Nitrogen metabolism in *Aspergillus fumigatus* with emphasis on the oligopeptide transporter (OPT) gene family.

Stickstoffmetabolismus in *Aspergillus fumigatus* mit Schwerpunkt auf der Oligopeptidtransporter (OPT) Genfamilie.

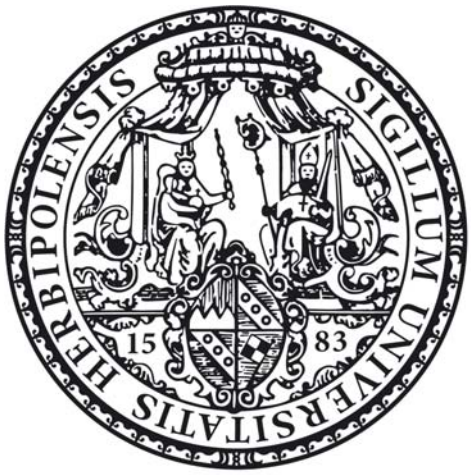

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TABLE OF CONTENTS

1. SUMMARY

The saprophytic filamentous fungus *Aspergillus fumigatus* has been gaining importance as an opportunistic human pathogen over the past decades. Advances in modern medicine have created a growing group of patients susceptible to infection with *A. fumigatus*, often contracting potentially deadly invasive aspergillosis. The virulence of this pathogen appears to be a multifactorial trait, a combination of physiological characteristics that enables the fungus to infect immunocompromised humans.

This work concentrates on the nitrogen metabolism of *A. fumigatus*, which is essential for meeting the nutritional needs inside the human host. Using DNA microarrays, the transcriptional response during growth on three different secondary nitrogen sources was examined, which revealed the metabolic versatility of *A. fumigatus*, especially when challenged with proteins as the sole source of nitrogen. In-depth transcriptional profiling of the eight-member oligopeptide transporter (OPT) gene family underlined the importance of oligopeptide transport for growth on complex nitrogen sources like BSA or collagen.

Heterologous expression of the *opt* genes in *Saccharomyces cerevisiae* showed their functionality as oligopeptide transporters, and characterized their substrate specificity. Using a Cre/*loxP* based genetic tool, a complete deletion of all *opt* genes in *A. fumigatus* was achieved. The resultant strain exhibited diminished growth on medium where the oligopeptide GPGG was the sole nitrogen source, but did not show any other *in vitro* phenotype. The *opt* deletion strain was not attenuated in virulence in a murine model of pulmonary aspergillosis, suggesting that the OPT gene family is not necessary for successful infection. The connection of oligopeptide transport and extracellular proteolytic activity was investigated by deleting the genes encoding Dpp4 and Dpp5, two dipeptidyl peptidases, or PrtT, the transcriptional regulator of major secreted proteases, in the complete *opt* deletion background. In contrast to the deletion of *dpp4* and *dpp5*, which did not result in any additional phenotype, the absence of *prtT* led to a drastic growth defect on porcine lung agar. This suggests a synergistic action of extracellular proteolytic digest of proteins and transport of oligopeptide degradation products into the cell.

Finally, this work established the bacterial β-Rec/*six* site-specific recombination system as a novel genetic tool for targeted gene deletion in *A. fumigatus*.

ZUSAMMENFASSUNG

Bedingt durch die medizinischen Fortschritte der vergangenen Jahrzehnte, hat sich die Zahl der Infektionen mit dem saprophytischen Schimmelpilz *Aspergillus fumigatus* drastisch erhöht. Die Virulenz von *A. fumigatus* für immungeschwächte Personen scheint hierbei auf einer Kombination an physiologischen Merkmalen und Fähigkeiten des Pilzes zu beruhen, weniger auf spezifischen Virulenzfaktoren.

Diese Arbeit widmet sich dem Stickstoffmetabolismus von *A. fumigatus*, welcher essentiell für die Ernährung des Pilzes innerhalb des menschlichen Wirtes ist. Mittels DNA Microarrays gelang es die Reaktion des Pilzes auf das Vorhandensein dreier sekundärer Stickstoffquellen auf transkriptioneller Ebene zu erforschen, wobei sich besonders in Gegenwart von Protein die metabolische Vielseitigkeit von *A. fumigatus* zeigte. Tiefergehende transkriptionelle Studien der Oligopeptidtransporter (OPT) Genfamilie unterstrichen die Relevanz des Oligopeptidtransportes, während des Wachstums auf komplexen Stickstoffquellen wie BSA oder Collagen.

Expression der *opt* Gene in *Saccharomyces cerevisiae* half deren Funktionalität als Oligopeptidtransporter und deren Substratspezifität zu untersuchen. Mittels eines Cre/*loxP* basierten Systems gelang es, sämtliche 8 *opt* Gene in *A. fumigatus* zu deletieren. Der daraus resultierende Stamm zeigte vermindertes Wachstum auf Medium mit dem Oligopeptid GPGG als einziger Stickstoffquelle, wuchs sonst allerdings wie der Wildtyp. Der Stamm zeigte keine verminderte Virulenz in einem Mausmodell für pulmonale Aspergillose, was darauf hindeutet, dass die OPT Genfamilie für einen erfolgreichen Infektionsverlauf nicht von nöten ist. Durch Deletion im OPT defizienten Stammhintergrund, entweder der zwei Dipeptidylpeptidasen Dpp4 und Dpp5, oder des transkriptionellen Regulators einiger zentraler sekretierter Proteasen PrtT, wurde die Verbindung zwischen Oligopeptidtransport und extrazellulärem Proteinabbau untersucht. Während die Deletion der Dipeptidylpeptidasen zu keinem weiteren Wachstumsphänotyp führte, resultierte das Entfernen des *prtT* Gens in einem drastischen Wachstumsdefekt auf einem Lungenagarmedium. Dies legt den Schluss nahe, dass sekretierte Proteasen und Oligopeptidtransporter synergistisch zusammenwirken, um extrazelluläres Protein als Nährstoffquelle zu erschließen.

Schlussendlich gelang es in dieser Arbeit ebenfalls, das bakterielle β-Rec*/six* basierte Rekombinationssystem als genetisches Werkzeug zur gezielten Genmanipulation von *A. fumigatus* zu etablieren.

2. INTRODUCTION

2.1. *Aspergillus fumigatus*

The mold *Aspergillus fumigatus* is a filamentous fungus that belongs to the phylum Ascomycota and it is ubiquitous in nature. As a saprophytic microorganism it plays an important role in the recycling of nitrogen and carbon from dead organic matter. Thus, its main habitat is the soil, but it can colonize a wide variety of ecological niches, such as compost or hay stacks (Mullins *et al.*, 1976; Latge, 1999).

It was long thought that *A. fumigatus* was a member of the *fungi imperfecti*, a deuteromycete lacking a sexual cycle. This view has been changed with the recent discovery of its sexual form, which was named *Neosartorya fumigata* (O'Gorman *et al.*, 2009). Besides this, *A. fumigatus* mainly propagates *via* its asexual lifecycle, whose hallmark is the production of great numbers of asexual spores, so called conidia. These conidia are grey-green in colour, covered with a hydrophobic coat and are relatively small, being 2 to 3 μm in diameter. They harbour a single haploid nucleus and are shed from the tips of conidiophores, which are specialized structures within the mycelial mass. These conidiphores, which are often distinct for a given species, extend at a right angle from a foot cell and contain hundreds of conidia at their tips. There is no active mechanism for spore dispersal, *A. fumigatus* relies on air currents to dislodge and carry away the conidia. When a conidium reaches a permissable environment it may germinate and in time form a whitish mycelium, which defines the vegetative growth state. The mycelium is made up from a net of hyphae, which elongate at their tips and branch at regular intervals. The hyphal tube is septated and each compartment typically contains several nuclei (Latge, 1999; Brakhage and Langfelder, 2002).

2.2. Diseases caused by *A. fumigatus*

In contrast to the vast majority of saprophytic filamentous fungi, *A. fumigatus* can cause disease in humans. While it is possible for immunocompetent persons to aquire aspergillosis, immunocompromised humans represent the main pool of patients (Ellis, 1999). It is this growing group of at-risk people that has most contributed to the rise of *A. fumigatus* to become a major fungal pathogen during the past two decades (McNeil *et al.*, 2001; Tong *et al.*, 2009).

The most dangerous and potentially life threatening form of disease is Invasive Aspergillosis (IA). The immune system of these patients is often impaired by conditions such as leukaemia, chronic granulomatous disease, neutropenia or prolonged steroid treatment after a solid organ transplantation. The main route of transmission is the lung, as the *A. fumigatus* spores are small enough to reach the alveoli and can start colonizing the host (Invasive Pulmonary Aspergillosis). Another common entry point are the nasal cavities and the sinuses (Acute Invasive Rhinosinusitis). From the initial site of infection the disease can disseminate and infect other organs like the kidneys, the liver or the brain, with the central nervous system being the most common site of secondary infection. The spread of the disease is helped by the angioinvasive nature of *Aspergillus*, which leads to invasion of blood vessels and dissimination throughout the body. The mortality of proven invasive aspergillosis is between 60% and 90% depending on the pre-existing condition of the patient and the site of infection (Brakhage and Langfelder, 2002; Clemons *et al.*, 2005; Hope *et al.*, 2005; Singh and Paterson, 2005; Latge and Steinbach, 2009).

In addition to this most severe form of aspergillosis there are other diseases caused by *A. fumigatus*: allergic bronchopulmonary aspergillosis (ABPA), chronic pulmonary aspergillosis and the aspergilloma ("fungus ball").

