spores on cantilevers under static conditions and partly under flow conditions has been demonstrated.

References for chapter 6

1. Alvarez M, Lechuga LM (2010) Microcantilever-based platforms as biosensing tools. *Analyst* **135:** 827-836.

2. Thaysen J, Marie R, Boisen A (2001) Cantilever-based bio-chemical sensor integrated in a microliquid handling system. *MEMS 2001,14th IEEE International Conference on Micro Electro Mechanical Systems* 401-404.

3. Alvarez M, Carrascosa LG, Zinoviev K, Plaza JA, Lechuga LM (2009) Biosensors Based on Cantilevers. *Methods Mol Biol* **504:** 51-71.

4. Lang HP, Gerber C (2008) Microcantilever sensors. *Top Curr Chem* **285:** 1-27.

5. Xu S, Mutharasan R (2009) Cantilever biosensors in drug discovery. *Expert Opin Drug Discovery* **4:** 1237-1251.

6. Nordstrom M, Keller S, Lillemose M, Johansson A, Dohn S, Haefliger D, Blagoi G, Havsteen-Jakobsen M, Boisen A (2008) SU-8 cantilevers for bio/chemical sensing; Fabrication, characterisation and development of novel read-out methods. *Sensors* **8:** 1595-1612.

7. Meyer G, Amer NM (1988) Novel Optical Approach to Atomic Force Microscopy. *Appl Phys Lett* **53:** 1045-1047.

8. Gunter RL, Zhine R, Delinger WG, Manygoats K, Kooser A, Porter TL (2004) Investigation of DNA sensing using piezoresistive microcantilever probes. *Ieee Sens J* **4:** 430-433.

9. Alvarez M, Carrascosa LG, Moreno M, Calle A, Zaballos A, Lechuga LM, Martinez A, Tamayo J (2004) Nanomechanics of the formation of DNA self-assembled monolayers and hybridization on microcantilevers. *Langmuir* **20:** 9663-9668.

10. McKendry R, Zhang JY, Arntz Y, Strunz T, Hegner M, Lang HP, Baller MK, Certa U, Meyer E, Guntherodt HJ, Gerber C (2002) Multiple label-free biodetection and quantitative DNA-binding assays on a nanomechanical cantilever array. *Proc Nat Acad Sci USA* **99:** 9783-9788.

11. Fritz J, Baller MK, Lang HP, Rothuizen H, Vettiger P, Meyer E, Guntherodt HJ, Gerber C, Gimzewski JK (2000) Translating biomolecular recognition into nanomechanics. *Science* **288:** 316-318.

12. Zhang J, Lang HP, Huber F, Bietsch A, Grange W, Certa U, McKendry R, Guntgerodt HJ, Hegner M, Gerber C (2006) Rapid and label-free nanomechanical detection of biomarker transcripts in human RNA. *Nat Nanotechnol* **1:** 214-220.

13. Lee JH, Kim TS, Yoon KH (2004) Effect of mass and stress on resonant frequency shift of functionalized Pb(Zr0.52Ti0.48)O-3 thin film microcantilever for the detection of C-reactive protein. *Appl Phys Lett* **84:** 3187-3189.

14. Moulin AM, O'Shea SJ, Badley RA, Doyle P, Welland ME (1999) Measuring surface-induced conformational changes in proteins. *Langmuir* **15:** 8776-8779.

15. Savran CA, Knudsen SM, Ellington AD, Manalis SR (2004) Micromechanical detection of proteins using aptamer-based receptor molecules. *Anal Chem* **76:** 3194-3198.

16. Campbell GA, Medina MB, Mutharasan R (2007) Detection of *Staphylococcus* enterotoxin B at picogram levels using piezoelectric-excited millimeter-sized cantilever sensors. *Sens Actuators, B* **126:** 354-360.

17. Saif MTA, Sager CR, Coyer S (2003) Functionalized biomicroelectromechanical systems sensors for force response study at local adhesion sites of single living cells on substrates. *Ann Biomed Eng* **31:** 950-961.

18. Gunter RL, Delinger WG, Manygoats K, Kooser A, Porter TL (2003) Viral detection using an embedded piezoresistive microcantilever sensor. *Sens Actuators, A* **107:** 219-224.

19. Gfeller KY, Nugaeva N, Hegner M (2005) Micromechanical oscillators as rapid biosensor for the detection of active growth of Escherichia coli. *Biosens Bioelectron* **21:** 528-533.

20. Campbell GA, Delesdernier D, Mutharasan R (2007) Detection of airborne *Bacillus anthracis* spores by an integrated system of an air sampler and a cantilever immunosensor. *Sens Actuators, B* **127:** 376-382.

21. Weeks BL, Camarero J, Noy A, Miller AE, Stanker L, De Yoreo JJ (2003) A microcantilever-based pathogen detector. *Scanning* **25:** 297-299.

22. Nugaeva N, Gfeller KY, Backmann N, Duggelin M, Lang HP, Guntherodt HJ, Hegner M (2007) An antibody-sensitized microfabricated cantilever for the growth detection of *Aspergillus niger* spores. *Microsc Microanal* **13:** 13-17.

23. Nugaeva N, Gfeller KY, Backmann N, Lang HP, Duggelin M, Hegner M (2005) Micromechanical cantilever array sensors for selective fungal immobilization and fast growth detection. *Biosens Bioelectron* **21:** 849-856.

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

25. Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, Arroyo J, Berriman M, Abe K, Archer DB, Bermejo C, Bennett J, Bowyer P, Chen D, Collins M, Coulsen R, Davies R, Dyer PS, Farman M, Fedorova N, Fedorova N, Feldblyum TV, Fischer R, Fosker N, Fraser A, Garcia JL, Garcia MJ, Goble A, Goldman GH, Gomi K, Griffith-Jones S, Gwilliam R, Haas B, Haas H, Harris D, Horiuchi H, Huang J, Humphray S, Jimenez J, Keller N, Khouri H, Kitamoto K, Kobayashi T, Konzack S, Kulkarni R, Kumagai T, Lafon A, Latge JP, Li W, Lord A, Lu C, Majoros WH, May GS, Miller BL, Mohamoud Y, Molina M, Monod M, Mouyna I, Mulligan S, Murphy L, O'Neil S, Paulsen I, Penalva MA, Pertea M, Price C, Pritchard BL, Quail MA, Rabbinowitsch E, Rawlins N, Rajandream MA, Reichard U, Renauld H, Robson GD, Rodriguez de CS, Rodriguez-Pena JM, Ronning CM, Rutter S, Salzberg SL, Sanchez M, Sanchez-Ferrero JC, Saunders D, Seeger K, Squares R, Squares S, Takeuchi M, Tekaia F, Turner G, Vazquez de Aldana CR, Weidman J, White O, Woodward J, Yu JH, Fraser C, Galagan JE, Asai K, Machida M, Hall N, Barrell B, Denning DW (2005) Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature* **438:** 1151-1156.

26. Galagan JE, Calvo SE, Cuomo C, Ma LJ, Wortman JR, Batzoglou S, Lee SI, türkmen M, Spevak CC, Clutterbuck J, Kapitonov V, Jurka J, Scazzocchio C, Farman M, Butler J, Purcell S, Harris S, Braus GH, Draht O, Busch S, D'Enfert C, Bouchier C, Goldman GH, Bell-Pedersen D, Griffiths-Jones S, Doonan JH, Yu J, Vienken K, Pain A, Freitag M, Selker EU, Archer DB, Peñalva MA, Oakley BR, Momany M, Tanaka T, Kumagai T, Asai K, Machida M, Nierman WC, Denning DW, Caddick M, Hynes M, Paoletti M, Fischer R, Miller B, Dyer P, Sachs MS, Osmani SA, Birren BW (2005) Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. *Nature* **438:** 1105-1115.

27. Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, Turner G, de Vries RP, Albang R, Albermann K, Andersen MR, Bendtsen JD, Benen JA, van den Berg M, Breestraat S, Caddick MX, Contreras R, Cornell M, Coutinho PM, Danchin EG, Debets AJ, Dekker P, van Dijck PW, van DA, Dijkhuizen L, Driessen AJ, d'Enfert C, Geysens S, Goosen C, Groot GS, de Groot PW, Guillemette T, Henrissat B, Herweijer M, van den Hombergh JP, van den Hondel CA, van der Heijden RT, van der Kaaij RM, Klis FM, Kools HJ, Kubicek CP, van Kuyk PA, Lauber J, Lu X, van der Maarel MJ, Meulenberg R, Menke H, Mortimer MA, Nielsen J, Oliver SG, Olsthoorn M, Pal K, van Peij NN, Ram AF, Rinas U, Roubos JA, Sagt CM, Schmoll M, Sun J, Ussery D, Varga J, Vervecken W, van de Vondervoort PJ, Wedler H, Wosten HA, Zeng AP, van Ooyen AJ, Visser J, Stam H (2007) Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat Biotechnol* **25:** 221-231.

28. Andersen MR, Nielsen J (2009) Current status of systems biology in Aspergilli. *Fungal Genet Biol* **46:** S180-S190.

29. Rank C, Larsen TO, Frisvad JC, Machida M, Gomi K (2010) Functional Systems Biology of *Aspergillus*. In: Machida M, Gomi K, editors. *Aspergillus*: Molecular Biology and Genomics. Norfolk, UK: Caister Academic Press. pp. 173-198.

30. Bakare N, Rickerts V, Bargon J, Just-Nubling G (2003) Prevalence of *Aspergillus fumigatus* and other fungal species in the sputum of adult patients with cystic fibrosis. *Mycoses* **46:** 19-23.

31. Nagano Y, Millar BC, Johnson E, Goldsmith CE, Elborn JS, Rendall J, Moore JE (2007) Fungal infections in patients with cystic fibrosis. *Rev Med Microbiol* **18:** 11-16.

32. Frisvad JC, Samson RA (2004) *Penicillium* subgenus *Penicillium* - A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. *Stud Mycol* **49:** 1-173.

33. Dohn S, Greve A, Svendsen WE, Boisen A (2010) The influence of refractive index change and initial bending of cantilevers on the optical lever readout method. *Rev Sci Instrum* **81:** 065104.

34. Blagoi G, Keller S, Johansson A, Boisen A, Dufva M (2008) Functionalization of SU-8 photoresist surfaces with IgG proteins. *Appl Surf Sci* **255:** 2896-2902.

7 Overall discussion and conclusion

The focus of this thesis was to create an overview of *Aspergillus* hydrophobins and achieve a deeper understanding of the roles of hydrophobins in *Aspergillus* species. Very little is known about *Aspergillus* hydrophobins, and the few studies that exist on *Aspergillus* hydrophobins focus on a limited number of hydrophobins. Part of this thesis dealt with *Aspergillus-P. aeruginosa* interactions. Similarly little is known about the potential interactions between Aspergilli and *P. aeruginosa* and the possible involvement of hydrophobins.
The results obtained in this project have been presented in the previous four chapters. In this chapter the obtained results and conclusions drawn from this PhD study will be discussed.

Aspergillus species display a varying number of hydrophobins

In the last 10 years several full genome sequences from Aspergilli have been published[1–5] providing us with a unique opportunity to putatively identify hydrophobins in the sequenced Aspergilli. Using a bioinformatics approach, nine full genome sequences from seven Aspergilli were analysed for hydrophobins providing, for the first time, a complete overview of putative hydrophobins in the full genome sequenced Aspergilli (**chapter 3**). For a protein to be defined as a hydrophobin in this study, the protein had to fulfil the criteria of having a minimum of eight cysteines, two cysteine pairs and an intact characteristic cysteine pattern. Furthermore, the protein had to be an appropriate size compared to previously characterized proteins. These criteria were based on guidelines set by Wessels[6] and the structure of hydrophobins HFBI, HFBII and EAS[7–10]. We assume that hydrophobins need to meet these criteria to maintain a globular overall fold similar to the known hydrophobin structures. This particular structure may be necessary for the hydrophobins in order to fulfil several proposed biological functions. By selecting hydrophobins based on our criteria, 50 putative hydrophobins were found in the Aspergilli. Of the identified hydrophobins, 20 (on species level) had not been mentioned in other published studies. Each *Aspergillus* species contained between two and eight putative hydrophobins, which was in agreement with Sunde *et al.*[11] who predicted that most fungal species contain between two and seven hydrophobins.

In this study (**chapter 3**), a Perl program was used which enabled the listing of all proteins in the *Aspergillus* sequences displaying, for each protein, their size, number of cysteines, number of cysteine pairs and cysteine distribution if similar to previous found hydrophobins.

Only proteins not fulfilling our criteria were omitted. Yang *et al.*[12] had previously used a combination of primary sequence analysis (of sequences annotated as hydrophobins) and BLAST searches in the NCBI database to create several motifs used to identify new hydrophobins. However, by using this method only hydrophobins containing the generated motifs would be identified, thereby possible missing hydrophobins not containing these motifs. We identified several hydrophobins, which did not resemble other identified hydrophobins (e.g. no other hydrophobins are listed in BLAST searches). These hydrophobins would possibly not be picked up using BLAST searches and would therefore not be included in the generated motifs resulting in the motifs becoming biased.

Recently, Littlejohn *et al.*[13] used concurrent BLAST searches (of known *Aspergillus* hydrophobins and the hydrophobins identified in our paper) on several protein sequence databases to expand the list of *Aspergillus* hydrophobins identified in our paper (paper 1). Each species was found to contain an additional two - five putative hydrophobins resulting in the identification of an additional 27 hydrophobins. However, if the extra hydrophobins (identified by Littlejohn *et al*.) are examined, many do not fulfil our criteria. Several of the hydrophobins have too few or numerous cysteines, are very large in size, have numerous cysteine pairs or do not contain an intact cysteine pattern resulting in only 10 hydrophobins remaining (ACLA_066600, AFLA_064900, AFLA_101340, AFUA_7G00970, AN5290, AN7327, AO090020000095, AO0907010000512, ATEG_05178 and ATEG_07140). These hydrophobins do, however, fulfil our criteria, but were missed in our approach. This may be, as pointed out by Littlejohn *et al*.[13], due to the use of full genome sequences from only a single protein sequence database, as we only used sequences obtained from the Central *Aspergillus* Data Repository (CADRE). It may have been beneficial to use several databases such as the Uniprot protein knowledge database or the NCBI (National Centre for Biotechnology Information) database, thereby possibly finding more hydrophobins due to differences in sequence annotation between the databases. Despite Littlejohn *et al.*[13] using several protein sequence databases, they have nevertheless missed two hydrophobins identified by us in *A. flavus*. They used concurrent BLAST searches, but this approach may not identify hydrophobins differentiating significantly in sequence from other known hydrophobins. It seems that generation of a definitive list of hydrophobins is not easily accomplished, but may require a combination of several approaches. By using the designed Perl program on protein sequences from different databases, combined with BLAST and

motifs searches a complete list of *Aspergillus* hydrophobins may be possible. This list would gradually expand as more genomes become available.

Many *Aspergillus* hydrophobins cannot be classified

Traditionally, Wessels[14] divided hydrophobins into two classes (class I and class II) based on their distinct hydropathy pattern and physical properties. As the majority of *Aspergillus* hydrophobins have not been physically isolated and characterized, a differentiation into class was performed using the cysteine spacing pattern and hydropathy patterns (**chapter 3**). Approximately half of the identified hydrophobins were classified as class I hydrophobins, while the other half could not be classified based on cysteine spacing pattern and hydropathy patterns and were found to be intermediate forms.

Interestingly, a single hydrophobin (ATEG_04730) from *A. terreus* was classified as a class II hydrophobin (**chapter 3**). Comparison of ATEG_04730 to known class II hydrophobins (HFBI and HFBII) showed a higher sequence identity than to known class I hydrophobins (RodA, SC3 and EAS). Furthermore, ATEG_04730 clustered with class II hydrophobins in a phylogenetic analysis. In agreement with our results, Littlejohn *et al.*[13] only found a single class II hydrophobin in the examined Aspergilli, namely ATEG_04730. By using a similar approach to differentiate *Aspergillus* hydrophobins into classes, they confirmed our findings that several hydrophobins cannot be classified as class I or class II hydrophobins. As previously stated, many of the *Aspergillus* hydrophobins have not been physically isolated and it could be speculated that the unclassifiable hydrophobins may exhibit physical properties between the two distinct classes. Our studies show, that the identification of several intermediate forms blurs the original classification system, so an extension of the classical system may be appropriate as more hydrophobins are isolated and characterized.

Characterization of *Aspergillus nidulans* hydrophobins

Aspergillus nidulans has six hydrophobins containing eight cysteines in the characteristic pattern[15] (**chapter 3**). Only two hydrophobins, RodA and DewA, have previously been characterized[16,17], resulting in little knowledge about the roles of different hydrophobins in Aspergilli. We created hydrophobin deletion strains (*rodA*Δ, *dewA*Δ, AN0940Δ, AN1837Δ,

AN6401Δ, *rodA*Δ*dewA*Δ,) in *A. nidulans* to examine the role of different hydrophobins (**chapter 4**). The AN7539Δ strain is still under construction and was not included.

First, we examined the visual phenotypes of the deletion strains, but as only the strains containing a *rodA* deletion displayed a phenotype, no indication of the location of the other hydrophobins could be obtained. The *rodA*Δ and *rodA*Δ*dewA*Δ strains displayed conidia with a wet and sticky phenotype, which could not easily be dispersed into the air. This phenotype had previously been described in an *A. nidulans rodA*Δ strain[17], an *A. fumigatus rodA* deletion strain[18] and an *Neurospora crassa eas* mutant[19] and is associated with the lack of the rodlet layer. Therefore, the hydrophobins AN0940, AN1837 and AN6401 do not seem essential for rodlet formation, but may still be located on the conidium cell wall similar to DewA[16]. To further examine if AN0940, AN1837 and AN6401 are involved in rodlet formation, the surfaces of the reference strain and the FGSC A4 wild type strain were initially examined by Scanning Electron Microscopy (SEM). These strains should display visible rodlets on the conidium surface, but as not rodlets could be seen, SEM was aborted. The involvement of AN0940, AN1837 and AN6401 in rodlet formation still remains elusive.

A phenotype described for *A. nidulans dewA*Δ strains is the ability to be wetted by detergent[16]. We observed a similar phenotype for our *dewA*Δ strain (**chapter 4**). Both the *rodA* deletion strains (*rodA*Δ and *rodA*Δ*dewA*Δ) were also rapidly wetted by detergent, while the other hydrophobin deletion strains (AN0940Δ, AN1837Δ and AN6401Δ) did not display an easily wetted phenotype. This indicates that if the hydrophobins AN0940, AN1837 and AN6401 are present on the conidium cell wall, they may not have dominant roles or may be able to compensate for each other. These hydrophobins may only be present in the mycelium cell wall or may be secreted.

The hydrophobins RodA and DewA in *A. nidulans* have been shown to be involved in hydrophobicity of the strain[16,20]. We measured the water contact angles of our constructed hydrophobin deletion strains to determine the colony surface hydrophobicity of the strains (**chapter 4**). In contrast to Dynesen *et al.*[20], we did not observe any reduction of hydrophobicity in the *rodA*Δ and *dewA*Δ strains by water contact angle measurements. Interestingly, we observed that water deposited on the surface of the *rodA*Δ*dewA*Δ strain passed the spores, and was absorbed into the underlying mycelium and agar within few minutes. This had previously been observed in an *A. fumigatus rodA*Δ strain[18]. The lack of

other hydrophobins did not change the surface colony hydrophobicity of the strain significantly. This indicates that the hydrophobins AN0940, AN1837 and AN6401 are not required for overall colony surface hydrophobicity, but may have other functions in the fungus. Water contact angle measurements on pure mycelium or conidia, will provide insight into the hydrophobicity of single fungal structures.

The involvement of hydrophobins in a fungus' ability to escape an aqueous environment has been proposed by Wösten *et al.*[21] By secreting hydrophobins from submerged hyphae into the surrounding aqueous environment, a lowering of the water surface tension is achieved allowing the fungus to breach into the air. This has been demonstrated for hydrophobins from *S. commune*[22–25], but whether a similar mechanism is used in Aspergilli remains to be determined. An initial assessment of different wild type *Aspergillus* species to escape an aqueous environment was conducted by submerging immature colonies under water and showed that both *A. fumigatus*, *A. niger* and *A. oryzae* could breach to the air. Next the different hydrophobin mutant strains were submerged to examine the roles of *A. nidulans* hydrophobins in allowing the fungus to escape the aqueous environment (**chapter 4**). Interestingly, neither the lack of a single hydrophobin nor both hydrophobins RodA and DewA impaired the fungus' ability to escape the water, as all strains produced mature colonies atop the water after 4-5 days of growth. This implies that single *Aspergillus* hydrophobins are not required for breaching into the air. Therefore *Aspergillus* hydrophobins may complement each other or individual mechanisms may be used by different fungal species.

Studies on hydrophobins from *Cladosporium fulvum* have previously shown that hydrophobins have different roles in the fungus and are expressed on different structures[26,27]. Similarly, Beauvais *et al*.[28] have shown that *A. fumigatus* hydrophobins are expressed under different conditions. To examine if hydrophobins are able to complement each other or are coupled in expression, expression levels of the six hydrophobins were examined in the *rodA* and *dewA* deletion strains by qRT-PCR (**chapter 4**). Firstly, the deletion of *rodA* in the *rodA*Δ strain resulted in all other hydrophobins (including *dewA*) having a reduced gene showing that the lack of rodlet layer appears to effect the expression of all other hydrophobins. In the *dewA*Δ strain the AN1837, AN6401 and AN7539 hydrophobins had reduced expression similar to the *rodA*Δ strain, showing that these three hydrophobins show comparable reduced

expression levels irrespectively of which conidial hydrophobin (*rodA* or *dewA*) has been deleted. In contrast the expression level of *rodA* was not changed in the *dewA*Δ strain. This may be due to RodA being the major outer wall hydrophobin on the conidia, while DewA has been proposed to be a part of the inner layer[16]. It seems that the presence of *dewA* is not a prerequisite for the expression of *rodA,* which is confirmed by the observation of rodlets in *dewA*Δ strains[16], but in contrast the lack of *rodA* affects *dewA* expression.
In the *rodA*Δ*dewA*Δ strain the deletion of both *rodA* and *dewA* resulted in the hydrophobins AN0940, AN1837 and AN6401 only having a minimal change in expression and slight down-regulation indicating that the deletion of the two hydrophobins does not reduce the expression of these hydrophobins to the same extent compared to the single deletion strains. These hydrophobins may become increasingly important as more hydrophobins are missing. Interestingly, the deletion of both *rodA* and *dewA*, resulted in an increased expression of AN7539, which was in contrast to the reduced expression observed in the single deletion strains. This hydrophobin may thus fulfil some of the functions of the hydrophobins RodA and DewA, when both hydrophobins have been deleted.

We were unsuccessful in determining the roles of hydrophobins AN0940, AN1837 and AN6401 in *A. nidulans*, but nevertheless found that these hydrophobins were not required for a number of different biological functions in the fungus including colony surface hydrophobicity and escaping aqueous environments. It would be interesting to further examine the hydrophobins from our *A. nidulans* strains and determine the location and roles of hydrophobins AN0940, AN1837 and AN6401.

Pseudomonas aeruginosa suppresses growth of *Aspergillus* species

P. aeruginosa and *Aspergillus* species both infect the lungs of cystic fibrosis (CF) patients[29,30], where *A. fumigatus* is the most common isolated filamentous fungi. However, other Aspergilli can also infect CF patients[31,32]. Despite, the common isolation of both organisms few studies have focused on a possible interaction between the two.

Using a purpose developed assay enabling balanced growth between Aspergilli and *P. aeruginosa*, we examined the interactions between *P. aeruginosa* and several *Aspergillus* species (**chapter 5**). Previous studies have dealt with interactions between *P. aeruginosa* and

Aspergilli, but have focused primarily on the major pathogen *A. fumigatus*[33–35]. Our study is the first to include several *Aspergillus* species allowing the examination of variations in interactions between the different Aspergilli and *P. aeruginosa*. We found that *P. aeruginosa* suppressed growth of all tested Aspergilli. This suppression was accompanied by an increase in production of antifungal compounds called phenazines by *P. aeruginosa* in the contact zone to the fungus. Interestingly, only *A. flavus*, *A. niger* and *A. oryzae* triggered the production of the phenazines, while no response could be detected in the presence of *A. fumigatus* showing that *P. aeruginosa* responds differentially towards different fungal species. Phenazines have been found to contribute to increased lung tissue damage and necrosis[36,37], and therefore the presence of the Aspergilli may contribute to disease progression. Furthermore, *A. fumigatus* can produce several classes of secondary metabolites (gliotoxins, fumagillins and helvolic acid) known to damage lung tissues and impair the ciliary beat frequency[38]. Therefore all tested Aspergilli may negatively affect disease development in CF patients either due to own production of secondary metabolites or triggering secondary metabolite production in *P. aeruginosa*. Several studies show that there is a significantly decrease in lung function in CF patients colonized with both organisms compared to patients colonized with only *A. fumigatus* or *P. aeruginosa*, respectively[39–41]. Why the impairment of lung function is increased in patients colonized by several organisms has not been elucidated, but could involve increased production of secondary metabolites from the colonizing organisms in response to each other or enhanced production of mucus.

The involvement of hydrophobins in human disease has only limited been studied. The hydrophobin RodA from *A. fumigatus* has been shown to make fungal spores immunological inert, thereby "hiding" the spores from the immune system.[42] We examined the possible role of hydrophobins in fungal-bacterial interactions namely the interactions between *A. nidulans* hydrophobin mutant strains and *P. aeruginosa* (**chapter 5**) using the plate assay developed in this project. We observed that all hydrophobin deletion strains produced similar repertoires of secondary metabolites. In the interaction with *P. aeruginosa*, all hydrophobin deletion strains were suppressed by the bacterium. *P. aeruginosa* increased its phenazine production as previously seen for other Aspergilli in response to the presence of the hydrophobin deletion strains. No major differences could be seen in the secondary metabolite response of *P. aeruginosa* to the different hydrophobin deletion strains and control strains indicating that hydrophobins do not seem change the macroscopic or chemical interaction between *A.*

nidulans and *P. aeruginosa*. If there is change in the interaction pattern microscopically remains to be determined, but would involve SEM. Furthermore, hydrophobins vary between species (**chapter 3**) and other hydrophobins in e.g. *A. fumigatus* may display a different pattern.

P. aeruginosa and *A. fumigatus* have been shown to have a close spatial relationship with the distribution of *P. aeruginosa* cells on the fungal hyphae[43,44]. We similarly observed that *P. aeruginosa* and *A. fumigatus* had a close spatial relationship with *P. aeruginosa*, as the organisms grew as close to each other as possible (**chapter 5**). To further examine the interactions between Aspergilli and *P. aeruginosa*, we attempted to develop a cantilever-lab-on-chip system (**chapter 6**) allowing measurements of physical interaction and possible biofilm formation between the two species. By using the hydrophobin deletion strains, an examination of the role of hydrophobins in the physical interactions would likewise be possible. Nugaeva *et al*.[45,46] had previously used cantilevers to follow the germination of *A. niger* conidia demonstrating the potential of cantilevers. Unfortunately, despite several efforts to construct a leak free closed-chamber cantilever lab-on-a-chip system, this was not accomplished. We did, however, successfully immobilize *A. oryzae* conidia on the cantilevers and perform preliminary germination experiments showing the potential in using cantilevers in studying mixed populations.

Common mutations in *P. aeruginosa* changes the interaction with *A. fumigatus*

During the course of a chronic CF infection, *P. aeruginosa* changes genetically due to loss-of-function mutations and changes in phenotype[47,48]. Common mutations include loss of motility and inactivation of several genes including *lasR* (quorum sensing regulator), *rpoN* (alternative sigma factor) and *mucA* (resulting in mucoid phenotype) genes[47,49–52].

We used several *P. aeruginosa* mutants to examine the effect of common mutations on the *P. aeruginosa-Aspergillus* interaction (**chapter 5**). Interestingly, we observed that the *rpoN* and *mucA* mutations rendered *P. aeruginosa* susceptible to *A. fumigatus* allowing the fungus to grow over the bacterial strains. Lack of both genes resulted in the bacterium being completely overgrown by *A. fumigatus*, where the mucoid *P. aeruginosa* formed the base of a lawn of *A. fumigatus*. Using our *A. nidulans* control and hydrophobin deletion strains, we observed that

P. aeruginosa turned mucoid in the contact zone with *A. nidulans*, but remained non-mucoid further away from the fungus (**chapter 5**). The mucoid phenotype of *P. aeruginosa* results in a reduced pulmonary clearance in animal models[53], thus it can be speculated that the presence of *A. nidulans* could result in lower clearance of *P. aeruginosa* or a beneficial environment for *A. fumigatus* colonisation. Other mutations including lack of motility, rhamnolipids and pyoverdins did not change the interaction between *A. fumigatus* and *P. aeruginosa* and could suppress *A. fumigatus* (**chapter 5**). Interestingly, examination of the interactions between the *rpoN* deletion strain and other Aspergilli (*A. niger*, *A. oryzae* and *A. flavus*) revealed that the fungi were suppressed by the bacterium and the production of several phenazines were increased by *P. aeruginosa* to the presence of the three fungi. In contrast, no increase in phenazine production was observed in response to *A. fumigatus* demonstrating that different mechanisms are used towards different fungal species and that genetic adaption of *P. aeruginosa* to the CF lung during chronic CF infections may result in a changed interaction pattern with *A. fumigatus*.

