

Aspergillus hydrophobins - Identification, classification and characterization

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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.

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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
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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 ($rodA\Delta$, $dewA\Delta$, AN0940\Delta, AN1837 Δ , AN6401 Δ) 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 $rodA\Delta$ strains, known to be involved in rodlet formation, and the $dewA\Delta$ 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 ($rodA\Delta$, $dewA\Delta$, AN0940 Δ , AN1837 Δ , AN6401 Δ) 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 $rodA\Delta$ stammer, hvor

rodA er kendt for at være involveret i dannelsen af "rodlets", og i $dewA\Delta$ 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
СМВ	Center for Microbial Biotechnology
СҮА	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 individuals³. 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 efficiency⁴. 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 society⁵.

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.

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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).

Chapter 1



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.* 2002¹⁴.

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 development⁴. Upon maturation these fruiting bodies contain thousands of sexual spores termed ascospores. These 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 day¹⁶. Healthy individuals readily clear inhaled conidia, while immunocompromised individuals may acquire severe pulmonary infections¹⁶. 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 fungus¹⁸. 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 production²², and also in the production of enzymes for baking and brewing²³. 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 Organisation²².

Aspergillus niger is likewise widely used in industry and many enzymes from *A. niger* are listed as GRAS by the FDA²⁴. *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 cattle²⁹.

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 years⁶⁻¹⁰ 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 microorganisms³³. 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 fungi³⁹. 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.

Chapter 1

Hydrophobins in general

Hydrophobins are small hydrophobic proteins, approximately 100-150 amino acids in size⁴⁰. 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 bodies⁴¹. 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 functions³⁹. 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 not⁴⁵. 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.

Class	hydro	phobins
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SC4	CNSG-PVQCCNETTTVANAQ-KQGLLGGLLGVVVGPITGLVGLNCSPISVVGVLTGNSCTA-QTVCCDHVTQNGLVNVGC
PRI2	CNNG-SLQCCNSSMTQDRGNLQIAQGVLGGLLGGLLGLGLGLLDLVDLNALIGVQCSPISIVGNANTCTQ-QTVCCSNNNFNGLIALGC
SC3	CTTG-SLSCCNQVQSASSSPVTALLGLLGIVLDLNVLVGISCSPLTVIGVGGSGCSA-QTVCCENTQFNGLINIGC
ABH1	CDVG-EIHCCDTQQTPDHTSAAASGLLGVPINLGAFLGFDCTPISVLGVGGNNCAA-QPVCCTGNQFTALINA-LDC
EAS	CSID-DYKPYCCQSMSGPAGSPGLLNLIPVDLSASLGCVUGVIGSQCGA-SVKCCKDDVTNTGNSFLIINA-ANC
HCF1	CAVGSQISCCTTNSSGSDILGNVLGGSCLLDNVSLISSLNSNCPAGNTFCCPS-NQDGTLNINVSC
MPG1	CGAEKVVSCCNSKELKNSKSGAEIPIDVLSGECKNIPINILTINQLIPINNFCSD-TVSCCSGEQIGLVNIQC
RODA	CGDQAQLSCCNKATYAG-DVTDIDEGILAGTLKNLIGGGSGTEGLGLFNQCSKLDLQIPVIGIPIQALVNQKCKQ-NIACCQNSPSDASGSLIGLGLPC

Class II hydrophobins

HFBI	CPPG-LFSNPQCCATQVLGLIGLDCKVPSQNVYDGTDFRNVCAKTGA-QP	LCCVAP-VAGQALLC
HFBII	CPTG-LFSNPLCCATNVLDLIGVDCKTPTIAVDTGAIFQAHCASKGS-KP	LCCVAP-VADQALLC
SRH1	CPNG-LYSNPQCCGANVLGVAALDCHTPRVDVLTGPIFQAVCAAEGGKQP	LCCVVP-VAGQDLLC
CU	CTGL-LQKSPQCCNTDILGVANLDCHGPPSVPTSPSQFQASCVADGGRSA	RCCTLS-LLGLALVC
CRP	CSST-LYSEAQCCATDVLGVADLDCETVPETPTSASSFESICATSG-RDA	KCCTIP-LLGQALLC
MGP	CSGLYGSAQCCATDILGLANLDCGQPSDAPVDADNFSEICAAIG-QRA	RCCVLP-ILDQGILC
HCF6	CPANRVPQCCQLSVLGVADVTCASPSSGLTSVSAFEADCANDG-TTA	QCCLIP-VLGLGLFC
HYD4	CPDGGLIGTPQCCSLDLVGVLSGECSSPSKTPNSAKEFQEICAASG-QKA	RCCFLSEVFTLGAFC

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.* 2008⁴⁷.

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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 low⁴⁰, 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 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

proteins³⁹. All eight cysteines are involved in disulphide bridges³⁸ and form four disulphide bridges⁴⁹. 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 sheet⁵⁰. 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 Nterminal 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	l characterized	Aspergillus	hydrophobins
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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 criteria⁴⁰ 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 predicted GPI were to be (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 anchor⁵⁹. 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 conditions⁵³. By examining the full genome sequence of *A. fumigatus*, an additional hydrophobin is found, AFUA_2G14661, displaying eight cysteines and a signal sequence⁹. 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

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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 masses³⁶. 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 water⁶⁴. 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 layer⁵⁵. The phenotypes observed for the *rodA* deletion strains have likewise been seen in other fungal species. The conidia of the easilywetted (*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 strain⁶⁵, a phenotype also observed for *M. grisea* colonies lacking the Mpg1 hydrophobin⁶⁶.

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 layer⁵⁸.



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, 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 hydrophobic⁴⁰. They coat air channels in fruiting bodies allowing gas exchange and prevent collapse and water filling of the channels in humid environments⁶⁷. 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 environments⁴⁰. 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* species⁷¹ 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 interfaces⁹⁰. 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 rodlets⁹¹. 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 rodlets³⁶. 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.* 1994⁵⁵.

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*) membranes⁹⁷. 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 surface⁹⁹. 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 growth⁴². 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.* 1999⁹⁹.

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 yields¹⁰⁴. 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

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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 scale¹⁰⁶. If hydrophobins can be produced in significant low-cost amounts, it is certainly possible that the application of hydrophobins will gradually expand.

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	y	
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

Table 2: Potential	industrial	applications	for hydro	phobins
Lable 2. 1 Otential	maasuna	upplications	101 Ilyulo	phooms

Fungal-bacterial interactions

Bacteria and fungi are abundantly found in many different environments and commonly coexist. 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 species¹²¹. 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.*, 2011¹²².

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 patients¹²¹. 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 patients¹²⁵. Furthermore, CF patients colonized with *Aspergillus* and *Candida albicans* or both *Aspergillus*, *Pseudomonas* and *C. albicans* have decreased lung functions¹²⁷. 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* are less explored. Part of this thesis deals with

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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 fungi². 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 expressed¹³¹, but are only expressed when advantageous to the organism itself¹²⁸. 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 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 properties¹³⁶. 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 metabolites¹³⁵. 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 genome¹³², and Aspergilli have been found to contain between 30 - 40 clusters per species¹⁴². Many of the biosynthetic pathways are to a large extent similar¹³⁹, 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* species¹³⁹ 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 animals¹³³. Especially *P. aeruginosa* can cause a number of infections in humans, but most commonly infect immunocompromised individuals¹³³. A patient group often encountering *P. aeruginosa* infections are cystic fibrosis patients, where over 80 % are chronically infected¹²⁵.

Pseudomonas species can similarly to the *Aspergillus* species produce many different secondary metabolites, where a recent review by Gross and Loper¹³³ provides an excellent overview. The secondary metabolites are important for among others nutrient acquisition, virulence and competition with other organisms¹³³. 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 derivatives¹⁴⁷. 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 medium¹⁴⁴. 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 characterized¹⁴⁹. 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 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 uncertainty¹⁵⁹. 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 mode¹⁴⁸.

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 algae¹⁵⁰. 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 patients¹²⁵. 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 culture¹⁶². 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 effect¹⁶⁵. 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 germination¹⁶⁶. 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.* 2010¹⁶⁶.

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 hours¹⁶⁷. 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* hyphae¹⁶⁸. *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 $> 100 \mu g/ml$ for pyocyanin and 50 μ g/ml for 1-hydroxyphenazine, while MICs for *C. albicans* were > 100 μ g/ml for pyocyanin and 25µg/ml for 1-hydroxyphenazine¹⁷⁴. This finding was supported by Blyth, who showed that the germination of A. *fumigatus* conidia was inhibited by pyocyanin¹⁶³, while Mangan demonstrated that 1-hydroxyphenazine could inhibit germination of spores from A. fumigatus, A. terreus, A. niger and A. flavus¹⁶². 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 cultures¹⁷⁵. 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 5methyl-phenazinium-1-carboxylate (5MPCA) and phenazine methosulphate (PMS) induced the red pigmentation^{175,176}. Similarly, the production of a red pigment (proposed to be 3hydroxyphenazine-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 pigment¹⁷⁷. Nevertheless, the pigment had different properties than the pigment observed in *C. albicans-P. aeruginosa* cocultures¹⁷⁵.