ABPA is a hypersensitivity disease caused by an abnormal inflammatory response to the conidia of *A. fumigatus*, which, if left untreated, can lead to severe lung damage. It is often a complication of classic asthma and cystic fibrosis, and has its root cause in a misdirected, predominantely T_h2 $CD4^+$ immune response to *Aspergillus* antigens (Stevens *et al.*, 2000; Greenberger, 2002).

In chronic pulmonary aspergillosis patients, *A. fumigatus* manages to establish itself in a body niche less accessible to the immune system, and the infection can remain asymptomatic for years. In time the *Aspergillus* colony can grow in size until it

becomes a macroscopic aspergilloma, made up from hyphal matter, fibrin, mucus and cellular debris. Aspergillomas in the lung are typical for patients who have an underlying pulmonary disease such as cystic fibrosis or cavitary tuberculosis. In these cases the fungal infection is limited to a ball of mycelium, situated in cavities inside the lung, which can develop into invasive pulmonary aspergillosis if the immune system is further weakened, but often does not. Aspergillomas can also form in other body cavities, such as the ear canal or the sinuses (fungal sinusitis), which can lead to the destruction of facial bones and in unfortunate cases spread to the brain and lead to cerebral aspergillosis (Stevens *et al.*, 2000).

Treatment of aspergillosis is hampered by the fact that there are only few drugs available. One is amphotericin B, which works by binding to the ergosterols of the fungal cell membrane, disrupting its function as a barrier to the extracellular environment. The major drawback of this drug is its high toxicity, as it also binds cholesterol, thus damaging mammalian cells as well (Clements and Peacock, 1990; Brajtburg and Bolard, 1996). Another class of drugs are the triazoles, which inhibit ergosterol biosynthesis. Against *A. fumigatus* itraconazole, voriconazole and posaconazole have proven useful. These drugs have the advantage to be more fungus-specific and less toxic to mammalian cells. Disadvantages are the evolution of resistance, especially against itraconazole, and the fact that the triazoles are merely fungistatic, unlike amphotericin B, which is fungicidal (Denning *et al.*, 1997; Pfaller *et al.*, 2002; Espinel-Ingroff *et al.*, 2005; Meletiadis *et al.*, 2007).

2.3. Virulence factors of *A. fumigatus*

In addition to being an ubiquitous, saprophytic organism that degrades mostly plant material, *A. fumigatus* is an opportunistic, fungal pathogen that is able to infect and kill primarily immunocompromised patients. This places *A. fumigatus* within a small group of fungi that are able to colonize and infect humans, which raises the question what enables it to do so. In contrast to many other human pathogens, *A. fumigatus* seems to lack dedicated virulence factors, molecular functions that distinguish it from non pathogenic fungi. Rather it seems that it has a number of traits that in combination make *A. fumigatus* virulent under certain circumstances (Tekaia and Latge, 2005; Askew, 2008)(see Fig. 1).

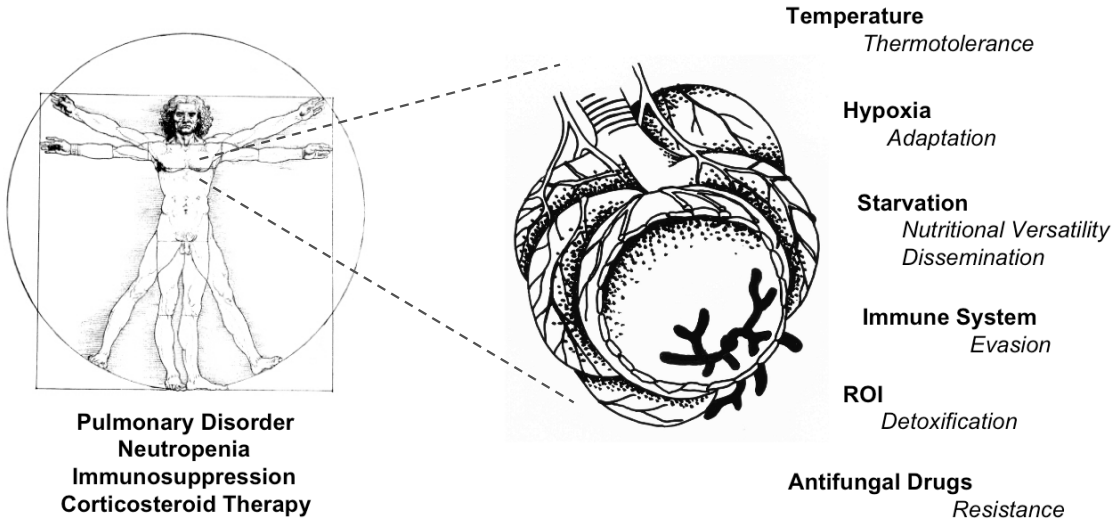

Fig. 1: Interdependence of host and fungal factors during Invasive Aspergillosis
On the left are human risk factors for developing invasive aspergillosis. On the right are stress factors that *A. fumigatus* encounters when colonizing the lungs (bold) and the fungal response to each stress (italic). ROI refers to reactive oxygen intermediates.

For one, *A. fumigatus* is quite thermotolerant, growing very well at 37°C and being able to grow even at 55°C. This sets it apart from other *Aspergilli*, like *A. niger* or *A. flavus* (Maheshwari *et al.*, 2000). There are a number of genes that have been shown to be necessary for thermotolerant growth such as the ribosome biogenesis protein CgrA, the O-mannosyltransferase Pmt1 or ThtA, a protein of unknown function (Bhabhra *et al.*, 2004; Chang *et al.*, 2004; Zhou *et al.*, 2007). Only the *cgrA* deletion showed impaired growth and virulence at 37°C, and overall thermophyly appears to be a characteristic that cannot be narrowed down to a small number of factors, but is a general feature of *A. fumigatus* proteins.

Another factor that has been associated with virulence is pigmentation. One important pigment is melanin, which has been recognized as a virulence factor as it quenches reactive oxygen species and protects against damage by macrophages and neutrophils (Jahn *et al.*, 2000; Langfelder *et al.*, 2003). A deletion of *pksP*, which is central to melanin synthesis, results in white coloured spores, an altered conidial morphology, which leads to a more rapid ingestion by human neutrophils, and it shows reduced pathogenicity compared to wildtype (Langfelder *et al.*, 1998; Tsai *et al.*, 1998; Langfelder *et al.*, 2001). On the other hand, deletion of *abr2*, also involved in that biosynthetic pathway, did not lead to attenuation of virulence, somewhat questioning the significance of pigments in the infection process again (Sugareva *et al.*, 2006).

In the lungs, *A. fumigatus* is attacked by immune cells, which use reactive oxygen intermediates (ROI) as a defence mechanism. Thus, resistance against this

stressor might be a feature favouring *A. fumigatus* when colonizing the human host. A number of genes have been shown to have an effect on sensitivity to oxidative stress, such as catalases, the PKA regulatory subunit PkaR or the transcription factor Yap1. While a double deletion mutant of *cat1/cat2* was only mildly attenuated in virulence and a deletion of *yap1* did not have any effect, a *pkaR* deletion did show a significantly reduced virulence in an immunosuppressed mouse model (Paris *et al.*, 2003; Zhao *et al.*, 2006; Lessing *et al.*, 2007). Fungal superoxide dismutases (SODs) detoxify superoxide anions, and as such support *A. fumigatus'* survival under conditions of oxygen stress. There are four SODs in *A. fumigatus*, one of which (*SOD4*) is essential. While deletion of the other three *SOD* genes leads to a heightened sensitivity to menadione and an increased sensitivity to killing by alveolar macrophages of immunocompetent mice, there was no significant difference in virulence between the triple deletion mutant and the wildtype in murine aspergillosis models in immunocompromised animals (Lambou *et al.*, 2010). Thus, the role of the anti-oxidant reponse of *A. fumigatus* in its virulence remains ambiguous, but it does not seem to play a dominant role (Askew, 2008).

Microbial infection sites often turn into a hypoxic microenvironment, due to tissue necrosis and the influx of immune effector cells. It could be shown, that *A. fumigatus* adapts to this environment using the sterol-regulatory element binding protein SrbA, which transcriptionally regulates genes associated with sterol biosynthesis and hyphal morphology. An *srbA* deletion mutant is unable to grow under hypoxic conditions, shows increased susceptibility to azole class antifungal drugs, and most significantly, is avirulent in murine models of invasive pulmonary aspergillosis (Willger *et al.*, 2008; Wezensky and Cramer, 2010).

When dealing with the host immune system, other fungal factors might also be important. One is secondary metabolites that directly influence the immune response. The best-studied example is gliotoxin, which can be detected in sera from patients with aspergillosis. Gliotoxin has immunosuppressive and cytocidal properties, as it inhibits phagocytosis by macrophages and can induce their apoptosis. The disruption of the *gliP* biosynthetic gene had no effect on virulence in neutropenic mice. However, when a different immunosuppressed mouse model was used, one which was non-neutropenic, the *gliP* deletion mutant was significantly less virulent than the wildtype, indicating that neutrophils are the major target of this mycotoxin (Kamei and Watanabe, 2005; Lewis *et al.*, 2005; Kupfahl *et al.*, 2006; Orciuolo *et al.*, 2007; Sugui *et al.*, 2007). Another secondary metabolite investigated is hemolysin, which can also be detected in infected

patients and which can disrupt blood cells. However, it could not be established that it is indeed a virulence factor (Ebina *et al.*, 1985; Malicev *et al.*, 2007). More generally, the disruption of *laeA*, a global regulator of secondary metabolite production, led to hypovirulence in mice. As LaeA influences roughly 10% of the *A. fumigatus* transcriptome, this effect is much too general to draw definite conclusions about the role of secondary metabolites in the infection process (Bok *et al.*, 2005; Perrin *et al.*, 2007).