The *lasR* mutant has reduced expression of many virulence factors including the phenazine, pyocyanin[54]. We found, that a *lasR* mutant could suppress growth of all tested Aspergilli (**chapter 5**). Interestingly, a large accumulation of pyocyanin was seen in the contact zone between the *lasR* mutant and *A. oryzae* and *A. flavus*, respectively. Similarly, Cugini *et al*.[54] observed an increase in pyocyanin production in a *lasR* mutant, when inoculated onto *C. albicans*, while Diggle *et al.*[55] observed that a *lasR* mutant regained the ability to produce pyocyanin at late time points. This demonstrates that *lasR* mutant strains lacking certain virulence factors may regain the ability to produce these virulence factors using alternative pathways under conditions favouring their production as in competitive environments with other organisms. The interactions between different species, including fungal-bacterial species is highly complicated and diverse, resulting in the need of much more research within this area to answer all the questions.

Conclusion

This project consisted of two parts. The aim of the first and major part was to achieve a deeper understanding of the roles of hydrophobins in *Aspergillus* species. A bioinformatics approach was used and showed that varying numbers of hydrophobins are found within the

Aspergillus species. Furthermore, many of the identified hydrophobins could not be classified into the original two hydrophobin classes, but displayed intermediate forms.

By developing hydrophobin deletion strains in *A. nidulans*, the different hydrophobins were characterised and their roles were elucidated. If the known conidial hydrophobins (*rodA* and *dewA*) were deleted, the AN1837, AN6401 and AN7539 hydrophobins similarly displayed reduced expression. The deletion of *rodA* resulted in reduced expression of *dewA*, but not vice versa. Interestingly, the hydrophobin AN7539 was up-regulated in the *rodA*Δ*dewA*Δ strain. Only *rodA* and *dewA* displayed visual phenotypes indicating that, if the AN0940, AN1837 and AN6401 hydrophobins are present on the conidia cell wall, they do not play dominant roles. These may only be present in the mycelium cell wall or are secreted. Furthermore, it was found that lack of hydrophobins AN0940, AN1837 and AN6401 did not affect several biological functions including surface hydrophobicity, spore dispersal or the ability of the different strains to breach a water-air interface. The roles of hydrophobins AN0940, AN1837 and AN6401 still remains to be determined.

The aim of the second part of this project was to provide insight into *Aspergillus-P. aeruginosa* interactions. *P. aeruginosa* was able to suppress all tested Aspergilli. This suppression may be mediated by the increased production of phenazines by *P. aeruginosa* in response to the presence of some Aspergilli. Several hydrophobin deletion strains were suppressed in a similar manner by *P. aeruginosa* and the suppression could not be discriminated using the different strains, showing that hydrophobins most likely do not play a major role in *A. nidulans–P. aeruginosa* interactions. Furthermore, different *P. aeruginosa* mutants were used to elucidate the mechanisms involved in the suppression of the fungus showing that factors involved in RpoN pathway may be involved. Interestingly, a *rpoN* mutant could not suppress growth of *A. fumigatus*, but successfully inhibited other Aspergilli. The genetic adaption of *P. aeruginosa* to the CF lung during chronic CF infections seems to result in bacteria becoming more susceptible towards competition from *A. fumigatus*, showing differential interaction patterns during a CF infection. Additional research is needed to explore these mechanisms.

References for chapter 7

1. Yu J, Payne GA, Nierman WC, Machida M, Bennett JW, Campbell BC, Robens JF, Bhatnagar D, Dean RA, Cleveland TE (2008) *Aspergillus flavus* genomics as a tool for studying the mechanism of aflatoxin formation. *Food Addit Contam Part A* **25:** 1152-1157.

2. Machida M, Yamada O, Gomi K (2008) Genomics of *Aspergillus oryzae*: Learning from the History of Koji Mold and Exploration of Its Future. *DNA Res* **15:** 173-183.

3. Fedorova ND, Khaldi N, Joardar VS, Maiti R, Amedeo P, Anderson MJ, Crabtree J, Silva JC, Badger JH, Albarraq A, Angiuoli S, Bussey H, Bowyer P, Cotty PJ, Dyer PS, Egan A, Galens K, Fraser-Liggett CM, Haas BJ, Inman JM, Kent R, Lemieux S, Malavazi I, Orvis J, Roemer T, Ronning CM, Sundaram JP, Sutton G, Turner G, Venter JC, White OR, Whitty BR, Youngman P, Wolfe KH, Goldman GH, Wortman JR, Jiang B, Denning DW, Nierman WC (2008) Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*. *PLoS Genet* **4:** e1000046.

4. Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, Arroyo J, Berriman M, Abe K, Archer DB, Bermejo C, Bennett J, Bowyer P, Chen D, Collins M, Coulsen R, Davies R, Dyer PS, Farman M, Fedorova N, Fedorova N, Feldblyum TV, Fischer R, Fosker N, Fraser A, Garcia JL, Garcia MJ, Goble A, Goldman GH, Gomi K, Griffith-Jones S, Gwilliam R, Haas B, Haas H, Harris D, Horiuchi H, Huang J, Humphray S, Jimenez J, Keller N, Khouri H, Kitamoto K, Kobayashi T, Konzack S, Kulkarni R, Kumagai T, Lafon A, Latge JP, Li W, Lord A, Lu C, Majoros WH, May GS, Miller BL, Mohamoud Y, Molina M, Monod M, Mouyna I, Mulligan S, Murphy L, O'Neil S, Paulsen I, Penalva MA, Pertea M, Price C, Pritchard BL, Quail MA, Rabbinowitsch E, Rawlins N, Rajandream MA, Reichard U, Renauld H, Robson GD, Rodriguez de CS, Rodriguez-Pena JM, Ronning CM, Rutter S, Salzberg SL, Sanchez M, Sanchez-Ferrero JC, Saunders D, Seeger K, Squares R, Squares S, Takeuchi M, Tekaia F, Turner G, Vazquez de Aldana CR, Weidman J, White O, Woodward J, Yu JH, Fraser C, Galagan JE, Asai K, Machida M, Hall N, Barrell B, Denning DW (2005) Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature* **438:** 1151-1156.

5. Galagan JE, Calvo SE, Cuomo C, Ma LJ, Wortman JR, Batzoglou S, Lee SI, türkmen M, Spevak CC, Clutterbuck J, Kapitonov V, Jurka J, Scazzocchio C, Farman M, Butler J, Purcell S, Harris S, Braus GH, Draht O, Busch S, D'Enfert C, Bouchier C, Goldman GH, Bell-Pedersen D, Griffiths-Jones S, Doonan JH, Yu J, Vienken K, Pain A, Freitag M, Selker EU, Archer DB, Peñalva MA, Oakley BR, Momany M, Tanaka T, Kumagai T, Asai K, Machida M, Nierman WC, Denning DW, Caddick M, Hynes M, Paoletti M, Fischer R, Miller B, Dyer P, Sachs MS, Osmani SA, Birren BW (2005) Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. *Nature* **438:** 1105-1115.

6. Wessels JGH (1997) Hydrophobins: Proteins that change the nature of the fungal surface. *Adv Microb Physiol* **38:** 1-45.

7. Kwan AH, Winefield RD, Sunde M, Matthews JM, Haverkamp RG, Templeton MD, Mackay JP (2006) Structural basis for rodlet assembly in fungal hydrophobins. *Proc Natl Acad Sci U S A* **103:** 3621-3626.

8. Hakanpaa J, Linder M, Popov A, Schmidt A, Rouvinen J (2006) Hydrophobin HFBII in detail: ultrahigh-resolution structure at 0.75 A. *Acta Crystallogr D Biol Crystallogr* **62:** 356-367.

9. Hakanpaa J, Szilvay GR, Kaljunen H, Maksimainen M, Linder M, Rouvinen J (2006) Two crystal structures of *Trichoderma reesei* hydrophobin HFBI - the structure of a protein amphiphile with and without detergent interaction. *Protein Sci* **15:** 2129-2140.

10. Hakanpaa J, Paananen A, Askolin S, Nakari-Setala T, Parkkinen T, Penttila M, Linder MB, Rouvinen J (2004) Atomic resolution structure of the HFBII hydrophobin, a self-assembling amphiphile. *J Biol Chem* **279:** 534-539.

11. Sunde M, Kwan AH, Templeton MD, Beever RE, Mackay JP (2008) Structural analysis of hydrophobins. *Micron* **39:** 773-784.

12. Yang K, Deng Y, Zhang C, Elasri M (2006) Identification of new members of hydrophobin family using primary structure analysis. *BMC Bioinformatics* **7:** S16.

13. Littlejohn KA, Hooley P, Cox PW (2012) Bioinformatics predicts diverse *Aspergillus* hydrophobins with novel properties. *Food Hydrocolloids* **27:** 503-516.

14. Wessels JGH (1994) Developmental Regulation of Fungal Cell-Wall Formation. *Annu Rev Phytopathol* **32:** 413-437.

15. de Groot PW, Brandt BW, Horiuchi H, Ram AF, de Koster CG, Klis FM (2009) Comprehensive genomic analysis of cell wall genes in *Aspergillus nidulans*. *Fungal Genet Biol* **46:** S72-S81.

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

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

18. Thau N, Monod M, Crestani B, Rolland C, Tronchin G, Latge JP, Paris S (1994) Rodletless mutants of *Aspergillus fumigatus*. *Infect Immun* **62:** 4380-4388.

19. Beever RE, Dempsey GP (1978) Function of rodlets on the surface of fungal spores. *Nature* **272:** 608-610.

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

21. Wosten HA, van Wetter MA, Lugones LG, van der Mei HC, Busscher HJ, Wessels JG (1999) How a fungus escapes the water to grow into the air. *Curr Biol* **9:** 85-88.

22. Asgeirsdottir SA, de Vries OM, Wessels JG (1998) Identification of three differentially expressed hydrophobins in *Pleurotus ostreatus* (oyster mushroom). *Microbiology* **144:** 2961-2969.

23. Wessels JGH, De VO, Asgeirsdottir SA, Schuren F (1991) Hydrophobin Genes Involved in Formation of Aerial Hyphae and Fruit Bodies in *Schizophyllum*. *Plant Cell* **3:** 793-799.

24. Wessels JGH, de Vries OM, Asgeirsdottir SA, Springer J (1991) The thn mutation of *Schizophyllum commune*, which suppresses formation of aerial hyphae, affects expression of the Sc3 hydrophobin gene. *J Gen Microbiol* **137:** 2439-2445.

25. Wessels JGH (1992) Gene expression during fruiting in *Schizophyllum commune*. *Mycol Res* **96:** 609-620.

26. Lacroix H, Whiteford JR, Spanu PD (2008) Localization of *Cladosporium fulvum* hydrophobins reveals a role for HCf-6 in adhesion. *FEMS Microbiol Lett* **286:** 136-144.

27. Whiteford JR, Lacroix H, Talbot NJ, Spanu PD (2004) Stage-specific cellular localisation of two hydrophobins during plant infection by the pathogenic fungus *Cladosporium fulvum*. *Fungal Genet Biol* **41:** 624-634.

28. Beauvais A, Schmidt C, Guadagnini S, Roux P, Perret E, Henry C, Paris S, Mallet A, Prevost MC, Latge JP (2007) An extracellular matrix glues together the aerial-grown hyphae of *Aspergillus fumigatus*. *Cell Microbiol* **9:** 1588-1600.

29. Bakare N, Rickerts V, Bargon J, Just-Nubling G (2003) Prevalence of *Aspergillus fumigatus* and other fungal species in the sputum of adult patients with cystic fibrosis. *Mycoses* **46:** 19-23.

30. Bauernfeind A, Bertele RM, Harms K, Horl G, Jungwirth R, Petermuller C, Przyklenk B, Weisslein-Pfister C (1987) Qualitative and quantitative microbiological analysis of sputa of 102 patients with cystic fibrosis. *Infection* **15:** 270-277.

31. Mortensen KL, Jensen RH, Johansen HK, Skov M, Pressler T, Howard SJ, Leatherbarrow H, Mellado E, Arendrup MC (2011) *Aspergillus* species and other molds in respiratory samples from patients with cystic fibrosis: a laboratory-based study with focus on *Aspergillus fumigatus* azole resistance. *J Clin Microbiol* **49:** 2243-2251.

32. Mortensen KL, Johansen HK, Fuursted K, Knudsen JD, Gahrn-Hansen B, Jensen RH, Howard SJ, Arendrup MC (2011) A prospective survey of *Aspergillus* spp. in respiratory tract samples: prevalence, clinical impact and antifungal susceptibility. *Eur J Clin Microbiol Infect Dis* **30:** 1355-1363.

33. Kerr JR (1994) Suppression of fungal growth exhibited by *Pseudomonas aeruginosa*. *J Clin Microbiol* **32:** 525-527.

34. Mangan A (1969) Interactions between some aural *Aspergillus* species and bacteria. *J Gen Microbiol* **58:** 261-266.

35. Blyth W, Forey A (1971) The influence of respiratory bacteria and their biochemical fractions on *Aspergillus fumigatus*. *Sabouraudia* **9:** 273-282.

36. Wilson R, Sykes DA, Watson D, Rutman A, Taylor GW, Cole PJ (1988) Measurement of *Pseudomonas aeruginosa* phenazine pigments in sputum and assessment of their contribution to sputum sol toxicity for respiratory epithelium. *Infect Immun* **56:** 2515-2517.

37. Lau GW, Ran H, Kong F, Hassett DJ, Mavrodi D (2004) *Pseudomonas aeruginosa* pyocyanin is critical for lung infection in mice. *Infect Immun* **72:** 4275-4278.

38. Amitani R, Taylor G, Elezis EN, Llewellyn-Jones C, Mitchell J, Kuze F, Cole PJ, Wilson R (1995) Purification and characterization of factors produced by *Aspergillus fumigatus* which affect human ciliated respiratory epithelium. *Infect Immun* **63:** 3266-3271.

39. Amin R, Dupuis A, Aaron SD, Ratjen F (2010) The effect of chronic infection with *Aspergillus fumigatus* on lung function and hospitalization in patients with cystic fibrosis. *Chest* **137:** 171-176.

40. Navarro J, Rainisio M, Harms HK, Hodson ME, Koch C, Mastella G, Strandvik B, McKenzie SG (2001) Factors associated with poor pulmonary function: cross-sectional analysis of data from the ERCF. European Epidemiologic Registry of Cystic Fibrosis. *Eur Respir J* **18:** 298-305.

41. Chotirmall SH, O'Donoghue E, Bennett K, Gunaratnam C, O'Neill SJ, McElvaney NG (2010) Sputum *Candida albicans* presages FEV decline and hospital-treated exacerbations in cystic fibrosis. *Chest* **138:** 1186-1195.

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

43. Blyth W (1971) Modifications in the ultrastructure of *Aspergillus fumigatus* due to the presence of living cells of *Pseudomonas aeruginosa*. *Sabouraudia* **9:** 283-286.

44. Mowat E, Rajendran R, Williams C, McCulloch E, Jones B, Lang S, Ramage G (2010) *Pseudomonas aeruginosa* and their small diffusible extracellular molecules inhibit *Aspergillus fumigatus* biofilm formation. *FEMS Microbiol Lett* **313:** 96-102.

45. Nugaeva N, Gfeller KY, Backmann N, Duggelin M, Lang HP, Guntherodt HJ, Hegner M (2007) An antibody-sensitized microfabricated cantilever for the growth detection of *Aspergillus niger* spores. *Microsc Microanal* **13:** 13-17.

46. Nugaeva N, Gfeller KY, Backmann N, Lang HP, Duggelin M, Hegner M (2005) Micromechanical cantilever array sensors for selective fungal immobilization and fast growth detection. *Biosens Bioelectron* **21:** 849-856.

47. Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, Miller SI, Ramsey BW, Speert DP, Moskowitz SM, Burns JL, Kaul R, Olson MV (2006) Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc Natl Acad Sci U S A* **103:** 8487-8492.

48. Yang L, Jelsbak L, Marvig RL, Damkiaer S, Workman CT, Rau MH, Hansen SK, Folkesson A, Johansen HK, Ciofu O, Hoiby N, Sommer MO, Molin S (2011) Evolutionary dynamics of bacteria in a human host environment. *Proc Natl Acad Sci U S A* **108:** 7481-7486.

49. Mahenthiralingam E, Campbell ME, Speert DP (1994) Nonmotility and phagocytic resistance of *Pseudomonas aeruginosa* isolates from chronically colonized patients with cystic fibrosis. *Infect Immun* **62:** 596-605.

50. Hoiby N (1975) Prevalence of mucoid strains of *Pseudomonas aeruginosa* in bacteriological specimens from patients with cystic fibrosis and patients with other diseases. *Acta Pathol Microbiol Scand Suppl* **83:** 549-552.

51. Heurlier K, Denervaud V, Pessi G, Reimmann C, Haas D (2003) Negative control of quorum sensing by RpoN (sigma54) in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **185:** 2227-2235.

52. Totten PA, Lara JC, Lory S (1990) The rpoN gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. *J Bacteriol* **172:** 389-396.

53. Boucher JC, Yu H, Mudd MH, Deretic V (1997) Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: characterization of muc mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection. *Infect Immun* **65:** 3838-3846.

54. Cugini C, Morales DK, Hogan DA (2010) *Candida albicans*-produced farnesol stimulates *Pseudomonas* quinolone signal production in LasR-defective *Pseudomonas aeruginosa* strains. *Microbiology* **156:** 3096-3107.

55. Diggle SP, Winzer K, Chhabra SR, Worrall KE, Camara M, Williams P (2003) The *Pseudomonas aeruginosa* quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates *rhl*-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. *Mol Microbiol* **50:** 29-43.

Appendix 1
Materials and methods for chapter 4

Alignment of hydrophobins and generation of hydropathy patterns

Sequences of the *A. nidulans* hydrophobins (AN0940, AN1837, AN6401, AN7539, AN8006 and AN8803) were obtained from the UniProt Protein Knowledgebase (www.uniprot.org). A multiple sequence alignment of the hydrophobins was conducted using MAFFT in the program Jalview[1]. Sequence similarity searches of the *A. nidulans* hydrophobins were conducted using the BLAST function on the UniProt Protein Knowledgebase. Hydropathy patterns were determined using ProtScale on the ExPASy Proteomics Server[2] by using the Kyte and Doolittle scale[3] and a nine amino acid window. The hydropathy patterns were aligned around the cysteine pairs by placing gaps in the sequences where the hydrophilic and hydrophobic regions alternated. Only sequence from the first to the eighth cysteine was used to create the hydropathy patterns.

Strains

The *A. nidulans* strain IBT29539 (*argB2*, *pyrG89*, *veA1*, *nkuA*Δ) was used as host for all deletion-strain constructions and strain IBT28738 (*argB2*, *pyrG89*, *veA1*, *nkuA-trS::AFpyrG*) was used for the construction of overexpression (OEx) strains. Deletion strains were crossed with IBT25456 (*biA1*, *veA1*) to restore genetic markers and the *nkuA* deficiency. NID750 (*veA1*) was used as reference strain for assays using the deletion strains, while NID191 (*argB2*, *pyrG89*, *veA1*, *nkuA*Δ::AF*pyrG*, IS1::PgpdA::TtrpC::*argB*) served as reference strain for the OEx strain constructs. All *A. nidulans* strains are listed in table 1. *Escherichia coli* strain DH5α was used for plasmid propagation.

Media

Minimal media (MM), supplements and 5-FOA were as described in Nielsen *et al.*[4]. For analysis of secondary metabolite production in *A. nidulans*, the following four media were used: MM, Czapek Yeast extract Agar (CYA)[5], Yeast Extract Sucrose agar (YES)[5] and Wickerhams Antibiotic Test Medium (WATM)[6]. Spore suspension (SS) consisted of 0.5 % Tween 80 and 0.5 % agar. All chemicals were from Sigma-Aldrich (St. Louis, Mo, USA) except for agar from Sorbigel (Hendaye, France).

PCR

All PCR reactions were carried out with the PfuX7 (Nørholm, 2010) using 1x HF buffer (Finnzymes, Finland) and 0.2 mM dNTPs (Sigma) applying the following standard conditions: 98 °C for 2 min, 35x (98 °C for 10 s, 60 °C for 30 s, 72 °C 2 min); 5 min at 72 °C. Standard Fusion-PCR was carried out at 98 °C for 1 min, 5x (98 °C for 10 s, 55 °C for 2 min, 72 °C 1:30 min); 30x (98 °C for 10 s, 60 °C for 30 s, 72 °C 2:30 min) ending with 5 min at 72 °C. Spore-PCR analysis was as standard: 98 °C for 15 min, 35x (98 °C for 10 s, (64-55) °C for 30 s, 72 °C 3 min); 5 min at 72 °C adding less than 1000 conidia per reaction tube. All oligonucleotides (Sigma-Aldrich) used in this study and primer pairs are listed in table 2, and for std PCR 0.8 µM was used, whereas 0.4 µM was used for fusion-PCR and Spore-PCR.

Construction of *A. nidulans* hydrophobin deletion strains

Individual gene deletions and genetic transformation of *A. nidulans* protoplasts were carried out as bi-partite previously described by Nielsen *et al.*[4,7]. Up- and downstream target sequences were amplified from *A. nidulans* IBT29539 gDNA. The plasmid pDEL2[4] was used for amplifying the AF*pyrG* marker These fragments were used for genetic transformation of *A. nidulans* IBT29539 protoplasts. All *A. nidulans* transformants were subsequently streak purified on 5-FOA media to eliminate the *pyrG* marker and verified by Spore-PCR. Completed strains were finally crossed[8] with IBT25456 to restore genetic markers and verified by Spore-PCR analysis.

Construction of *A. nidulans* hydrophobin over-expression strains

The *A. nidulans* hydrophobin over-expression strains were constructed using USER cloning for insertion in IS1[9]. Created PCR fragments (see table 2 for primer pairs) were USER cloned into a pU1111 based vector and subsequently transformed into competent *E. coli* DH5α cells. Purified DNA was digested with NotI and transformed into *A. nidulans* IBT28738 protoplasts.

Morphology

All *A. nidulans* hydrophobin deletion strains were cultivated on MM and WATM for five days at 37 °C. Plates were made in triplicate and examined macroscopically and under stereomicroscope. Sellotape preparations[10] were made of each plate and examined microscopically. A minimum of 10

images were compared for each plate, resulting in a comparison of a minimum of 30 images for each strain.

To examine spore surfaces for rodlets, the reference strain was examined using Scanning Electron Microscopy (SEM), which was performed using a FEI Nova 600 NanoSEM (FEI, Hillsboro, Oregon) operated at 3 kV. To minimize charging problems a thin layer of gold (~5 Å) was sputter deposited on the sample with an Anatech Hummer sputter coater (Anatech, Union City, California).

qRT-PCR

Total RNA from *rodA*Δ, *dewA*Δ, *rodA*Δ*dewA*Δ and the reference strain (grown for four days at 37 °C on MM) was isolated with Qiagen Plant RNAeasy kit. The samples were disrupted by a TissueLyser LT (Qiagen) using 45 Hz for 1 min. 10 µg of RNA was DNAse I (Qiagen) treated after manufacturer's protocol, with addition of 10U of RNAguard RNAse inhibitor (Amersham). 1 µg of DNAse I treated RNA samples were used in cDNA synthesis by the Phusion RT-PCR Kit (Finnzymes) according to manufacturer's protocol. The subsequent qRT-PCR was performed in a Chromo 4TM Detector/PTC-200 (MJ Research) using the SYBR® Green JumpStart Taq ReadyMix (Sigma). Two genes (*actA* (AN6542) encoding actin and *hhtA* (AN0733) encoding histone protein H3) were chosen as internal standards for normalization of expression levels. Only one of them, *hhtA*, was used for the fold change calculations. Primer combinations for the qPCR and sequences are listed in table 3. All PCR primer pairs were evaluated by running PCR on gDNA prior to qPCR. Moreover, two types of control samples were initially included for the qPCR; the DNAse treated RNA sample used for the reverse transcriptase reaction, and a template-free reaction to test for primer-dimer influence on the overall fluorescence. The final individual cDNA samples were added to the reactions as 5 times diluted samples. Samples were run in triplicates. The program was 94 °C for 2 min and cycling conditions 40 times; 94 °C for 10 s, 60 °C for 15 s, 72 °C for 30 s. A melting curve from 65 °C to 95 °C with reads every 0.2 min was ending the program to evaluate the purity of the reaction products. The fluorescence threshold values, C(t), was determined through the OpticonMonitor 3.1 software (MJ Research). The relative expression levels was approximated by $2^{-\Delta\Delta C(t)}$ as $\Delta\Delta C(t) = \Delta C(t)\text{normalized} - \Delta C(t)\text{calibrator}$. The $\Delta C(t)\text{normalized} = \Delta C(t)\text{target gene} - \Delta C(t)\text{internal\_std}$ and the calibrator C(t) values were the corresponding values from the reference strain.

Water Contact Angle Measurements

Spores were harvested in SS from five days old colonies grown on MM plates and 100 µl were plated out in a Petri dish containing app. 20 ml of solid MM. The plates were incubated at 37 °C for four days until a homogeneous compact layer of spores had developed. A square (1 cm x 3 cm) was cut out and placed on a microscope slide. Water contact angles were measured at set time intervals after placing 10 µl drops of water on the colony surface and using a Drop shape analysis system DSA 10 Mk2 (Krüss, Hamburg, Germany). All experiments were done in triplicate. For each drop and time point 10 measurements were made.

Ability to breach a water-air interface

Strains were inoculated on MM and grown for two days at 20 °C until an immature colony lacking spores was visible. 30 ml of MQ-water was added to each plate submerging the preformed colonies and incubated at 20 °C. The plates were studied each day for seven days. All experiments were done in triplicate.