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-C₁₂-HSL and C₄-HSL, which act as signalling molecules in the quorum sensing systems¹⁸⁰. Interestingly, two *P. aeruginosa* quorum sensing knockout-strains displayed reduced inhibitory effect on the germination of *A. fumigatus* conidia compared to the wild type strain¹⁶⁶. In *C. albicans* purified 3-oxo-C₁₂-HSL could inhibit filamentation, while C₄-HSL did not have any effect¹⁷⁰. Similarly, farnesol, dodecanol and C₁₂-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 biomass¹⁶⁶. 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 farnesol¹⁸³. 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*ns¹⁸⁴.

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 fungalinfections 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 conditions¹⁰². 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) cells¹⁸⁷. Lack of the RodB hydrophobin only had a minor effect⁵⁸.

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 biofilm¹⁹⁴. In another study examination of static, aerial grown hyphae (resembling biofilm growth) only showed expression of the *rodB* and *rodE* genes⁵³. 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).

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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 fungi¹. 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-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-Cys8⁴ (see figure 3 in chapter 1).

Based on their distinct hydropathy patterns (figure 1) and physical properties, hydrophobins were traditionally divided into two classes³.



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 Ascomycetes¹. 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 hydrophobins². 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 hydrophobins⁷.

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-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 server¹⁷, 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

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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.

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	Ι	RolA ^a
	AO090020000588	15231	151	8	CN(7)CCN(39)CN(17)CN(5)CCN(17)C	Ι	New
A. niger CBS 513.88	An03g02360 ^b	12486	122	8	CN(6)CCN(32)CN(25)CN(5)CCN(4)C	Ι	
	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	Ι	
	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	Ι	
	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	JGI128530	10803	105	7	Fragment (similar to An07g03340)	Intermediate	(New)
11100 1015	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	Ι	(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	Ι	(New)
	JGI43184	20381	201	9	CN(8)CCN(33)CN(11)CN(5)CCN(16)C	Intermediate	(New)
E. nidulans	AN7539.2°	10798	109	8	CN(5)CCN(32)CN(6)CN(5)CCN(13)C	Intermediate	
1050111	AN8803.2°	15625	157	8	CN(7)CCN(39)CN(18)CN(5)CCN(17)C	Ι	$\operatorname{Rod} A^d$
	AN6401.2 ^c	16131	162	8	CN(6)CCN(38)CN(22)CN(5)CCN(35)C	Intermediate	
	AN8006.2°	13183	135	8	CN(6)CCN(31)CN(23)CN(5)CCN(6)C	Ι	DewA ^e
	AN1837.2 ^c	13397	135	8	CN(7)CCN(39)CN(18)CN(5)CCN(17)C	Ι	
	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	Ι	$RodC^{f}$
Ar295	AFUA_5G09580	16153	159	8	CN(7)CCN(39)CN(21)CN(5)CCN(17)C	Ι	${\rm RodA}^{{\rm f/g}}$
	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	Ι	$RodB^{f/h}$
	AFUA_5G03280	19825	190	9	CN(7)CCN(33)CN(11)CN(5)CCN(14)C	Ι	$\operatorname{RodF}^{\mathrm{f}}$
A. fumigatus A1163	AFUB_016640	14300	140	8	CN(7)CCN(36)CN(18)CN(5)CCN(18)C	Ι	(RodB New)
	AFUB_057130	16153	159	8	CN(7)CCN(39)CN(21)CN(5)CCN(17)C	Ι	(RodA New)
	AFUB_080740	15996	155	8	CN(7)CCN(39)CN(21)CN(5)CCN(17)C	Ι	(RodC New)
	AFUB_051810	19825	190	9	CN(7)CCN(33)CN(11)CN(5)CCN(14)C	Intermediate	(RodF New)
<i>A. terreus</i> 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	Ι	New

Table 1:	Identified Aspergillus hydrophobins.

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	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	Ι	New
	AFLA_014260	14304	145	8	CN(8)CCN(38)CN(10)CN(5)CCN(21)C	Ι	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	Ι	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	Ι	New
	ACLA_072820	16127	158	8	CN(7)CCN(39)CN(21)CN(5)CCN(17)C	Ι	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

¹In the eight cysteine pattern, "N", signifies any other amino acid than cysteine.

²In 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 ProtScale¹⁹ 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 doublets². Twenty-three of the identified hydrophobins displayed a characteristic class I hydropathy plot (not shown) and displayed a class I cysteine pattern⁷ 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 pattern⁷ 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.fr¹⁸. 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.fr¹⁸. 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 Wessels² 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 Wessels³. 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.

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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 development¹ and may be expressed at different stages of development². 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 interface³. 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 surfaces¹.

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).

	10	2	0	30	40	50	60	70	80	90
AN8803/1-157 MKF AN8006/1-135 MRF AN0940/1-101 MHL AN1837/1-135 MKF AN6401/1-162 MQF AN7539/1-109 MKV	SIAAAVVA IVSLLAFT STSAAAIL IGLATLSL TIASLI ATALSVLA	FAASVAALF AAATATALF - ALSLAGP - FALASAD - ATAVLGL - VAGS	PAHD SQF AG AS TMAG QMAS	NGVGNKGNS AAKNAKLAT RPYFCPLAL AAPHAPGSS ALPSA	NVKFPVPENV SAAF L DAKF L KFPVPDSI NAAI I 	TVKQASDKC -AKQAEGTT QVPY TVAEGSSKC SQAQEQNTC NSEKRQSDC	GDQAQLSCCN CNVGSIACCN CCE GDQAQLSCCN GN-AHLSCCE IGPLLCCG	KATYAGDTTT SP AET GF VP DVKYGGDTTV ST SLTTPLDPLV	VDEGLLS NNDSLLSG ARDSKVSFEG VQKGIAA DNSVSLTQ DPILELVG	GALSGL LLGAGLLNGL GLLSDL EEEEGLLHLL
10 AN8803/1-157 I GA AN8006/1-135 SGN AN0940/1-101 AN1837/1-135 L GA AN6401/1-162 GGT AN7539/1-109	10 GSGAEG TG GSAAEG SSVLSDGL	110 LGLFDQCSK SACAK LGAFSGCSK LGKYSGCSS	120 (LDVAVLI (ASLIDQL VNCIDVS (LDISLLI SLASVEGILG IDAAAIV	130 GIQDLV- GLLALV- SDEDFV- GIEDIL- AGGNQGLV- GSVGLLC	140 NQK KT KQK SGQ HAYDDT	150 - C KQN I A VC KN I VA - CPKGGTPK - C KQN I A - C NNHVA - C TSEPQ	160 CCQNSPSSAD CCPE CCYS CCAKSGGSAD CCDAGD CCTE	170 GNLIG GDLVG NELVGFLEPG ANLLG	180 	190
AN8803/1-157 PCV. AN8006/1-135 NCV. AN0940/1-101 ICT AN1837/1-135 PCI. AN6401/1-162 PCV AN7539/1-109 GCG	200 AL AVDNAGAG TEVGD AL PV PL	GSIL TKAE GVDK GSIL -QVL EHGH								

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-C). The hydrophobins vary in size from $\sim 10 - 17$ kDa and contain $\sim 100 - 150$ 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 acids¹¹.

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.

% 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 %	

Table 1: Identity between A. nidulans hydrophobins

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. <i>niaulans</i> hydrophobins to hydrophobins from other full genome sequenced Asp	spergilli
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A. nidulans	Hydrophobins from other Aspergilli with highest percentage of					
hydrophobin	identical amino	hydrophobins ¹				
RodA	75 % AFUA_5G09580	69 % ACLA_010960	66 % An07g03340			
	A. fumigatus	A. clavatus	A. niger			
DewA	44 % AFLA_060780	41 % An03g02400	41 % An03g02360			
	A. flavus	A. niger	A. niger			
AN0940	38 % An01g10940					
	A. niger					
AN1837	60 % AFUA_5G09580	57 % ACLA_010960	55 % An07g03340			
	A. fumigatus	A. clavatus	A. niger			
AN6401	33 % ACLA_018290	33 % An07g03340	31 % AFUA_1G17250			
	A. clavatus	A. niger	A. fumigatus			
AN7539	74 % ACLA_072820	71 % AFUA_2G14661	56 % ATEG_07808			
	A. clavatus	A. fumigatus	A. terreus			

¹The 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 $rodA\Delta dewA\Delta$. 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 (MM¹²) and rich media; WATM (Wickerhams Antibiotic Test Medium¹³), YES (Yeast Extract Sucrose agar¹⁴) and CYA (Czapek Yeast extract Agar¹⁴) 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 $rodA\Delta$ 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 ,

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displayed conidia with dark brown colour. Conidia were similarly to the $rodA\Delta$ 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 $rodA\Delta$ mutant enhanced the phenotype observed in the $rodA\Delta$ strain as the spore clumping was more pronounced with larger clumps.