When growing inside the human body, any pathogen has to find the nutrients to support its growth. There is a growing body of evidence that suggests that its metabolic versatility is key to *A. fumigatus'* success as a pathogen. One key nutrient is iron, which *A. fumigatus* acquires using siderophores. These were shown to be important *in vivo*, as siderophores like SidA are essential for successful infection (Schrettl *et al.*, 2004; Schrettl *et al.*, 2007). A similar case could be made for zinc aquisition, as the deletion of the transcriptional activator ZafA, which regulates zinc homeostasis, led to attenuation of virulence in a murine model of invasive aspergillosis (Moreno *et al.*, 2007).

Apart from these micronutrients, nitrogen assimilation *in vivo* has also attracted research. The aquisition of this macronutrient will be elaborated on later, so the emphasis here will be on the sensory and regulatory proteins involved in this process. One is AreA, a transcription factor involved in a broad range of nitrogen sources other than the preferred ammonium and glutamine, another is the Ras-related protein RhbA, which is also needed for growth on poor nitrogen sources. Deletion mutants of both are hypovirulent in a mouse model of invasive aspergillosis (Hensel *et al.*, 1998; Panepinto *et al.*, 2003). CpcA, the transcriptional activator of the cross pathway control (CPC) system of amino acid biosynthesis does also play a role in *A. fumigatus'* virulence, as a *cpcA* deletion mutant exhibited attenuated virulence in a mouse infection model. This suggests that there is some degree of amino acid starvation inside the mammalian host. Interestingly the upstream signalling eIF2α kinase CpcC is dispensable for pathogenicity, despite increased sensitivity to amino acid starvation conditions (Krappmann *et al.*, 2004; Sasse *et al.*, 2008).

Other, more general fungal factors such as the structure of the cell wall or the conidia surface have also been investigated as possible virulence factors or targets for anti-fungal drugs. While there have been some virulence phenotypes associated with this, these traits are hardly specific for *A. fumigatus* compared to its closely related, much less pathogenic "cousins" like *A. nidulans* (Tekaia and Latge, 2005; Askew, 2008). Overall, the picture emerges that indeed it is not one or two specific

characteristics of *A. fumigatus* that make it so dangerous to immunocompromised humans, but rather a mix of traits that normally aid the fungus to live its saprophytic lifestyle, which in a peculiar way lead to its virulence.

2.4. Nitrogen catabolite repression

Fungi need to react efficiently to varying nitrogen sources, in order to compete in nature. The necessary regulatory mechanisms are best understood for *S. cerevisiae*, and the insights gained from this model organism often direct research in other fungi.

In *S. cerevisiae* four regulators, Gln3, Gat1(Nil1), Dal80 and Deh1(Nil2), regulate all known nitrogen catabolite pathways, in a system known as nitrogen catabolite repression (NCR) (Hofman-Bang, 1999). They are all GATA-type zinc finger proteins, and they bind to promoters that contain the consensus sequence 5'-GATAA-3' (Coffman *et al.*, 1997). The regulated catabolic pathways include the ones for glutamine, glutamate, proline, urea, arginine, GABA (γ-aminobutyrate), ornithine and allantoine (Magasanik and Kaiser, 2002). Additionally, the expression of a number of permeases, such as the general amino acid permease Gap1 or the proline permease Put4 is also influenced by the NCR in yeast (Xu *et al.*, 1995; Stanbrough and Magasanik, 1996).

The NCR is based on the division of nitrogen sources into preferred, rich (primary) nitrogen sources, such as glutamine, asparagine or ammonium, and alternative, poor (secondary) nitrogen sources, like arginine, urea, or proline. In the presence of primary nitrogen sources, the genes necessary for the catabolism of secondary nitrogen sources are under transcriptional repression, which is relieved once the rich nitrogen source is exhausted (Wiame *et al.*, 1985). In this process, Gln3 and Gat1 are positive regulators of gene expression, while Dal80 and Deh1 act negatively. In addition to a complex network of cross-regulation among these 4 transcription factors themselves, which fine tunes the exact response of this system to external stimuli and internal nutritional needs, the NCR is also connected to other regulatory pathways (Magasanik and Kaiser, 2002). Especially the TOR (target of rapamycine) pathway exerts an influence on the NCR, by controlling the localization of Gln3 in the cytoplasm or the nucleus, and thus affecting the transcriptional expression of NCR target genes (Beck and Hall, 1999).

Among aspergilli, *A. nidulans* is the model organism for the nitrogen catabolite repression. The central regulator of the NCR is the GATA transcription factor AreA, which is involved in the transcriptional regulation of more than 100 genes implicated in the utilization of nitrogen sources (Arst and Cove, 1973). It is a global transcriptional activator, which is conserved in filamentous ascomycetes, and an *areA* deletion leads to the inability of the fungus to grow on nitrogen sources other than ammonium or glutamine (Kudla *et al.*, 1990; Wilson and Arst, 1998). Activity of AreA under nitrogen replete conditions is regulated on multiple levels, such as reduced stability of its own mRNA, disruption of a positive autoregulation loop, and the interaction with the negative regulator NmrA (Langdon *et al.*, 1995; Andrianopoulos *et al.*, 1998; Morozov *et al.*, 2001). The activity of AreA is also influenced by its cellular localization, as AreA is found evenly distributed in the cell under all tested nitrogen conditions, but exhibits strong nuclear accumulation under fully nitrogen-deprived conditions (Todd *et al.*, 2005).

During growth on nitrate, AreA needs another, nitrate-specific transcription factor, NirA, which upon entry of nitrate into the cell accumulates in the nucleus and together with AreA forms a transcriptional complex that activates the genes required for nitrate utilization (Burger *et al.*, 1991; Narendja *et al.*, 2002). AreA is also involved in the activation of trancription of the *prn* gene cluster during growth on proline, by influencing the transcription of the gene *prnB*, a proline permease (Sophianopoulou *et al.*, 1993). Additionally, the regulation of *prnB* is controlled by the proline specific transcriptional activator PrnA, and most interestingly also by CreA, the transcriptional regulator that mediates carbon catabolite repression (Sharma and Arst, 1985; Dowzer and Kelly, 1991; Gomez *et al.*, 2003). This illustrates the integration of nitrogen and carbon catabolite repression in *A. nidulans*.

2.5. Nutrition of *A. fumigatus* inside the mammalian host

The primary route of *A. fumigatus* into the human host is *via* the lungs. Conidia are inhaled and travel deep into the lungs down to the level of the alveoli, where they land on the pulmonary surface. This is then the environment where the conidia have to survive, first swelling, germinating and finally developing into mature hyphae. In order to do so, nutrients are necessary, which have to be provided by the host.

The lung surface is covered by a surfactant fluid, which is a complex of 90% lipids, 10% proteins and miniscule amounts of carbohydrates. Phosphatidylcholine is the main phospholipid of the surfactant, the protein fraction is made up mainly of the four proteins SP-A to SP-D (Ding *et al.*, 2001; Paananen *et al.*, 2002). Thus, it is tempting to assume that *A. fumigatus* will primarily feed on lipids of the lung surfactant. However, it was found that this is not the case, as strains missing isocitrate lyase, a key enzyme of the glyoxylate bypass essential for growth on lipids and fatty acids, remained as virulent as the wildtype (Schobel *et al.*, 2007). This contrasts with the situation in another fungal pathogen *Candida albicans*, where a strain defective in isocitrate lyase showed dramatically reduced virulence (Lorenz and Fink, 2001; Lorenz and Fink, 2002).

There are, however, indications that amino acids and proteins play a large role in *A. fumigatus'* nutrition during pulmonary infection. First, lysine auxotrophic strains were avirulent in a murine mouse model of invasive aspergillosis (Liebmann *et al.*, 2004). It was also found that lysine auxotrophic *hcsA* deletion mutants which lacked homocitrate synthase, an enzyme essential for lysine biosynthesis, where unable to germinate on unhydrolyzed protein as the sole nitrogen source, unless minimal amounts of free lysine were present in the medium. These mutants were also strongly attenuated in a murine model of bronchopulmonary aspergillosis, but virulence was partially restored by addition of lysine to the drinking water (Schobel *et al.*, 2010). Moreover, the deletion of the enzyme methylcitrate synthase, which is needed to detoxify intermediary products of the valine, isoleucine and methionine catabolism, led to attenuated virulence in a murine infection model, suggesting that amino acids do serve as important nutrients inside the mammalian lung (Maerker *et al.*, 2005; Ibrahim-Granet *et al.*, 2008).

Given the fact that the lung surfactant does not contain significant amounts of free amino acids, *A. fumigatus* does most likely have to use host proteins as a source of nutrition (Fig. 2).