Tables for materials and methods

Table 1: Strains used in this study

Strain	**Genotype**	**Source**
FGSC A4 (IBT4887)	Wild-type	CMB fungal collection
*nkuA*Δ (IBT29539)	*argB2, pyrG89, veA1, nkuA*Δ	Nielsen *et al.* 2008
nkuA-trS (IBT28738)	*argB2, pyrG89, veA1, nkuA-trS*::AF*pyrG*	Nielsen *et al.* 2008
G051 (IBT25456)	*biA1, veA1*	CMB fungal collection
*rodA*Δ (auxotrophic, NID179)	*argB2, pyrG89, veA1, nkuA*Δ, *rodA*Δ	This study
*dewA*Δ (auxotrophic, NID238)	*argB2, pyrG89, veA1, nkuA*Δ, *dewA*Δ	This study
*rodA*Δ*dewA*Δ (auxotrophic, NID 295)	*argB2, pyrG89, veA1, nkuA*Δ, *rodA*Δ, *dewA*Δ	This study

AN0940Δ (auxotrophic, NID781)	*argB2*, *pyrG89*, *veA1*, *nkuA*Δ, AN0940Δ	This study
AN1837Δ (auxotrophic, NID780)	*argB2*, *pyrG89*, *veA1*, *nkuA*Δ, AN1837Δ	This study
AN6401Δ (auxotrophic, NID764)	*argB2*, *pyrG89*, *veA1*, *nkuA*Δ, AN6401Δ	This study
Reference strain for knockouts (NID750)	*veA1*	This study
*rodA*Δ (AN8803Δ, NID600)	*veA1*, *rodA*Δ	This study
*dewA*Δ (AN8006Δ, NID601)	*veA1*, dew*A*Δ	This study
*rodA*Δ*dewA*Δ (NID602)	*veA1*, *dewA*Δ, *rodA*Δ	This study
AN0940Δ (NID666)	*veA1*, AN0940Δ	This study
AN1837Δ (NID667)	*veA1*, AN1837Δ	This study
AN6401Δ (NID668)	*veA1*, AN6401Δ	This study
Reference strain for over-expression strains (NID191)	*argB2*, *pyrG89*, *veA1*, *nkuA*Δ*::*AF*pyrG*, IS1::PgpdA-TtrpC::*argB*	CMB fungal collection
rodA OEx (NID770)	*argB2*, *pyrG89*, *veA1*, *nkuA-trS::*AF*pyrG*, IS1::PgpdA-AN8803-TtrpC::*argB*	This study
dewA OEx (NID684)	*argB2*, *pyrG89*, *veA1*, *nkuA-trS::*AF*pyrG*, IS1::PgpdA-AN8006-TtrpC::*argB*	This study
AN7539 OEx (NID771)	*argB2*, *pyrG89*, *veA1*, *nkuA-trS::*AF*pyrG*, IS1::PgpdA-AN7539-TtrpC::*argB*	This study
AN6401 OEx (NID895)	*argB2*, *pyrG89*, *veA1*, *nkuA-trS::*AF*pyrG*, IS1::PgpdA-AN6401-TtrpC::*argB*	This study
AN1837 OEx (NID896)	*argB2*, *pyrG89*, *veA1*, *nkuA-trS::*AF*pyrG*, IS1::PgpdA-AN1837-TtrpC::*argB*	This study
AN0940 OEx. (NID893)	*argB2*, *pyrG89*, *veA1*, *nkuA-trS::*AF*pyrG*, IS1::PgpdA-AN0940-TtrpC::*argB*	This study

Table 2: Oligonucleotides for strain construction

Purpose	Name	Oligonucleotide sequence
Construction of *rodA*Δ	ANrodA-chk-Up-F	GGGACAGGAGTTGAGAGG
	ANrodA-del-Up-F	CGCTGGGAGCTGAAGCTG
	ANrodA-del-Up-Ra	gatccccgggaattgccatgGAGAATGAGGGACTGGACTGTC
	ANrodA-del-Dw-Fa	aattccagctgaccaccatgGCTTATCTCCGAATACTGG
	ANrodA-del-Dw-R	CTCTGGACCTTGTTCTTGG
	ANrodA-chk-Dw-R	CTGTATGAAAGCCCCGATG
Construction of *dewA*Δ	ANdewA-chk-Up-F	ACTCAATCCGTGCCTACA
	ANdewA-del-Up-F	GCGATGCTAGTGGTTGTT
	ANdewA-del-Up-Ra	gatccccgggaattgccatgGAGATGCTTGTGATGGAAGG
	ANdewA-del-Dw-Fa	aattccagctgaccaccatgCTTGGAGAGAACAGACGG
	ANdewA-del-Dw-R	CGTTTCTGTCTTCCGTGG
	ANdewA-chk-Dw-R	GTCCTGAAGTCCTGAACC
Construction of AN0940Δ	AN0940-chk-Up-F	CCACAGAAGAGCTCTGTGC
	AN0940-del-Up-F	GGAGTACTACTACTGGGAATCGG
	AN0940-del-Up-Ra	gatccccgggaattgccatgGAGTGGCTTGGCAGGATAGAG
	AN0940-del-Dw-Fa	aattccagctgaccaccatgGGCGGTTCCGAGGATCAG
	AN0940-del-Dw-R	GGGCAGGCAGAGATTGGTG
	AN0940-chk-Dw-R	CAATATACGCCGAGCCAACCC
Construction of AN1837Δ	AN1837-chk-Up-F	CAGCAGTGACCACAGGATTC
	AN1837-del-Up-F	GTTATCAGGTGGCGCAATCTAC
	AN1837-del-Up-Ra	gatccccgggaattgccatgGAGGTAGGTGAGAGAGGACG
	AN1837-del-Dw-Fa	aattccagctgaccaccatgGCGGTTGATGTTGTAACTTCAG
	AN1837-del-Dw-R	CGTTGAGAGACGTAATCGGC
	AN1837-chk-Dw-R	CCTTGGAGACGGTCGAGATAG
Construction of AN6401Δ	AN6401-chk-Up-F	GAACTAACCTTCTGGATCCGTC
	AN6401-del-Up-F	GGATCATATTGCGCGGTCGG
	AN6401-del-Up-Ra	gatccccgggaattgccatgGGTGTCTGGTAAGATGCTGCG
	AN6401-del-Dw-Fa	aattccagctgaccaccatgGAGAGTCTCGGTCTCCTGGAC
	AN6401-del-Dw-R	CCGGTTGTGACATGGATAGGTG
	AN6401-chk-Dw-R	CTGCAACGCCAGATTATAACG
Construction of AF*pyrG*	pDEL-Up-F-Ad	catggcaattcccggggatcTGGATAACCGTATTACCGCC
	AFpyrG-int-F3	TGATACAGGTCTCGGTCCC
	AFpyrG-int-R	GGAAGAGAGGTTCACACC
	pDEL-Dw-R-Ad	catggtggtcagctggaattTGCCAAGCTTAACGCGTACC
Construction of AN8803-OEx	FW_AN8803	AGAGCGA**U**AAGACTTTCATAGACAGTCCAGTC
	RV_AN8803	TCTGCGA**U**AGGCAGTAATGAAGCAATCAAG
Construction of AN8006-OEx	FW_AN8006	AGAGCGA**U**GCAATTCAGCAATTCAGCAATCCA
	RV_AN8006	TCTGCGA**U**CAAATCCGAACAACACCAATTATT
Construction of AN0940-OEx	FW_AN0940	AGAGCGA**U**ATGCATCTTTCCACCTCCGCT
	RV_AN0940	TCTGCGA**U**CTACTTGTCAACGCCATCACCAAC
Construction of AN1837-OEx	FW_AN1837	AGAGCGA**U**GCAGACTAAACGTCACGTCCCA

	RV_AN1837	TCTGCGA**U**GGATTATTAATGTATTTATTCGAACGAATTT
Construction of AN6401-OEx	FW_AN6401	AGAGCGA**U**ATGCAATTCACAATCGCTTCCC
	RV_AN6401	TCTGCGA**U**TTAGAGAACCTGGACAGGA
Construction of AN7539-OEx	FW_AN7539	AGAGCGA**U**ATGAAGGTCGCCACTGCC
	RV_AN7539	TCTGCGA**U**TTAGTGGCCGTGCTCCAG
Check primers for IS1 in OEx strains	AN-IS1-Up-chk-F	GGGAAAGACATCTGATCAGCG
	AN-IS1-Dw-chk-R	GAGCCTGGTCAAAGTGGG
	AN-IS1-Up-gapchk-F2	GGACAACGGGAAGAGGCTCAG
	AN-IS1-Dw-gapchk-R2	GGAGAGGGAGAGAAGAAGAAGGG

Table 3: Oligonucleotides for qRT-PCR

Name	**Oligonucleotide sequence**
AN8803-qRT-F	CACGTACGCCGGTGACAC
AN8803-qRT-RV	GAGTTCTGGCAGCAGGCAATG
AN8006-qRT-F	CTCTCCTCGCCTTCACTGC
AN8006-qRT-RV	CCGAGCAGACCGCTCAAC
AN0940-RT-F	CTTCGTGAAGACATGTCCCAAGG
AN0940-RT-R	CGCCATCACCAACCTCAGTC
AN1837-RT-F	GGTGCTTTCAGCGGTTGCTC
AN1837-RT-R	GAGCCAAGGGCAATGCAGG
AN6401-qRT-F	GTACTCTGGCTGCTCTTCGC
AN6401-qRT-RV	GAGGCCGTTCTGTATACCATCAG
AN7539-qRT-F	GCTGTGGTAGCCTTACTACTCCT
AN7539-qRT-RV	CGCAGCCAAGACCGAGAG
hhtA-qRT-F	GTGCTCTCCAGGAGTCCG
hhtA-qRT-RV	GAGGCGACGAGCAAGCTG
actA-qRT-F	GACGTCCGTAAGGATCTGTACG
actA-qRT-RV	GCGGTGGACGATCGAAGG

Appendix 2
Materials and methods for chapter 5

Interactions between *A. nidulans* hydrophobins deletion strains and *P. aeruginosa* PAO1

The interaction between the hydrophobin deletion strains and *Pseudomonas aeruginosa* PAO1[11] was assessed using a plating assay described in paper 2. Wickerhams antibiotic test medium (WATM) was used as media as it supported growth of both *Aspergillus* and *Pseudomonas aeruginosa*. *Aspergillus* spores were harvested in a suspension consisting of 0.5 % Tween80 and 0.5 % agar and diluted to $1x10^6$ spores/ml. The suspension was streaked out horizontally on to the left side of sterile WATM plates and incubated over night at 37 °C. After 24 hours incubation a *P. aeruginosa* overnight culture was diluted to $1x10^8$ CFU/ml and streaked out four times perpendicular to the fungal streak. Control plates containing *P. aeruginosa* or *Aspergillus* alone were included. Plates were hereafter incubated at 37 °C for five days.

The plate was divided into four zones. Two zones in the fungus and two zones in the bacterium. Zone 1 was defined in the *Aspergillus* as far away from the *Pseudomonas* as possible. Zone 2 in the *Aspergillus* as close to *Pseudomonas* as possible. Zone 3 in the *Pseudomonas* as close to the *Aspergillus* as possible and zone 4 in the *Pseudomonas* as far from the *Aspergillus* as possible.

Extracts were prepared by cutting four plugs of 6 mm from each zone. The plugs were transferred to 2 ml vials, 1 ml of methanol: dichloromethane:ethyl acetate (1:2:3 v/v/v) with 0.5 % formic acid was added and the vials placed in an ultrasonication bath for 60 min. The extract was transferred to new vials and evaporated to dryness. The residues were dissolved in 500 µl methanol and ultrasonicated for 10 min. Extracts were finally filtered through a PTFE 0.45 µm syringe filter into a new vial and used for analysis.

Extracts were analysed by UHPLC-qTOFMS on a Dionex RSLC Ultimate 3000 (Dionex, Sunnyvale, CA) equipped with diode-array detector and a Kinetex C_{18} column (100 x 2.1 mm, 2.6 µm; Phenomenex, Torrence CA). Separation was performed using a linear water-acetonitril (CH_3CH) gradient starting from 10 % CH_3CH to 100 % over 7 min at a flow rate of 0.4 ml/min. Both water and CH_3CH were buffered with 20 mM formid acid. The UHPLC was coupled to a maXis G3 quadrupole time of flight mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray (ESI) ion source. The instrument was operated in ESI^+ at a resolution of 40 000 FMWH and scanning m/z 100-1000. Data analysis for identification of microbial secondary metabolites is described in [12–14]. The instrument generally produces multi-charged ions

above molecular masses of 700-800 Da. For unbiased data analysis the data files were analyzed in random, molecular features detected, peaks aligned and compared using the Bruker Profile Analysis 2.0 software (Bruker Daltonics, Bremen, Germany). All experiments were done in biological duplicates.

References for appendix 1 and 2

1. Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ (2009) Jalview Version 2-a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **25:** 1189-1191.

2. Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkens MR, et al. (2005) Protein Identification and Analysis Tools on the ExPASy Server. In: Walker JM, editors. The Proteomics Protocols Handbook. Humana Press Inc. pp. 571-607.

3. Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157:** 105-132.

4. Nielsen JB, Nielsen ML, Mortensen UH (2008) Transient disruption of non-homologous end-joining facilitates targeted genome manipulations in the filamentous fungus *Aspergillus nidulans*. *Fungal Genet Biol* **45:** 165-170.

5. Frisvad JC, Samson RA (2004) *Penicillium* subgenus *Penicillium* - A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. *Stud Mycol* **49:** 1-173.

6. Raper, K. B. and Thom, C. (1949) A manual of the penicillia. Baltimore: Williams & Wilkens Co. 920 p.

7. Nielsen ML, Albertsen L, Lettier G, Nielsen JB, Mortensen UH (2006) Efficient PCR-based gene targeting with a recyclable marker for *Aspergillus nidulans*. *Fungal Genet Biol* **43:** 54-64.

8. Todd RB, Davis MA, Hynes MJ (2007) Genetic manipulation of *Aspergillus nidulans*: meiotic progeny for genetic analysis and strain construction. *Nat Protoc* **2:** 811-821.

9. Hansen BG, Salomonsen B, Nielsen MT, Nielsen JB, Hansen NB, Nielsen KF, Regueira TB, Nielsen J, Patil KR, Mortensen UH (2011) Versatile enzyme expression and characterization system for *Aspergillus nidulans*, with the *Penicillium brevicompactum* polyketide synthase gene from the mycophenolic acid gene cluster as a test case. *Appl Environ Microbiol* **77:** 3044-3051.

10. Samson, RA, Houbraken, J., Thrane, U., Frisvad, JC, and Andersen, B. (2010) Food and Indoor Fungi. Utrech: CBS-KNAW Fungal Biodiversity Centre Utrech. 390 p.

11. Holloway BW, Morgan AF (1986) Genome organization in *Pseudomonas*. *Annu Rev Microbiol* **40:** 79-105.

12. Smedsgaard J (1997) Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. *J Chromatogr A* **760:** 264-270.

13. Frisvad JC, Rank C, Nielsen KF, Larsen TO (2009) Metabolomics of *Aspergillus fumigatus*. *Med Mycol* **47:** S53-S71.

14. Nielsen KF, Mogensen JM, Johansen M, Larsen TO, Frisvad JC (2009) Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. *Anal Bioanal Chem* **395:** 1225-1242.

Paper 1

Hydrophobins from *Aspergillus* species cannot be clearly divided into two classes

Britt G Jensen, Mikael R Andersen, Mona H Pedersen, Jens C Frisvad, Ib Søndergaard

Paper published in *BMC Research Notes*, 2010 3:344

Jensen *et al. BMC Research Notes* 2010, **3**:344
http://www.biomedcentral.com/1756-0500/3/344

BMC Research Notes

SHORT REPORT Open Access

Hydrophobins from *Aspergillus* species cannot be clearly divided into two classes

Britt G Jensen*, Mikael R Andersen, Mona H Pedersen, Jens C Frisvad, Ib Søndergaard

Abstract

Background: Hydrophobins are a family of small secreted proteins with a characteristic pattern of eight cysteine residues found exclusively in filamentous fungi. They have originally been divided into two classes based on their physical properties and hydropathy patterns, and are involved in the attachment of hyphae to hydrophobic structures, the formation of aerial structures and appear to be involved in pathogenicity.

Findings: Analysis of nine genome sequences from seven Aspergilli revealed fifty hydrophobins, where each species displayed between two to eight hydrophobins. Twenty of the identified hydrophobins have not previously been described from these species. Apart from the cysteines, very little amino acid sequence homology was observed. Twenty-three of the identified hydrophobins could be classified as class I hydrophobins based on their conserved cysteine spacing pattern and hydropathy pattern. However twenty-six of the identified hydrophobins were intermediate forms. Notably, a single hydrophobin, ATEG_04730, from *Aspergillus terreus* displayed class II cysteine spacing and had a class II hydropathy pattern.

Conclusion: Fifty hydrophobins were identified in *Aspergillus*, all containing the characteristic eight cysteine pattern. *Aspergillus terreus* exhibited both class I and class II hydrophobins. This is the first report of an *Aspergillus* species with the potential to express both class I and class II hydrophobins. Many of the identified hydrophobins could not directly be allocated to either class I or class II.

Background

Hydrophobins are a family of small proteins found uniquely in filamentous fungi [1]. The currently characterised hydrophobins are approximately 100 AA in size and have little amino acid sequence homology except from eight conserved cysteines in a characteristic pattern [2,3]. The eight cysteines form four disulfide bonds in the pattern Cys1-Cys6, Cys2-Cys5, Cys3-Cys4, Cys7-Cys8 and especially the Cys3-Cys4 loop can vary considerably in length [4]. Based on their distinct hydropathy patterns and physical properties, hydrophobins are traditionally divided into two classes [3]. Class I hydrophobins form highly insoluble membranes in water, organic solvents and 2% SDS, while the membranes formed by class II hydrophobins easily can be dissolved in aqueous ethanol (60%) or 2% SDS [2]. Class I hydrophobins have been identified in Ascomycetes and Basiodiomycetes, while class II hydrophobins have only been identified in Ascomycetes [1]. Typically, a single fungal species only expresses either class I or class II hydrophobins, however previous studies have shown that few species have the ability to express both class I and class II hydrophobins [5,6]. In class I hydrophobins the cysteine doublets are followed by hydrophilic amino acids, while hydrophobic amino acids are observed after the cysteine doublets in class II hydrophobins [2]. Furthermore, considerable variation is seen in the cysteine spacing of class I hydrophobins, while less variation is seen for class II hydrophobins [7]. In this study, we examine nine full genome sequenced Aspergilli for new hydrophobins.

Results and Discussion

Identification of hydrophobins

Nine full genome sequenced *Aspergillus* species were used to search for new hydrophobins. A total of 50 potential hydrophobins were identified (Table 1) based on the criteria of minimum eight cysteines, two cysteine

* Correspondence: brgj@bio.dtu.dk
Center for Microbial Biotechnology, Department of Systems Biology, Technical University of Denmark, Søltofts Plads, Building 221/223, DK-2800 Kgs. Lyngby, Denmark

Jensen *et al. BMC Research Notes* 2010, **3**:344
http://www.biomedcentral.com/1756-0500/3/344

Table 1 Aspergillus hydrophobins

Species	Gene	Size (Da)	n(AA)	n(cys)	Eight cysteine pattern	Theoretical class	Common name
A. oryzae RIB40	AO090012000143	14304	145	8	CN{8}CCN{38}CN{10}CN{5}CCN{21}C	I	RolA[a]
	AO090020000588	15231	151	8	CN{7}CCN{39}CN{17}CN{5}CCN{17}C	I	New
A. niger CBS 513.88	An03g02360[b]	12486	122	8	CN{6}CCN{32}CN{25}CN{5}CCN{4}C	I	
	An03g02400[b]	13063	131	8	CN{6}CCN{31}CN{23}CN{5}CCN{6}C	Intermediate	
	An04g08500[b]	14397	146	8	CN{7}CCN{39}CN{20}CN{5}CCN{17}C	I	
	An15g03800[b]	13225	130	8	CN{5}CCN{32}CN{6}CN{5}CCN{13}C	Intermediate	
	An01g10940[b]	10693	100	8	CN{14}CCN{17}CN{11}CN{7}CCN{8}C	Intermediate	
	An07g03340[b]	16207	162	8	CN{7}CCN{39}CN{21}CN{5}CCN{17}C	I	
	An09g05530[b]	20465	202	9	CN{8}CCN{33}CN{11}CN{5}CCN{16}C	Intermediate	
	An08g09880[b]	9169	91	9	CN{7}CCN{16}CN{6}CN{5}CCN{10}C	Intermediate	
A. niger ATCC 1015	JGI128530	10803	105	7	Fragment (similar to An07g03340)	Intermediate	(New)
	JGI35683	10693	100	8	CN{14}CCN{17}CN{11}CN{7}CCN{8}C	Intermediate	(New)
	JGI45683	13063	131	8	CN{6}CCN{31}CN{23}CN{5}CCN{6}C	Intermediate	(New)
	JGI45685	13716	132	8	CN{6}CCN{32}CN{25}CN{5}CCN{14}C	I	(New)
	JGI53462	13224	130	8	CN{5}CCN{32}CN{6}CN{5}CCN{13}C	Intermediate	(New)
	JGI194815	14397	146	8	CN{7}CCN{39}CN{20}CN{5}CCN{17}C	I	(New)
	JGI43184	20381	201	9	CN{8}CCN{33}CN{11}CN{5}CCN{16}C	Intermediate	(New)
E. nidulans FGSC A4	AN7539.2[c]	10798	109	8	CN{5}CCN{32}CN{6}CN{5}CCN{13}C	Intermediate	
	AN8803.2[c]	15625	157	8	CN{7}CCN{39}CN{18}CN{5}CCN{17}C	I	RodA[d]
	AN6401.2[c]	16131	162	8	CN{6}CCN{38}CN{22}CN{5}CCN{35}C	Intermediate	
	AN8006.2[c]	13183	135	8	CN{6}CCN{31}CN{23}CN{5}CCN{6}C	I	DewA[e]
	AN1837.2[c]	13397	135	8	CN{7}CCN{39}CN{18}CN{5}CCN{17}C	I	
	AN0940.2[c]	10594	101	8	CN{13}CCN{17}CN{12}CN{7}CCN{8}C	Intermediate	
A. fumigatus AF293	AFUA_8G07060	15996	155	8	CN{7}CCN{39}CN{21}CN{5}CCN{17}C	I	RodC[f]
	AFUA_5G09580	16153	159	8	CN{7}CCN{39}CN{21}CN{5}CCN{17}C	I	RodA[f/g]
	AFUA_2G14661	12928	125	8	CN{5}CCN{32}CN{6}CN{5}CCN{13}C	Intermediate	New
	AFUA_1G17250	14299	140	8	CN{7}CCN{36}CN{18}CN{5}CCN{18}C	I	RodB[f/h]
	AFUA_5G03280	19825	190	9	CN{7}CCN{33}CN{11}CN{5}CCN{14}C	I	RodF[f]
A. fumigatus A1163	AFUB_016640	14300	140	8	CN{7}CCN{36}CN{18}CN{5}CCN{18}C	I	(RodB New)
	AFUB_057130	16153	159	8	CN{7}CCN{39}CN{21}CN{5}CCN{17}C	I	(RodA New)
	AFUB_080740	15996	155	8	CN{7}CCN{39}CN{21}CN{5}CCN{17}C	I	(RodC New)
	AFUB_051810	19825	190	9	CN{7}CCN{33}CN{11}CN{5}CCN{14}C	Intermediate	(RodF New)
A. terreus NIH 2624	ATEG_10285	13978	129	8	CN{5}CCN{28}CN{14}CN{8}CCN{13}C	Intermediate	New
	ATEG_08089	18936	177	8	CN{8}CCN{33}CN{11}CN{5}CCN{14}C	Intermediate	New
	ATEG_07808	11677	115	8	CN{5}CCN{32}CN{6}CN{5}CCN{13}C	Intermediate	New
	ATEG_06492	17374	175	8	CN{7}CCN{40}CN{16}CN{5}CCN{17}C	I	New
	ATEG_04730	11797	121	8	CN{10}CCN{11}CN{16}CN{8}CCN{10}C	II	New
A. flavus NRRL 3357	AFLA_094600	8377	83	8	CN{7}CCN{16}CN{6}CN{5}CCN{9}C	Intermediate	New
	AFLA_131460	10867	106	8	CN{5}CCN{32}CN{6}CN{5}CCN{13}C	Intermediate	New
	AFLA_060780	27807	251	8	CN{6}CCN{30}CN{23}CN{5}CCN{4}C	I	New
	AFLA_014260	14304	145	8	CN{8}CCN{38}CN{10}CN{5}CCN{21}C	I	New
	AFLA_063080	9362	87	9	CN{5}CCN{17}CN{7}CN{7}CCN{12}C	Intermediate	New
	AFLA_098380	23415	217	10	CN{7}CCN{39}CN{17}CN{5}CCN{44}C	I	New
	AFLA_064900	9147	91	10	CN{7}CCN{15}CN{6}CN{5}CCN{8}C	Intermediate	New
A. clavatus NRRL 1	ACLA_001890	10214	100	8	CN{7}CCN{16}CN{6}CN{5}CCN{26}C	Intermediate	New
	ACLA_048810	18458	182	8	CN{7}CCN{33}CN{11}CN{5}CCN{15}C	Intermediate	New
	ACLA_010960	14671	145	8	CN{7}CCN{39}CN{21}CN{5}CCN{17}C	I	New
	ACLA_072820	16127	158	8	CN{7}CCN{39}CN{21}CN{5}CCN{17}C	I	New
	ACLA_018290	12820	126	8	CN{5}CCN{32}CN{6}CN{5}CCN{13}C	Intermediate	New
	ACLA_007980	14558	144	8	CN{7}CCN{36}CN{18}CN{5}CCN{17}C	Intermediate	New

[a] described by [9],[b] mentioned by [10], [c] mentioned by [11], [d] described by [12], [e] described by [8], [f] described by [13], [g] RodA described by [14,15], [h] RodB described by [16]

Jensen *et al. BMC Research Notes* 2010, **3**:344
http://www.biomedcentral.com/1756-0500/3/344

pairs, a size of app. 100 AA and the cysteine pattern. On species level twenty of the identified hydrophobins have not previously been mentioned in other studies, while the number increases to thirty-one on strain level. The number of identified hydrophobins within the species varied from two to eight between the nine species. All identified hydrophobins had theoretical signal sequences and therefore have the possibility of being secreted. They contain approximately 100 - 200 amino acids and are 8 - 30 kDa in size. Furthermore, they contain eight to ten cysteines, where excess cysteines (above eight) are located before or after the conserved cysteine spacing pattern. Beauvais *et al*. (2007) have classified AFUA_8G05890 and AFUA_5G01490 as hydrophobins. As AFUA_8G05890 has 11 cysteines, no signal sequence and both proteins lack the conserved cysteine pattern, we disregard these proteins as hydrophobins as they do not fulfil our criteria. Other *Aspergillus* hydrophobins previously identified fulfilled the criteria [8-16] and have likewise been found and included in this study.

Forty-five of the identified proteins contained domains classifying them as hydrophobins by Pfam. The remaining five hydrophobins could not be classified. Four of these (An01g10940, JGI35683, AN0940.2, AFLA_063080) can be differentiated from the rest in displaying a distinctive cysteine pattern. They have a similar cysteine pattern of CN{5-13}CCN{17}CN{7-12}CN{7}CCN{8-12}C (where N signifies any other amino acid than cysteine) and group together in the phylogenetic tree (Additional file 1), but still with other hydrophobins. They also have hydropathy patterns that differ from both class I and class II hydrophobins and can therefore theoretically not be placed in either class. Furthermore, their hydropathy patterns differ from each other, so they do not form a new class either. The fifth hydrophobin (ATEG_10285) differs in having a different cysteine spacing compared to all other identified hydrophobins, but still clusters with other hydrophobins in the phylogenetic tree (Additional file 1). Forty-four of the identified hydrophobins displayed class I cysteine spacing pattern, but only twenty-four had a characteristic class I hydropathy plot resulting in only twenty-three identified class I hydrophobins (see Additional file 2 and Table 1). Only one identified hydrophobin displayed a characteristic class II cysteine spacing pattern and had a class II hydropathy pattern, while the rest (twenty-six) were intermediate forms. However, as the majority of the identified hydrophobins have not physically been isolated and characterised, a differentiation into type of class is only provisional. As many of the identified hydrophobins displayed intermediate forms, they may also exhibit solubility characteristics between the two known classes. As these intermediate forms blur the original classification, it could be speculated, whether an extension of the classical two class system would be in place as more fungal genomes become available.

An examination of the multiple alignment (Additional file 3) of the putative hydrophobins revealed very low similarity between the hydrophobins. Apart from the eight cysteines a proline was observed in the majority of the sequences (82%) situated in close proximity to the theoretical signal sequence cleavage site. This proline may be involved in the correct cleavage of the signal sequence and thereby influence the eventual secretion of the hydrophobins. Tryptophan is rarely seen in hydrophobins [2], and only twelve of the identified hydrophobins from Aspergilli contained between 1-5 tryptophan residues.

Several groups are revealed in the phylogenetic tree (Additional file 1) and it seems that hydrophobins cluster according to their cysteine spacing pattern. A common feature in 44 of the 50 hydrophobins is a conserved spacing of five amino acids between the fifth and sixth cysteines, while the remaining six hydrophobins contain either seven or eight amino acids. This spacing of five cysteines is also observed in other known class I hydrophobins (eg. SC3, EAS and MPG1) [7] and may be a common feature in class I hydrophobins.

Previously Yang *et al*. (2006) [17] used primary structure analysis to identify new members of the hydrophobin family. By searching the Uniprot Knowledgebase using the key word hydrophobin followed by a BLAST against the NCBI database, Yang *et al*. retrieved several sequences. However, by using the above mentioned method putative hydrophobin sequences may be missed as hydrophobins have high sequence diversity, and may not resemble known hydrophobins sufficiently to be picked up by a BLAST. In our search we found five hydrophobins (An01g10940, JGI35683, AN0940.2, AFLA_063080, ATEG_10285), which do not resemble the other identified hydrophobins. If these hydrophobins are used to conduct a BLAST, no known hydrophobins appear in the results. So if the method described by Yang *et al*. was used, these putative hydrophobins would likely have been missed. Furthermore, Yang *et al*. uses their identified sequences to create motifs, and thereby identify nine new hydrophobins including five *E. nidulans* (*A. nidulans*) hydrophobins. In our approach we only sort our putative hydrophobins by the criteria of size, number of cysteines and the eight cysteine pattern, thereby not eliminating any hydrophobins even if they do not contain any common motifs.