The AN0940, AN1837 and AN6401 mutant strains (AN0940 Δ , AN1837 Δ and AN6401 Δ) 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 , AN0940 Δ , AN1837 Δ and AN6401 Δ 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 $rodA\Delta$ and $rodA\Delta dewA\Delta$ strains displayed a darker colony colour compared to the other strains. (C) Conidial heads of $rodA\Delta$ and $rodA\Delta dewA\Delta$ 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 detergent⁷. 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, AN0940 Δ , AN1837 Δ and AN6401 Δ 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 AN0940 Δ , AN1837 Δ , AN6401 Δ and AN7539 Δ 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 foldchange 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 $rodA\Delta$, $dewA\Delta$ and $rodA\Delta dewA\Delta$ strains using the reference as a control. The data shown are means \pm 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 AN7539 Δ) 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 hydrophobic¹⁷.

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 $146^{\circ} \pm 4^{\circ}$. No statistical significant change of surface hydrophobicity of the *rodA* Δ , *dewA* Δ , AN0940 Δ , AN1837 Δ and AN6401 strains compared to our reference was measured as these strains displayed water contact angles from $142^{\circ} \pm 8^{\circ}$ to $154^{\circ} \pm 3^{\circ}$. 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 $rodA\Delta dewA\Delta$) displayed high hydrophobicity with constant water contact angles in the first minute. The data shown are means \pm standard errors from three experiments. (B) Hydrophobicity of the $rodA\Delta dewA\Delta$ 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.

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Using the hydrophobin deletion strains, our results indicate that lack of a single hydrophobin or two ($rodA\Delta dewA\Delta$) 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 environments¹ and may therefore be expressed at different stages of fungal development². *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 hydrophobins¹, 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 $rodA\Delta$ and $rodA\Delta dewA\Delta$ strains had different phenotypes compared to the reference strain, where the $rodA\Delta dewA\Delta$ strain displayed a more severe phenotype than the $rodA\Delta$ 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 ($dewA\Delta$, AN0940 Δ , AN1837 Δ , AN6401 Δ and reference) displayed dry spores, were not easily wetted and resisted suspension in water. This indicates that these hydrophobins, as previously shown for DewA⁷, 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 strains⁷ 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 AN0940 Δ , AN1837 Δ and AN6401 Δ strains did not display a detergent wettable phenotype, indicating that these hydrophobins may not have dominant roles on conidia if

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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 layer⁷. 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 strains⁷, 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 AN7539∆ 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 hydrophobicity⁸. In agreement with our results, Dynesen *et al.*⁸ did not observe any difference in surface hydrophobicity between the $dewA\Delta$ and reference strain, but did in contrast to our results see a decrease in surface hydrophobicity in the $rodA\Delta$ strain. Our $rodA\Delta dewA\Delta$ strain allowed passage of water to the underlying agar, a phenotype previously described in an A. fumigatus rodA Δ strain²⁵. 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 AN0940 Δ , AN1837 Δ and AN6401 Δ 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 tension³. 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 environments¹. 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.

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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 function³.

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* strains⁴. *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 motility⁷ and inactivation of the anti-sigma factor MucA resulting in a mucoid phenotype⁸. 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 species¹⁸. 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. aeruginosa¹. Many CF patients harbour several A. fumigatus genotypes in their lungs^{24–26}, with some strains being chronically present, while others are found more transiently²⁶. Bakare *et al.* found that 64% of A. fumigatus positive patients also had P. aeruginosa infections², 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 patients²⁸.

Both Aspergillus species and Pseudomonas aeruginosa are frequently isolated from CF patients², 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* PAO1³⁹ 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 patients¹⁸, 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.

					WAT	ſM me	dium						LB medium ¹
A. fumigatus strain	Type of strain	Color of <i>P.</i> <i>aeruginosa</i> 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

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

¹Gliotoxin is mainly produced on low carbohydrate containing media⁴¹ and therefore LB medium was used. ND: $< 20 \text{ ng/cm}^2$

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 transiently²⁶.

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.

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				

 Table 2: P. aeruginosa mutant strains used

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.

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	-	-

Table 3: Suppression of P. aeruginosa strains

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-1carboxylic 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-1carboxylic acid and phenazine-1-carboxamide⁴⁹ 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. 1hydroxyphenazine 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).

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. annuain and lasP	Quorum sensing	Phenazine-1-carboxamide and phenazine-1-carboxylic					
r. aeruginosa iask	mutant	acid reduced in mutant					
D gamuginagg maN	Degulatory mutant	Reduced expression of several phenazines and quinolones					
r. aeruginosa rpon	Regulatory mutant	in mutant					
P. aeruginosa mucA	Mucoid mutant	No major difference in secondary metabolite profile					

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.

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 factors⁶.



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 factors⁶. 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 ($rodA\Delta$, $dewA\Delta$, AN0940 Δ , AN1837 Δ , AN6401 Δ 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, Blyth³³ likewise found a close spatial relationship between A. fumigatus and P. aeruginosa, but the presence of P. aeruginosa affected ultrastructures of A. fumigatus hyphae³³. In mature A. fumigatus biofilm, P. aeruginosa cells have been found distributed throughout the filamentous network with a minimal effect on A. fumigatus biomass⁶¹. 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 functions⁶. 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 virulence⁶, including a reduced pulmonary clearance in animal models and increased alginate production⁶². 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 virulent⁶³ 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 compounds⁵¹, 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-1carboxylate (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
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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 index⁷⁰. 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 pathogenicity⁵⁴, 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 antifungal 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.

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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 DNA⁸⁻¹¹, RNA¹² and proteins^{11,13-15}. Furthermore, cantilevers have been used to detect *Staphylococcus* enterotoxin B¹⁶, Bovine capillary endothelial cells¹⁷, virus¹⁸, bacteria¹⁹⁻²¹ and fungi^{22,23}. Most bio-chemical reactions are performed in static mode⁶ 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 $39 \times 39 \times 5$ 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 $39 \times 39 \times 1.5$ 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 $11.9 \times 11.9 \times 1$ 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 μ m wide, 500 μ m long and 5.5 μ m

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 broth³² 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 plate³² 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 μ l/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 systems³³. 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

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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-ona-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.

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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 published¹⁻⁵ 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 Wessels⁶ and the structure of hydrophobins HFBI, HFBII and EAS⁷⁻¹⁰. 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, Wessels¹⁴ 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 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 pattern¹⁵ (**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 ($rodA\Delta$, $dewA\Delta$, AN0940 Δ , AN1837 Δ ,

AN6401 Δ , *rod* $\Delta dewA\Delta$,) in *A. nidulans* to examine the role of different hydrophobins (**chapter 4**). The AN7539 Δ 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 strain¹⁷, an *A. fumigatus rodA* deletion strain¹⁸ and an *Neurospora crassa eas* mutant¹⁹ 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 DewA¹⁶. 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 detergent¹⁶. 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 (AN0940 Δ , AN1837 Δ and AN6401 Δ) 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 strain¹⁸. 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. commune²²⁻²⁵, 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* 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* strain the AN1837, AN6401 and AN7539 hydrophobins had reduced expression similar to the *rodA* 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 layer¹⁶. 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 strains¹⁶, but in contrast the lack of *rodA* affects *dewA* expression.

In the $rodA\Delta dewA\Delta$ 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 downregulation 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 frequency³⁸. 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 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-offunction 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 models⁵³, 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. funigatus* colonisation. Other mutations including lack of motility, rhamnolipids and pyoverdins did not change the interaction between *A. funigatus* and *P. aeruginosa* and could suppress *A. funigatus* (**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, pyocyanin⁵⁴. 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.

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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 Jalview¹. 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 Server² by using the Kyte and Doolittle scale³ 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 deletionstrain 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 ::AFpyrG, 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 DH5 α 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 μ M was used, whereas 0.4 μ M 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 pDEL2⁴ 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 crossed⁸ 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 IS1⁹. Created PCR fragments (see table 2 for primer pairs) were USER cloned into a pU1111 based vector and subsequently transformed into competent *E. coli* DH5 α 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 preparations¹⁰ 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 $rodA\Delta$, $dewA\Delta$, $rodA\Delta dewA\Delta$ 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 Tag 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 $\Delta\Delta C(t) = \Delta C(t)$ normalized $\Delta C(t)$ calibrator. The $\Delta C(t)$ normalized $= \Delta C(t)$ target gene- $\Delta C(t)$ 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 μ l 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 μ l 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∆ (IBT29539)	argB2, pyrG89, veA1, nkuA Δ	Nielsen et al. 2008
nkuA-trS (IBT28738)	argB2, pyrG89, veA1, nkuA-trS::AFpyrG	Nielsen et al. 2008
G051 (IBT25456)	biA1, veA1	CMB fungal collection
<i>rodA</i> ∆ (auxotrophic, NID179)	argB2, pyrG89, veA1, nkuA Δ , rodA Δ	This study
$dewA\Delta$ (auxotrophic, NID238)	argB2, pyrG89, veA1, nkuA Δ , dewA Δ	This study
$rodA\Delta dewA\Delta$ (auxotrophic, NID 295)	argB2, pyrG89, veA1, nkuA Δ , rodA Δ , dewA Δ	This study