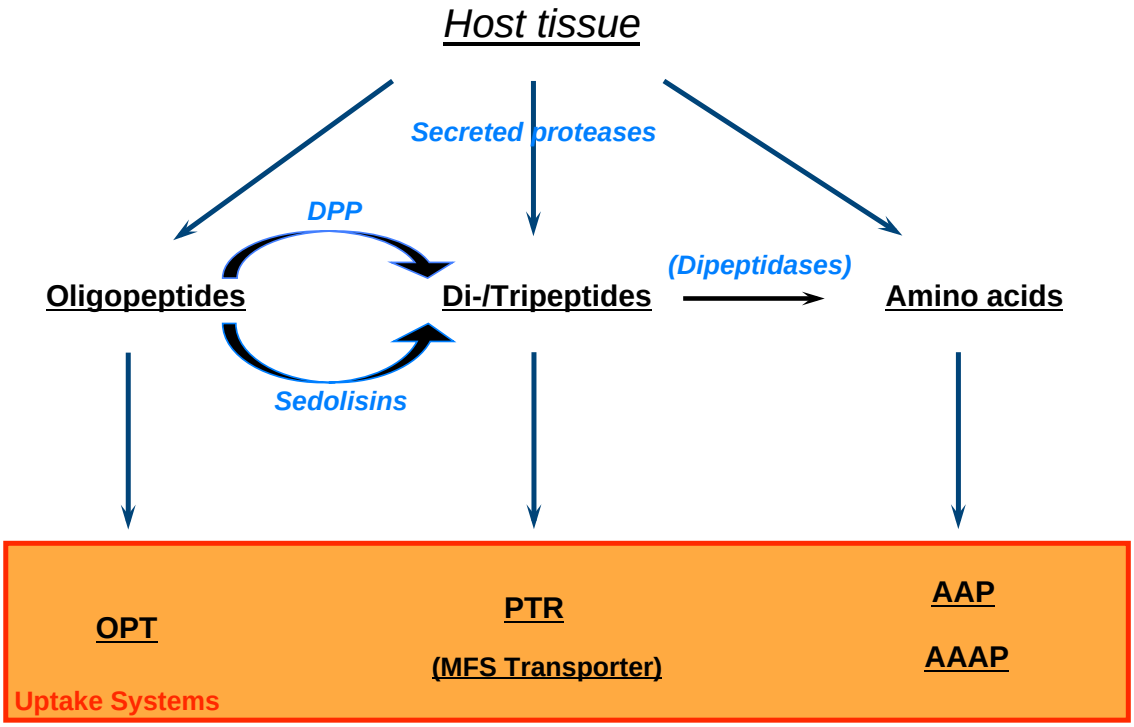

Fig. 2: Schematic representation of the possible nutrient flow during growth of *A. fumigatus* inside the human host
The proteins of the human lungs are digested by secreted proteases giving rise to different primary digestion products: oligopeptides, di-/tripeptides and free amino acids. These are taken up by specialized uptake system such as the oligopeptide transporters (OPT), peptide transporters (PTR), major facilitator superfamily (MFS) transporters or amino acid permeases (AAP and AAAP). More complex primary digestion products like oligopeptides might be further broken down by enzymes like dipeptidyl peptidases (DPP) or sedolisins, possibly down to single amino acids by dipeptidases.

The following pages will elaborate on the process of how *A. fumigatus* might use the mammalian lungs as a nutrient source, detailing the possible role of each player involved.

2.6. Secreted proteases of *A. fumigatus*

The genome of *Aspergillus fumigatus* contains a large number of proteases, more than 100, of which 46 possess a signal sequence for secretion. This enables the fungus to grow well on medium where protein is the only carbon and nitrogen source and also supports its saprophytic lifestyle (Reichard *et al.*, 1990; Latge and Steinbach, 2009). Depending on how they degrade peptides, proteases can be classified as either endo- or exoproteases.

When grown on protein as the sole nitrogen source, *A. fumigatus* secretes three major endoproteases: the aspartic protease Pep1 (Reichard *et al.*, 1994), the metalloprotease Mep (Monod *et al.*, 1993), and the alkaline protease Alp1 (Monod *et al.*, 1991). Deletion mutants were generated for each encoding gene and it turned out, that an *alp/mep* knockout strain did not show any extracellular proteolytic activity at neutral pH. The single *alp* and *mep* deletion strains showed a reduced activity of 30% and 70%, respectively (Monod *et al.*, 1993; Jaton-Ogay *et al.*, 1994).

There are however many more endoproteases encoded by the *A. fumigatus* genome, such as seven aspartic proteases of the pepsin-like protease family, which are active at acidic pH (Latge and Steinbach, 2009). While four of these remain hypothetical, three have been characterized: Pep1, the orthologue to aspergillopepsin I in *A. niger* (Berka *et al.*, 1990), Pep2, which was found in the cell wall fraction of *A. fumigatus* (Reichard *et al.*, 2000), and CtsD which is also a secreted protease (Vickers *et al.*, 2007). Overall seven secreted metallo-endoproteases are present in the genome, among which Mep1 is the only characterized member. Mep1 is most active at neutral pH, was shown to cleave collagen and has an elastinolytic activity (Monod *et al.*, 1993; Markaryan *et al.*, 1994). Finally, the genome contains seven secreted serine endoproteases. Here Alp1, a subtilisin, is best known. Alp1 is extracellular and cleaves collagen as well as elastin at neutral pH (Monod *et al.*, 1991; Frosco *et al.*, 1992). Most interestingly, it cleaves components of the human complement system (Behnsen *et al.*, 2010). Alp2, which is primarily vacuolar but can also be isolated from the cell wall fraction (Reichard *et al.*, 2000), and SedA, an endoproteolytic sedolisin most active at a slightly acidic pH, have also been described experimentally (Reichard *et al.*, 2006).

Exopeptidases can digest a peptide either from the N-terminus or from the C-terminus and are thus called aminopeptidases or carboxypeptidases respectively. Lap1 and Lap2 are two aminopeptidases that have been well studied as recombinant enzymes. Both are active over a rather broad pH spectrum of 6.5 to 10.5 and they remove any amino acid from the N-terminus of a peptide as long as proline is not in the second position (Monod *et al.*, 2005). There are two other related aminopeptidases present in the genome, but they have not been characterized yet (Latge and Steinbach, 2009). Ten genes encoding secreted serine carboxypeptidases can be found in the genome of *A. fumigatus*. Of these, only two, AfuCp1 and AfuCp2, have been characterized as recombinant proteins, hydrolyzing an artificial substrate preferably at slightly acidic pH

(Zaugg *et al.*, 2008). While dipeptidases do exist in the genome of *A. fumigatus*, none have been experimentally verified so far (Latge and Steinbach, 2009).

While the above mentioned exopeptidases cleave single amino acids off substrates, there are two families of exopeptidases that remove larger molecules. First, there are three secreted tripeptidyl peptidases, SedB, SedC and SedD of the sedolisin family, which remove tripeptides from the N-terminus of substrates at slightly acidic pH (Reichard *et al.*, 2006). Sedolisins are challenged by substrates with proline in the third- or fourth to last position, e.g. X-X-Pro or X-X-X-Pro. In this case the prolyl peptidase AfuS28 acts on these sequences (Sriranganadane *et al.*). Furthermore there are two members of the dipeptidyl peptidase family, Dpp4 and Dpp5. Dpp5, which was previously identified as one of the two major antigens for serodiagnosis in aspergillosis (Biguet *et al.*, 1967), cleaves off dipeptidesat neutral pH from the N-terminus of peptide chains, preferentially when the penultimate amino acid is alanine (Beauvais *et al.*, 1997). Dpp4 on the other hand, has a strong substrate preference if proline is in the second to last position, and it was also shown to bind and process collagen. Additionally, several human hormone peptides were digested by Dpp4, which might be significant when infecting a human host (Beauvais *et al.*, 1997; Grouzmann *et al.*, 2002). The importance of the Dpps lies in the fact that for a number of aminopeptidases such as Lap1 and Lap2, proline in the penultimate position poses a large problem, which leads to the termination of digestion (Monod *et al.*, 2005). This might heighten the need for Dpps when high-proline substrates such as collagen are the only available nitrogen source.

Knowledge about the regulation of extracellular proteases, especially the molecular mechanisms behind their expression is still slim. It is known that the expression of the three major endoproteases Pep1, Mep and Alp1 is induced on medium with protein as the sole nitrogen source and completely inhibited by free amino acids or small peptides in the medium (Latge and Steinbach, 2009). The three sedolisins SedB, SedC and SedD could be detected in medium where hemoglobin is the sole nitrogen source (Reichard *et al.*, 2006). The two Dpps were expressed in culture medium that only contain proteins (Beauvais *et al.*, 1997; Beauvais *et al.*, 1997). *A. fumigatus* also expresses different sets of proteases depending on the pH: neutral pH promotes the secretion of neutral and alkaline endoproteases, Lap2 or Dpp4, acidic pH leads to the secretion of Pep1, SedB or SedD (Sriranganadane *et al.*, 2010). On a regulatory level, there is one transcription factor, PrtT, which controls expression of a number of secreted

proteases, including main proteases such as Alp1, Mep, Pep1, Dpp4 or SedB. However, transcription of other protease encoding genes like *dpp5*, *lap1* or *lap2* were not under the control of PrtT, suggesting at least one more regulatory pathway (Bergmann *et al.*, 2009; Sharon *et al.*, 2009).

In summary, it becomes obvious that *A. fumigatus* is very well equipped with extracellular proteolytic activity, which places it in a comfortable position to use human tissue as growth substrate. Whether or not secreted proteases play a role in the virulence of *A. fumigatus* is a matter of debate. There are several findings that support this view. First, it could be shown that protease secretion does happen *in vivo* and that serum stimulates fungal growth and leads to a drastic increase in protease secretion (Moutaouakil *et al.*, 1993; Markaryan *et al.*, 1994; Gifford *et al.*, 2002). Antibodies against the proteases Mep, Pep and Dpp5 can be found in the sera of aspergilloma patients and are used as diagnostic markers (Monod *et al.*, 1993; Sarfati *et al.*, 2006). *A. fumigatus* does have an elastinolytic activity which has been linked to its ability to cause invasive aspergillosis in mice (Kothary *et al.*, 1984). Lastly, Alp1 degrades human complement factors C3, C4 and C5, which could help in the evasion of complement attack and aid in avoiding host phagocytosis (Sturtevant and Latge, 1992; Behnsen *et al.*).