Class I and class II hydrophobins of *Aspergillus terreus*

In *Aspergillus terreus* five different hydrophobins were identified. ATEG_06492 displayed a characteristic class I hydrophobin cysteine spacing pattern (CN{7}CCN{40}CN{16}CN{5}CCN{17}C), whereas a class II hydrophobin

Jensen *et al. BMC Research Notes* 2010, **3**:344
http://www.biomedcentral.com/1756-0500/3/344

spacing pattern was observed for ATEG_04730 (CN{10}CCN{11}CN{16}CN{8}CCN{10}C). Comparison of ATEG_06492 and ATEG_04730 to hydropathy patterns of known class I and class II hydrophobins indicates that *A. terreus* has genes for both class I and class II hydrophobins (Figure 1). The hydrophobins SC3 (*Schizophyllum commune)*, EAS (*Neurospora crassa*) and RodA (*Aspergillus fumigatus*) are known class I hydrophobins, where the cysteine doublets are followed by a stretch of hydrophilic amino acids. Likewise, the cysteine doublets in ATEG_06492 are followed by a stretch of hydrophilic amino acids contrasting ATEG_04730, where hydrophobic

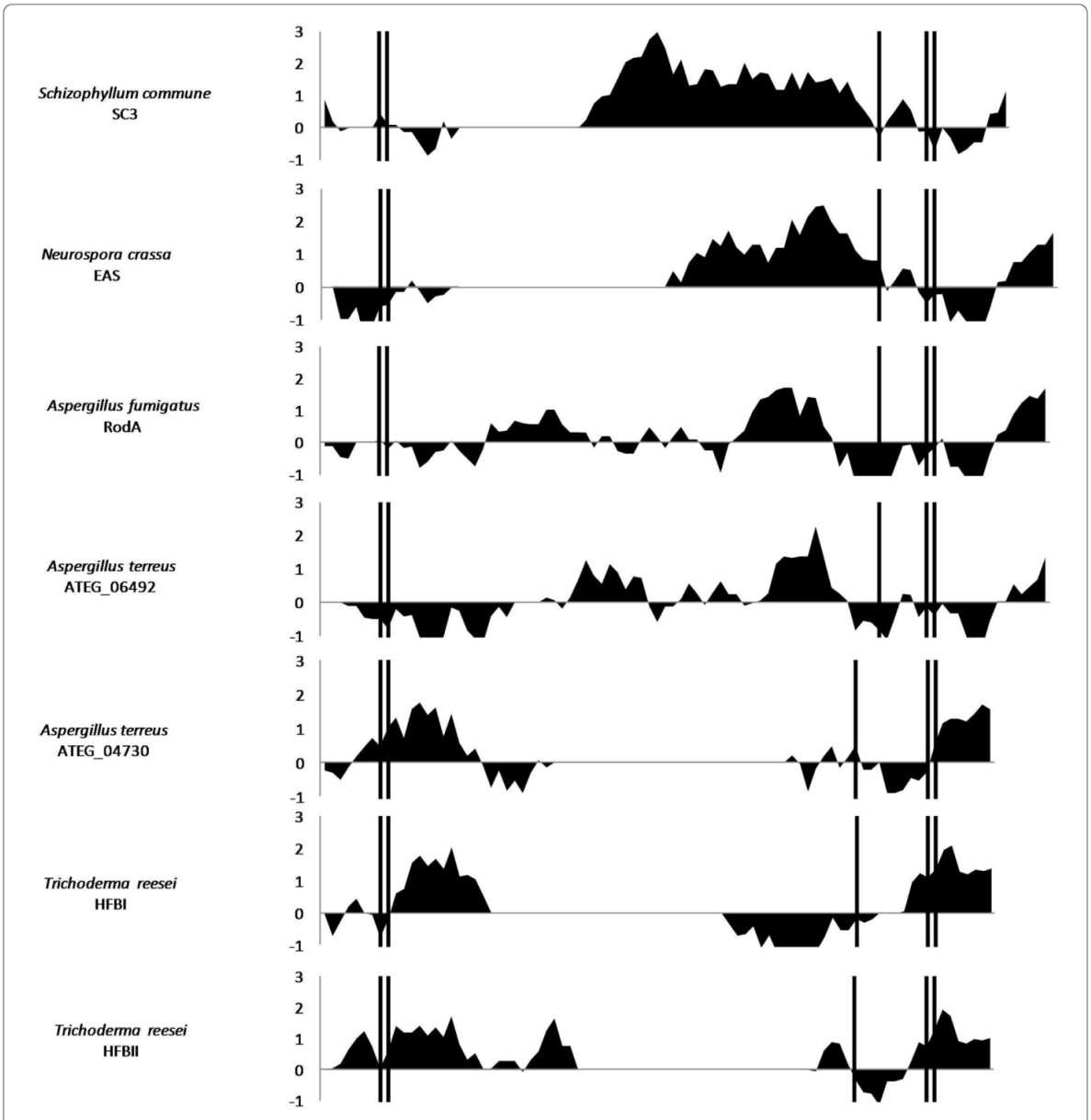


Figure 1 Hydropathy patterns. Hydropathy patterns of SC3 from *S. commune*, EAS from *N. crassa*, RodA from *A. fumigatus*, HFBI and HFBII from *T. reesei* and proteins ATEG_06492 and ATEG_04730 from *A. terreus*. The amino acids of the hydrophobins are shown along the x-axis, where cysteines are indicated by vertical lines. Hydrophobic amino acids are shown above the x-axis, while hydrophilic amino acids are shown below. Only the part of the sequence from the first to the eighth cysteine was used to create the hydropathy pattern.

Jensen *et al. BMC Research Notes* 2010, **3**:344
http://www.biomedcentral.com/1756-0500/3/344

amino acids follow the cysteine doublets. Similarly, the cysteine doublets are followed by hydrophobic amino acids in the known class II hydrophobins HFBI and HFBII from *Trichoderma reesei*. Therefore ATEG_06492 displays a characteristic class I hydropathy pattern, while ATEG_04730 displays a class II hydropathy pattern. Comparison of ATEG_04730 to class II hydrophobins HFBI and HFBII showed 37% and 35% sequence identity, while comparison to class I hydrophobins RodA, SC3 and EAS showed 21%, 16% and 20% sequence identity. In contrast ATEG_06492 showed 20% and 29% sequence identity to class II hydrophobins HFBI and HFBII, but 51%, 21% and 24% to class I hydrophobins RodA, SC3 and EAS. Furthermore, a phylogenetic analysis (Figure 2) revealed that ATEG_04730 clusters with HFBI and HFBI, while ATEG_06492 clusters with RodA, EAS and SC3, strongly indicating that ATEG_04730 can be classified as a class II hydrophobin, while ATEG_06492 is classified as a class I hydrophobin. As neither ATEG_06492 nor ATEG_04730 have physically been isolated or characterised, these can obviously only tentatively be classified as a class I and a class II hydrophobin respectively. This is the first report of an *Aspergillus* species with the potential to express both class I and class II hydrophobins.

Conclusion

Analysis of nine genome sequences from seven Aspergilli revealed fifty hydrophobins, where each species displayed between two and eight hydrophobins. Twenty of the identified hydrophobins have not previously been described from these species. All identified hydrophobins contained two cysteine pairs, were approximately 100-200 AA in size, and displayed the common eight cysteine pattern. Besides the cysteines, very little amino acid sequence homology was observed. Twenty-three of the identified hydrophobins could be classified as class I hydrophobins based on their conserved cysteine spacing pattern and hydropathy pattern, but the majority seem to be intermediate forms. A single hydrophobin, ATEG_04730, from *Aspergillus terreus* displayed a clear class II cysteine spacing and had a class II hydropathy pattern. Furthermore, this hydrophobin grouped together with other known class II hydrophobins in a phylogenetic analysis, showing a close phylogenetic relationship to these. As *Aspergillus terreus* also has the potential to express a class I hydrophobin, this is the first reported case of an *Aspergillus* species with the potential to express both class I and class II hydrophobins.

Methods

Availability of genomic data

The sequences of *Aspergillus oryzae* RIB40, *Aspergillus niger* CBS 513.88, *Emericella nidulans* FGSC A4, *Aspergillus fumigatus* AF293, *Aspergillus fumigatus* A1163, *Aspergillus terreus* NIH 2624, *Aspergillus flavus* NRRL 3357 and *Aspergillus clavatus* NRRL 1 were obtained from the Central Aspergillus Data Repository (CADRE) [18], while the sequence of *Aspergillus niger* ATCC 1015 was obtained from DOE Joint Genome Institute.

Identification of putative hydrophobins

A Perl program was constructed to search the nine *Aspergillus* genomes for putative hydrophobins by identification of the common C..CC..C..C..CC..C cysteine motif [2,3]. The identified putative hydrophobins were further sorted for size and number of cysteine residues resulting in fifty putative hydrophobins. The identified putative hydrophobin sequences were used to conduct a BLAST search against the NCBI (National Center for Biotechnology Information) non-redundant (nr) database to differentiate between known and newly identified hydrophobins. The sequences were examined for domains using Pfam to verify their function as hydrophobins [19] and the presence of and location of signal peptide cleavage sites using SignalP 3.0 to examine their theoretical ability to be secreted [20].

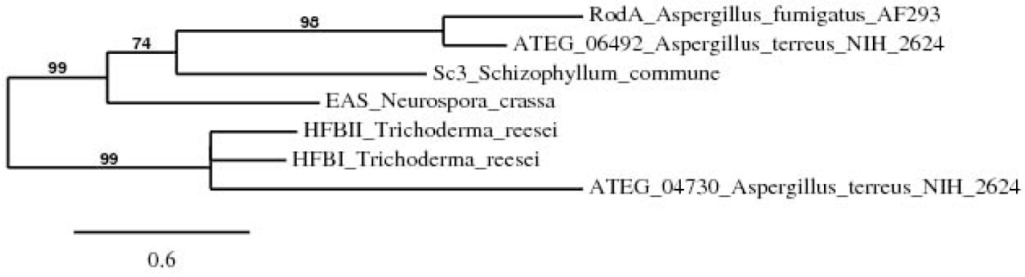


Figure 2 Phylogenetic tree of class I and class II hydrophobins. Sequences of SC3 (*S. commune*), EAS (*N. crassa*), RodA (*A. fumigatus*), HFBI and HFBII (*T. reesei*) were obtained from the National Center for Biotechnology Information (NCBI). The phylogenetic tree was constructed based on a multiple alignment of identified hydrophobins using Phylogeny.fr [22]. Branches with support values less than 50% were collapsed.

Protein sequence analysis

A multiple sequence alignment of the identified hydrophobin sequences was conducted using MUSCLE [21] and based on this alignment a phylogenetic tree was constructed [22-25].

Generation of hydropathy plots

Hydropathy patterns were determined using the hydropathy scale set by Kyte and Doolittle [26]. A nine amino acid window was used and data was extracted using Protscale on the ExPASy Proteomics Server [27]. The hydropathy patterns were aligned around the cysteine pairs placing gaps in the sequences where the hydrophobic and hydrophilic regions alternate. Only the part of the sequence from the first cysteine to the eight was used for examining the hydropathy pattern.

Additional material

Additional file 1: Phylogenetic tree of identified hydrophobins in Aspergilli. The phylogenetic tree was constructed based on a multiple alignment of identified hydrophobins using Phylogeny.fr (Dereeper *et al.*, 2008). Branches with support values less than 50% were collapsed. N signifies any other amino acid than cysteine.

Additional file 2: Theoretical class of identified hydrophobins based on cysteine spacing and hydropathy plot. The hydropathy plots were created using ProtScale (Gasteiger *et al.* 2005).

Additional file 3: Multiple alignment of putative hydrophobins in Aspergilli. Comparison of hydrophobins identified in full genome sequenced Aspergilli. Amino acid residues are colored by conservation (> 80%). Figure created using Jalview [28].

Acknowledgements

We acknowledge The Danish Research Council for Technology and Production Sciences for support (project 09-064225).

Authors' contributions

BGJ carried out the sequence analysis, blast searches, domain searches, phylogenetic analysis, created hydropathy patterns and drafted the manuscript. MRA wrote the Perl program used for searching the *Aspergillus* sequences and participated in data analysis. MHP, JCF and IS gave general direction and manuscript revisions. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

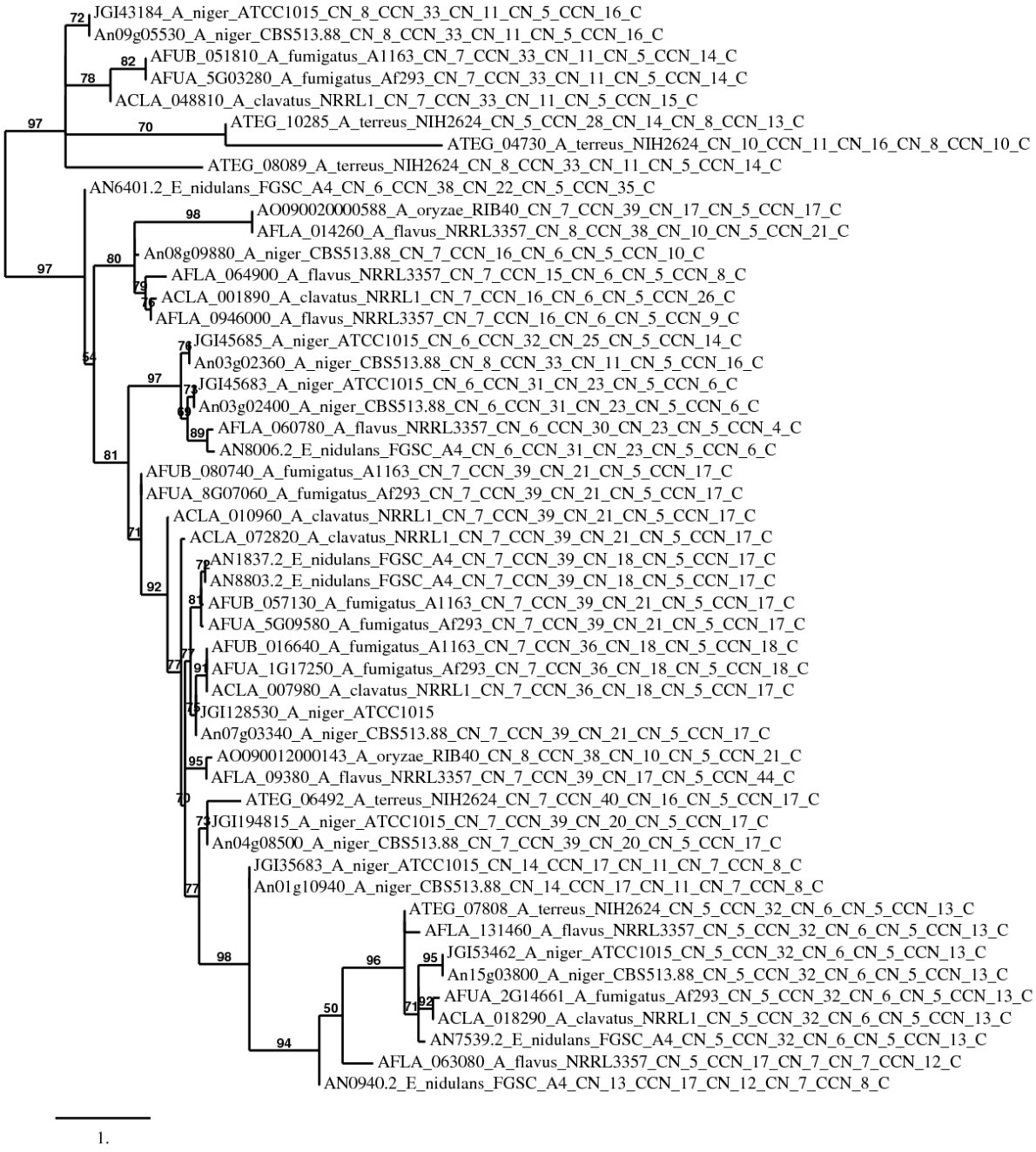
Received: 16 August 2010 Accepted: 23 December 2010
Published: 23 December 2010

References

1. Wosten HA: **Hydrophobins: multipurpose proteins.** *Annu Rev Microbiol* 2001, **55**:625-646.
2. Wessels JGH: *Hydrophobins: Proteins that change the nature of the fungal surface* LONDON: ACADEMIC PRESS LTD; 1997.
3. Wessels JGH: **Developmental Regulation of Fungal Cell-Wall Formation.** *Annual Review of Phytopathology* 1994, **32**:413-437.
4. Kwan AH, Winefield RD, Sunde M, Matthews JM, Haverkamp RG, Templeton MD, *et al*: **Structural basis for rodlet assembly in fungal hydrophobins.** *Proc Natl Acad Sci USA* 2006, **103**:3621-3626.
5. Linder MB, Szilvay GR, Nakari-Setala T, Penttila ME: **Hydrophobins: the protein-amphiphiles of filamentous fungi.** *FEMS Microbiol Rev* 2005, **29**:877-896.
6. Segers GC, Hamada W, Oliver RP, Spanu PD: **Isolation and characterisation of five different hydrophobin-encoding cDNAs from the fungal tomato pathogen Cladosporium fulvum.** *Mol Gen Genet* 1999, **261**:644-652.
7. Kershaw MJ, Talbot NJ: **Hydrophobins and repellents: proteins with fundamental roles in fungal morphogenesis.** *Fungal Genet Biol* 1998, **23**:18-33.
8. Stringer MA, Timberlake WE: **dewA encodes a fungal hydrophobin component of the Aspergillus spore wall.** *Mol Microbiol* 1995, **16**:33-44.
9. Takahashi T, Maeda H, Yoneda S: **The fungal hydrophobin RolA recruits polyesterase and laterally moves on hydrophobic surfaces.** *Molecular Microbiology* 2005, **57**:1780.
10. Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, *et al*: **Genome sequencing and analysis of the versatile cell factory Aspergillus niger CBS 513.88.** *Nat Biotechnol* 2007, **25**:221-231.
11. de Groot PW, Brandt BW, Horiuchi H, Ram AF, de Koster CG, Klis FM: **Comprehensive genomic analysis of cell wall genes in Aspergillus nidulans.** *Fungal Genet Biol* 2009, **46(Suppl 1)**:S72-S81.
12. Stringer MA, Dean RA, Sewall TC, Timberlake WE: **Rodletless, a new Aspergillus developmental mutant induced by directed gene inactivation.** *Genes Dev* 1991, **5**:1161-1171.
13. Beauvais A, Schmidt C, Guadagnini S, Roux P, Perret E, Henry C, *et al*: **An extracellular matrix glues together the aerial-grown hyphae of Aspergillus fumigatus.** *Cellular Microbiology* 2007, **9**:1588-1600.
14. Parta M, Chang Y, Rulong S, Pinto-DaSilva P, Kwon-Chung KJ: **HYP1, a hydrophobin gene from Aspergillus fumigatus, complements the rodletless phenotype in Aspergillus nidulans.** *Infect Immun* 1994, **62**:4389-4395.
15. Thau N, Monod M, Crestani B, Rolland C, Tronchin G, Latge JP, *et al*: **rodletless mutants of Aspergillus fumigatus.** *Infect Immun* 1994, **62**:4380-4388.
16. Paris S, Debeaupuis JP, Crameri R, Carey M, Charles F, Prevost MC, *et al*: **Conidial hydrophobins of Aspergillus fumigatus.** *Appl Environ Microbiol* 2003, **69**:1581-1588.
17. Yang K, Deng Y, Zhang C, Elasri M: **Identification of new members of hydrophobin family using primary structure analysis.** *BMC Bioinformatics* 2006, **7(Suppl 4)**:S16.
18. Mabey JE, Anderson MJ, Giles PF, Miller CJ, Attwood TK, Paton NW, *et al*: **CADRE: the Central Aspergillus Data REpository.** *Nucleic Acids Res* 2004, **32**:D401-D405.
19. Finn RD, Mistry J, Tate J, Coggill P, Heger A, Pollington JE, *et al*: **The Pfam protein families database.** *Nucleic Acids Res* 2010, **38**:D211-D222.
20. Bendtsen JD, Nielsen H, von HG, Brunak S: **Improved prediction of signal peptides: SignalP 3.0.** *J Mol Biol* 2004, **340**:783-795.
21. Edgar RC: **MUSCLE: a multiple sequence alignment method with reduced time and space complexity.** *BMC Bioinformatics* 2004, **5**:113.
22. Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, *et al*: **Phylogeny.fr: robust phylogenetic analysis for the non-specialist.** *Nucleic Acids Res* 2008, **36**:W465-W469.
23. Chevenet F, Brun C, Banuls AL, Jacq B, Christen R: **TreeDyn: towards dynamic graphics and annotations for analyses of trees.** *BMC Bioinformatics* 2006, **7**:439.
24. Guindon S, Lethiec F, Duroux P, Gascuel O: **PHYML Online–a web server for fast maximum likelihood-based phylogenetic inference.** *Nucleic Acids Res* 2005, **33**:W557-W559.
25. Anisimova M, Gascuel O: **Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative.** *Syst Biol* 2006, **55**:539-552.
26. Kyte J, Doolittle RF: **A simple method for displaying the hydropathic character of a protein.** *J Mol Biol* 1982, **157**:105-132.
27. Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkens MR, Appel RD, *et al*: **Protein Identification and Analysis Tools on the ExPASy Server.** In *The Proteomics Protocols Handbook*. Edited by: Walker JM. Humana Press Inc; 2005:571-607.
28. Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ: **Jalview Version 2-a multiple sequence alignment editor and analysis workbench.** *Bioinformatics* 2009, **25**:1189-1191.

doi:10.1186/1756-0500-3-344
Cite this article as: Jensen *et al.*: Hydrophobins from *Aspergillus* species cannot be clearly divided into two classes. *BMC Research Notes* 2010 **3**:344.

Additional material



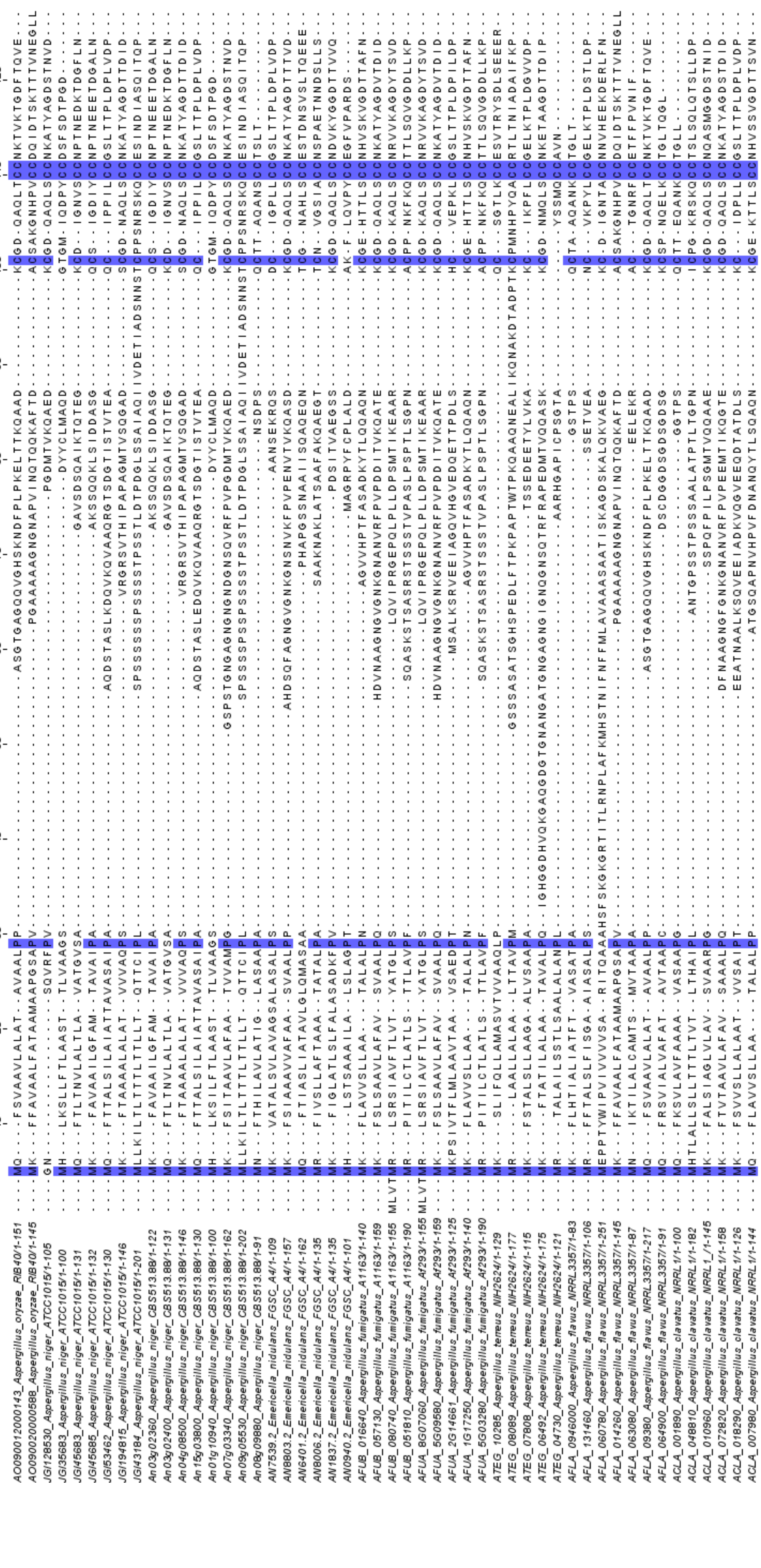
Additional file 1. Phylogenetic tree of identified hydrophobins in Aspergilli

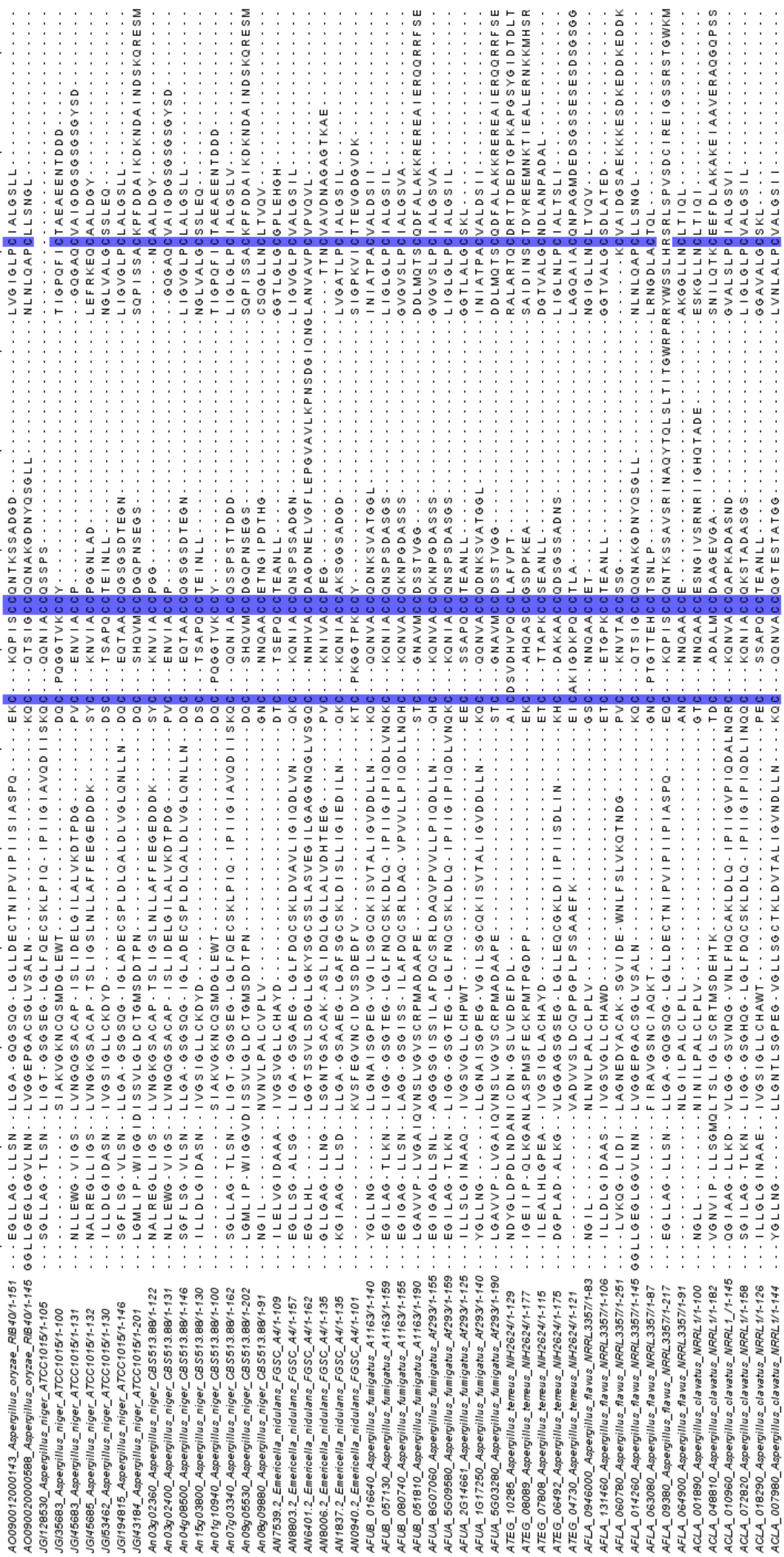
The phylogenetic tree was constructed based on a multiple alignment of identified hydrophobins using Phylogeny.fr (Dereeper *et al.*, 2008). Branches with support values less than 50 % were collapsed. N signifies any other amino acid than cysteine.