AN0940∆ (auxotrophic, NID781)	argB2, pyrG89, veA1, nkuA Δ , AN0940 Δ	This study
AN1837∆ (auxotrophic, NID780)	argB2, pyrG89, veA1, nkuA Δ , AN1837 Δ	This study
AN6401∆ (auxotrophic, NID764)	argB2, pyrG89, veA1, nkuA Δ , AN6401 Δ	This study
Reference strain for knockouts (NID750)	veA1	This study
<i>rodA</i> ∆ (AN8803∆, NID600)	veA1, rodA Δ	This study
<i>dewA</i> Δ (AN8006Δ, NID601)	$veAI$, dewA Δ	This study
$rodA\Delta dewA\Delta$ (NID602)	$veA1$, $dewA\Delta$, $rodA\Delta$	This study
AN0940Δ (NID666)	<i>veA1</i> , AN0940Δ	This study
AN1837Δ (NID667)	<i>veA1</i> , AN1837∆	This study
AN6401Δ (NID668)	$veAI$, AN6401 Δ	This study
Reference strain for over- expression strains (NID191)	argB2, pyrG89, veA1, nkuA∆::AFpyrG, IS1::PgpdA- TtrpC::argB	CMB fungal collection
rodA OEx (NID770)	argB2, pyrG89, veA1, nkuA-trS::AFpyrG, IS1::PgpdA- AN8803-TtrpC::argB	This study
dewA OEx (NID684)	argB2, pyrG89, veA1, nkuA-trS::AFpyrG, IS1::PgpdA- AN8006-TtrpC::argB	This study
AN7539 OEx (NID771)	argB2, pyrG89, veA1, nkuA-trS::AFpyrG, IS1::PgpdA- AN7539-TtrpC::argB	This study
AN6401 OEx (NID895)	argB2, pyrG89, veA1, nkuA-trS::AFpyrG, IS1::PgpdA-AN6401-TtrpC::argB	This study
AN1837 OEx (NID896)	argB2, pyrG89, veA1, nkuA-trS::AFpyrG, IS1::PgpdA- AN1837-TtrpC::argB	This study
AN0940 OEx. (NID893)	argB2, pyrG89, veA1, nkuA-trS::AFpyrG, IS1::PgpdA-AN0940-TtrpC::argB	This study

 Table 2: Oligonucleotides for strain construction

Purpose	Name	Oligonucleotide sequence
Construction of $rodA\Delta$	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 $dewA\Delta$	ANdewA-chk-Up-F	ACTCAATCCGTGCCTACA
	ANdewA-del-Up-F	GCGATGCTAGTGGTTGTT
	ANdewA-del-Up-Ra	gatecccgggaattgccatgGAGATGCTTGTGATGGAAGG
	ANdewA-del-Dw-Fa	aattccagctgaccaccatgCTTGGAGAGAACAGACGG
	ANdewA-del-Dw-R	CGTTTCTGTCTTCCGTGG
	ANdewA-chk-Dw-R	GTCCTGAAGTCCTGAACC
Construction of AN0940∆	AN0940-chk-Up-F	CCACAGAAGAGCTCTGTGC
	AN0940-del-Up-F	GGAGTACTACTGGGAATCGG
	AN0940-del-Up-Ra	gatccccgggaattgccatgGAGTGGCTTGGCAGGATAGAG
	AN0940-del-Dw-Fa	aattccagctgaccaccatgGGCGGTTCCGAGGATCAG
	AN0940-del-Dw-R	GGGCAGGCAGAGATTGGTG
	AN0940-chk-Dw-R	CAATATACGCCGAGCCAACCC
Construction of AN1837∆	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 AN6401∆	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 AFpyrG	pDEL-Up-F-Ad	catggcaattcccggggatcTGGATAACCGTATTACCGCC
	AFpyrG-int-F3	TGATACAGGTCTCGGTCCC
	AFpyrG-int-R	GGAAGAGAGGTTCACACC
	pDEL-Dw-R-Ad	catggtggtcagctggaattTGCCAAGCTTAACGCGTACC
Construction of AN8803-OEx	FW_AN8803	AGAGCGAUAAGACTTTCATAGACAGTCCAGTC
	RV_AN8803	TCTGCGAUAGGCAGTAATGAAGCAATCAAG
Construction of AN8006-OEx	FW_AN8006	AGAGCGAUGCAATTCAGCAATTCAGCAATCCA
	RV_AN8006	TCTGCGAUCAAATCCGAACAACACCAATTATT
Construction of AN0940-OEx	FW_AN0940	AGAGCGAUATGCATCTTTCCACCTCCGCT
	RV_AN0940	TCTGCGAUCTACTTGTCAACGCCATCACCAAC
Construction of AN1837-OEx	FW_AN1837	AGAGCGAUGCAGACTAAACGTCACGTCCCA

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	RV_AN1837	TCTGCGAUGGATTATTAATGTATTTATTCGAACGAATTT
Construction of AN6401-OEx	FW_AN6401	AGAGCGAUATGCAATTCACAATCGCTTCCC
	RV_AN6401	TCTGCGAUTTAGAGAACCTGGACAGGA
Construction of AN7539-OEx	FW_AN7539	AGAGCGAUATGAAGGTCGCCACTGCC
	RV_AN7539	TCTGCGAUTTAGTGGCCGTGCTCCAG
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* PAO1¹¹ 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 1×10^6 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 1×10^8 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 μ l methanol and ultrasonicated for 10 min. Extracts were finally filtered through a PTFE 0.45 μ m 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 μ m; Phenomenex, Torrence CA). Separation was performed using a linear water-acetonitril (CH₃CH) gradient starting from 10 % CH₃CH to 100 % over 7 min at a flow rate of 0.4 ml/min. Both water and CH₃CH 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.

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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

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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,

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Center for Microbial Biotechnology, Department of Systems Biology, Technical University of Denmark, Søltofts Plads, Building 221/223, DK-2800 Kgs. Lyngby, Denmark 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



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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		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	1	(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	1	(New)
	JGI43184	20381	201	9	CN{8}CCN{33}CN{11}CN{5}CCN{16}C	Intermediate	(New)
E. nidulans FGSC A4	AN7539.2 [℃]	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	1	DewA ^e
	AN1837.2 ^c	13397	135	8	CN{7}CCN{39}CN{18}CN{5}CCN{17}C	1	
	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	1	RodC ^f
	AFUA_5G09580	16153	159	8	CN{7}CCN{39}CN{21}CN{5}CCN{17}C	1	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	1	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	1	(RodB New)
-	AFUB_057130	16153	159	8	CN{7}CCN{39}CN{21}CN{5}CCN{17}C	1	(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	1	New
	ATEG_04730	11797	121	8	CN{10}CCN{11}CN{16}CN{8}CCN{10}C	Ш	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	1	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
<i>A. clavatus</i> 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	L	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]

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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 twentyfour 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

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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 HFBI 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.

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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..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.

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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].

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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.

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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.