On the other hand, there are some experimental results that cast doubt on the importance of secreted proteases in the infection process. Most importantly, up to now no difference in pathogenicity has been observed in strains with impaired extracelluar proteolytic activity. Neither strains carrying single mutations of the main proteolytic functions *alp*, *mep* and *pep*, nor a double *alp/mep* mutant which completely lacks *in vitro* proteolytic activity at neutral pH, show any less virulence than the wildtype, and histopathological studies of infected lungs showed that mycelial growth was not impaired in these strains (Monod *et al.*, 1993; Tang *et al.*, 1993; Jaton-Ogay *et al.*, 1994; Reichard *et al.*, 1997). The deletion of *prtT*, encoding a transcription factor that controls expression of a number of secreted proteases, also did not result in attenuated virulence (Bergmann *et al.*, 2009; Sharon *et al.*, 2009). However, as there is great functional redundancy among the proteases of *A. fumigatus* so that a number of proteases could complement the loss of a small set of missing proteins, this cannot be taken as definitive evidence against the importance of proteases in infection. A second point raised is that when looking at the tissue of humans who had died from invasive

aspergillosis, no vessel wall elastinolysis could be observed. This questions the need for elastinolytic activity for successful infection (Denning *et al.*, 1992).

In summary, the case for or against a central role of secreted proteases in the infection process remains open. Compared to other, closely related fungi which are far less pathogenic, such as *Aspergillus oryzae*, *A. fumigatus* does not differ greatly in its protease armamentarium (Latge and Steinbach, 2009). However, as the virulence of *A. fumigatus* is most likely of multifactorial nature, the significance of secreted proteolytic activity for disease in mammals cannot be ruled out yet.

2.7. Amino acid and peptide uptake systems

All realms of life share the ability to take up free amino acids into the cell and transporters that are able to do so can be found in many transporter families (Saier, 2000; Saier, 2000). In fungi, most amino acid permeases belong to the amino acid permease (AAP) family, a subfamily of the amino acid/polyamine/organocation (APC) superfamily, which has members in bacteria, archaea and eukaryotes (Andre, 1995; Young *et al.*, 1999). Most research has been focused on *S. cerevisiae*, which has, depending on the strictness of definition, 18 to 24 members of the AAP family (Regenberg *et al.*, 1999; Jack *et al.*, 2000). Much less is known about the situation in filamentous fungi, but members of the AAP family and/or the amino acid auxin permease (AAAP) family have been described in *Neurospora crassa*, *Penicillium chrysogenum* and *Aspergillus nidulans* (Stuart *et al.*, 1988; Margolis-Clark *et al.*, 2001; Trip *et al.*, 2004; Apostolaki *et al.*, 2009).

Peptide uptake is mediated by a much smaller number of transporters. In eukaryotes these transporters belong to the major facilitator superfamily (MFS), the peptide transporter (PTR) family, which is also known as proton-dependent oligopeptide transporter (POT) family, or the oligopeptide transporter family (OPT) (Pao *et al.*, 1998; Saier, 2000). Peptide transport of the MFS family is rather poorly characterized. In eukaryotes Dal5p of *S. cerevisiae* is the only described dipeptide-transporting MFS, whose substrate spectrum seems to be complementary to that of the PTR transporter Ptr2p (Pao *et al.*, 1998; Cai *et al.*, 2007).

2.8. The peptide transport (PTR) system

The PTR family can be found in a wide range of organisms, from prokaryotes such as *Lactococcus lactis* (Hagting *et al.*, 1994) or *Escherichia coli* (Harder *et al.*, 2008), to lower eukaryotes like *Saccharomyces cerevisiae* (Perry *et al.*, 1994) or *Candida albicans* (Basrai *et al.*, 1995), all the way to plants like *Arabidopsis thaliana* (Steiner *et al.*, 1994) and even humans (Fei *et al.*, 1994). The transporters of the PTR system transport di- and tripeptides (Steiner *et al.*, 1995), are energized by a proton-motive force and are predicted to have 12 transmembrane domains (Hauser *et al.*, 2001). Based on sequence similarity, it was initially hypothesized that the PTR family was a member of the much broader MFS family, but later the PTR system was defined as its own distinct family (Pao *et al.*, 1998; Hauser *et al.*, 2001).

The PTR system, and especially its regulation, is best characterized in the yeast *S. cerevisiae*. In this organism, Ptr2p is an integral membrane protein with 12 transmembrane domains and constitutes the di-/tripeptide transporter. The expression of *PTR2* seems to be partially controlled *via* nitrogen catabolite repression (Marzluf, 1997), but, more specifically, *PTR2* expression is under the control of a number of other proteins, in particular *PTR1* (Alagramam *et al.*, 1995) and the SPS (Ssy1p-Ptr3p-Ssy5p) signal transduction pathway (Forsberg and Ljungdahl, 2001).

PTR1, which is also known as *UBR1*, is an essential component of the "N-end rule pathway", which constitutes a ubiquitin-dependent protein degradation system (Bartel *et al.*, 1990; Alagramam *et al.*, 1995). As such, Ptr1p together with the specific ubiquitin conjugating enzyme Ubc2p can aid in the degradation of "N-end rule" substrates, which in the context of the PTR system is Cup9p (Dohmen *et al.*, 1991; Varshavsky, 1996). Cup9p represses *PTR2* expression by binding to its promotor. If however, Cup9p is ubiquitinated by Ptr1p and subsequently degraded, the *PTR2* promotor is free and *PTR2* expression can take place (Byrd *et al.*, 1998). There are hints that internalized peptides might bind to Ptr1p accelerating the degradation of Ubc9p, thus indirectly inducing *PTR2* expression (Turner *et al.*, 2000; Du *et al.*, 2002).

PTR3 is part of the SPS system, which consists of the amino acid sensor Ssy1p located in the plasma membrane and the cytoplasmatic Ssy5p and Ptr3p, which interact with the cytoplasmatic N-terminus of Ssy1p (Forsberg and Ljungdahl, 2001; Poulsen *et al.*, 2005). Downstream of the SPS are Stp1p and Stp2p, two related transcription factors, which regulate the expression of *PTR2* and branched-chain amino acid

permeases (de Boer *et al.*, 2000; Andreasson and Ljungdahl, 2002). Both transcription factors are synthesized in an inactive form which, upon addition of micromolar amounts of certain amino acids to the medium, is activated by endoproteolytic processing of their N-terminal domain, mediated by Ptr3p and Ssy5p. The truncated Stp1p and Stp2p then enter the nucleus and upregulate expression of, among other genes, *PTR2* (Bernard and Andre, 2001; Forsberg and Ljungdahl, 2001; Andreasson and Ljungdahl, 2002; Andreasson and Ljungdahl, 2004). There are additional proteins involved in this process, such as the AAP specific chaperone Shr3p which assists in the correct localization of AAPs (Kota and Ljungdahl, 2005) and two kinases (Yck1p and Yck2p) which affect the phosphorylation status of Ptr3p and thus SPS function (Liu *et al.*, 2008).

In *C. albicans,* the *PTR2* orthologue is *CaPTR2,* which can functionally complement the dipeptide transport defect in a *S. cerevisiae ptr2Δ* deletion mutant (Basrai *et al.*, 1995). Additionally, another putative di-/tripeptide transporter (*PTR22*) could be identified by its sequence, but it remains to be further characterized (Reuss and Morschhauser, 2006). *C. albicans* also seems to possess components of the SPS sensor - Csy1p as the orthologue to Ssy1p, CaPtr3p and CaSsy5p, which together might make up a functional SPS sensor complex (Brega *et al.*, 2004; Martinez and Ljungdahl, 2005). Very much like Shr3p in *S. cerevisiae*, the CaCsh3p is necessary for the correct localization of AAPs such as Csy1p in *C. albicans.* In a mouse infection model, a *CSH3* deletion mutant was attenuated in virulence, which strongly suggests that high-capacity amino acid transport is necessary for successful colonization of the mammalian host (Martinez and Ljungdahl, 2004). Furthermore, CaStp1p and CaStp2p were found and functionally analyzed in *C. albicans*. It was also found that these proteins were synthesized in an inactive form and activated by proteolytic processing. Activated CaStp1p induces the expression of proteins needed for degradation of extracellular protein (*SAP2*) and the uptake of the peptide degradation products (*OPT1, OPT3, PTR2*), while the processed CaStp2p activates amino acid permeases (AAPs). Interestingly, CaStp1p is only expressed in the presence of micromolar concentrations of amino acids, but hardly detectable when amino acids are abundant in the growth medium (Martinez and Ljungdahl, 2005). This gives rise to the speculation that CaStp2p drives the expression of AAPs under high amino acid concentrations, and once the amino acids get exhausted, CaStp1p induces the expression of genes needed for growth on more complex nitrogen sources.

In *A. thaliana*, more than 50 PTR encoding homologues can be found in the genome (Karim *et al.*, 2007), but only four PTR transporters have been analyzed in detail and proven to be di-/tripeptide transporters (Tsay *et al.*, 2007; Komarova *et al.*, 2008). Only little is known about the regulation of the PTR family, research focused more on the function of the PTR transporters. There are indications that PTR transporters do play a role in taking up dipeptides at the rhizosphere, but PTRs are associated more with developmental processes like germination and senescence (Komarova *et al.*, 2008). One PTR encoding gene, *AtPTR3*, has also been associated with plant wounding, defense against bacterial pathogens and salt stress, however the mechanism behind this remains unclear (Karim *et al.*, 2005; Karim *et al.*, 2007).