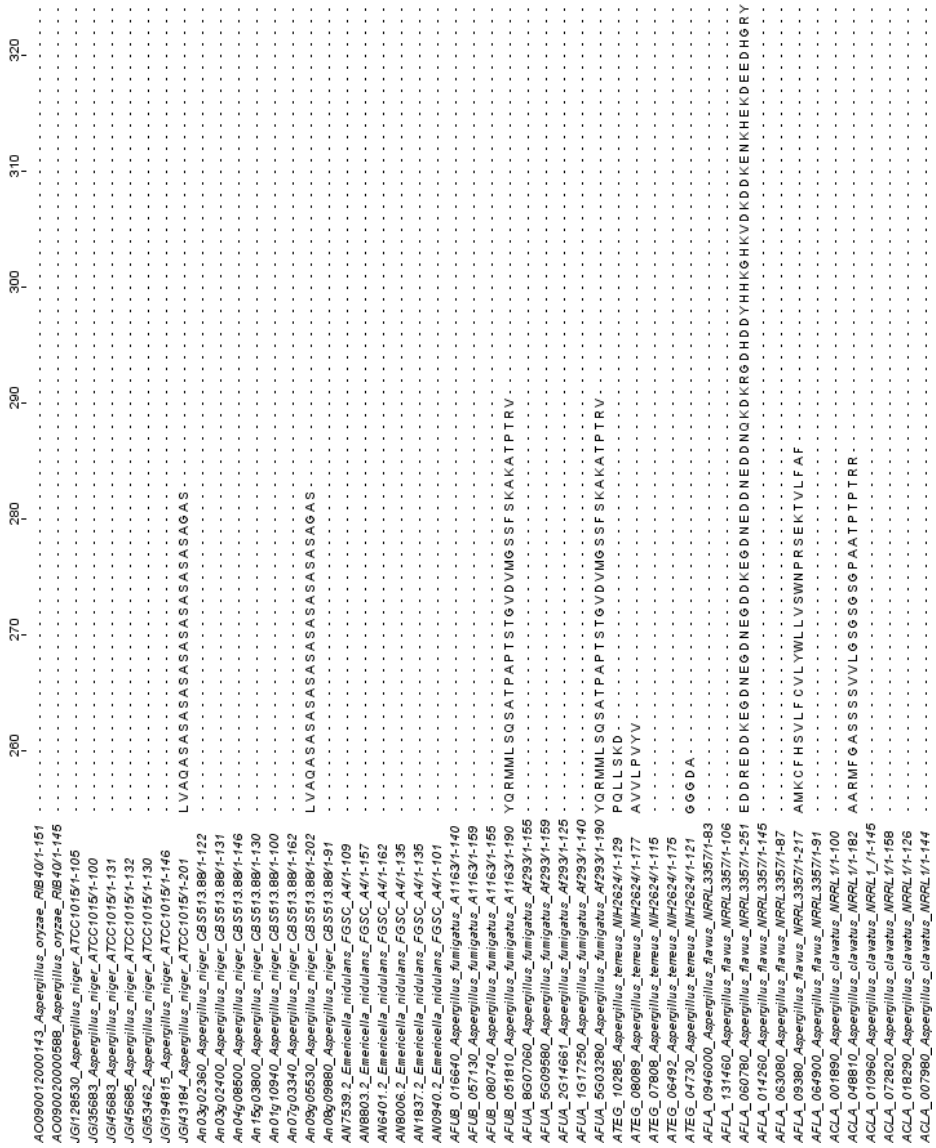
Species	Gene	Eight cysteine pattern	Class based on cysteine pattern	Class based on hydropathy plot	Theoretical class
A. oryzae RIB40	AO090012000143	CN{8}CCN{38}CN{10}CN{5}CCN{21}C	I	I	I
	AO090020000588	CN{7}CCN{39}CN{17}CN{5}CCN{17}C	I	I	I
A. niger CBS 513.88	An03g02360	CN{6}CCN{32}CN{25}CN{5}CCN{4}C	I	I	I
	An03g02400	CN{6}CCN{31}CN{23}CN{5}CCN{6}C	I	Intermediate	Intermediate
	An04g08500	CN{7}CCN{39}CN{20}CN{5}CCN{17}C	I	I	I
	An15g03800	CN{5}CCN{32}CN{6}CN{5}CCN{13}C	I	II	Intermediate
	An01g10940	CN{14}CCN{17}CN{11}CN{7}CCN{8}C	-	Intermediate	Intermediate
	An07g03340	CN{7}CCN{39}CN{21}CN{5}CCN{17}C	I	I	I
	An09g05530	CN{8}CCN{33}CN{11}CN{5}CCN{16}C	I	Intermediate	Intermediate
	An08g09880	CN{7}CCN{16}CN{6}CN{5}CCN{10}C	I	Intermediate	Intermediate
A. niger ATCC 1015	JGI128530	Fragment (similar to An07g03340)	I	Intermediate	Intermediate
	JGI35683	CN{14}CCN{17}CN{11}CN{7}CCN{8}C	-	Intermediate	Intermediate
	JGI45683	CN{6}CCN{31}CN{23}CN{5}CCN{6}C	I	Intermediate	Intermediate
	JGI45685	CN{6}CCN{32}CN{25}CN{5}CCN{14}C	I	I	I
	JGI53462	CN{5}CCN{32}CN{6}CN{5}CCN{13}C	I	II	Intermediate
	JGI194815	CN{7}CCN{39}CN{20}CN{5}CCN{17}C	I	I	I
	JGI43184	CN{8}CCN{33}CN{11}CN{5}CCN{16}C	I	Intermediate	Intermediate
E. nidulans FGSC A4	AN7539.2	CN{5}CCN{32}CN{6}CN{5}CCN{13}C	I	II	Intermediate
	AN8803.2	CN{7}CCN{39}CN{18}CN{5}CCN{17}C	I	I	I
	AN6401.2	CN{6}CCN{38}CN{22}CN{5}CCN{35}C	I	Intermediate	Intermediate
	AN8006.2	CN{6}CCN{31}CN{23}CN{5}CCN{6}C	I	I	I
	AN1837.2	CN{7}CCN{39}CN{18}CN{5}CCN{17}C	I	I	I
	AN0940.2	CN{13}CCN{17}CN{12}CN{7}CCN{8}C	-	II	Intermediate
A. fumigatus AF293	AFUA_8G07060	CN{7}CCN{39}CN{21}CN{5}CCN{17}C	I	I	I
	AFUA_5G09580	CN{7}CCN{39}CN{21}CN{5}CCN{17}C	I	I	I
	AFUA_2G14661	CN{5}CCN{32}CN{6}CN{5}CCN{13}C	I	II	Intermediate
	AFUA_1G17250	CN{7}CCN{36}CN{18}CN{5}CCN{18}C	I	I	I
	AFUA_5G03280	CN{7}CCN{33}CN{11}CN{5}CCN{14}C	I	I	I
A. fumigatus A1163	AFUB_016640	CN{7}CCN{36}CN{18}CN{5}CCN{18}C	I	I	I
	AFUB_057130	CN{7}CCN{39}CN{21}CN{5}CCN{17}C	I	I	I
	AFUB_080740	CN{7}CCN{39}CN{21}CN{5}CCN{17}C	I	I	I
	AFUB_051810	CN{7}CCN{33}CN{11}CN{5}CCN{14}C	I	II	Intermediate
A. terreus NIH 2624	ATEG_10285	CN{5}CCN{28}CN{14}CN{8}CCN{13}C	-	I	Intermediate
	ATEG_08089	CN{8}CCN{33}CN{11}CN{5}CCN{14}C	I	Intermediate	Intermediate
	ATEG_07808	CN{5}CCN{32}CN{6}CN{5}CCN{13}C	I	Intermediate	Intermediate
	ATEG_06492	CN{7}CCN{40}CN{16}CN{5}CCN{17}C	I	I	I
	ATEG_04730	CN{10}CCN{11}CN{16}CN{8}CCN{10}C	II	II	II
A. flavus NRRL 3357	AFLA_094600	CN{7}CCN{16}CN{6}CN{5}CCN{9}C	I	II	Intermediate
	AFLA_131460	CN{5}CCN{32}CN{6}CN{5}CCN{13}C	I	Intermediate	Intermediate
	AFLA_060780	CN{6}CCN{30}CN{23}CN{5}CCN{4}C	I	I	I
	AFLA_014260	CN{8}CCN{38}CN{10}CN{5}CCN{21}C	I	I	I
	AFLA_063080	CN{5}CCN{17}CN{7}CN{7}CCN{12}C	-	Intermediate	Intermediate
	AFLA_098380	CN{7}CCN{39}CN{17}CN{5}CCN{44}C	I	I	I
	AFLA_064900	CN{7}CCN{15}CN{6}CN{5}CCN{8}C	I	II	Intermediate
A. clavatus NRRL 1	ACLA_001890	CN{7}CCN{16}CN{6}CN{5}CCN{26}C	I	Intermediate	Intermediate
	ACLA_048810	CN{7}CCN{33}CN{11}CN{5}CCN{15}C	I	II	Intermediate
	ACLA_010960	CN{7}CCN{39}CN{21}CN{5}CCN{17}C	I	I	I
	ACLA_072820	CN{7}CCN{39}CN{21}CN{5}CCN{17}C	I	I	I
	ACLA_018290	CN{5}CCN{32}CN{6}CN{5}CCN{13}C	I	Intermediate	Intermediate
	ACLA_007980	CN{7}CCN{36}CN{18}CN{5}CCN{17}C	I	II	Intermediate

Additional file 2. Theoretical class of identified hydrophobins based on cysteine spacing and hydropathy plot.

The hydropathy plots were created using ProtScale (Gasteiger *et al.* 2005).







Additional file 3. Multiple alignment of putative hydrophobins in Aspergilli

Comparison of hydrophobins identified in full genome sequenced Aspergilli. Amino acid residues are colored by conservation (>80 %). Figure created using Jalview (Waterhouse *et al.* 2009)

Paper 2

Interactions between *Aspergillus fumigatus* and *Pseudomonas aeruginosa* are mediated by soluble chemical mediators involved in the RpoN pathway

Britt G Jensen, Lars Jelsbak, Thomas Pedersen, Søren Damkiær, Mona H Pedersen, Ib Søndergaard, Jens C Frisvad, Kristian F Nielsen.

Manuscript in preparation for resubmission to PloS ONE after a favourable review.

Interactions between *Aspergillus fumigatus* and *Pseudomonas aeruginosa* are mediated by soluble chemical mediators involved in the RpoN pathway

Britt Guillaume Jensen[1], Lars Jelsbak[2], Thomas Pedersen[3], Søren Damkiær[2], Mona Højgaard Pedersen[1], Ib Søndergaard[1], Jens Christian Frisvad[1], Kristian Fog Nielsen[1§]

[1]Center for Microbial Biotechnology, Department of Systems Biology, Technical University of Denmark, Søltofts Plads, Building 221, DK-2800 Kgs. Lyngby, Denmark

[2]Center for Systems Microbiology, Department of Systems Biology, Technical University of Denmark, Matematiktorvet, Building 301, DK-2800 Kgs. Lyngby, Denmark

[3]Department of Micro- and Nanotechnology, Technical University of Denmark, Ørsteds Plads, Building 344, DK-2800 Kgs. Lyngby, Denmark

[§]Corresponding author

Email addresses:

BGJ: brgj@bio.dtu.dk

LJ: lj@bio.dtu.dk

TP: thomas.pedersen@nanotech.dtu.dk

SD: sdp@bio.dtu.dk

MHP: mohp@bio.dtu.dk

IS: ib@biolearning.dk

JCF: jcf@bio.dtu.dk

KFN: kfn@bio.dtu.dk

Abstract

Aspergillus species and *Pseudomonas aeruginosa* are both frequently isolated from cystic fibrosis (CF) patients, however little is known about the possible interactions of these organisms. Here we developed an assay allowing examination of interactions between different Aspergilli and *P. aeruginosa* on solid medium using chemical analysis of secondary metabolites. We show that *P. aeruginosa* PAO1 suppressed growth of all tested Aspergilli. The suppression of growth by *P. aeruginosa* was correlated with an increased production of phenazines by *P. aeruginosa* in the presence of Aspergilli. However, no increased production of phenazines was seen in response to *A. fumigatus*, indicating variation in sensitivity to phenazines or that other bacterial exoproducts may also contribute to the growth suppression. Interestingly, the Aspergilli did not alter their secondary metabolite profile in response to the presence of *P. aeruginosa*. Mutations that inactivate the function of regulatory proteins MucA and RpoN are frequently observed among *P. aeruginosa* isolates from chronic CF patients. We show that both a *mucA* mutant (overproduction of exopolysaccharide alginate) and an *rpoN* mutant (reduced production of several virulence factors) of *P. aeruginosa* were unable to suppress growth of *A. fumigatus*, but could suppress other Aspergilli. Moreover, an increase in phenazines and quinolones by *P. aeruginosa rpoN* was observed in response to *A. niger, A. oryzae* and *A. flavus*, but not *A. fumigatus.* This indicates that the dynamics of the bacterial-fungal interaction may change in the later stages of CF infections, where the genetically adapted *P. aeruginosa* strains are more susceptible towards competition from *A. fumigatus.*

Introduction

Cystic fibrosis (CF) is a chronic inherited genetic disorder characterized by recurrent infections of the lower respiratory tract due to an impaired mucociliary clearance [1]. CF is caused by a mutation in the gene encoding the cystic fibrosis transmembrane regulator (CFTR) leading to a dysfunction of a chloride ion channel [2]. In the respiratory tract the mutations in the CFTR gene results in the thickening of bronchial mucus providing a growth environment for bacteria and fungi. Over time the recurrent infections and inflammation lead to a severely impaired lung function [1].

Pseudomonas aeruginosa is the most dominant bacterium found in CF lungs and colonizes

approximately 80 % of CF patients [3,4]. In general CF patients harbour between $1x10^7$ – $1x10^9$ CFU *P. aeruginosa*/g sputum [3], and as CF patients reach early childhood, most CF patients eventually become chronically infected by *P. aeruginosa* strains [2]. *P. aeruginosa* changes genetically during the course of chronic CF lung infections resulting in loss-of-function mutations and change in phenotypes [5,6]. These phenotypes can among others include loss of motility [7] and loss of pyoverdine production [8]. Mutations that inactivate the anti-sigma factor MucA result in overproduction of the exopolysaccharide alginate and conversion to the frequently observed mucoid phenotype [9]. Loss-of function mutations are also frequently found in *lasR*, which encodes a transcriptional regulator of quorum sensing [5,10]. Quorum sensing signaling molecules (3-oxo-C_{12}-HSL and C_4-HSL) produced by the bacteria have been detected in the sputum of CF patients [11]. Inactivation of LasR results in a decrease in expression of many virulence factors including pyocyanin [12–15]. Common mutations in the alternative sigma factor, RpoN, similarly affect many virulence factors including synthesis of pili, flagella, pyocyanin and rhamnolipids [16–19]. *P. aeruginosa* can additionally produce a large array of extracellular secondary metabolites including phenazines and quinolones [12,20,21]. 2-heptyl-3-hydroxy-4-quinolone (PQS) functions as a signaling molecule and has also been found *in vivo* in lungs of CF patients infected by *P. aeruginosa* [22]. *P. aeruginosa* PAO1 can produce at least four different phenazines; pyocyanin, 1-hydroxyphenazine, phenazine-1-carboxylic acid and phenazine-1-carboxamide [23]. Both phenazines and PQS have been demonstrated to have antifungal activity [21,24–26].

Aspergillus spp. are ubiquitously found in nature, and fungal spores are inhaled by most individuals every day. Healthy individuals can easily clear the fungi from their airways, but *Aspergillus* species can cause disease in immunocompromised hosts and individuals with underlying pulmonary diseases, where *Aspergillus fumigatus* is responsible for approximately 90% of human infections [27]. In patients with impaired lung functions, *Aspergillus* can cause allergic bronchopulmonary aspergillosis (ABPA), which is a hypersensitive response to the fungi. *A. fumigatus*, frequently found in house dust [28], is the most common isolated filamentous fungus in CF patients, however the prevalence varies between sites and centres. In Denmark *A. fumigatus* was found in 37% (2007) and 33% (2009) of CF patients [29], in France 57% [30], in Germany 6% [3], 46% [4] and 58% [31] and in USA 25% [32] and 36% [33] of CF patients. Many CF patients harbour several *A.*

fumigatus genotypes in their lungs [34–36], with some strains being chronically present, while others are found more transiently [36]. Colonization by *A. fumigatus* is uncommon in young CF patients and is normally a secondary infection following bacterial infections [37]. The prevalence of *A. fumigatus* infections increases with age [31], and *A. fumigatus* is the most persistent organism to colonize CF patients next to *P. aeruginosa* [3]. Bakare *et al.* found that 64% of *A. fumigatus* positive patients also had *P. aeruginosa* infections [4], and patients with *A. fumigatus* more frequently had *P. aeruginosa* [30,38]. *A. fumigatus* can produce several different secondary metabolites including fumiquinazolines, fumigaclavines, fumagillin, helvolic acid, pseurotins and gliotoxin [39]. The latter is the most important virulence factor and has been found in human lung tissue [40]. It has been shown to significantly lower the ciliary beat frequency and damage human respiratory epithelium *in vitro* [41]. Other *Aspergillus* species found in cystic fibrosis patients are *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus versicolor* and *Aspergillus terreus* [29,30,37,42]. Most are possibly only found transiently, but *A. terreus* seems to be able to chronically colonize the airways of CF patients [37].

Leclair and Hogan [43] have recently provided an excellent overview of current knowledge on mixed bacterial-fungal infections in CF patients, but despite frequent isolations of both *Aspergillus* and *P. aeruginosa* from CF patients [4], few studies have focused on a possible interaction between the species in mixed populations [43–47]. An interaction between *Aspergillus* and *P. aeruginosa* in CF patients may lead to changes in behaviour of one of the organisms, changes in secondary metabolite profiles, increased inflammatory response and worsening of underlying pulmonary disease. As a result of genetic adaption to the CF environment mutational changes in *P. aeruginosa* may alter the interactions between *A. fumigatus* and *P. aeruginosa* during the course of a CF infection. The aim of this study was to examine the interactions between *P. aeruginosa* and different Aspergilli, especially *A. fumigatus*, using morphological studies and chemical analysis for secondary metabolites.

Results

Development of an assay to investigate *Aspergillus* and *Pseudomonas* interactions.

In order to find an appropriate medium that supports balanced growth of both *Aspergillus* and *Pseudomonas*, three different media; Luria broth (LB) [48], Yeast Extract Sucrose agar

(YES) [49] and Wickerhams Antibiotic Test Medium (WATM) [50], were tested. LB is a bacterial nutritionally rich medium, which supported the growth of *Pseudomonas* well, while the growth of *Aspergillus* was restricted due to very slow growth compared to *Pseudomonas.* YES and WATM are fungal nutritionally rich media. YES supported growth of both *Aspergillus* and *Pseudomonas,* however, WATM showed the most equal growth of *Aspergillus* and *Pseudomonas* (data not shown) and was chosen as standard medium.

To investigate possible interactions between *Aspergillus* and *Pseudomonas aeruginosa* a standard plating assay was developed (figure 1 and Materials & Methods). *Aspergillus* was streaked on the left side of the WATM plate, while *P. aeruginosa* was streaked on the right allowing the organisms to initially colonize separately. After five days incubation the plates were examined and divided into four zones; two zones in the *Aspergillus* and two zones in *P. aeruginosa*, allowing comparison and examination of the interactions and secondary metabolite production.

Levels of phenazines are increased by *P. aeruginosa* in response to some Aspergilli, but not *A. fumigatus*

P. aeruginosa and different *Aspergillus* species commonly infect cystic fibrosis patients [4,30,37]. In order to investigate the interaction between *P. aeruginosa* and Aspergilli, we used the standard plating method (figure 1 and Materials & Methods) and HPLC methods (UV/Vis and MS detection) to examine the production of secondary metabolites. Our results show that *P. aeruginosa* PAO1 suppressed the growth of all tested Aspergilli including; *A. fumigatus, A. niger, A. oryzae* and *A. flavus* (figure 2A). For *A. oryzae* and *A. flavus*, *P. aeruginosa* produced a greenish compound (presumably pyocyanin) in the contact zone to the Aspergilli. An increase in pyocyanin production was measured by HPLC in *P. aeruginosa* in the contact zone of *A. oryzae* and *A. flavus* (data not shown). No coloration was observed for *P. aeruginosa* in the contact zone of *A. fumigatus* or *A. niger*, nor was the production of pyocyanin increased. *P. aeruginosa* seemed growth restricted in the contact zone with *A. niger*. This may be a result of citric acid produced by *A. niger*, previously shown to inhibit *P. aeruginosa* [51]. HPLC analysis revealed that *P. aeruginosa* increased production of two additional phenazines in the contact zone (zone 3, figure 2B) of *A. oryzae* and *A. flavus* compared to zone 4, a single phenazine in response to *A. niger*, while *A. fumigatus* did not seem to affect *P. aeruginosa* (figure 2B). All analyses were done as

biological duplicates. The *P. aeruginosa* production of phenazine-1-carboxamide in zone 3 was increased three and six times in response to *A. oryzae* ($P<0.1$) compared to levels of phenazine-1-carboxamide found in *P. aeruginosa* alone (control). The phenazine-1-carboxylic acid production was only slightly increased. *P. aeruginosa* phenazine-1-carboxylic acid production in zone 3 was increased six and seven times in response to *A. niger* ($P<0.05$) and four and five times in response to *A. flavus* ($P<0.05$). Phenazine-1-carboxamide production was only slightly increased in response to *A. flavus* ($P<0.1$), but not to *A. niger*. Neither phenazine-1-carboxamide nor phenazine-1-carboxylic acid production was increased in response to *A. fumigatus*. For *A. niger, A. oryzae* and *A. flavus* several other unidentified compounds with similar UV-chromophores to the phenazines were observed in zone 3. These findings show that *P. aeruginosa* responds to the presence of some Aspergilli by increasing the production of phenazine compounds. Interestingly, it does not appear that the growth inhibitory effect of *P. aeruginosa* requires an increased production of phenazines, as growth of *A. fumigatus* was inhibited in a similar way as other Aspergilli. However, two commercially available phenazines; 1-hydroxyphenazine (MIC = 100 µg/ml) and pyocyanin (MIC > 150 µg/ml) could inhibit *A. fumigatus*. Surprisingly, no changes in the chemical profile of secondary metabolite levels were observed for any of the four Aspergilli, indicating that the Aspergilli produce the same metabolites and levels irrespectively of the presence of *P. aeruginosa* (data not shown).

Colonization of the airways of cystic fibrosis patients by *Aspergillus* usually follows bacterial infections [37], however few cases are seen were *Aspergillus* infections occur before *Pseudomonas* infections [52]. Furthermore, the occurrence of *Aspergillus* seems to be a risk factor for developing chronic *P. aeruginosa* infections [53]. Differences in the interaction between *P. aeruginosa* and *Aspergillus* may therefore be observed depending on which organism colonizes first. However, no major differences was observed macroscopically or in chemical profile between plates having differential inoculation times (*Pseudomonas* first, *Pseudomonas* and *Aspergillus* simultaneously or *Aspergillus* first) (data not shown).

To further examine the growth suppressing effect of *P. aeruginosa* on the Aspergilli, five day old WATM plates containing *A. fumigatus* or *A. flavus* and *P. aeruginosa* were moved to 60 °C for 1 hour and subsequently moved back to 37 °C (supplementary figure S1). This heat-treatment kills *P. aeruginosa*, but not the *Aspergillus* and should further inactivate

potential enzymatic activity. *A. fumigatus* completely overgrew heat-killed *P. aeruginosa* showing that the presence of living *P. aeruginosa* is necessary for suppression of *A. fumigatus*. This was further confirmed by HPLC with both UV/Vis and MS detection, which did not show any changes in secondary metabolite profile after switching the temperature from 37 °C to 60 °C (supplementary figure S2). Similar observations were observed for *A. flavus* (supplementary figure S1). The green compound produced by *P. aeruginosa* in response to the presence of *A. flavus* could no longer be seen, however *A. flavus* could only grow over heat-killed *P. aeruginosa* to a slight extent.

A *P. aeruginosa rpoN* mutant cannot suppress growth of *A. fumigatus*

The development of chronic CF lung infections is associated with genetic adaptation and evolution of the infecting *P. aeruginosa* strains [5,6], and several genes have repeatedly been found to be inactivated by mutation in *P. aeruginosa* isolates sampled from different CF patients and clinical settings [5,6]. Examples of such genes include the *lasR*, *rpoN* and *mucA* genes which all encode proteins with regulatory functions. To examine the effect of these frequently occurring regulatory mutations on the *P. aeruginosa–Aspergillus* interaction, we analyzed the interaction between *P. aeruginosa* knock-out mutants and *A. fumigatus* AF293 using both morphological and chemical analysis.

We found that both the *lasR* mutant strain and *mucA* mutant strain suppressed growth of *A. fumigatus* (figure 3). However, the *mucA* strain was slightly overgrown by *A. fumigatus* in the interaction zone after five days incubation, increasing slightly over time. The *mucA* mutant had a similar metabolite profile compared to PAO1, while phenazine-1-carboxamide and phenazine-1-carboxylic acid was reduced in the *lasR* mutant (supplementary figure S3). Interestingly, a mutant defective in the alternative sigma factor RpoN suppressed growth of *A. fumigatus* after three days incubation (supplementary figure S4), but already after 4-6 days of growth the *rpoN* mutant started to be overgrown by *A. fumigatus* resulting in the *rpoN* mutant being nearly completely overgrown after nine days. It seemed that *A. fumigatus* preferred growing on top of the *rpoN* mutant rather than in between the streaks as seen in other mutants tested (figure 3). Examination of the secondary metabolite profile of the *rpoN* mutant showed that the *rpoN* mutant did not produce several quinolones and phenazines including pyocyanin (supplementary figure S3). A double mutant (*rpoN/mucA*) was introduced next, and this mutant was completely overgrown by *A. fumigatus* after seven days

of incubation resulting in the mucoid *P. aeruginosa* forming the base of a lawn of *A. fumigatus* (data not shown).

In addition to pyocyanin, the alternative sigma factor RpoN controls production of multiple virulence factors including pili, flagella, and rhamnolipids [16–19]. We next examined the individual contribution of these factors to the growth inhibition of *A. fumigatus*. Similarly to PAO1, motility mutants lacking either type IV pili (*pilA* mutant) or the polar flagellum (*fliM* mutant) were able to suppress or partly suppress *A. fumigatus* after five days incubation indicating that lack of motility does not seem to change the interaction between *P. aeruginosa* and *A. fumigatus* (figure 3). Long-term incubation (13 days) resulted in *A. fumigatus* growing in between the *P. aeruginosa pilA* mutant streaks and turning a darker colour. No change was observed on the *fliM* mutant plates over time (supplementary figure S4). Secondary metabolite profiles of the *pilA* mutant and *fliM* mutant resembled PAO1 showing that the mutations had no affect on secondary metabolite profiles (supplementary figure S5).

Rhamnolipids are involved in surface motility, immune modulation and virulence [54]. *rhlA* is involved in rhamnolipid synthesis [55], however, the *rhlA* mutant strain still suppressed *A. fumigatus* after five days of incubation (figure 3). During long-term incubation *A. fumigatus* grew in between the *rhlA* mutant strain streaks, but did not grow over it (supplementary figure S4). As expected, chemical analysis showed that the *rhlA* mutant did not produce rhamnolipid (data not shown).

To acquire Fe^{3+} ions from the environment, *P. aeruginosa* utilizes siderophores, called pyoverdines, encoded by the gene *pvdA* [56]. We used a *P. aeruginosa pvdA* mutant to examine if growth inhibition of *A. fumigatus* was related to competition for iron. However, the *P. aeruginosa pvdA* mutant similar to PAO1 suppressed growth of *A. fumigatus*. No differences in secondary metabolite profile were observed for the *pvdA* mutant compared to PAO1 (supplementary figure S5). It would be expected to see pyoverdin lacking in the secondary metabolite profile of the *pvdA* mutant, but pyoverdin was not detected in PAO1, which may be due to the choice of medium. Production of pyoverdin by *P. aeruginosa* may require use of an iron-free medium, which is often the case for siderophores.

A *P. aeruginosa rpoN* mutant suppresses growth of other Aspergilli and increases production of phenazines

To further examine the effect of frequently occurring regulatory mutations on the *P. aeruginosa–Aspergillus* interaction, we included the Aspergilli; *A. niger, A. oryzae* and *A. flavus* and examined the interaction between them and a number of *P. aeruginosa* knock-out mutants using both morphological and chemical analysis. All analyses were done in duplicate.

Our data so far demonstrated that a mutant defective in the alternative sigma factor RpoN could not suppress the growth of *A. fumigatus*, but was overgrown by the fungus. Unexpectedly, neither *A. niger, A. oryzae* nor *A. flavus* could overgrow the *rpoN* mutant (figure 4A). Furthermore, chemical analysis revealed that several phenazines and quinolones were increased in response to the three Aspergilli (figure 4C, data not shown). These included among others phenazine-1-carboxamide, phenazine-1-carboxylic acid, 2-n-Heptyl-4-oxy-quinoline/2-n-Heptyl-(1H)-quinolin-4-one and 2-Heptyl-3-hydroxy-4(3H)-quinolinone. As no increase in phenazine production was detected against *A. fumigatus* (figure 4C), these data suggest that *P. aeruginosa* responds differentially toward different Aspergilli, and that the increased phenazine production observed for co-culture with *A. niger, A. oryzae* or *A. flavus* is independent of RpoN.