Paper 1

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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	Ι	Ι	Ι
	AO090020000588	CN{7}CCN{39}CN{17}CN{5}CCN{17}C	Ι	Ι	Ι
A. niger CBS 513.88	An03g02360	CN{6}CCN{32}CN{25}CN{5}CCN{4}C	Ι	Ι	Ι
	An03g02400	CN{6}CCN{31}CN{23}CN{5}CCN{6}C	Ι	Intermediate	Intermediate
	An04g08500	CN{7}CCN{39}CN{20}CN{5}CCN{17}C	Ι	Ι	Ι
	An15g03800	CN{5}CCN{32}CN{6}CN{5}CCN{13}C	Ι	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	Ι	Ι	Ι
	An09g05530	CN{8}CCN{33}CN{11}CN{5}CCN{16}C	Ι	Intermediate	Intermediate
	An08g09880	CN{7}CCN{16}CN{6}CN{5}CCN{10}C	Ι	Intermediate	Intermediate
A. niger ATCC 1015	JGI128530	Fragment (similar to An07g03340)	Ι	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	Ι	Intermediate	Intermediate
	JGI45685	$CN{6}CCN{32}CN{25}CN{5}CCN{14}C$	Ι	Ι	Ι
	JGI53462	$CN{5}CCN{32}CN{6}CN{5}CCN{13}C$	Ι	II	Intermediate
	JGI194815	$CN{7}CCN{39}CN{20}CN{5}CCN{17}C$	Ι	Ι	Ι
	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	l	II	Intermediate
	AN8803.2	$CN{7}CCN{39}CN{18}CN{5}CCN{17}C$	I	1	1
	AN6401.2	CN{6}CCN{38}CN{22}CN{5}CCN{35}C	l	Intermediate	Intermediate
	AN8006.2	CN{6}CCN{31}CN{23}CN{5}CCN{6}C	l	l	I
	AN1837.2	$CN{7}CCN{39}CN{18}CN{5}CCN{17}C$	1	l H	
A (AF202	AN0940.2	$CN{13}CCN{17}CN{12}CN{7}CCN{8}C$	-	II T	Intermediate
A. fumigatus AF293	AFUA_8G07060	$CN{7}CON{39}CN{21}CN{5}CCN{17}C$	I T	l	I
	AFUA_5G09580	$CN{7}CON{39}CN{21}CN{5}CCN{17}C$	I	1	1
	AFUA_2G14661	$CN{5}CCN{32}CN{6}CN{5}CCN{13}C$	I	11	Intermediate
	AFUA_IG1/250	$CN{7}CCN{30}CN{18}CN{5}CCN{18}C$	I	I	I
A fumicatus A1162	AFUA_3003280	CN[7]CCN[26]CN[18]CN[5]CCN[18]C	I	I	I
A. jumiguius A1105	AFUD_010040	CN[7]CCN[20]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	$CN_{7}CCN_{3}CN_{1}CN_{5}CCN_{4}CN_{7}CCN_{4}CN_{5}CCN_{5}CCN_{4}CN_{5}CCN_{5}CCN_{4}CN_{5}CCN_{5$	I	п	Intermediate
A terreus NIH 2624	ATEG 10285	$CN{5}CCN{28}CN{14}CN{8}CCN{13}C$	-	I	Intermediate
11. 10110105 1 (111 202 1	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	Ι	Ι
	ATEG 04730	CN{10}CCN{11}CN{16}CN{8}CCN{10}C		II	П
A. flavus NRRL 3357	AFLA 094600	CN{7}CCN{16}CN{6}CN{5}CCN{9}C	Ι	II	Intermediate
5	AFLA 131460	CN{5}CCN{32}CN{6}CN{5}CCN{13}C	Ι	Intermediate	Intermediate
	AFLA_060780	CN{6}CCN{30}CN{23}CN{5}CCN{4}C	Ι	Ι	Ι
	AFLA_014260	CN{8}CCN{38}CN{10}CN{5}CCN{21}C	Ι	Ι	Ι
	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	Ι	Ι	Ι
	AFLA_064900	CN{7}CCN{15}CN{6}CN{5}CCN{8}C	Ι	II	Intermediate
A. clavatus NRRL 1	ACLA_001890	CN{7}CCN{16}CN{6}CN{5}CCN{26}C	Ι	Intermediate	Intermediate
	ACLA_048810	CN{7}CCN{33}CN{11}CN{5}CCN{15}C	Ι	II	Intermediate
	ACLA_010960	CN{7}CCN{39}CN{21}CN{5}CCN{17}C	Ι	Ι	Ι
	ACLA_072820	CN{7}CCN{39}CN{21}CN{5}CCN{17}C	Ι	Ι	Ι
	ACLA_018290	CN{5}CCN{32}CN{6}CN{5}CCN{13}C	Ι	Intermediate	Intermediate
	ACLA_007980	CN{7}CCN{36}CN{18}CN{5}CCN{17}C	Ι	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).