2.9. The oligopeptide transporter (OPT) system

The OPT family was named after the first identified substrates, oligopeptides of three to five amino acids in length (Lubkowitz *et al.*, 1997; Lubkowitz *et al.*, 1998). It was defined by sequence similarity, the common function of oligopeptide transport, and certain conserved motifs to distinguish it from other transport systems, such as the PTR system. Later, using *hidden Markov modeling* (HMM), members of the OPT family were found in a wide range of organisms from bacteria and archaea to plants and fungi, but not in the kingdom of Animalia (Lubkowitz, 2006). Among fungi, OPT members could be experimentally found in *Schizosaccharomyces pombe* (Lubkowitz *et al.*, 1998), *Candida albicans* (Lubkowitz *et al.*, 1997; Reuss and Morschhauser, 2006), *Schizophyllum commune* (Lengeler and Kothe, 1999), *Saccharomyces cerevisiae* (Lubkowitz *et al.*, 1998; Bourbouloux *et al.*, 2000; Hauser *et al.*, 2000), *Yarrowia lipolytica* (Gonzalez-Lopez *et al.*, 2002), and *Colletotrichum gloeosporioides* (Chague *et al.*, 2009), but have also been predicted in *Aspergillus nidulans* (Wiles *et al.*, 2006). Among plants, members of the OPT family have been described in *Zea mays* (Curie *et al.*, 2001; Yen *et al.*, 2001) and *Brassica juncea* (Bogs, 2003), though most research has been carried out in *Arabidopsis thaliana* (Koh *et al.*, 2002).

What has emerged from these studies is that a) OPTs are driven by the symport of protons (Osawa *et al.*, 2006), b) there are two distinct clades, the peptide transport (PT) clade and the yellow stripe (YS) clade (Lubkowitz, 2006) and c) the substrates of the PT clade are mostly peptides of three to eight amino acids (Lubkowitz *et al.*, 1997; Reuss and Morschhauser, 2006), possibly up to thirteen (Pike *et al.*, 2009) amino acids

in length, whereas the substrates of the YS clade are primarily metal-chelating peptides (Curie *et al.*, 2001; Wintz *et al.*, 2003; Lubkowitz, 2006; Stacey *et al.*, 2008).

In plants, OPTs are suggested to play a number of roles; one is in metal acquisition through the transport of phytosiderophores, glutathione and nicotianamine (NA), which bind ions like iron, zinc and copper (Wintz *et al.*, 2003; Cagnac *et al.*, 2004; Stacey *et al.*, 2008). A link between OPTs and developmental processes has been established (Stacey *et al.*, 2006) and it has been proposed that OPTs are involved in long-distance transport of peptides as nitrogen sources, although this has not yet been experimentally proven (Stacey *et al.*, 2006; Waterworth and Bray, 2006).

In fungi, most research has been performed on *S. cerevisiae* and *C. albicans*. There are three proposed members of the OPT family in *S. cerevisiae*: *ScOPT1*, *ScOPT2* and *YGL114w*. *YGL114w* shows high similarity to the Yellow Stripe 1 (YS1) of *Zea mays*, which is an iron-phytosiderophore transporter (Curie *et al.*, 2001) and which later was classified as an OPT based on sequence analysis (Yen *et al.*, 2001), an interpretation that has recently been challenged (Wiles *et al.*, 2006). Interestingly, *YGL114w* could be found in the vacuolar membrane (Wiederhold *et al.*, 2009), and a *Δygl114w* deletion mutant exhibited a strong growth defect on synthetic complete (SC) medium (Wiles *et al.*, 2006).

ScOPT1 was first discovered along with *isp4* in *S. pombe*, however its functionality as an oligopeptide transporter could not be established then (Lubkowitz *et al.*, 1998). *ScOPT1* was later described as an enkephalin transporter, able to transport various tetra- and pentapeptides (Hauser *et al.*, 2000), and in parallel as a high affinity glutathione transporter (Bourbouloux *et al.*, 2000), which is induced by sulfur starvation and repressed by addition of cysteine to the growth medium creating an intriguing link to sulfur metabolism (Miyake *et al.*, 2002). Additionally, Ptr3p and Ssy1p are required for *ScOPT1* expression in a sulfur-free environment, suggesting a surprising connection between the PTR system and the OPT family (Wiles *et al.*, 2006). The same link between sulfur metabolism and a member of the OPT family could be shown for *S. pombe*, where the disruption of the *ScOPT1* homologue *SpOPT1* led to the inability to grow on medium where glutathione is the sole sulfur source (Dworeck *et al.*, 2009). In *S. pombe SpOPT1* is located in the cell membrane (Dworeck *et al.*, 2009).

The functionality of *ScOPT2* as an oligopeptide transporter could be established early on, when it was described that a forced expression of the transporter led to growth on KLGL as the sole leucine and lysine source in an otherwise growth deficient strain,

and the ability of *ScOPT2* to transport the toxic peptides KLAEth and KLLAEth was shown (Lubkowitz *et al.*, 1998). Additionally, *ScOPT2* has been associated with drug detoxification, such as polyamines and bleomycin. However, the importance of *ScOPT2* appears to lie in its role in proper formation of a mature, functional vacuole, and not in the transport activity of *ScOPT2* (Aouida *et al.*, 2009).

Finally, both *ScOPT1* and *ScOPT2* were also shown to be expressed under sporulation conditions (Velculescu *et al.*, 1997; Chu *et al.*, 1998), a fact in accordance with the oligopeptide transporter *mtd1* in *S. commune*, whose transcription is also induced under sexual development conditions (Lengeler and Kothe, 1999).

CaOPT1 was the founding member of the OPT family in *C. albicans* when its ORF was expressed in an *S. cerevisiae* di-/tripeptide transport mutant and the protein confered the ability to grow on the tetrapeptide KLLG and as well as sensitivity to toxic tetra- and pentapeptides (Lubkowitz *et al.*, 1997). Later, the OPT family was expanded to eight members, with seven additional *OPT* genes showing a significant sequence similarity to the original *CaOPT1*, one of which (*CaOPT8*) displaying a high similarity to the *S. cerevisiae YGL114w gene* product. The *OPT* genes were differentially induced when grown on medium with BSA as the sole nitrogen source, and for *CaOPT1* - *CaOPT5* the function as peptide transporters could be experimentally established. The *OPT* genes showed individual substrate preferences and were able to transport oligopeptides of up to eight amino acids in length. A triple deletion mutant of *CaOPT1*, *CaOPT2* and *CaOPT3* was unable to grow in medium with BSA as the sole nitrogen source, however even a quintuple deletion mutant of *CaOPT1* - *CaOPT5* was not attenuated in virulence in a mouse model of disseminated candidiasis (Reuss and Morschhauser, 2006).

It is not uncommon for transporter families to contain members with a sensor function, which in some cases lost their transporter function altogether (Forsberg and Ljungdahl, 2001). Examples are the amino acid permease Gap1p for a functional sensor/transporter (Donaton *et al.*, 2003), and the amino acid permease homologue Ssy1p, which has lost its transporter function and is now a sensor only (Didion *et al.*, 1998; Iraqui *et al.*, 1999; Klasson *et al.*, 1999). In the OPT family, only one example was found, where an OPT might be implicated in sensing. In *Yarrowia lipolytica* the deletion of the *CaOPT1* homologue *YlOPT1* led to the almost complete transcriptional downregulation of two proteases (*XPR2* and *AXP1*) under otherwise inducing

conditions as well as diminished hyphal formation, although the exact mechanism of *YlOPT1* remains elusive (Gonzalez-Lopez *et al.*, 2002).

2.10. Gene targeting in *A. fumigatus*

Uncovering what exactly makes *A. fumigatus* pathogenic is hampered by two main factors: the absence of clearly defined virulence determinants (Tekaia and Latge, 2005; Askew, 2008) and the high genetic redundancy in many molecular functions, such as transporters, proteases or superoxid dismutases (Latge and Steinbach, 2009; Lambou *et al.*, 2010). The latter fact makes it a big challenge to investigate *A. fumigatus* genetically since single or even double gene deletions might be phenotypically silent due to the ability of the fungus to metabolically compensate for their loss (Krappmann, 2006).

There are well established genetic tools that make handling and genetically modifying aspergilli feasible (Goldman and Osmani, 2008). Gene deletions are ordinarily carried out by replacing a target gene with a selectable marker cassette *via* homologous recombination at the target gene locus. This process has been simplified with the introduction of *A. fumigatus* strains deficient in illegitimate recombination, which greatly increases the otherwise poor rate of homologous recombination in a wildtype strain (da Silva Ferreira *et al.*, 2006; Krappmann *et al.*, 2006). Still, there is only a very limited number of dominant resistance markers available for *A. fumigatus*, namely against the antifungal agents hygromycin, phleomycin and pyrithiamine (Punt and van den Hondel, 1992; Kubodera *et al.*, 2002). Using these markers, a maximum of three genes could be deleted, with the added drawback of having three heterologous genes with potentially unanticipated phenotypic side effects in the fungal genome. To circumvent these difficulties when working with aspergilli, first marker recycling *via* genetic markers that are flanked by repetitive sequences and which under certain selective conditions allows for marker excision were introduced (d'Enfert, 1996; Krappmann and Braus, 2003).

By now, more advanced methods became available in other fungi, the bacteriophage P1 based Cre/*loxP* system (Hamilton and Abremski, 1984) or the *S. cerevisiae* Flp/*FRT* system (Sadowski, 1986; Kilby *et al.*, 1993). These systems rely on enzymes, classified as tyrosine recombinases, that bind to short recognition sequences, the Cre recombinase recognizing *loxP* sequences, the Flp recombinase recognizing *FRT*

sites. In case two of these sites flank a certain stretch of DNA, the recombinase then facilitates site specific rebombination at these sites, removing that DNA sequence in between.