The *lasR* mutant strain was able to suppress growth of *A. fumigatus* and similarly suppressed growth of *A. oryzae*, *A. niger* and *A. flavus* (figure 4A). In the contact zone of the *P. aeruginosa lasR* mutant and *A. oryzae/A. flavus* a large accumulation of a greenish compound was observed. This had similarly been observed in the contact zone of *P. aeruginosa* PAO1 and *A. flavus/A. oryzae*, as described above, however the *lasR* mutant seemed to produce larger amounts of the compound, found to be pyocyanin. Chemical analysis revealed an increase in production of all four phenazines, including pyocyanin, 1-hydroxyphenazine, phenazine-1-carboxamide and phenazine-1-carboxylic acid (figure 4B and 4D) by *P. aeruginosa lasR* towards *A. oryzae* and *A. flavus*. For *A. niger* an increase in phenazine-1-carboxylic acid was observed (data not shown), while a minimal increase in phenazine-1-carboxylic acid was observed in response to *A. fumigatus.* No differences in quinolone production were observed.

Finally, we tested the *mucA* mutant (figure 4A) and the remaining mutants (*fliM, pilA, pvdA* and *rhlA*). All mutants could suppress growth of *A. oryzae, A. niger* and *A. flavus*

(supplementary figure S6) as previously observed in *A. fumigatus* AF293.

Clinical and environmental *A. fumigatus* strains interact similarly with *P. aeruginosa*

As *A. fumigatus* is the most common *Aspergillus* species infecting cystic fibrosis patients [27], further understanding of the interaction between different strains of *A. fumigatus* and *P. aeruginosa* is needed. Isolates of *A. fumigatus* can produce many different secondary metabolites and a variation in secondary metabolite profiles between strains has been observed [39]. To elucidate differences in the interaction between *P. aeruginosa* and *A. fumigatus* species, six clinical and four environmental *A. fumigatus* strains were tested against *P. aeruginosa* PAO1 and secondary metabolite profiles were examined using UHPLC methods.

All *A. fumigatus* strains were inhibited by *P. aeruginosa* (figure 5A), nevertheless *A. fumigatus* conidium heads and conidiophores appeared normal. All *A. fumigatus* strains had normal round spores, except TUBF-32, which displayed elliptical spores slightly bigger than the rest.

Strains AF41, A37941, CBS 144.89 and NRRL1979 triggered the production of a green compound by *P. aeruginosa* in the contact zone (figure 5A). AF250 triggered the production of a yellow compound, while the remaining strains did not trigger any visual response in *P. aeruginosa.* Five strains (AF293, ATCC 201531, TUBF-32, AFIR 974 and TUBF-440) were very dusty (2-3 mm spores almost floating on top of the culture), while the remaining strains were less. It seemed that the less dusty strains triggered the production of the green compound in *P. aeruginosa,* while the very dusty strains did not (figure 5A and table 1). Examination of *A. fumigatus* AF293 and *P. aeruginosa* PAO1 by Scanning Electron Microscopy (SEM) (figure 5B) revealed that the organisms grew towards each other, but only had a very small interaction zone, where both organisms were present. The density of *A. fumigatus* conidia heads decreased towards *P. aeruginosa,* while *P. aeruginosa* had a rather sharp edge towards *A. fumigatus* as the fungus became denser. In the interaction zone the few *A. fumigatus* conidia heads appeared normal (data not shown). All *A. fumigatus* strains were examined for differences in secondary metabolite profiles and as expected a variation in secondary metabolite profile was observed between strains (see table 1). Several metabolite classes were detected, where fumiquinazolins, fumagillins and sterols were detected in all strains. Furthermore, fumigaclavins (90%), pseurotins (60%), trypacidins

(70%), helvolic acid (90%), pyripyropenes (90%), fumitremorgins (70%) and tryptoquivalins (90%) were detected. Gliotoxin has previously been found in the lungs of CF patients [40] and four clinical strains and two environmental strains were found to produce gliotoxin (table 1). The environmental strain TUBF-440 displayed the highest amount of gliotoxin of 0.65 µg/cm^2, while the remaining strains produced levels varying from 0.04 – 0.19 µg/cm^2. Differences in secondary metabolite profile did not correlate with the source of the strain (clinical vs. environmental), interaction zone colour (green vs. no colour) nor spore appearance (dusty). This was further confirmed by principal component analysis of the UHPLC-TOFMS data, where no clear groupings were observed.

As described above, *P. aeruginosa* PAO1 increased production of two phenazines in the contact zone of *A. oryzae* and *A. flavus*, a single phenazine in response to *A. niger*, while *A. fumigatus* did not trigger an increase in phenazine production. Similarly, no difference in the production of phenazines by *P. aeruginosa* was observed in the contact zone with any of the *A. fumigatus* strains (data not shown), indicating that *A. fumigatus* strains do not trigger the production of phenazines by *P. aeruginosa*. Furthermore, no *A. fumigatus* strain affected production of 2-heptyl-3-hydroxy-4(3H)-quinolone in *P. aeruginosa* PAO1 (data not shown). We also observed that *A. fumigatus* AF293 grew over the *P. aeruginosa* mutant strain *rpoN*. Similarly, all strains of *A. fumigatus* tested here were able to grow over the *rpoN* mutant (data not shown).

Discussion

We have developed an assay allowing the examination of Aspergilli-*Pseudomonas* interactions. This assay is based upon solid medium, which of course differs from the environment found in the lungs. Liquid culture may have been a more accurate assay, but again this differs from the environment in the lungs, which do not contain large amounts of liquid. Our assay supports growth of both organisms and allows examinations of the interactions in different zones resulting in a unique opportunity to study the effect of the two organisms on each other.

We tested the interaction between *P. aeruginosa* and several *Aspergillus* species demonstrating that growth of all tested Aspergilli (*A. fumigatus*, *A. niger*, *A. oryzae* and *A. flavus*) were suppressed by *P. aeruginosa*. Previous studies have tested the interaction of *P.*

aeruginosa and Aspergilli showing varying results. Kerr demonstrated a partially or non-inhibitory effect of *A. fumigatus* by *P. aeruginosa* strains [45], Mangan demonstrated an inhibitory effect of *P. aeruginosa* on *A. fumigatus* and *A. terreus* in broth culture [46], while Blyth and Forey showed an inhibitory effect of *A. fumigatus* by *P. aeruginosa* on medium plates [44]. *P. aeruginosa* has likewise been shown to inhibit other fungal species, including *Cryptococcus* species [26], *Trichopyton* species [57], *Saccharomyces cerevisiae* [45] and several *Candida* species [45,58]. Hogan *et al.* [59] demonstrated that *P. aeruginosa* formed a dense biofilm on *Candida albicans* filaments and subsequently killed the fungus. In this study, a close spatial relationship between *A. fumigatus* AF293 and *P. aeruginosa* PAO1 was found, while SEM revealed that the organisms only had a very small interaction zone, where both organisms were present. In agreement with our observations, Blyth [47] likewise found a close spatial relationship between *A. fumigatus* and *P. aeruginosa* showing that most bacteria were adherent to fungal hyphae. However, the presence of *P. aeruginosa* affected ultrastructures of *A. fumigatus* hyphae [47]. Mowat *et al.* showed that *P. aeruginosa* cells were distributed throughout the filamentous network in mature *A. fumigatus* biofilms and that a minimal effect was seen on mature *A. fumigatus* biomass, when exposed to live *P. aeruginosa* cells [60]. Therefore it seems that *P. aeruginosa* and *A. fumigatus* can have a close spatial relationship, when coming in contact, but preferably occupy their own area. Similarly, it seems that other Aspergilli, although probably only found transiently [37], preferably occupy their own area.

We observed an increase in phenazine production by *P. aeruginosa* in the contact zone of several *Aspergillus* species. Both phenazine-1-carboxylic acid and phenazine-1-carboxamide were increased by *P. aeruginosa* in response to *A. flavus* and *A. oryzae*, while only phenazine-1-carboxylic acid was increased in response to *A. niger*. This increase in production was consistent regardless of which organism (*Aspergillus* or *Pseudomonas*) had been allowed to colonize first. Both phenazine-1-carboxylic acid and phenazine-1-carboxamide are known antifungal compounds [21], and the increase of the phenazines is a response from *P. aeruginosa* to the presence of the Aspergilli. The increase did, however, not prevent the Aspergilli from growing as close to the *Pseudomonas* as possible, indicating that the phenazines may both be a signal to the Aspergilli of the existence of the *Pseudomonas* and an attempt to inhibit the fungi. Despite the Aspergilli being suppressed by *P. aeruginosa*, no changes in *Aspergillus* secondary metabolite profile could be detected in

any of the Aspergilli. Phenazine-1-carboxylic acid is a precursor to the three other phenazines (phenazine-1-carboxamide, 1-hydroxyphenazine and pyocyanin) [23] and Kerr *et al*. demonstrated that pyocyanin and 1-hydroxyphenazine can inhibit *A. fumigatus* [24]. Similarly, we observed that pure pyocyanin and 1-hydroxyphenazine could inhibit *A. fumigatus*. Interestingly, *A. fumigatus* strains did not trigger any increase of phenazines by *P. aeruginosa*, nor were any differences in *A. fumigatus* secondary metabolites observed, resulting in no detectable secondary metabolic effect on either organism. Even though it does not seem that the two organisms affect each other, it has been shown that there is a significant decrease in lung function in CF patients colonized with both organisms compared to patients colonized with only *A. fumigatus* or *P. aeruginosa,* respectively [38,61]. Similarly, it has been observed that CF patients colonized with both *C. albicans* and *Aspergillus* or *Pseudomonas*, *C. albicans* and *Aspergillus* have decreased lung functions and body mass index [62]. Increased lung damage has also been observed by the presence of oropharyngeal bacteria (e.g. streptococci and staphylococci) in the CF lung, which resulted in increased *P. aeruginosa* virulence due to an up regulation of several virulence genes [63].

Several studies indicate that phenazines seem to play a role in antagonistic interactions among fungi. Phenazine-1-carboxylate secreted by *Pseudomonas fluorescens* suppresses *Gaeumannomyces graminis* [64], while phenazine-1-carboxamide produced by *Pseudomonas chlorophis* and *P. aeruginosa* can suppress *Fusarium oxysporum* and *Pythium splendens* [65,66]. Similarly, Rella *et al.* [26] demonstrated that *P. aeruginosa* inhibited growth of *Cryptococcus* species by producing pyocyanin and PQS (2-heptyl-3,4-dihydroxyquinolone). Gibson *et al.* [25] described the accumulation of a red pigment in *Candida albicans* cells and subsequently killing of the cells, when cocultured with *P. aeruginosa.* The formation of red pigment required 5-methyl-phenazinium-1-carboxylate (5MPCA) produced by *P. aeruginosa*. Morales *et al.* [67] demonstrated that phenazine methosulphate likewise killed *C. albicans* and induced accumulation of red pigment.

Both clinical and environmental strains of *A. fumigatus* have been shown to produce gliotoxin. Kupfahl *et al.* demonstrated that 98 % of tested clinical strains produced gliotoxin, while 96 % of tested environmental strains produced gliotoxin [68]. Gliotoxin at concentrations above 0.2 µg/ml have been shown to significantly lower the ciliary beat frequency. [41]. Furthermore, gliotoxin production has been shown to be enhanced during *in vitro* biofilm formation [69], and can damage human respiratory epithelium [41]. We tested

ten *A. fumigatus* strains and found gliotoxin in six out of ten strains. These strains may therefore be able to reduce the ciliary beat frequency and cause epithelium damage. Both fumagillin and helvolic acid have likewise been shown to impair the ciliary beat frequency, but higher concentrations are required compared to gliotoxin [41]. Fumagillins were produced by all tested *A. fumigatus* strains, while helvolic acid was produced by nine strains. Therefore the presence of *A. fumigatus* may likewise contribute to an enhanced pulmonary infection due to a further reduced ciliary beat frequency. Further reduction of the ciliary beat frequency will likewise delay the clearance of the inhaled conidia, possibly allowing an enhanced time frame for the *Aspergillus* and other organisms to colonize the CF lung. Fumagillins and fumiquinazolins are common secondary metabolites produced by *A. fumigatus* [39] and was found to be produced by all tested *A. fumigatus* strains. Whether these metabolites can be responsible for the lacking production of phenazines by *P. aeruginosa* towards *A. fumigatus* strains remains elusive, but is not likely, as these secondary metabolites do not diffuse very well. Other factors are probably involved.

Genetic adaption and evolution of *P. aeruginosa* is common during the course of chronic CF lung infections and several genes have been found to be inactivated by mutations in clinical *P. aeruginosa* isolates [5,6,9]. Common mutations include the *lasR*, *rpoN* and *mucA* genes which all encode proteins with regulatory functions. We analyzed the interaction between *P. aeruginosa* knock-out mutants and *Aspergillus* species and found differential inhibition patterns between the strains. *A. fumigatus* AF293 was able to partially grow over a mucoid *P. aeruginosa* strain, while *A. flavus, A. oryzae* and *A. niger* were suppressed. *mucA* mutants display a reduced pulmonary clearance in animal models and have increased alginate production [70]. Interestingly, *A. fumigatus* AF293 completely grew over a *P. aeruginosa rpoN* mutant, while *A. flavus, A. oryzae* and *A. niger* were suppressed. *rpoN*, an alternative sigma factor, is required for initial promoter recognition and consequently for transcription of a subset of genes by *P. aeruginosa* RNA polymerase [16,17]. Several virulence factors are affected in a *rpoN* mutant including the synthesis of pili, flagella, pyocyanin and rhamnolipids [16–19]. *rpoN* mutants are also less virulent [71] and form poorer biofilms [18,59] compared to wildtype strains. Individual contribution of motility, rhamnolipid and pyoverdin to the suppression of *Aspergillus* was examined, but did not seem to play a role as all Aspergilli were suppressed by these *P. aeruginosa* mutants. *A. fumigatus* could however grow in between the *pilA, rpoN* and *rhlA* strains over time. Both the *pilA* and *fliM* strains

have mutations in single motility genes, while the *rpoN* strain is affected in general motility. Consequently motility and mucoidy are factors necessary to suppress *A. fumigatus* sufficiently, demonstrated by the complete overgrowth of the double mutant (*rpoN/mucA*) by *A. fumigatus*. Therefore we examined the differential interaction between the *rpoN* mutant and the different Aspergilli by chemical analysis. Our data show that the *rpoN* mutant in mono-culture is defective in production of several quinolones and phenazines including pyocyanin compared to wild-type *P. aeruginosa*. We speculate that this reduced secondary metabolite production removes the inhibition of *A. fumigatus* and allows it to grow over the *P. aeruginosa rpoN* mutant. Interestingly, we found that production of several phenazines and quinolones were increased in the *rpoN* mutant in response to *A. flavus, A. oryzae* and *A. niger*, but not *A. fumigatus*. This finding suggests that the increased phenazine production observed for co-culture with *A. niger, A. oryzae* or *A. flavus* is independent of RpoN and that regulation of phenazine production most likely is multifactorial.

In contrast to the *mucA* and *rpoN* deletion strains, a *lasR* mutant suppressed growth of all tested Aspergilli including *A. fumigatus*. This supports findings by Mowat *et al.* [60], who previously demonstrated that two quorum sensing knockout strains (PAO1:Δ*LasR* and PAO1:Δ*LasI*) inhibited *A. fumigatus* biofilm formation. When comparing interactions between the *lasR* mutant and the Aspergilli, we observed that phenazines (including pyocyanin, 1-hydroxyphenazine, phenazine-1-carboxamide and phenazine-1-carboxylic acid) were increased in response to the presence of *A. oryzae, A. flavus* and *A. niger*, while no response was seen to the presence of *A. fumigatus.* In the contact zone of the *lasR* mutant and *A. oryzae/A. flavus* a large accumulation of a greenish compound was observed. This accumulation was higher in the *lasR* mutant compared to PAO1 and is likely pyocyanin. Cugini *et al.*[72] similarly observed a large enhancement of pyocyanin by a *lasR* mutant, when inoculated onto a *C. albicans* lawn. Finally, a double mutant of *mucA/rpoN* was completely overgrown by *A. fumigatus* AF293.

In conclusion *P. aeruginosa* interacted differently towards *A. fumigatus* than other Aspergilli including *A. niger, A. oryzae* and *A. flavus.* Furthermore, our results indicate that regulatory mutations frequently observed in *P. aeruginosa* during long-term CF infections change bacterial-fungal interactions as measured in defined culture conditions in the laboratory. Further studies are required to investigate if such genetically adapted *P. aeruginosa* strains are less competitive towards *A. fumigatus* in the CF airways, and if there are clinical effects

associated with particular mutations in *P. aeruginosa*.

Materials and methods

Strains and media

The *Aspergillus* strains were from the Center for Microbial Biotechnology (CMB), IBT culture collection and all Aspergillus used in this study are listed in table 2. *Pseudomonas aeruginosa* PAO1 [73] was used as the model organism in this study. *P. aeruginosa* strains with knock-out mutations in *lasR*, *rpoN* and *mucA* were constructed by allelic displacement in the PAO1 wild-type (S. Damkiær and L. Jelsbak, unpublished). Strains with knock-out mutations in *pilA* [74], *fliM* [74], *pvdA* [75], *rhlA* [76] have previously been described.
Wickerhams antibiotic test medium (WATM) [50] was used as standard media. Luria broth medium (LB) [48]) was used to examine gliotoxin production by *Aspergillus fumigatus* isolates. Yeast Extract Sucrose agar (YES) [49] was initially used to examine growth of *Aspergillus* and *Pseudomonas.*

Standard plating method

Wickerhams antibiotic test medium (WATM) [50] was chosen as solid media as both *Aspergillus* and *Pseudomonas aeruginosa* grew well on this media. *Aspergillus* spores were harvested in a suspension consisting of 0.5 % Tween80 and 0.5 % agar and diluted to $1x10^6$ spores/ml. The suspension was streaked on to sterile WATM plates as indicated on figure 1A and incubated over night at 37 °C. After 24 hours incubation a *P. aeruginosa* overnight culture was diluted to $1x10^8$ CFU/ml and streaked out four times perpendicular to the fungal streak. Control plates containing *P. aeruginosa* or *Aspergillus* alone were included. Plates were hereafter incubated at 37 °C for five days.

Definition of zones on standard plate

Four zones were defined on each plate, see figure 1B. Zone 1 was defined in the *Aspergillus* as far away from the *Pseudomonas* as possible. Zone 2 in the *Aspergillus* as close to *Pseudomonas* as possible. Zone 3 in the *Pseudomonas* as close to the *Aspergillus* as possible and zone 4 in the *Pseudomonas* as far from the *Aspergillus* as possible.

Standard method for chemical analysis of strains

Extracts were prepared by cutting four plugs of 6 mm from each zone using a cork drill. The plugs were transferred to 2 ml vials, 1 ml of methanol: dichloromethane:ethyl acetate (1:2:3 v/v/v) with 0.5 % formic acid was added and the vials placed in an ultrasonication bath for 60 min. The extract was transferred to new vials and evaporated to dryness. The residues were dissolved in 500 µl methanol and ultrasonicated for 10 min. Extracts were finally filtered through a PTFE 0.45 µm syringe filter into a new vial and used for analysis.

Analysis of *A. oryzae, A. niger, A. flavus* and *A. fumigatus* AF293.

Extracts were analysed by two methods: I) High performance liquid chromatography-diode array detection (HPLC-DAD) using an Agilent 1100 system equipped with 3 µm, 10 cm, 2mm ID Luna C_{18} column using a 15→100 % water-acetonitrile gradient as described in detail in Nielsen *et al.* [77]; and II) Ultra High performance liquid chromatography-diode array detection (UHPLC-DAD) on a Kinetex C_{18} column (150 x 2.1 mm, 2.6 µm; Phenomenex, Torrence, CA) using a linear water-acetonitril gradient starting from 15 % CH_3CH to 100 % over 7 min at a flow rate of 0.8 ml/min. Both water and CH_3CH were buffered with 50 ppm trifluoroacetic acid. HPLC-DAD-TOFMS confirmation was performed on an Agilent 1100 system using a Luna C_{18} column and coupled to a Micromass LCT oaTOF equipped with an electrospray source, also describe in details in Nielsen *et al.* [77].

Analysis of clinical and environmental isolates of *A. fumigatus*

Extracts were analyzed by UHPLC-DAD described above and UHPLC-qTOFMS on a Dionex RSLC Ultimate 3000 (Dionex, Sunnyvale, CA) equipped with diode-array detector and a Kinetex C_{18} column (100 x 2.1 mm, 2.6 µm; Phenomenex, Torrence CA). Separation was performed using a linear water-acetonitril (CH_3CH) gradient starting from 10 % CH_3CH to 100 % over 7 min at a flow rate of 0.4 ml/min. Both water and CH_3CH were buffered with 20 mM formid acid. The UHPLC was coupled to a maXis G3 quadrupole time of flight mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray (ESI) ion source. The instrument was operated in ESI^+ at a resolution of 40 000 FMWH and scanning m/z 100-1000. Data analysis for identification of microbial secondary metabolites is described in [39,77,78]. The instrument generally produces multi-charged ions above

molecular masses of 700-800 Da. For unbiased data analysis the data files were analyzed in random, molecular features detected, peaks aligned and compared using the Bruker Profile Analysis 2.0 software (Bruker Daltonics, Bremen, Germany).

Examination of *Pseudomonas* mutants

The "standard plating method" was used to examine the interaction between different *P. aeruginosa* mutants and *Aspergillus*. *P. aeruginosa* metabolites were extracted using "the standard method for chemical analysis" described above and analyzed by UHPLC-DAD and UHPLC-qTOFMS also described above.

Differences in infection order

To examine differences in secondary metabolite profiles, three sets of WATM plates were made. The first set was inoculated as described in "the standard plating method" with *Aspergillus* on day 1 and *Pseudomonas* on day 2. The second set was inoculated with both *Aspergillus* and *Pseudomonas* on day 1, while the third set was inoculated with *Pseudomonas* on day 1 and *Aspergillus* on day 2. Plates were subsequently incubated at 37 °C for five days and extracted using the "standard method for chemical analysis of strains" previously described. Analysis was performed by UHPLC-DAD using a Dionex RSLC Ultimate 3000 (Dionex, Sunnyvale, CA) equipped with diode-array detection as previously described.

Production of gliotoxin by *A. fumigatus* strains

Gliotoxin is produced at highest concentrations on media with low C/N ratio [39]. Therefore *A. fumigatus* strains were 3-point inoculated onto LB plates and incubated at 37 °C for nine days. Extracts were prepared by cutting three plugs of 6 mm from a single colony using a cork drill as previously described by Smedsgaard [78]. To compensate for extraction losses a series of agar plugs were transferred to 2 ml vials, and spiked with 10 µl gliotoxin from a dilution row of pure gliotoxin (Fluka) in acetonitril and left for 1 h to allow the gliotoxin to diffuse into the agar. Samples were then extracted as above. 1 ml of methanol: dichloromethane:ethyl acetate (1:2:3 v/v/v) with 0.5 % formic acid was added and the vials were placed in an ultrasonication bath for 60 min. The extract was transferred to new vials and evaporated to dryness. The residues were dissolved in 500 µl methanol and

ultrasonicated for 10 min. Extracts were finally filtered through a PFTE 0.45 µm syringe filter into a new vial and used for analysis. The gliotoxin content was quantitated by UHPLC-DAD as described above using 1/x weighted linear regression of the peak areas compared to the spiked plugs.

Pictures and Scanning Electron Microscopy

All pictures were taken using a standard 18 megapixel digital camera. Scanning Electron Microscopy (SEM) was performed using a FEI Nova 600 NanoSEM (FEI, Hillsboro, Oregon) operated at 3 kV. To minimize charging problems a thin layer of gold (~5 Å) was sputter deposited on the sample with an Anatech Hummer sputter coater (Anatech, Union City, California).

Author contributions

Conceived and designed experiments: BGJ LJ MHP IS JCF KFN. Constructed the *P. aeruginosa* mutants: SD. Performed the experiments: BGJ. Performed SEM: TP BGJ. Analyzed the data: BGJ JCF KFN. Wrote the paper: BGJ. Read and improved draft: BGJ LJ TP SD MHP IS JCF KFN.

Acknowledgments

We thank Dr. Liang Yang for discussion.

Reference list

1. Horre R, Symoens F, Delhaes L, Bouchara JP (2010) Fungal respiratory infections in cystic fibrosis: a growing problem. Med Mycol 48 Suppl 1: S1-S3.

2. Govan JR, Deretic V (1996) Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol Rev 60: 539-574.

3. Bauernfeind A, Bertele RM, Harms K, Horl G, Jungwirth R, Petermuller C, Przyklenk B, Weisslein-Pfister C (1987) Qualitative and quantitative microbiological analysis of sputa of 102 patients with cystic fibrosis. Infection 15: 270-277.

4. Bakare N, Rickerts V, Bargon J, Just-Nubling G (2003) Prevalence of *Aspergillus fumigatus* and other fungal species in the sputum of adult patients with cystic fibrosis. Mycoses 46: 19-23.

5. Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, Miller SI, Ramsey BW, Speert DP, Moskowitz SM, Burns JL, Kaul R, Olson MV (2006) Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc Natl Acad Sci U S A 103: 8487-8492.

6. Yang L, Jelsbak L, Marvig RL, Damkiaer S, Workman CT, Rau MH, Hansen SK, Folkesson A, Johansen HK, Ciofu O, Hoiby N, Sommer MO, Molin S (2011) Evolutionary dynamics of bacteria in a human host environment. Proc Natl Acad Sci U S A 108: 7481-7486.

7. Mahenthiralingam E, Campbell ME, Speert DP (1994) Nonmotility and phagocytic resistance of *Pseudomonas aeruginosa* isolates from chronically colonized patients with cystic fibrosis. Infect Immun 62: 596-605.

8. De VD, De CM, Cochez C, Jansen S, Tummler B, Meyer JM, Cornelis P (2001) Study of pyoverdine type and production by *Pseudomonas aeruginosa* isolated from cystic fibrosis patients: prevalence of type II pyoverdine isolates and accumulation of pyoverdine-negative mutations. Arch Microbiol 175: 384-388.

9. Hoiby N (1975) Prevalence of mucoid strains of *Pseudomonas aeruginosa* in bacteriological specimens from patients with cystic fibrosis and patients with other diseases. Acta Pathol Microbiol Scand Suppl 83: 549-552.

10. Hoffman LR, Kulasekara HD, Emerson J, Houston LS, Burns JL, Ramsey BW, Miller SI (2009) *Pseudomonas aeruginosa* lasR mutants are associated with cystic fibrosis lung disease progression. J Cyst Fibros 8: 66-70.

11. Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, Greenberg EP (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature 407: 762-764.

12. Pesci EC, Milbank JB, Pearson JP, McKnight S, Kende AS, Greenberg EP, Iglewski BH (1999) Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 96: 11229-11234.

13. Gambello MJ, Iglewski BH (1991) Cloning and characterization of the *Pseudomonas aeruginosa* lasR gene, a transcriptional activator of elastase expression. J Bacteriol 173: 3000-3009.

14. Juhas M, Eberl L, Tummler B (2005) Quorum sensing: the power of cooperation in the world of *Pseudomonas*. Environ Microbiol 7: 459-471.

15. D'Argenio DA, Wu M, Hoffman LR, Kulasekara HD, Deziel E, Smith EE, Nguyen H, Ernst RK, Larson Freeman TJ, Spencer DH, Brittnacher M, Hayden HS, Selgrade S, Klausen M, Goodlett DR, Burns JL, Ramsey BW, Miller SI (2007) Growth phenotypes of *Pseudomonas aeruginosa* lasR mutants adapted to the airways of cystic fibrosis patients. Mol Microbiol 64: 512-533.

16. Totten PA, Lara JC, Lory S (1990) The rpoN gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. J Bacteriol 172: 389-396.

17. Ishimoto KS, Lory S (1989) Formation of pilin in *Pseudomonas aeruginosa* requires the

alternative sigma factor (RpoN) of RNA polymerase. Proc Natl Acad Sci U S A 86: 1954-1957.

18. Thompson LS, Webb JS, Rice SA, Kjelleberg S (2003) The alternative sigma factor RpoN regulates the quorum sensing gene rhlI in *Pseudomonas aeruginosa*. FEMS Microbiol Lett 220: 187-195.

19. Heurlier K, Denervaud V, Pessi G, Reimmann C, Haas D (2003) Negative control of quorum sensing by RpoN (sigma54) in *Pseudomonas aeruginosa* PAO1. J Bacteriol 185: 2227-2235.

20. McGrath S, Wade DS, Pesci EC (2004) Dueling quorum sensing systems in *Pseudomonas aeruginosa* control the production of the Pseudomonas quinolone signal (PQS). FEMS Microbiol Lett 230: 27-34.

21. Gross H, Loper JE (2009) Genomics of secondary metabolite production by *Pseudomonas* spp. Nat Prod Rep 26: 1408-1446.