	무-	8-	е-	7	۳ ۹-	g-	60	P2	8-	08-	<u>6</u> -	문-	120
A0090012000143_Aspergillus_oryzae_RIB40/1-151 M	Q F SVAA	/LALAT · ·	AVAAL <mark>P</mark> P				- ASGTGAGQQ	VGHSKNDFPI	- PKELTTKQAAD			AQLTCC	ΝΚΤΥΚΤGDFTQVE···
A0090020000588_Aspergillus_oryzae_R/B40/1-145 • • • • M	K · · · F F A V A A I	- F А Т А А М А	APGSAPV				PGAA	AAAGNGNAP	/ I NQ TQQKAF TD		• • • • ACSAKG	NHPVCC	DQIDTSKTTTVNEGLL
JG/128530_Aspergillus_niger_ATCC1015/1-1056	N		SQVRFPV						PGDMTVKQAED			AQLSCC	NKATYAGDSTNVD
JG/35683_Aspergillus_niger_ATCC1015/1-100 · · · · · M	HLKSLLF	LAAST	TLVAAGS						РҮҮСЦМАQD		GTGM-I	QDPYCC	DSF SD TPGD · · · · · · ·
JG/45683_Aspergillus_niger_ATCC1015/1-131 · · · · M	10 F T L T N V I	- ALTLA	VATGVSA					GAV	SDSQAIKTQTEG		Kc D	GNVSCC	NPTNEDKTDGFLN
JG/45685_Aspergillus_niger_ATCC1015/1-132 M	KFAVAA	LGFAM-	TAVAIPA					AKS	SQQKLSIDDASG		0 0 0	GDIYCC	NPTNEEETDGALN
JG/53462_Aspergillus_niger_ATCC1015/1-130 · · · · M	Q F T T A L S	LA ATT/	VASAIPA			ΑΑ	2 D S T A S L K D Q	VKQVAAQRG	I SDGT I STVTEA			PPILCC	SLTTPLDPLVDP
JG/194815_Aspergillus_niger_ATCC1015/1-146 M	K F T A A A	ALALAT	VVVAQPS				Λ	RGRSVTHIP	APAGMTVSQGAD		sccp.v	AQLSCC	VKATYAGDTTDID
JG/43184_Aspergillus_niger_ATCC1015/1-201 · · · · M		. TTLLT.	attel P L			٥ 	PSSSSSSPSS	SSTPSSTLD.	[PDGLSSA AQ	IVDETIAD	SNNSTCPPSN	I R S K O C C	SINDIASQITQP
An 03y 02360_Aspergillus_niger_CB S513.88/1-122 M	KFAVAA	ILGFAM	TAVAIPA					AKS	SQQKLSIDDASG			GDIYCC	VPTNEEETDGALN
An 03g 02400_Aspergillus_niger_CBS513.88/1-131 M	Q · · · F T L T N V I	- ALTLA	VATGVSA					GAV	SDSQAIKTQTEG		· · · · K <mark>C</mark> D · · · I	GNVSCC	VPTNEDKTDGFLN
An 04g 08500_Aspergillus_niger_CB S513.88/1-146 M	K F T A A A	ALALAT.	VVVAQPS				Λ	RGRSVTHIP	APAGMTVSQGAD			AQLSCC	ΝΚΑΤΥΑGDTTDID
An 15y03800_Aspergillus_niger_CBS513.88/1-130 M	QFTTALS	ILAIATT/	VASAIPA			ΑΑ	QDSTASLEDQ	VKQVAAQRG.	I SDGT I STVTEA			IPPILCO	SLTTPLDPLVDP
An 01g 10940_Aspergillus_niger_CBS513.88/1-100 M	HLKSILF	LAAST	TLVAAGS						РҮҮСНМАQD		GTGM-I	QDPYCC	DSF SD TPGD · · · · · · ·
An 07g 03340_Aspergillus_niger_CB S513.88/1-162 M	K · · · F S I T A A	/LAFAA	TVVAMPG			GSPST	3 NGAGNGNGN	DGNSQVRFP	/PGDMTVKQAED		K00D.0	AQLSCC	NKATYAGDSTNVD
An 09y 05530_Aspergillus_niger_CB S513.88/1-202 M	ILLKILTLTTT	- T T L L T	QTTCIPL			SP	SSSPSSPSS	SSTPSSTLD.	r P D G L S S A I A Q I	IVDETIAD	SNNST <mark>C</mark> PPSN	ARSKOCC	SINDIASQITQP
An 08y 09880_Aspergillus_niger_CB S513.88/1-91 M	NFTHILA	/LATIG	LASAAPA						· · · · · · · · NSDPS			AQANSCC.	rslt
AN7539.2_Emericella_nidulans_FGSC_A4/1-109 M	K · · · VATALS	/LAVAGS/	LASAL PS	********	*********	******	*********	********	AANSEKRQS		D C I	I G P L L C C C	SLTTPLDPLVDP
ANBB03.2 Emericella_nidulans_FGSC_A4/1-157	K FSIAAN	/VAFAA	SVAAL P			0 H Y	SQFAGNGVGN	KGNSNVKFP	/PENVTVKQASD		KCGD.C	AQLSCC	ΝΚΑΤΥΑGDTTTVD
AN6401.2 Emericella nidulans FGSC A4/1-162	Q FTIASI	LATAVLO	LQMASAA					PHAPGSSI	VAAIISQAQEQN		T 00N	VAHLSCC	ESTDNSVSLTQEEE
ANB006.2 Emericella nidulans FGSC A4/1-135	R F I VSLL	AFTAAA	TATAL					SAAKNAKLA	SAAFAKQAEGT		· · · · T C N · · · V	GSIACC	NSPAETNNDSLLS
AN1837 2 Emericella nidulans FGSC A4/1-135	K FIGIA	LSIFALA	SADKEPV						PDSITVAFGSS		kogp.o	AOL SCOL	NDVKYGGDTTVV0
ANO40 9 Emained and Jack FGSC 44/4-104	H												
ACT DATE AND ADDRESS AND ADDRE								VEVUDTEN					
ACING 057430 Association functional Adda of 450						2	V N A A C N C V C N						
Ar-UB_080/40_Aspergillus_tumigatus_A1163/1-100_MLVIM		/ F L V · ·						PRGEPULFL	UPSMITEAAR		× ×		VKVVKAGDYISVD
AFUB_051810_Aspergrillus_tumigatus_A1163/1-190 IN		LATLS	TTLAVPF				SQASKSTSAS	RSTSSSTVP	ASEPSPTESGPN		ACPP	KF KO C C	TLSQVGDDLLKP
AFUA_BG07060_Aspergillus_fumigatus_Af293/1-155 ML V T M	R LSRS I A	/FTLVT · ·	YATGLES			• • •	۰۰۰۰ ۲۵۸	PRGEPQLPL	DPSMTIKEAAR		K00D-K	AQLSCC	VRVVKAGDYTSVD
AFUA_5G09580_Aspergillus_fumigatus_Af293/1-159 • • • • M	KFSLSAA	/LAFAV.	SVAALPO			0H	V N A A G N G V G N	KGNANVRFP	/PDDITVKQATE		K00D.0	AQLSCC	VKATYAGDVTDID
AFUA_2G14661_Aspergillus_fumigatus_Af293/1-125 • • • • M	KPSIVTFLML	ANTAA	VSAEDPT				MSALKSR	VEEIAGQVHO	VEDQETTPDLS		л <mark>О</mark> Н Л	/EPKL <mark>CC</mark>	3 S L T T P L D P I L D P
AFUA_1G17250_Aspergillus_fumigatus_Af293/1-140 · · · · M	K · · · FLAVVSI	- L A A	TALALPN					AGVVHPTFA	SADKYTLQQAQN		К <mark>С</mark> СЕ.Н	HTTLSCC	VHVSKVGDTTAFN
AFUA_5G03280_Aspergillus_fumigatus_Af293/1-190 • • • • M	R PITILC	FLATLS.	TTLAV <mark>P</mark> F				SQASKSTSAS	RSTSSSTVP	ASLPSPTLSGPN			NKF KQ C C	TTLSQVGDDLLKP
ATEG_10285_Aspergillus_terreus_NiH2624/1-129 M	KSLIFQI	LAMASVI	VVAAQLF									3GTLKCC	ESVTRYSDLSEEER
ATEG_08089_Aspergillus_terreus_NIH2624/1-177 M	R LAAI	- LALAA -	LTTAVPN			GSSSA	SATSGHSPED	LFTPKPAPTV	VTPKQAAQNEAL	IKQNAKDT	ADPTK <mark>C</mark> PMNH	H P Y Q A C C	RTLTNIADAIFKP
ATEG_07808_Aspergillus_terreus_NIH2624/1-115 M	K F STALSI	-LAAGA-4	LVSAA <mark>P</mark> A	* * * * * * * * *	* * * * * * * * * *	******	* * * * * * * * * *	1 · · · · · · · · ·	SSEDEETYLVKA		· · · · K <mark>C</mark> · · · ·	I K P F L C C	<pre>>ELKTPLDGVVDP</pre>
ATEG_06492_Aspergillus_terreus_NIH2624/1-175 M	KFTAT	ILALAA.	TAVAL PO	IGHGGDH	VQKGAQGDGT	GNANGAT	3 NGAGNGIGN	QGNSQTRFR	APEDMTVQQASK			MMQLSCC	ΝΚΕΤΑΑGDTTDIP
ATEG_04730_Aspergillus_terreus_NIH2624/1-121 M	R TALAII	SSTLSA/	LALANP	********	* * * * * * * * * *	******		AA	RHGAPICPSGTA		λ	SSMQ CO	AVN
AFLA_0946000_Aspergillus_flavus_NRRL3357/1-83 · · · · M	K · · · FLHTIAI	- I A T F T	VASATPA	* * * * * * * * *		******		********	GSTPS		QCTA.A	AQANKCC	roLT
AFLA_131460_Aspergillus_flavus_NRRL3357/1-106 M	R FFTALSI	-FISGA-/	I ASAL PS						· · · · SSETVEA		ν···νον····	/ K P Y L <mark>C C</mark>	<pre>>ELKTPLDSTLDP</pre>
AFLA_060780_Aspergillus_flavus_NRRL3357/1-251 M	EPPTYWIPVI	A S V V V	RITQAAA	HSFSKGKGR.	TITLRNPLAF	KMHSTNI	FNFFMLAVAA	ASAAT I SKA() D S K A L Q K V A E G		K <mark>C</mark> -D-I	GNTACC	NNVHEEKDERLFN
AFLA_014260_Aspergillus_flavus_NRRL3357/1-145 • • • • M	K F F AVAAI	-FATAAM/	APGSAPV	********	* * * * * * * * * *	******	PGAA	AAAGNGNAP	/ I NQ TQQKAF TD		· · · · A C SAKG	NHPVCC	DQIDTSKTTTVNEGLL
AFLA_063080_Aspergillus_flavus_NRRL3357/1-87 M	N · · · IKTILAI	CAMTS	MV T A A P A						EELEKR			CONRF CC	E T F F P V N I F
AFLA_09380_Aspergillus_flavus_NRRL3357/1-217 M	Q F SVAA	/LALAT	AVAAL <mark>P</mark> P				- ASGTGAGQQ	VGHSKNDFPI	-PKELTTKQAAD			AQLTCC	ΝΚΤΥΚΤGDFTQVE···
AFLA_064900_Aspergillus_flavus_NRRL3357/1-91 M	Q · · · F R S V I A I	VAFAT	AVTAAPC					DSC	DGGDSGDSGDSG		KCSP-N	NQELKCC	rettaet
ACLA_001890_Aspergillus_clavatus_NRRL1/1-100 · · · · • M	Q · · · F K S V L A	/FAAAA	VASAAPG			• • • • • •			GGTPS		QCTT.E	QANKCC.	rgLL
ACLA_04BB10_Aspergillus_clavatus_NRRL1/1-182 · · · · · M	HTLALLSLLT	ΓΤLΤVΤ	LTHAIPL			• • • • • •	T N A A N T	GPSSTPSSS/	AALATPTLTGPN			KRSKQCC	rsusqlqtsllbP
ACLA_010960_Aspergillus_clavatus_NRRL1_/1-145 · · · · M	K · · · FALSIA	• - V L A V - •	SVAARPG					SSPQFPII	- P SGMT VQQAAE		K <mark>0</mark> 6D.0	2 A Q L S C C I	VQASMGGDSTNID
ACLA_072820_Aspergillus_clavatus_NRRL1/1-158 · · · · M	K · · · F T V T A A	/LAFAV	SAAAL PO			Q · · · · · · D	FNAAGNGFGN	KGNANVRFP	/РЕЕМТІКОСТЕ			AQLSCC	VKATYAGDSTDID
ACLA_018290_Aspergillus_clavatus_NRRL1/1-126 · · · · M	K · · · F SVVSLI	- ALAAT	VVSAIPT			ш 	EATNAALKSQ	VEELADKVQ	VEEQDTATDLS			I D P L L C C	SLTTPLDPLVDP
ACLA_007980_Aspergillus_clavatus_NRRL1/1-144 · · · · · M	Q FLAVVSI	LAA	TALAL <mark>P</mark> P				ATGSQ	APNVHPVFDI	NANQYTL SQAQN			KTTLS <mark>CC</mark> I	NHVSSVGDTTSVN