For other eukaryotic organisms, such as plants or mammalian cells, similar systems which employ serine recombinases were developed (Diaz *et al.*, 1999; Grønlund *et al.*, 2007). Here site-specific recombination is catalized by a prokaryotic small β serine recombinase which binds to so called *six* recognition sequences (Rojo *et al.*, 1993; Rojo and Alonso, 1994; Canosa *et al.*, 1996).

The Cre/*loxP* based system has been widely used in *S. cerevisiae* and other yeasts (Güldener *et al.*, 1996; Wieczorke *et al.*, 1999; Krappmann *et al.*, 2000; Güldener *et al.*, 2002; Hegemann *et al.*, 2006), while the Flp/*FRT* system was established successfully in the human pathogen *C. albicans* (Morschhäuser *et al.*, 1999; Reuss *et al.*, 2004; Morschhäuser *et al.*, 2005). A distinct feature of the Flp/*FRT* system in *C. albicans* is that the *FLP* recombinase gene is part of the excisable marker cassette, which allows for gene replacement and marker recycling performing just one tranformation. In this system the *SAT1* flipper cassette, which confers resistance to nourseothricin, is lost simply by growing transformants in medium without selective pressure. Recently the Flp/*FRT* system has also been adopted for filamentous fungi, such as *Penicillium chrysogenum* or *Sordaria macrospora* (Kopke *et al.*, 2010)

In *Aspergilli*, the Cre/*loxP* based system was established in two different forms: one where the *cre* gene was introduced into the genome of the fungus (Forment *et al.*, 2006) and one where the recombinase gene was transiently expressed from an autonomously replicating plasmid which would be lost in the absence of selective pressure (Krappmann *et al.*, 2005). The second approach was implemented in *A. fumigatus*, which has the advantage that the Cre/*loxP* system can be used in any strain background, not just a strain that harbours the *cre* gene. Compared to the *C. albicans* Flp/*FRT* system the *A. fumigatus* Cre/*loxP* system has the distinct disadvantage that two transformations are needed, the first one replacing the target gene with the marker cassette and the second one introducing the *cre* carrying plasmid necessary for marker excision. Even so, the Cre/*loxP* system finally makes it possible to replace multiple genes or even entire gene families in *A. fumigatus*.

2.11. Aims of this work

Over the past decades *Aspergillus fumigatus* has steadily gained importance as a fungal human pathogen, infecting primarily immunocompromised patients and causing potentially deadly invasive aspergillosis. Still, the nutritional situation of *A. fumigatus* inside the human host is poorly understood, especially during the initial stages of infection.

This work concentrates on nitrogen metabolism in general, and oligopeptide transport in particular. In the first part, the transcriptional response of *A. fumigatus* towards different nitrogen sources is investigated, focusing on secondary nitrogen sources such as nitrate, proline and BSA.

The second part characterizes the oligopeptide transporter (OPT) gene family. Its response on the transcriptional level to different nitrogen sources is examined in detail. The substrate specificity of individual oligopeptide transporters is investigated by heterologous expression in *Saccharomyces cerevisiae*. Furthermore, multiple *opt* gene deletion mutants in *A. fumigatus* are created and phenotypically characterized, including tests in a murine model of pulmonary invasive aspergillosis.

In the third part of this work, a novel tool for targeted gene deletions in *Aspergillus fumigatus* is introduced, which bears clear advantages over the established Cre/*loxP* system.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Plasmids

The following abbreviations are used for the description of plasmids: *loxP*, the recognition site of the Cre recombinase; *pgpdA*, the constitutive promoter of the *A. nidulans gpdA* gene; *ble*, *Streptoalloteichus hindustanus ble* gene, which confers resistance towards phleomycin; *hph*, *Escherichia coli* phosphotransferase-encoding *hph* gene which confers resistance towards hygromycine; *tk*, thymidine kinase-encoding sequence from herpes simplex virus type 1; *trpC*, terminator of the *A. nidulans trpC* gene; *xylP*, promoter of the *Penicillium chrysogenum xylP* gene*; β-Rec*, small β serine recombinase; *ptrA*, pyrithiamine resistance gene of *A. oryzae*.

Tab. 1: Plasmids used in *A. fumigatus* experiments

Plasmid	Relevant Insert	Reference
pCR-BluntII-TOPO	general cloning vector	Invitrogen GmbH
pJET	general cloning vector	MBI Fermentas
pSK215	*niaD::cre* in pPTRII (Cre recombinase expression module in autonomously replicating Aspergillus vector pPTRII)	(Krappmann *et al.*, 2005)
pSK341	*loxP-phleo^R/tk-loxP, Aspergillus* marker cassette conferring resistance towards phleomycin, *phleo^R/tk* refers to [*pgpdA::ble/tk::trpCt*]	(Krappmann *et al.*, 2005)
pSK346	*abr2::hyg^R/tk* deletion cassette	this study
pSK397	*loxP-hyg^R/tk-loxP, Aspergillus* marker cassette conferring resistance towards hygromycin, *hyg^R/tk* refers to [p*gpdA::hph/tk::trpCt*]	(Krappmann *et al.*, 2005)
pSK462	*prtT:: loxP-phleo^R/tk-loxP* deletion cassette	(Bergmann *et al.*, 2009)
pSK463	*prtT:: <ptrA^R> A. fumigatus prtT* reconstitution cassette	(Bergmann *et al.*, 2009)
pSK479	codon optimized *β-rec* allele and *trpC* terminator between two *six* sites in the pMA-RQ vector	GeneArt AG
pSK485	*pxylP::β-rec::trpCt - ptrA*, β-rec allele under the control of the *xylP* promoter, *ptrA* gene conferring pyrithiamine resistance	this study
pSK509	*abr2::pxylP::β-rec::trpCt - ptrA* deletion cassette	this study
pSK510	*pksP::pxylP::β-rec::trpCt - ptrA* deletion cassette	this study
pSK511	*optA::loxP-phleo^R/tk-loxP* deletion cassette	this study

Plasmid	Relevant Insert	Reference
pSK512	*optB::loxP-phleo^R/tk-loxP* deletion cassette	this study
pSK513	*optC::loxP-phleo^R/tk-loxP* deletion cassette	this study
pSK514	*optD::loxP-phleo^R/tk-loxP* deletion cassette	this study
pSK515	*optE::loxP-phleo^R/tk-loxP* deletion cassette	this study
pSK516	*optF::loxP-phleo^R/tk-loxP* deletion cassette	this study
pSK517	*optB::loxP-hyg^R/tk-loxP* deletion cassette	this study
pSK518	*optC::loxP-hyg^R/tk-loxP* deletion cassette	this study
pSK519	*optD::loxP-hyg^R/tk-loxP* deletion cassette	this study
pSK520	*optE::loxP-hyg^R/tk-loxP* deletion cassette	this study
pSK521	*optF::loxP-hyg^R/tk-loxP* deletion cassette	this study
pSK522	*optG::loxP-hyg^R/tk-loxP* deletion cassette	this study
pSK523	*optH::loxP-hyg^R/tk-loxP* deletion cassette	this study
pSK524	*dpp4::loxP-phleo^R/tk-loxP* deletion cassette	this study
pSK525	*dpp5::loxP-hyg^R/tk-loxP* deletion cassette	this study
pSK526	*prtT:: loxP-hyg^R/tk-loxP* deletion cassette	this study

Tab. 2: Plasmids used in *S. cerevisiae* experiments

Plasmid	Relevant Insert	Reference
pSK51	pRS426 derivate (URA3, 2µm) carrying methionine-repressable *MET25* promoter	(Krappmann *et al.*, 2006)
pSK527	*A. fumigatus optA* cDNA in pCR-BluntII-TOPO	this study
pSK528	*A. fumigatus optA* cDNA in pSK51 (SalI)	this study
pSK529	*A. fumigatus optB* cDNA in pSK51 (SalI/NotI)	this study
pSK530	*A. fumigatus optC* cDNA in pSK51 (SalI/NotI)	this study
pSK531	*A. fumigatus optD* cDNA in pSK51 (NotI)	this study
pSK532	*A. fumigatus optE* cDNA in pSK51 (SalI/NotI)	this study
pSK533	*A. fumigatus optF* cDNA in pSK51 (SalI/NotI)	this study
pSK534	*A. fumigatus optG* cDNA in pSK51 (SalI/NotI)	this study
pSK535	*A. fumigatus optH* cDNA in pSK51 (SalI/NotI)	this study
pSK536	*C. albicans OPT1* cDNA in pSK51 (SalI/NotI)	this study

3.1.2. Strains

For cloning procedures the strain *Escherichia coli* DH5α (F-, *end*A1, *hsd*R17 [rk-, mk-], *sup*E44, *thi-1,rec*A1, *gyr*A96, *rel*A1, Δ[*arg*F-*lac*]U169, λ-, φ80d*lacZ*ΔM15) (Bethesda Research Laboratories, 1986) was used.

The following two tables list the *A. fumigatus* and *S. cerevisiae* strains used in this study.