22. Collier DN, Anderson L, McKnight SL, Noah TL, Knowles M, Boucher R, Schwab U, Gilligan P, Pesci EC (2002) A bacterial cell to cell signal in the lungs of cystic fibrosis patients. FEMS Microbiol Lett 215: 41-46.

23. Mavrodi DV, Bonsall RF, Delaney SM, Soule MJ, Phillips G, Thomashow LS (2001) Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. J Bacteriol 183: 6454-6465.

24. Kerr JR, Taylor GW, Rutman A, Hoiby N, Cole PJ, Wilson R (1999) *Pseudomonas aeruginosa* pyocyanin and 1-hydroxyphenazine inhibit fungal growth. J Clin Pathol 52: 385-387.

25. Gibson J, Sood A, Hogan DA (2009) *Pseudomonas aeruginosa-Candida albicans* interactions: localization and fungal toxicity of a phenazine derivative. Appl Environ Microbiol 75: 504-513.

26. Rella A, Yang MW, Gruber J, Montagna MT, Luberto C, Zhang YM, Del PM (2011) *Pseudomonas aeruginosa* Inhibits the Growth of *Cryptococcus* Species. Mycopathologia Epub ahead of print.

27. Latge JP (1999) *Aspergillus fumigatus* and aspergillosis. Clin Microbiol Rev 12: 310-350.

28. Nielsen KF (2003) Mycotoxin production by indoor molds. Fungal Genetics and Biology 39: 103-117.

29. Mortensen KL, Jensen RH, Johansen HK, Skov M, Pressler T, Howard SJ, Leatherbarrow H, Mellado E, Arendrup MC (2011) *Aspergillus* species and other molds in respiratory samples from patients with cystic fibrosis: a laboratory-based study with focus on *Aspergillus fumigatus* azole resistance. J Clin Microbiol 49: 2243-2251.

30. Paugam A, Baixench MT, Demazes-Dufeu N, Burgel PR, Sauter E, Kanaan R, Dusser D, Dupouy-Camet J, Hubert D (2010) Characteristics and consequences of airway colonization by filamentous fungi in 201 adult patients with cystic fibrosis in France. Med Mycol 48: S32-S36.

31. Valenza G, Tappe D, Turnwald D, Frosch M, Konig C, Hebestreit H, Abele-Horn M (2008) Prevalence and antimicrobial susceptibility of microorganisms isolated from sputa of patients with cystic fibrosis. J Cyst Fibros 7: 123-127.

32. Mroueh S, Spock A (1994) Allergic bronchopulmonary aspergillosis in patients with cystic fibrosis. Chest 105: 32-36.

33. Sudfeld CR, Dasenbrook EC, Merz WG, Carroll KC, Boyle MP (2010) Prevalence and risk factors for recovery of filamentous fungi in individuals with cystic fibrosis. J Cyst Fibros 9: 110-116.

34. Neuveglise C, Sarfati J, Debeaupuis JP, Vu TH, Just J, Tournier G, Latge JP (1997) Longitudinal study of *Aspergillus fumigatus* strains isolated from cystic fibrosis patients. Eur J Clin Microbiol Infect Dis 16: 747-750.

35. de Valk HA, Klaassen CH, Yntema JB, Hebestreit A, Seidler M, Haase G, Muller FM, Meis JF (2009) Molecular typing and colonization patterns of *Aspergillus fumigatus* in patients with cystic fibrosis. J Cyst Fibros 8: 110-114.

36. Vanhee LM, Symoens F, Bouchara JP, Nelis HJ, Coenye T (2008) High-resolution genotyping of *Aspergillus fumigatus* isolates recovered from chronically colonised patients with cystic fibrosis. Eur J Clin Microbiol Infect Dis 27: 1005-1007.

37. Pihet M, Carrere J, Cimon B, Chabasse D, Delhaes L, Symoens F, Bouchara JP (2009) Occurrence and relevance of filamentous fungi in respiratory secretions of patients with cystic fibrosis--a review. Med Mycol 47: 387-397.

38. Amin R, Dupuis A, Aaron SD, Ratjen F (2010) The effect of chronic infection with *Aspergillus fumigatus* on lung function and hospitalization in patients with cystic fibrosis. Chest 137: 171-176.

39. Frisvad JC, Rank C, Nielsen KF, Larsen TO (2009) Metabolomics of *Aspergillus fumigatus*. Med Mycol 47: S53-S71.

40. Lewis RE, Wiederhold NP, Chi J, Han XY, Komanduri KV, Kontoyiannis DP, Prince RA (2005) Detection of gliotoxin in experimental and human aspergillosis. Infect Immun 73: 635-637.

41. Amitani R, Taylor G, Elezis EN, Llewellyn-Jones C, Mitchell J, Kuze F, Cole PJ, Wilson R (1995) Purification and characterization of factors produced by *Aspergillus fumigatus* which affect human ciliated respiratory epithelium. Infect Immun 63: 3266-3271.

42. Nelson LA, Callerame ML, Schwartz RH (1979) Aspergillosis and atopy in cystic fibrosis. Am Rev Respir Dis 120: 863-873.

43. Leclair LW, Hogan DA (2010) Mixed bacterial-fungal infections in the CF respiratory tract. Med Mycol 48 Suppl 1: S125-S132.

44. Blyth W, Forey A (1971) The influence of respiratory bacteria and their biochemical fractions on *Aspergillus fumigatus*. Sabouraudia 9: 273-282.

45. Kerr JR (1994) Suppression of fungal growth exhibited by *Pseudomonas aeruginosa*. J Clin

Microbiol 32: 525-527.

46. Mangan A (1969) Interactions between some aural aspergillus species and bacteria. J Gen Microbiol 58: 261-266.

47. Blyth W (1971) Modifications in the ultrastructure of *Aspergillus fumigatus* due to the presence of living cells of *Pseudomonas aeruginosa*. Sabouraudia 9: 283-286.

48. Bertani G (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J Bacteriol 62: 293-300.

49. Frisvad JC, Samson RA (2004) *Penicillium* subgenus *Penicillium* - A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. Stud Mycol 49: 1-173.

50. Raper, K. B. and Thom, C. (1949) A manual of the penicillia. Baltimore: Williams & Wilkens Co. 920 p.

51. Nagoba BS, Deshmukh SR, Wadher BJ, Mahabaleshwar L, Gandhi RC, Kulkarni PB, Mane VA, Deshmukh JS (1998) Treatment of superficial pseudomonal infections with citric acid: an effective and economical approach. J Hosp Infect 40: 155-157.

52. Symoens F, Haase G, Pihet M, Carrere J, Beguin H, Degand N, Mely L, Bouchara JP (2010) Unusual *Aspergillus* species in patients with cystic fibrosis. Med Mycol 48 Suppl 1: S10-S16.

53. Pressler T, Frederiksen B, Skov M, Garred P, Koch C, Hoiby N (2006) Early rise of anti-*pseudomonas* antibodies and a mucoid phenotype of *pseudomonas aeruginosa* are risk factors for development of chronic lung infection--a case control study. J Cyst Fibros 5: 9-15.

54. Abdel-Mawgoud AM, Lepine F, Deziel E (2010) Rhamnolipids: diversity of structures, microbial origins and roles. Appl Microbiol Biotechnol 86: 1323-1336.

55. Soberon-Chavez G, Aguirre-Ramirez M, Sanchez R (2005) The *Pseudomonas aeruginosa* RhlA enzyme is involved in rhamnolipid and polyhydroxyalkanoate production. J Ind Microbiol Biotechnol 32: 675-677.

56. Visca P, Ciervo A, Orsi N (1994) Cloning and nucleotide sequence of the pvdA gene encoding the pyoverdin biosynthetic enzyme L-ornithine N5-oxygenase in *Pseudomonas aeruginosa*. J Bacteriol 176: 1128-1140.

57. Treat J, James WD, Nachamkin I, Seykora JT (2007) Growth inhibition of *Trichophyton* species by *Pseudomonas aeruginosa*. Arch Dermatol 143: 61-64.

58. Bandara HM, Yau JY, Watt RM, Jin LJ, Samaranayake LP (2010) *Pseudomonas aeruginosa* inhibits in-vitro *Candida* biofilm development. BMC Microbiol 10: 125.

59. Hogan DA, Kolter R (2002) *Pseudomonas-Candida* interactions: an ecological role for virulence factors. Science 296: 2229-2232.

60. Mowat E, Rajendran R, Williams C, McCulloch E, Jones B, Lang S, Ramage G (2010) *Pseudomonas aeruginosa* and their small diffusible extracellular molecules inhibit

Aspergillus fumigatus biofilm formation. FEMS Microbiol Lett 313: 96-102.

61. Navarro J, Rainisio M, Harms HK, Hodson ME, Koch C, Mastella G, Strandvik B, McKenzie SG (2001) Factors associated with poor pulmonary function: cross-sectional analysis of data from the ERCF. European Epidemiologic Registry of Cystic Fibrosis. Eur Respir J 18: 298-305.

62. Chotirmall SH, O'Donoghue E, Bennett K, Gunaratnam C, O'Neill SJ, McElvaney NG (2010) Sputum *Candida albicans* presages FEV decline and hospital-treated exacerbations in cystic fibrosis. Chest 138: 1186-1195.

63. Duan K, Dammel C, Stein J, Rabin H, Surette MG (2003) Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. Mol Microbiol 50: 1477-1491.

64. Thomashow LS, Weller DM (1988) Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. J Bacteriol 170: 3499-3508.

65. Chin AWT, Bloemberg GV, Mulders IH, Dekkers LC, Lugtenberg BJ (2000) Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. Mol Plant-Microbe Interact 13: 1340-1345.

66. Anjaiah V, Koedam N, Nowak-Thompson B, Loper JE, Hofte M, Tambong JT, Cornelis P (1998) Involvement of phenazines and anthranilate in the antagonism of *Pseudomonas aeruginosa* PNA1 and Tn5 derivatives toward *Fusarium* spp. and *Pythium* spp. Mol Plant-Microbe Interact 11: 847-854.

67. Morales DK, Jacobs NJ, Rajamani S, Krishnamurthy M, Cubillos-Ruiz JR, Hogan DA (2010) Antifungal mechanisms by which a novel *Pseudomonas aeruginosa* phenazine toxin kills *Candida albicans* in biofilms. Mol Microbiol 78: 1379-1392.

68. Kupfahl C, Michalka A, Lass-Florl C, Fischer G, Haase G, Ruppert T, Geginat G, Hof H (2008) Gliotoxin production by clinical and environmental *Aspergillus fumigatus* strains. Int J Med Microbiol 298: 319-327.

69. Bruns S, Seidler M, Albrecht D, Salvenmoser S, Remme N, Hertweck C, Brakhage AA, Kniemeyer O, Muller FM (2010) Functional genomic profiling of *Aspergillus fumigatus* biofilm reveals enhanced production of the mycotoxin gliotoxin. Proteomics 10: 3097-3107.

70. Boucher JC, Yu H, Mudd MH, Deretic V (1997) Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: characterization of muc mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection. Infect Immun 65: 3838-3846.

71. Hendrickson EL, Plotnikova J, Mahajan-Miklos S, Rahme LG, Ausubel FM (2001) Differential roles of the *Pseudomonas aeruginosa* PA14 rpoN gene in pathogenicity in plants, nematodes, insects, and mice. J Bacteriol 183: 7126-7134.

72. Cugini C, Morales DK, Hogan DA (2010) *Candida albicans*-produced farnesol stimulates *Pseudomonas* quinolone signal production in LasR-defective *Pseudomonas aeruginosa* strains. Microbiology 156: 3096-3107.

73. Holloway BW, Morgan AF (1986) Genome organization in *Pseudomonas*. Annu Rev Microbiol 40: 79-105.

74. Klausen M, Heydorn A, Ragas P, Lambertsen L, Aaes-Jorgensen A, Molin S, Tolker-Nielsen T (2003) Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. Mol Microbiol 48: 1511-1524.

75. Yang L, Nilsson M, Gjermansen M, Givskov M, Tolker-Nielsen T (2009) Pyoverdine and PQS mediated subpopulation interactions involved in *Pseudomonas aeruginos*a biofilm formation. Mol Microbiol 74: 1380-1392.

76. Pamp SJ, Tolker-Nielsen T (2007) Multiple roles of biosurfactants in structural biofilm development by *Pseudomonas aeruginosa*. J Bacteriol 189: 2531-2539.

77. Nielsen KF, Mogensen JM, Johansen M, Larsen TO, Frisvad JC (2009) Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. Anal Bioanal Chem 395: 1225-1242.

78. Smedsgaard J (1997) Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. J Chromatogr A 760: 264-270.

Tables

Table 1. Comparison of clinical and environmental *A. fumigatus* strains

		WATM medium												LB medium
***A. fumigatus* strain**	**Clinical or Environmental strain**	**Color of *P. aeruginosa* in contact zone**	**Dusty (*)**	**Fumigaclavins**	**Pseurotins**	**Tryptoquivalins**	**Fumiquinazolins**	**Trypacidins**	**Fumagillins**	**Helvolic acid**	**Sterols**	**Pyripyropenes**	**Fumitremorgins**	**Gliotoxin production (ug/cm^2)**
AF293	Clinical	-	+	+	+	+	+	+	+	+	+	+	-	0.04
AF41	Clinical	Green	-	+	-	+	+	+	+	+	+	+	+	-
A37941	Clinical	Green	-	-	-	-	+	-	+	+	+	-	-	-
CBS 144.89	Clinical	Green	-	+	+	+	+	-	+	+	+	+	+	0.18
ATCC 201531	Clinical	-	+	+	+	+	+	+	+	+	+	+	+	0.19
AF250	Clinical	Yellow	-	+	+	+	+	+	+	+	+	+	+	0.04
NRRL1979	Environmental	Green	-	+	-	+	+	+	+	+	+	+	+	-
TUBF-32	Environmental	-	+	+	+	+	+	-	+	+	+	+	-	0.05
AFIR974	Environmental	-	+	+	-	+	+	+	+	+	+	+	+	-
TUBF-440	Environmental	-	+/-	+	+	+	+	+	+	-	+	+	+	0.65

Table 2. Fungal strains used in this study.

Strains	Description	Source or reference
Aspergillus oryzae RIB40	Full-genome sequenced, from cereal	CMB, IBT culture collection
Aspergillus niger NRRL3	Full-genome sequenced, USA	CMB, IBT culture collection
Aspergillus flavus NRRL3357	Full-genome sequenced, from maize	CMB, IBT culture collection
Aspergillus nidulans FGSC A4	Full-genome sequenced, Glasgow strain	CMB, IBT culture collection
Aspergillus terreus NIH2624	Full-genome sequenced, from patient, USA	CMB, IBT culture collection
Aspergillus fumigatus AF293	Isolated from Aspergilloma, UK	CMB, IBT culture collection
Aspergillus fumigatus AF41	Isolated from pericardial tissue, USA	CMB, IBT culture collection
Aspergillus fumigatus TUBF-32	Isolated from grapes, Hungary	CMB, IBT culture collection
Aspergillus fumigatus AFIR974	Isolated from air, Ireland	CMB, IBT culture collection
Aspergillus fumigatus TUBF-440	Isolated from soil, Portugal	CMB, IBT culture collection
Aspergillus fumigatus A37941	Isolated from patient, Denmark	CMB, IBT culture collection
Aspergillus fumigatus CBS 144.89	Isolated from patient with aspergillosis, France	CMB, IBT culture collection
Aspergillus fumigatus ATCC 201531	Isolated from pleural fluid, USA	CMB, IBT culture collection
Aspergillus fumigatus AF250	Isolated from Salford Hope Hospital, UK	CMB, IBT culture collection
Aspergillus fumigatus NRRL1979	Isolated from soil/compost, USA	CMB, IBT culture collection

Figures

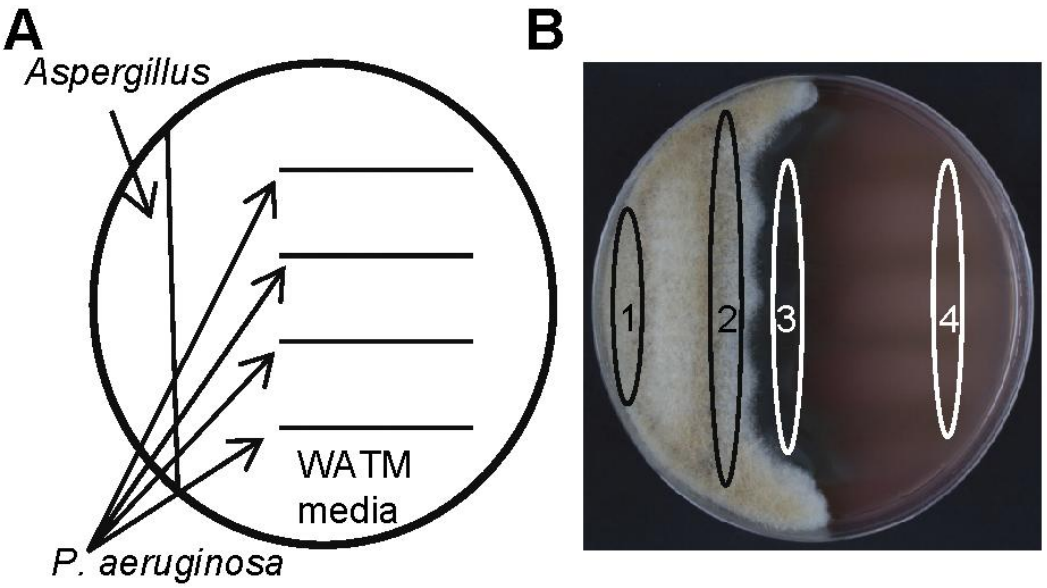


Figure 1. Standard plating method and zones. (A) The standard plating method was used throughout this study. *Aspergillus* was streaked on the left side of the plate, while *P. aeruginosa* was streaked four times perpendicular to the fungal streak on the right side. (B) The plate was divided into four zones, two zones in the *Aspergillus* and two zones in *Pseudomonas* allowing comparison of the interactions and secondary metabolite production different places on the plate.

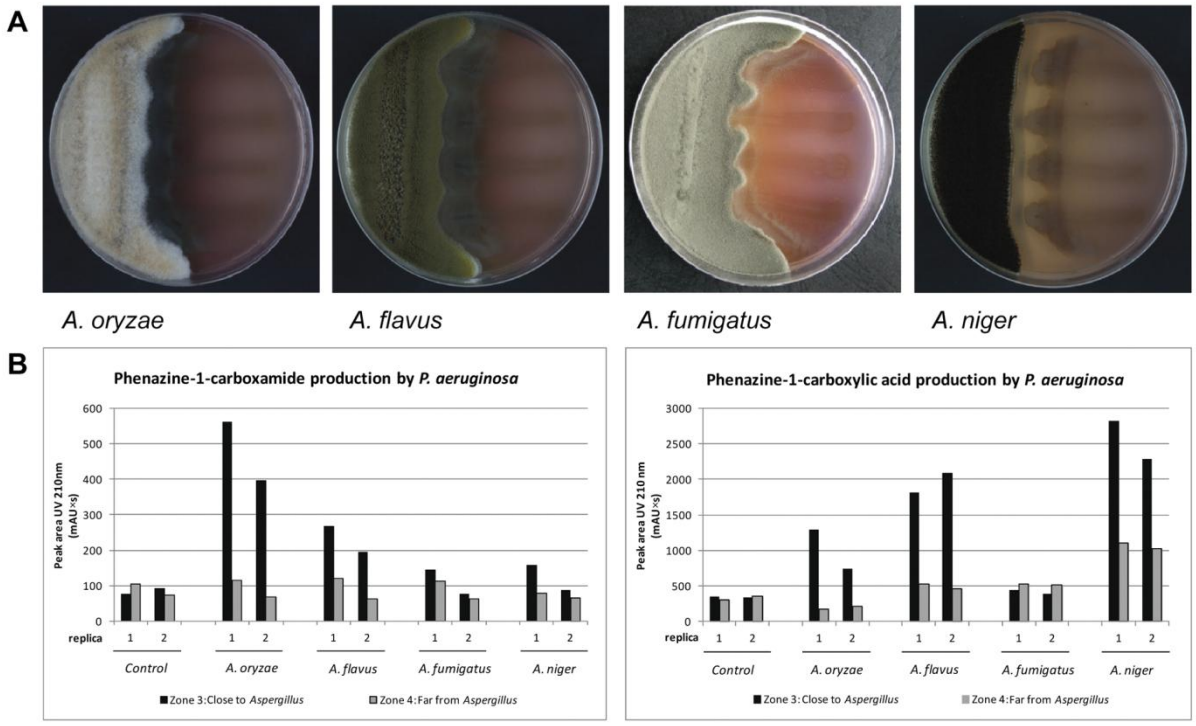


Figure 2. Phenazine production and suppression of *Aspergillus* growth by *P. aeruginosa*. (A) *P. aeruginosa* suppressed growth of all tested Aspergilli on WATM agar plates. Pictures were taken after five days of incubation at 37 °C. (B) Production of two phenazines (phenazine-1-carboxamid and phenazine-1-carboxylic acid) by *P. aeruginosa* was increased in the presence of *A. oryzae* and *A. flavus*, while phenazine-1-carboxylic acid was increased in the presence of *A. niger*. No increase was measured in response to *A. fumigatus*. Phenazines were measured in zone 3 and 4 by HPLC after five days of incubation and results from two independent biological replica experiments are shown. Control: *P. aeruginosa* only.

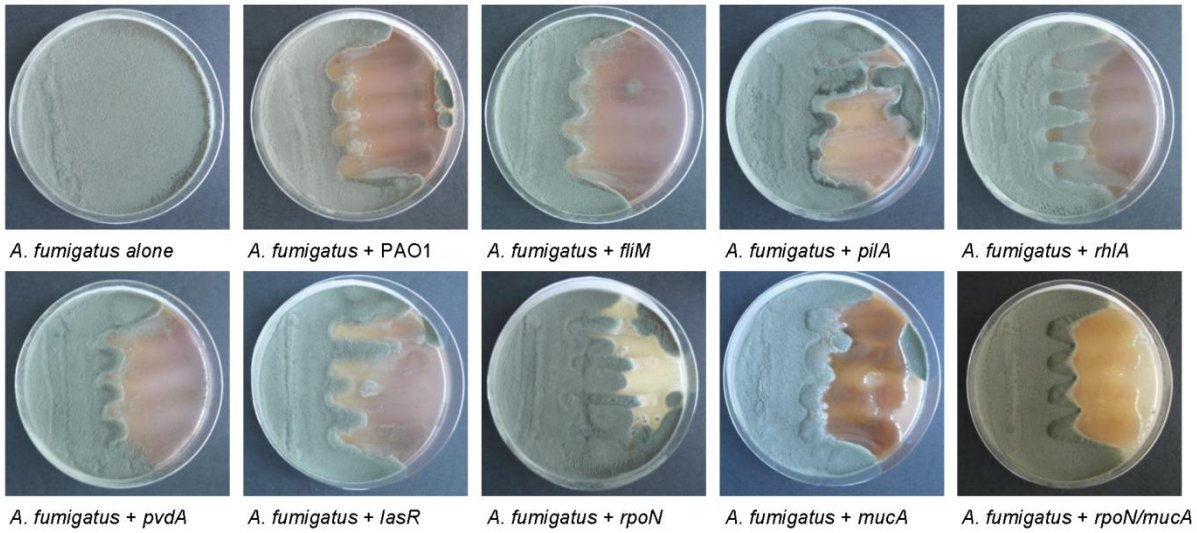


Figure 3. Interactions between *A. fumigatus* AF293 and different *P. aeruginosa* mutants. Eight *P. aeruginosa* mutants were tested against *A. fumigatus* AF293 using the standard plating method. Six mutants suppressed *A. fumigatus,* while the *rpoN*, *mucA* and *rpoN/mucA* mutants were unsuccessful. All pictures were taken in a LAF bench (after five days incubation at 37 °C) due to safety reasons, wherefore the colours of *A. fumigatus* AF293 varies due to insufficient photo setup.

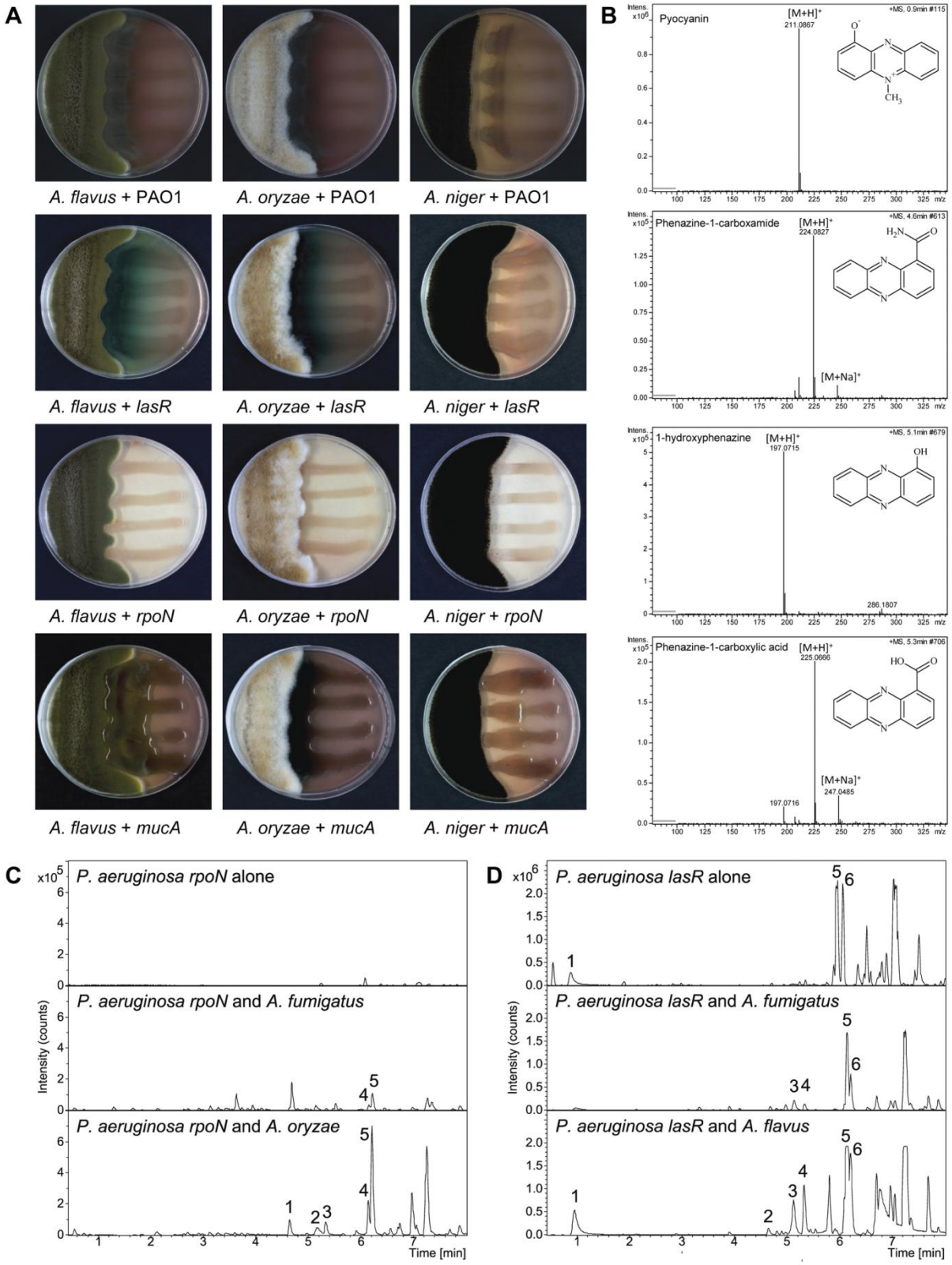


Figure 4. Interactions between *P. aeruginosa mucA, lasR and rpoN* and Aspergilli. The three *P. aeruginosa* mutants, *mucA*, *lasR* and *rpoN* were tested against *A. flavus, A. oryzae*

and *A. niger*. (A) All *P. aeruginosa* mutants could suppress the three Aspergilli. The *lasR* mutant triggered the production of a green compound, likely pyocyanin, by *P. aeruginosa* in response to *A. flavus* and *A. oryzae.* All plates were incubated at 37 °C for five days. (B) *P. aeruginosa* can produce four phenazines, namely pyocyanin, 1-hydroxyphenazine, phenazine-1-carboxylic acid and phenazine-1-carboxamide. (C) Comparison of the secondary metabolite profile of the *P. aeruginosa rpoN* mutant alone and together with *A. oryzae* in zone 3 revealed an increase in phenazines (1: phenazine-1-carboxamide, 2: 1-hydroxyphenazine and 3: phenazine-1-carboxylic acid) and several quinolones (4: 2-n-Heptyl-4-oxy-quinoline/2-n-Heptyl-(1H)-quinolin-4-one, 5: 2-Heptyl-3-hydroxy-4(3H)-quinolinone) by *P. aeruginosa* in response to *A. oryzae.* No phenazines could be detected in response to *A. fumigatus*, while quinolones were detected in lower amounts. All experiments were done in duplicate. (D) Comparison of the secondary metabolite profile of the *P. aeruginosa lasR* mutant alone and together with *A. flavus* in zone 3 revealed an increase in all four phenazines (1: pyocyanin, 2: phenazine-1-carboxamide, 3: 1-hydroxyphenazine and 4: phenazine-1-carboxylic acid) by *P. aeruginosa*. Phenazines could similar be detected in response to *A. fumigatus*, but at lower levels. Quinolones (5: 2-n-Heptyl-4-oxy-quinoline/2-n-Heptyl-(1H)-quinolin-4-one and 6: 2-Heptyl-3-hydroxy-4(3H)-quinolinone) were detected in all three cases. All experiments were done in duplicate.