	130	140	150	160	170	180	190	200	210	220	230	240	250
A0090012000143 Aspervillus oryzae RIB40/1-151	EGLLAG-LL	SN LLGA C	COSCO LGL	LDECTNIPV	IPIISIASPO	EKC.	KOPISCO	NTKSSADGD		ΓΛ(SIGLP <mark>C</mark> IALG	SLL	
A0090020000588_Aspergillus_oryzae_RIB40/1-145 G(3 L L G E G L G G V L	-NNLVGGEF	GACSGLVSA	Γ Ν Ν			QTSIGCCQ	QNAKGDNYQSG	L	NL	NLQAP <mark>C</mark> LLSN	GL	
JG/128530_Aspergillus_niger_ATCC1015/1-105	SGLLAG-TL	SNLIGT-C	SGSEG-LGL	FQECSKLPI(Q-IPIIGIAV	QDIISKQC.	QQNIACC	SSPS					
JG/35683_Aspergillus_niger_ATCC1015/1-100		SIAKV6	9 K N C Q S M D G L	EWT		DQC.	РД66ТVK <mark>сс</mark> Ү	* * * * * * * * * * *		1 L T I (ЗРДГІ <mark>с</mark> таеа	EENTDDD	
JGI45683_Aspergillus_niger_ATCC1015/1-131	· · NLLEWG · V	IGS LVNGQC	SACAP - ISL	IDELGILAL	VKDTPDG	PVC.	ENVIACO				SQGAQ <mark>C</mark> VAIG	DGSGSGSGYSD.	
JGI45685_Aspergillus_niger_ATCC1015/1-132	NALREGLL	IGS LVNGK(9 SACAP - TSL	IGSLNLLAF	FEEGEDDDK.	SYC.	KNVIACC	GGNLAD		· · · · · · · LE	F RKEQ <mark>C</mark> AALD	6 Y	
JG/53462_Aspergillus_niger_ATCC1015/1-130		ASN IVGSIC	SLLCKDYD			· · · · · D S C ·	TSAPOCCT			9N NG	LVALGCSSLE		
JG/194815_Aspergillus_niger_ATCC1015/1-146		LSN - LLGA - C	SSGSDG-1GL	ADECSPLDL	GALDLVGLQN		EQTAACCO	GSGSDTEGN			3 VGLPCLALG	SLL	Maldona
4009-003-200 Amomilius miner CP 5543 00/4429		START SOLATER				3 0				3			
Anoguzaou_Asperginus_inger_copport30001-122 AnO24000 Amomiliun minor CP 5543 00/4-434					VKDTDD6								
Anogozzao_Apergmus_mger_colorizao risi Anogo08500 Asnemillus niger CBS513 88/1-146	··SGFISG·VI	SNLGA-6	356506 - 16L	ADECSPIDIC	DAL DI VGI DN		- FOTAACOO	GSGSDTEGN			S C C C C C C C C C		
An 15003800 Aspervillus niger CB 5513.8871-130	· · ILLDLGID/	ASN IVGSIC	HLCKDYD.			· · · · D S C ·	- TSAPOCCT	EINLL·····		5N	LVALGOSSLE		
An 01g 10940_Aspergillus_niger_CBS513.88/1-100		SIAKV6	KNCQSMDGL	EWT		DQC.	PQGGTVK <mark>CC</mark> Y			1 L	3 P Q F I <mark>C</mark> T A E A	EENTDDD	
An 07g 03340_Aspergillus_niger_CB S513.88/1-162	· · SGLLAG · TL	SNLIGT-C	SGSEG - LGL	FQECSKLPI(Q-IPIIGIAV	QDIISKQC.	QQNIACCO	SSPSTTDDD			glglp <mark>c</mark> ialg	SLVSL	
An 09g 05530_Aspergillus_niger_CB S513.88/1-202	LGMLIP-W	IGG VD ISSVL(FLDCTGMSDD	T P N		DQC.	SHQVMCCD	GQPNSEGS		so	PISSA <mark>C</mark> KPFD	DAIKDKNDAIND	SKQRESM
An 08y 09880_Aspergillus_niger_CB S513.88/1-91		NVNVLF	PALCVPLV.			G N C .	NNQAACCE	TNGIPDTHG		cs	26 L L N <mark>C</mark> L T V Q	۰ν	
AN7539.2 Emericella_nidulans_FGSC_A4/1-109		AAA - IVGSVO	3 L L C H A Y D			DTC.	TSEPQCC	EANLL		66	TLGLG <mark>C</mark> GPLE	Н6Нн6	
ANBB03.2_Emericella_nidulans_FGSC_A4/1-157	EGLLSG-AL	-SGLIGA-(9 SGAEG - LGL	F DQ C S K L D V,	AVLIGIQDLV	N 0 K C	KQNIACC	NSPSSADGN			3 VG L P C V A L G	s L	
AN6401.2_Emericella_nidulans_FGSC_A4/1-162	EGLLHL	LGGTS;	SVLSDGLLGK	YSGCSSLAS	VEGILGAGGN	QGLVSGQC.	NNHVACCD	AGDNELVGFLE	PGVAVLKPNS	SDGIQNGLA	NVAVPCVPVQ	۸Γ	
AN8006.2_Emericella_nidulans_FGSC_A4/1-135	GLLGAG-LI	-NG L SGNT(9 SACAK - ASL	DQLGLLAL	VDHTEEG	PVC.	KNIVACCP	EG			TTNCVAVD	NAGAGTKAE	
AN1837.2_Emericella_nidulans_FGSC_A4/1-135	KGIAAG-LL	SDLLGA-(9 SAAEG - LGA	FSGCSKLDI	SLLIGIEDIL	N 0 K C .	KQNIACCA	KSGGSADGD		LV	3ATLPCIALG	S I L	
AN0940.2_Emericella_nidulans_FGSC_A4/1-101		KVSFE(VNCIDVSSD	EDFV····		KT C -	P K G G T P K <mark>C C</mark> Y			s.	3 P K V I C T T E V	GDGVDK	
AFUB_016640_Aspergillus_fumigatus_A1163/1-140 .	··· YGLLNG···	· · · · · LLGNA	ISGPEG - VGI	LSGCQKISV	TALIGVDDLL	N KQ C -	Q Q N V A C C Q	DNKSVATGGL -		N · · · · · · ·	IATPACVALD	S	
AFUB_057130_Aspergillus_fumigatus_A1163/1-159 .	· · EGILAG · TL	- KN L 166 - (SGTEG - LGL	FNQCSKLDL(Q - I P I I G I P I	α D L V N α K <mark>c</mark> .	KQNIACCQ	NSPSDASGS			glglp <mark>c</mark> ialg	S I L	
AFUB_080740_Aspergillus_fumigatus_A1163/1-155 .	· · EGIGAG · LI	SNLAGG-	956155-1LA	FDQCSRLDA	Q - VPVVLLPI	adllnah <mark>c</mark> .	KQNVACC	KNPGDASSS		GV	3 V S L P <mark>C</mark> I A L G	SVA	
AFUB_051810_Aspergillus_fumigatus_A1163/1-190	· · LGAVVP · L/	VGAIQVNSLV(VSCRPMADA	APE		STC.	GNAVMCCD	SSTVGG		0 0 · · · · · · D D	LMQTS <mark>C</mark> QDFA	LAKKREREAIER	QQRRFSE
AFUA_BG07060_Aspergillus_fumigatus_Af293/1-155	· · EGIGAGLL	SNLAGGGS(9 I S S I L A F D Q	CSRLDAQVP	VVLLP IQDLL	N Ω H C -	KQNVACC	KNPGDASSS		GV	3 V S L P <mark>C</mark> I A L G	sva	
AFUA_5G09580_Aspergillus_fumigatus_Af293/1-159 .	· · EGILAG · TL	. KN L 166 - (3 SG TEG - LG L	FNGCSKLDL(Q - I P I I G I P I	α D L V N α K <mark>c</mark> .	KQNIACC	NSPSDASGS			9 L G L P <mark>C</mark> I A L G	S I L	
AFUA_2G14661_Aspergillus_fumigatus_Af293/1-125 .	· · I LL SLGIN/	AAQ - IVGSV(9 L L C H P W T			EEC.	SSAPQ <mark>CC</mark> T	EANLL		99	TLALG <mark>C</mark> SKL -		
AFUA_1G17250_Aspergillus_fumigatus_Af293/1-140	··· YGLLNG···	LLGNA	ISGPEG - VGI	LSGCQKISV	TALIGVDDLL	N KQ C -	QQNVACC	DNKSVATGGL -		N • • • • • • •	IATPA <mark>C</mark> VALD	S	
AFUA_5G03280_Aspergillus_fumigatus_Af293/1-190 ·	· · LGAVVP · L/	VGAIQVNSLV	<pre>>VSCRPMADA</pre>	APE·····		STC.	GNAVMCCD	SSTVGG		0 0 · · · · · · D D	LMQTSCODFA	LAKKREREALER	QQRRFSE
ATEG_10285_Aspergillus_terreus_NiH2624/1-129	· · NDYGLDPDL	NDANICDN-C	SLVEDEFDL			A I C D	SVDHVPQCCL	AFVPT·····		RA	LARTQ <mark>C</mark> DRTT	DEDTGPKAPGSY	GIDTDLT
ATEG_08089_Aspergillus_terreus_NIH2624/1-177		KGANLASPM	SFECKPMTPG	D P P		EKC.	AHQASCCG	SDPKEA		SA	IDINSCTDYR	EEMNKTIEALER	NKKMHSR
ATEG_07808_Aspergillus_terreus_NH2624/1-115	I LEALHLG	PEA IVGSIC	<pre>>LACHAYD</pre>			ETC.	TTAPKCCS	EANLL		DG	TVALG <mark>C</mark> NDLA	NPADAL	
ATEG_06492_Aspergillus_terreus_NIH2624/1-175	DGPLAD-AL	- KG VLGGA	SGSEG-LGL	LEQCGKLDI	IPIISDLIN-	KHC-	DAKAACC	DSGSSADNS			3 L N L P <mark>C</mark> I A L T	SLI	
ATEG_04730_Aspergillus_terreus_NH2624/1-121	• • • • • • • • •	VADVV;	SLDCQPPGPL	PSSAAEFK.		E C A	KIGDKPQCCT	۲Α		LA(90AIACONPA	GMDEDSGSSESE	SDSGSGG
AFLA_0946000_Aspergillus_flavus_NRRL3357/1-83	NG I L	· · · · · · N L N V L I	PALCLPLV.		•	GSO.	NNQAACCE	Τ		9N NG	IGLLNCLTVQ	ν	
AFLA_131460_Aspergillus_flavus_NRRL3357/1-106	· · I L L D L G I D /	AAS I VGSV(9 L L C H A W D			ETC.	ETGPKCCT	EANLL		66	TVALG CSDLA	TED	
AFLA_060780_Aspergillus_flavus_NRRL3357/1-251	LVKQG-L	IDI LAGNEI	0 YACAK - SGV	IDE - WNLFS.	LVKQTNDG	PVC	· · KNVTACCS	se			K <mark>C</mark> VAID	G S A E K K K E S D K E	DDKEDDK
AFLA_014260_Aspergillus_flavus_NRRL3357/1-145 G(GLLGEGLGGVI	NN LVGGE	GACSGLVSA	r N				QNAKGDNYQSG		· · · · · · · · NLI	NLQAP <mark>C</mark> LLSN	6L	
AFLA_063080_Aspergillus_flavus_NRRL3357/1-87		FIRAV(SNCIAQKT -			GNC.	P T G T T E H C C T	SNLP		LRI	NGDLA <mark>C</mark> TQL ·		
AFLA_09380_Aspergillus_flavus_NRRL3357/1-217	··EGLLAG·LI	SNLLGA-(306506 - LGL	LDECTNIPV	I P I I P I A S P Q	EQ.	KQPISCCQ	NTKSSAVSRIN	ΑαΥΤαΙSLT	I TGWRPRRY	NSSLHRSRLS	PVSDCIREIGSS	RSTGWKM
AFLA_064900_Aspergillus_flavus_NRRL3357/1-91		· · · · · NLGIL	- ALCLPLL.				NNQAACCE			AK	GGLLNCLTIQ	Γ	
ACLA_001890_Aspergillus_clavatus_NRRL1/1-100	NGLL	· N I N I L I	PALCLPLV.			GT <mark>C</mark> .	NNQAACCE	SNGIVSRNRII	GHQTADE	S	KGLLN <mark>C</mark> LTIQ		
ACLA_048810_Aspergillus_clavatus_NRRL1/1-182	VGNVIP-LL	SGMQLTSLI	GLSCRTMSDE	HTK		TDC.	ADALMCCD	AAGEVGA		No	I LQ TKCEEFD	LAKAKEIAAVER	AQGQPSS
ACLA_010960_Aspergillus_clavatus_NRRL1_/1-145	QGIAAG-LI	-KD - VLGG - (SVNQG - VNL	FHQCAKLDL	Q-IPIIGVPI	QDALNORC-	- KONVACCO	APKADASND		0.0	ALSLPCIALG	SVI	
ACLA_072820_Aspergillus_clavatus_NRRL1/1-108		- KN L 166 - C	9 S G H Q G - L G L	FDGCSKLDL	0 - 1 F I I G I F I			KSTADASGS			3 LGLPUVALG	S L	
ACLA_018290_Aspergillus_clavatus_NRRL1/1-126	1 L L G L G I N J	AAE IVGSI(GLCHAWT.			Р. 	· · SSAPQCCT	EANLL		99	AVALGCSKL-		
ACLA_00/380_Asperginus_cravatus_nnmi / 1 - 1 + +	YGLLNG		- 26766 - 461	רצפטוארעע	ALIGVNULL	N	300 V V 00 0	6 E 0 A 66		×	NLALFUVALG		