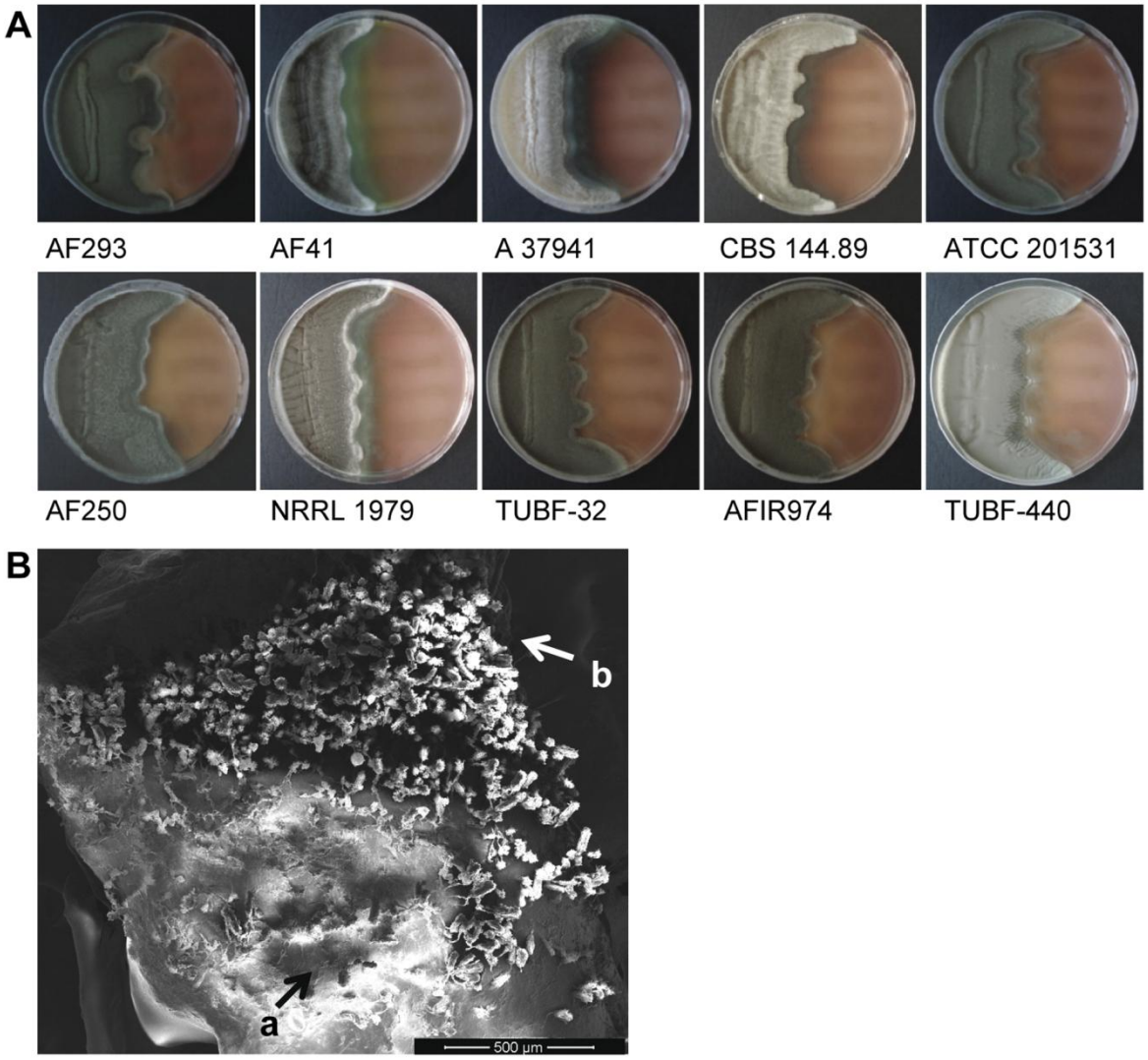


Figure 5. Interactions between *P. aeruginosa* PAO1 and different *A. fumigatus* isolates. (A) All *A. fumigatus* isolates were inhibited by *P. aeruginosa* after five days incubation at 37°C. (B) SEM analysis revealed a narrow close spacial interaction between *A. fumigatus* AF293 and *P. aeruginosa* PAO1 (a: *P. aeruginosa* (light area), b: *A. fumigatus*).

Supplementary figures

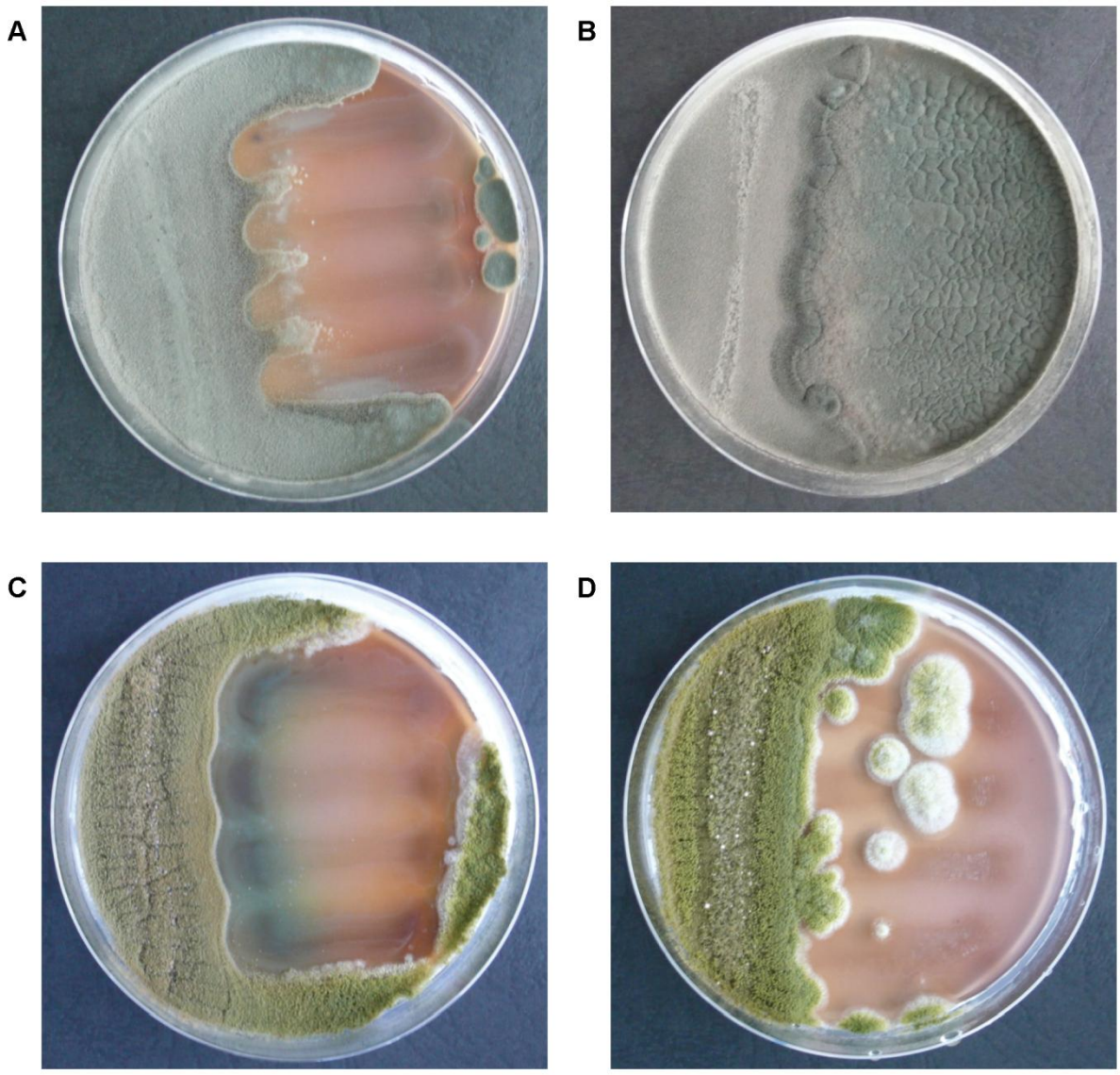


Figure S1. Effect of 60 °C on the interaction between *Aspergillus* and *P. aeruginosa*. (A) A. *fumigatus* was suppressed by *P. aeruginosa* after five days at 37 °C. (B) After 1 h incubation at 60 °C, followed by incubation at 37 °C, *A. fumigatus* completely grew over *P. aeruginosa*. (C) Similarly *Aspergillus flavus* was suppressed by *P. aeruginosa* after five days at 37 °C. (D) After 1 h incubation at 60 °C, followed by incubation at 37 °C, *A. flavus* was able to grow over *P. aeruginosa*.

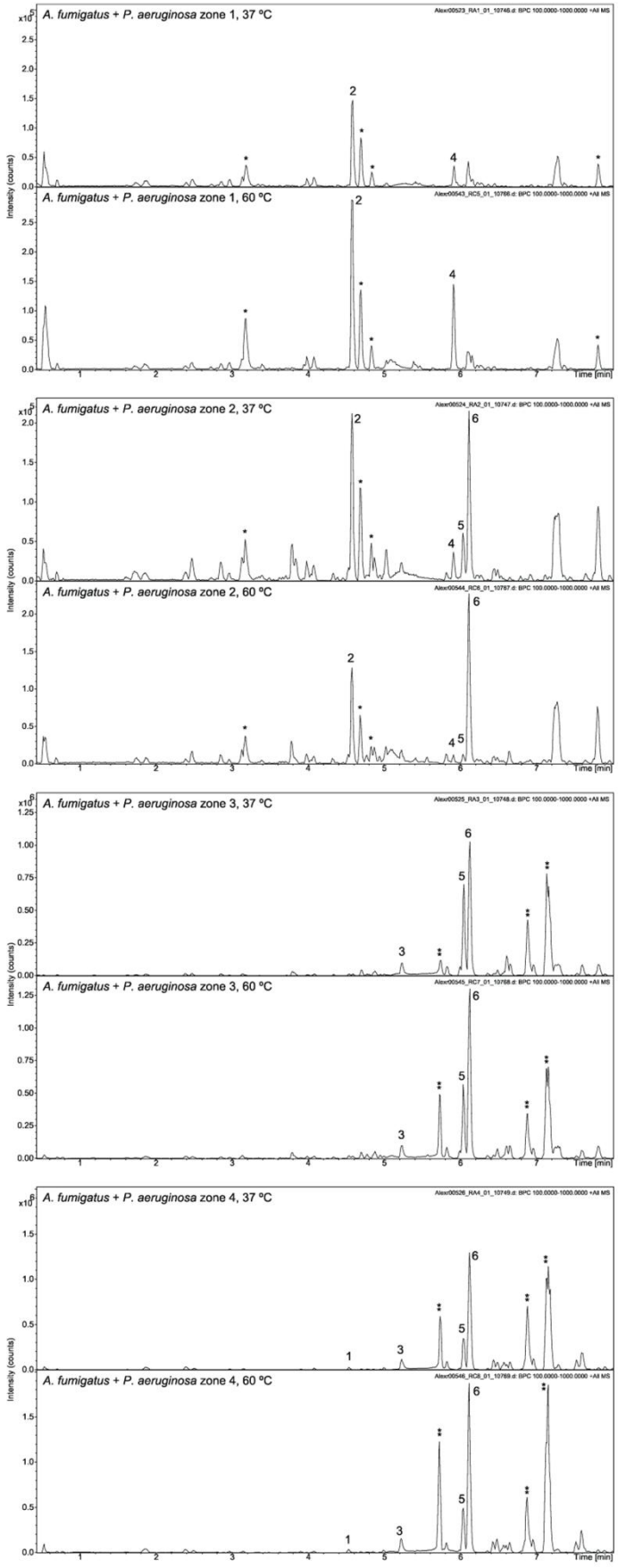

A. fumigatus + P. aeruginosa zone 1, 37 °C
A. fumigatus + P. aeruginosa zone 1, 60 °C
A. fumigatus + P. aeruginosa zone 2, 37 °C
A. fumigatus + P. aeruginosa zone 2, 60 °C
A. fumigatus + P. aeruginosa zone 3, 37 °C
A. fumigatus + P. aeruginosa zone 3, 60 °C
A. fumigatus + P. aeruginosa zone 4, 37 °C
A. fumigatus + P. aeruginosa zone 4, 60 °C
Intensity (counts)
Time [min]

Figure S2. Comparison of secondary metabolite profiles. Differences in secondary metabolite profile between plates incubated at only 37 °C and plates incubated at 37 °C with one hour at 60 °C were examined by chemical analysis. No differences in secondary metabolite profiles were observed between the plates. Peak 1: phenazine-1-carboxamide. Peak 2: fumigaclavine C. Peak 3: phenazine-1-carboxamide. Peak 4: trypacidin. Peak 5: 2-n-heptyl-(1H)-quinolon-4-one/2-n-heptyl-4-oxy-quinolon. Peak 6: 2-heptyl-3-hydroxy-quinolone. (⁎): Unidentified *A. fumigatus* secondary metabolite. (⁑): Unidentified *P. aeruginosa* secondary metabolite.

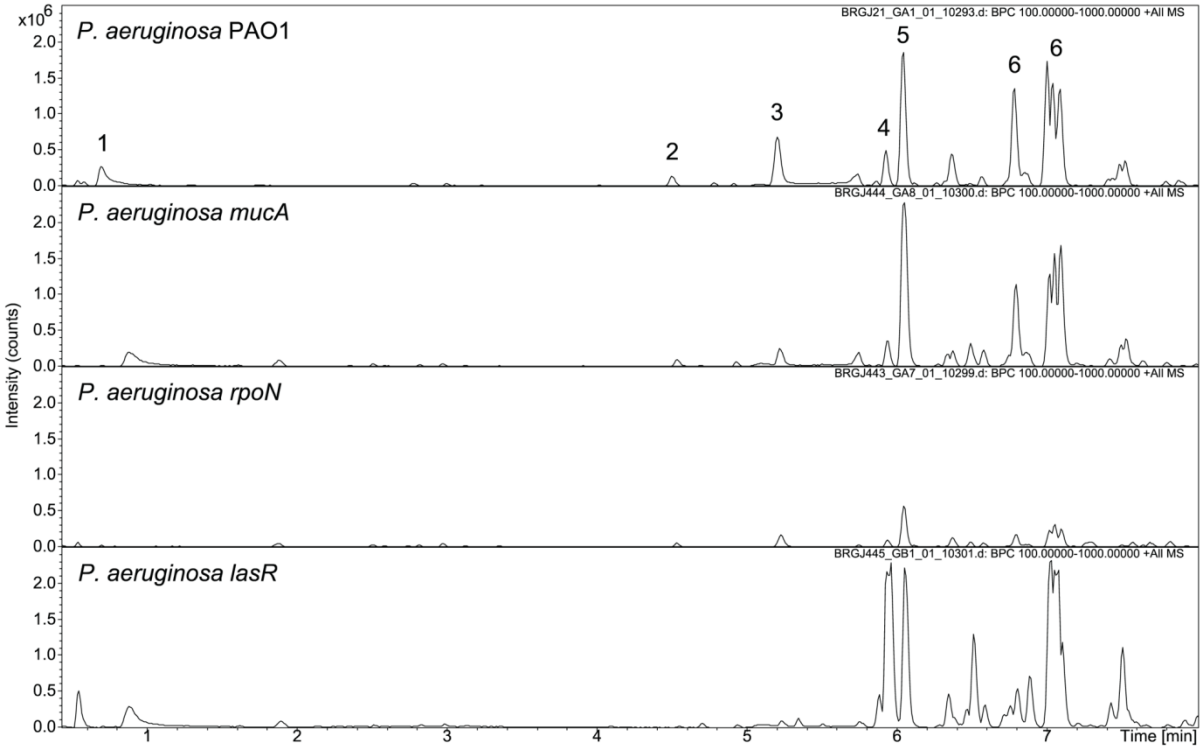


Figure S3. Comparison of secondary metabolite production of PAO1, *mucA*, *lasR* and *rpoN* mutant. Base peak chromatogram of extracts from *P. aeruginosa* PAO1, *mucA*, *lasR* and the *rpoN* mutant. Peaks are; 1: pyocyanin, 2: phenazine-1-carboxamide, 3: phenazine-1-carboxylic acid, 4: 2-n-Heptyl-4-oxy-quinoline/2-n-Heptyl-(1H)-quinolin-4-one, 5: 2-Heptyl-3-hydroxy-4(3H)-quinolinone, 6: unidentified quinolones with same elemental composition. No major differences could be observed between PAO1 and the *mucA* mutant. The phenazines, phenazines-1-carboxamide and phenazine-1-carboxylic acid were reduced in the *lasR* mutant, while no phenazines or quinolones could be detected in the *rpoN* mutant. All mutants had grown on WATM media for five days and secondary metabolites were extracted using the standard method for chemical analysis of strains (see materials and methods).

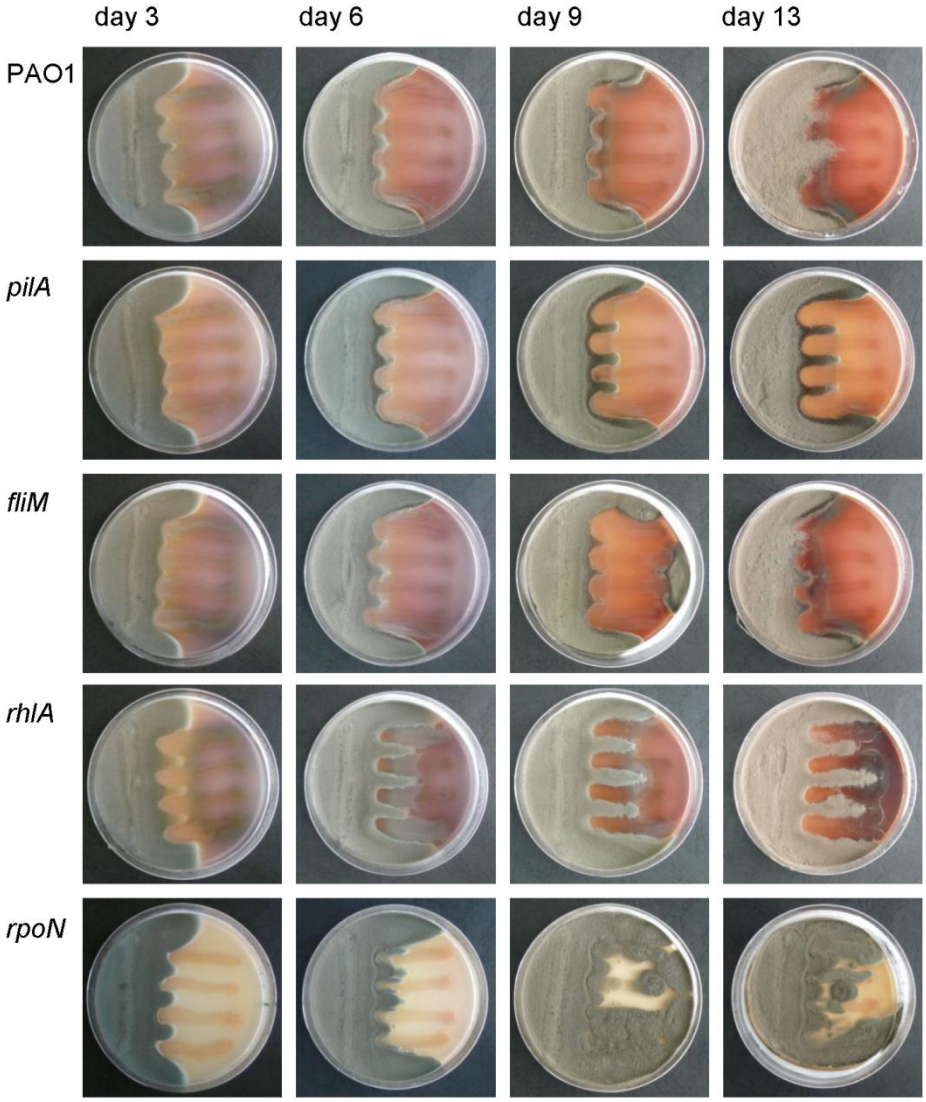


Figure S4. Suppression of *A. fumigatus* by *P. aeruginosa* over time. The suppression of *A. fumigatus* by *P. aeruginosa* was followed over 13 days. Several *P. aeruginosa* mutants still suppressed growth of *A. fumigatus* AF293 after 13 days at 37 °C. However the *rpoN* mutant began to be overgrown by *P. aeruginosa* after only six days incubation.

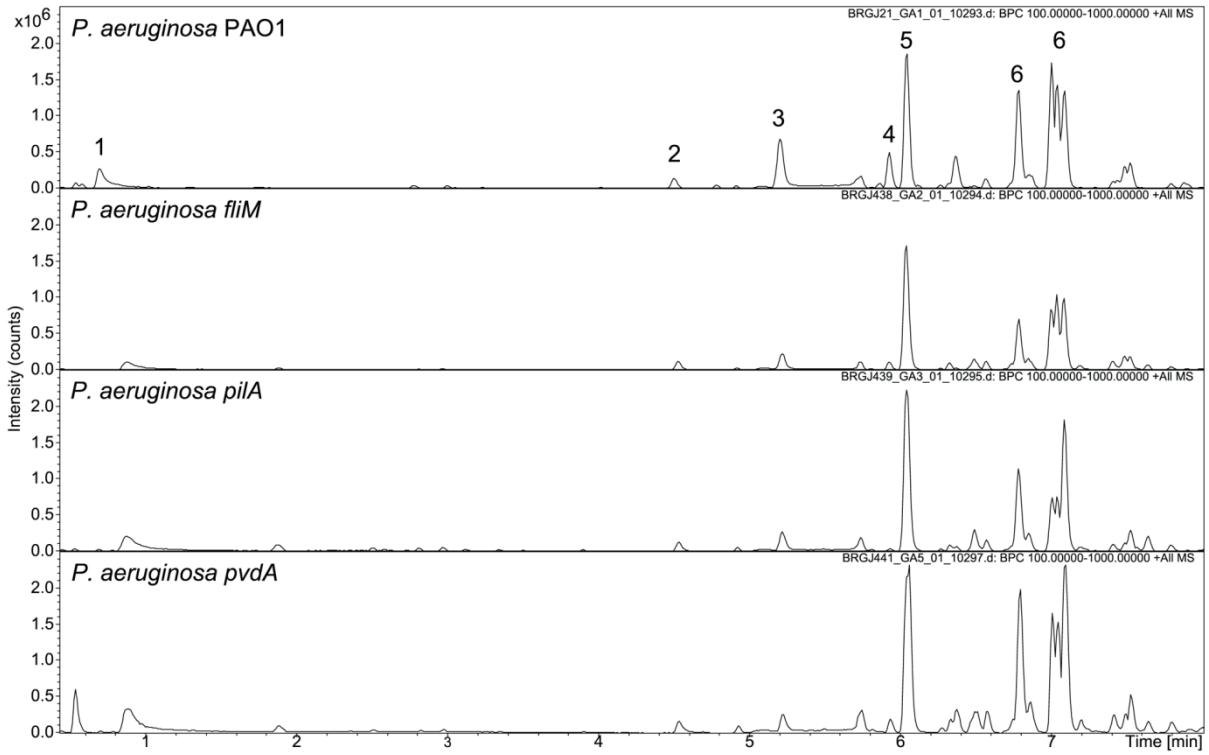


Figure S5. Comparison of secondary metabolite production. Base peak chromatogram of extracts from *P. aeruginosa* PAO1, *fliM*, *pilA* and the *pvdA* mutant. No major differences could be observed between the extracts. Peaks are; 1: pyocyanin, 2: phenazine-1-carboxamide, 3: phenazine-1-carboxylic acid, 4: 2-n-Heptyl-4-oxy-quinoline/2-n-Heptyl-(1H)-quinolin-4-one, 5: 2-Heptyl-3-hydroxy-4(3H)-quinolinone, 6: unidentified quinolones with same elemental composition. All mutants had grown on WATM media for five days and secondary metabolites were extracted using the standard method for chemical analysis of strains (see materials and methods).

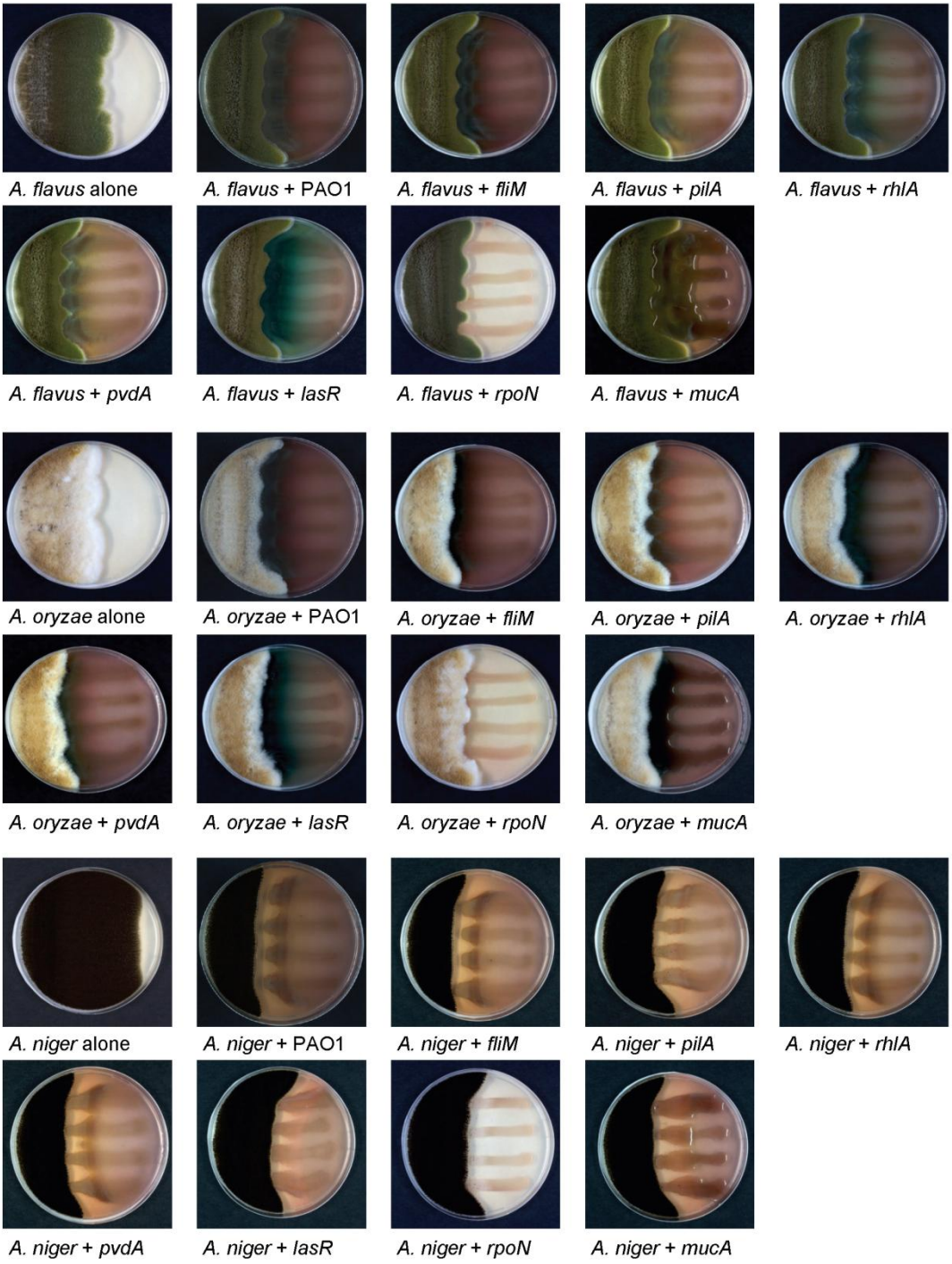


Figure S6. Interactions between *Aspergillus* species and *Pseudomonas aeruginosa* mutants. All *P. aeruginosa* mutants could suppress *A. oryzae*, *A. flavus* and *A. niger*. All *P. aeruginosa* mutants, except *rpoN*, produced a green compound (possibly pyocyanin) in the contact zone of *A. oryzae* and *A. flavus*. Especially the *lasR* mutant produced visually large amounts of this compound. *P. aeruginosa* was likewise slightly inhibited by *A. niger*, probably due to the production of citric acid. Plates had been incubated at 37 °C for five days.