	260	270	280	290	300	310	320
A0090012000143_Aspergillus_oryzae_R/B40/1-151							
A0090020000588_Aspergillus_oryzae_R/B40/1-145							
JG/128530_Aspergillus_niger_ATCC1015/1-105							
JG/35683_Aspergillus_niger_ATCC1015/1-100							
JGH2683_Aspergillus_niger_A/GC1015/1-131 JGH5685_Assemillus_nimer_ATCC4045/4-432							
JGH3969_Asperginus_niger_ALCC1013/1-132 JGH53462_Aspervillus_niger_ATCC1015/1-130							· · ·
JG/194815 Aspergillus niger ATCC1015/1-146							
JGI43184_Aspergillus_niger_ATCC1015/1-201	LVAQASASASASA	SASASASASAS	ASAGAS				
An 03g 02360_Aspergillus_niger_CB S513.8871-122							
An 03g 02400_Aspergillus_niger_CB S513.88/1-131							
An 04g 08500_Aspergillus_niger_CB S513.88/1-146							
An 15g03800_Aspergillus_niger_CB 5513.88/1-130							
An 01g 10940_ Aspergillus_niger_GB 5513.8871-100 An 07m 03340_Assemillus_nimer_GB 5513 8841-462							
An 09a 05530 Aspervillus niger CB 5513.8841-202	LVAQASASASASA	SASASASASA	ASAGAS				
An 08y 09880_Aspergillus_niger_CB S513.88/1-91							
AN7539.2_Emericella_nidulans_FGSC_A4/1-109							
AN8803.2_Emericella_nidulans_FGSC_A4/1-157							
AN6401.2_Emericella_nidulans_FGSC_A4/1-162							
AN8006.2_Emericella_nidulans_FGSC_A4/1-135							
AN1837.2_Emericella_nidulans_FGSC_A4/1-135							
AN0940.2_Emericella_nidulans_FGSC_A4/1-101							
AFUB_016640_Aspergillus_fumigatus_A1163/1-140							
AFUB_057130_Aspergillus_fumigatus_A1163⁄1-159							
AFUB_080740_Aspergillus_fumigatus_A1163/1-155							
AFUB_051810_Aspergillus_fumigatus_A1163'1-190	YQRMMLSQSATPA	PISTGVDVMO	SSFSKAKATP	TRV			
AFUA_8G07060_Aspergillus_fumigatus_Af293/1-155							
AF-UA_5G09580_Aspergillus_tumigatus_Af293/1-159							
AFUA_2014661_Aspergillus_1umigatus_A1293/1-120 AFUA_4047750_Annomillus_turin-turi A59624_440							
AFUA_101/200_Aspergillus_1umigatus_Af293/1-140 AFUA_6603390_Annomillun_fumicretur_Af393/4490	VODMMI SOSATDA	DTCTCVDVDV		T D V			
ATEG 10285 Assemillus terreus MH2624/1-129	POLLSKD						
ATEG 08089 Aspervillus terreus NIH2624/1-177	AVVLPVYV						
ATEG 07808 Aspergillus terreus NIH2624/1-115							
ATEG_06492_Aspergillus_terreus_NIH2624/1-175							
ATEG_04730_Aspergillus_temeus_NIH2624/1-121	GGGDA						
AFLA_0946000_Aspergillus_flavus_NRRL3357/1-83							
AFLA_131460_Aspergillus_flavus_NRRL3357/1-106							
AFLA_060780_Aspergillus_flavus_NRRL3357/1-251	EDDREDDKEGDNE	GDNEGDDKEG	DNEDDNEDDN	QKDKRGDHDD	у ЧНК G НК V D К	CD KENKHEKD	ЕЕРНGRY
AFLA_014260_Aspergillus_flavus_NRRL3357/1-145							
AFLA_063080_Aspergillus_flavus_NRRL3357/1-87							
AFLA_09380_Aspergillus_flavus_NRRL3357/1-217	AMKCFHSVLFCVL	YWLLVSWNPF	SEKTVLFAF.				
AFLA_064900_Aspergillus_flavus_NRRL3357/1-91							
ACLA_001890_Aspergillus_clavatus_NRRL1/1-100							
ACLA_048810_Aspergillus_clavatus_NRRL1/1-182	AARMFGASSSSVV	LGSGSGSGP/	ATPTPTRR				
ACLA_010960_Aspergillus_clavatus_NRRL1_/1-145							
ACLA_072820_Aspergillus_clavatus_NRRL1/1-158							
ACLA_018290_Aspergillus_clavatus_NKHL1/1-126							
ACLA_007980_Aspergillus_clavatus_NKHL1/1-144							

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

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Interactions between *Aspergillus fumigatus* and *Pseudomonas aeruginosa* are mediated by soluble chemical mediators involved in the RpoN pathway

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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 1×10^7 – 1x10⁹ 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-offunction 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 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-1carboxylic acid production was only slightly increased. P. aeruginosa phenazine-1carboxylic 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-1carboxamide 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 = $100 \mu g/ml$) 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 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 Fe³⁺ 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.

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 μ g/cm², while the remaining strains produced levels varying from 0.04 – 0.19 μ g/cm². 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 noninhibitory 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-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 μ g/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], *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 1×10^6 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 1×10^8 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 μ m, 10 cm, 2mm ID Luna C₁₈ column using a 15 \rightarrow 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 C₁₈ column (150 x 2.1 mm, 2.6 μ m; Phenomenex, Torrence, CA) using a linear water-acetonitril gradient starting from 15 % CH₃CH to 100 % over 7 min at a flow rate of 0.8 ml/min. Both water and CH₃CH were buffered with 50 ppm trifluoroacetic acid. HPLC-DAD-TOFMS confirmation was performed on an Agilent 1100 system using a Luna C₁₈ 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 (CH₃CH) gradient starting from 10 % CH₃CH to 100 % over 7 min at a flow rate of 0.4 ml/min. Both water and CH₃CH 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 μ l 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 μ l methanol and

ultrasonicated for 10 min. Extracts were finally filtered through a PFTE 0.45 μ m 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.

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Tables

		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 1. Comparison of clinical and environmental A. fumigatus strains

Table 2. Fungal strains used in this study.

Strains	Description	Source or reference
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Aspergulus oryzae RIB40	Full-genome sequenced, from cereal	CMB, IB1 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 60 °C, followed by incubation at 37 °C, *A. 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*.



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).

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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).

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