Tab. 3: *A. fumigatus* strains used in this study

Strain	Genotype	Reference
ATCC 46645	Wildtype, clinical isolate	Fungal Genetics Stock Center
AfS61	*prtT::loxP-phleo*^{R}	(Bergmann *et al.*, 2009)
AfS77	*akuA::loxP*	this study
AfS100	*optA::loxP-phleo*^{R}	this study
AfS101	*optA::loxP*	this study
AfS102	*optB::loxP-phleo*^{R}	this study
AfS103	*optC::loxP-phleo*^{R}	this study
AfS104	*optD::loxP-phleo*^{R}	this study
AfS105	*optE::loxP-phleo*^{R}	this study
AfS106	*optF::loxP-phleo*^{R}	this study
AfS107	*optG::loxP-hyg*^{R}	this study
AfS108	*optH::loxP-hyg*^{R}	this study
AfS109	*optA::loxP; optE::loxP-phleo*^{R}	this study
AfS110	*optA::loxP; optE::loxP-phleo*^{R}*; optG::loxP-hyg*^{R}	this study
AfS111	*optA::loxP; optE::loxP-phleo*^{R}*; optG::loxP*	this study
AfS112	*optA::loxP; optE::loxP-phleo*^{R}*; optG::loxP; optF::loxP-hyg*^{R}	this study
AfS113	*optA::loxP; optE::loxP-phleo*^{R}*; optG::loxP; optF::loxP*	this study
AfS114	*optA::loxP; optE::loxP-phleo*^{R}*; optF::loxP; optG::loxP; optB::loxP-hyg*^{R}	this study
AfS115	*optA::loxP; optE::loxP-phleo*^{R}*; optF::loxP; optG::loxP; optB::loxP*	this study
AfS116	*optA::loxP; optB::loxP; optE::loxP-phleo*^{R}*; optF::loxP; optG::loxP; optC::loxP-hyg*^{R}	this study
AfS117	*optA::loxP; optB::loxP; optE::loxP-phleo*^{R}*; optF::loxP; optG::loxP; optC::loxP*	this study
AfS118	*optA::loxP; optB::loxP; optC::loxP; optE::loxP-phleo*^{R}*; optF::loxP; optG::loxP; optD::loxP-hyg*^{R}	this study
AfS119	*optA::loxP; optB::loxP; optC::loxP; optE::loxP-phleo*^{R}*; optF::loxP; optG::loxP; optD::loxP*	this study
AfS120	*optA::loxP; optB::loxP; optC::loxP; optD::loxP ; optE::loxP-phleo*^{R}*; optF::loxP; optG::loxP; optH::loxP-hyg*^{R}	this study
AfS121	*optA::loxP; optB::loxP; optC::loxP; optD::loxP ; optE::loxP-phleo*^{R}*; optF::loxP; optG::loxP; optH::loxP*	this study
AfS122	*dpp4::loxP-phleo*^{R}	this study
AfS123	*dpp4::loxP*	this study
AfS124	*dpp5::loxP-hyg*^{R}	this study
AfS125	*dpp4::loxP; dpp5::loxP-hyg*^{R}	this study
AfS126	*dpp4::loxP; dpp5::loxP-hyg*^{R}*; optA::loxP-phleo*^{R}	this study
AfS127	*dpp4::loxP; dpp5::loxP; optA::loxP*	this study
AfS128	*dpp4::loxP; dpp5::loxP; optA::loxP; optG::loxP-hyg*^{R}	this study
AfS129	*dpp4::loxP; dpp5::loxP; optA::loxP; optG::loxP-hyg*^{R}*; optE::loxP-phleo*^{R}	this study
AfS130	*dpp4::loxP; dpp5::loxP; optA::loxP; optE::loxP-phleo*^{R} *; optG::loxP*	this study

Strain	Genotype	Reference
AfS131	*dpp4::loxP; dpp5::loxP; optA::loxP; optE::loxP-phleo^R; optG::loxP; optF::loxP-hyg^R*	this study
AfS132	*dpp4::loxP; dpp5::loxP; optA::loxP; optE::loxP-phleo^R; optG::loxP; optF::loxP*	this study
AfS133	*dpp4::loxP; dpp5::loxP; optA::loxP; optE::loxP-phleo^R; optF::loxP; optG::loxP; optB::loxP-hyg^R*	this study
AfS134	*dpp4::loxP; dpp5::loxP; optA::loxP; optE::loxP-phleo^R; optF::loxP; optG::loxP; optB::loxP*	this study
AfS135	*dpp4::loxP; dpp5::loxP; optA::loxP; optB::loxP; optE::loxP-phleo^R; optF::loxP; optG::loxP; optC::loxP-hyg^R*	this study
AfS136	*dpp4::loxP; dpp5::loxP; optA::loxP; optB::loxP; optE::loxP-phleo^R; optF::loxP; optG::loxP; optC::loxP*	this study
AfS137	*dpp4::loxP; dpp5::loxP; optA::loxP; optB::loxP; optC::loxP; optE::loxP-phleo^R; optF::loxP; optG::loxP; optD::loxP-hyg^R*	this study
AfS138	*dpp4::loxP; dpp5::loxP; optA::loxP; optB::loxP; optC::loxP; optE::loxP-phleo^R; optF::loxP; optG::loxP; optD::loxP*	this study
AfS139	*dpp4::loxP; dpp5::loxP; optA::loxP; optB::loxP; optC::loxP; optD::loxP ; optE::loxP-phleo^R; optF::loxP; optG::loxP; optH::loxP-hyg^R*	this study
AfS140	*dpp4::loxP; dpp5::loxP; optA::loxP; optB::loxP; optC::loxP; optD::loxP ; optE::loxP-phleo^R; optF::loxP; optG::loxP; optH::loxP*	this study
AfS141	*optA::loxP; optB::loxP; optC::loxP; optD::loxP ; optE::loxP-phleo^R; optF::loxP; optG::loxP; optH::loxP; prtT::loxP-hyg^R*	this study
AfS142	*optA::loxP; optB::loxP; optC::loxP; optD::loxP ; optE::loxP-phleo^R; optF::loxP; optG::loxP; optH::loxP; prtT<ptrA^R>*	this study

Tab. 4: *S. cerevisiae* strains used in this study

Strain	Genotype	Reference
SKY132	EuroScarf strain BY4742, MATα; *his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*	EuroScarf collection
SKY133	SKY132 contains plasmid pSK528	this study
SKY134	SKY132 contains plasmid pSK529	this study
SKY135	SKY132 contains plasmid pSK530	this study
SKY136	SKY132 contains plasmid pSK531	this study
SKY137	SKY132 contains plasmid pSK532	this study
SKY138	SKY132 contains plasmid pSK533	this study
SKY139	SKY132 contains plasmid pSK534	this study
SKY140	SKY132 contains plasmid pSK535	this study
SKY141	SKY132 contains plasmid pSK536	this study
SKY142	SKY132 contains plasmid pSK51	this study

3.1.3. Primers

All primers were obtained from Eurofins MWG Operon. Non-capital letters in a primer sequence indicate the position of introduced of restriction sites.

Tab. 5: Primers used in *A. fumigatus* gene deletion experiments

Primer	**Sequence**
THA1	5'- TATggccATCTAggccCAGGCATCGCTAATTAATCTAG-3'
THA2	5'- TATgttAACACCGCGCCCAGTTCTG-3'
THA3	5'- TATggccTGAGTggccAAAAGTATCTTCTGCAGAAGC-3'
THA4	5'- TATgttAACCTCCTCAGCTCAATGTC-3'
THA5	5'- TCGATAGGCTAATGAATGTCC-3'
THA6	5'- ACATCATCGTCACTGAGCAC-3'
THA7	5'- AGAGGAAGCAAGGGCAACAG-3'
THA8	5'- TATgttAACTTACCTCGGAAAGTCCAC-3'
THA9	5'- TATggccATCTAggccCTGAGAGGATGATTGAACGG-3'
THA10	5'- TATggccTGAGTggccCTTGATGCCGTGCAGATGGC-3'
THA11	5'- TATgttAACATGTCTCCATTGCGGAC-3'
THA12	5'- TACCAGCTAGCAATATGTTGG-3'
THA13	5'- TTAACTGGTTACCTATCAGGG-3'
THA14	5'- GAGGTCGTCGATTCCTGAAG-3'
THA15	5'- TATgttAACGCCGACATACTCCCTTTC-3'
THA16	5'- TATggccATCTAggccCAATGCTGAGGAAGACATCC-3'
THA17	5'- TATggccTGAGTggccGGATCCCTTAGGTACTTTTGC-3'
THA18	5'- TATgttAACATAATATGCGCGATGACAG-3'
THA19	5'- ACTCTGAGTAGTTTCCTCCG-3'
THA20	5'- TACAGTCTGTGGCATTTGGC-3'
THA21	5'- GAGTTCCGTGCAGGAACAAG-3'
THA22	5'- TATgttAACCAGCAATGGGCTTATGC-3'
THA23	5'- TATggccTGAGTggccGTGCTTTAAGCAGGGTCCAG-3'
THA24	5'- TATggccATCTAggccTACGGCGGTGGCAGAAATC-3'
THA25	5'- TATgttAACACCACTCGGGGCCGTG-3'
THA26	5'- TATgttAACGGCTTCCATCCGCGTTC-3'
THA27	5'- AGCTGAGAATCCGTTATCGC-3'
THA28	5'- GCTAGCAATGAGAATGCTGG-3'
THA29	5'- AGCGAGCTGGGATATCTACG-3'
THA30	5'- TATgttAACCAGCGTCCATGCCGTTG-3'
THA31	5'- TATggccTGAGTggccTGGTCCAACAGTGATACCGC-3'
THA32	5'- TATggccATCTAggccGAGGTTCGCGATTTGCTCAG-3'
THA33	5'- TATgttAACACTTGATTCGGCCATCG-3'
THA34	5'- CTAAGCATGCGCCAATAACG-3'
THA35	5'- TTACGTGGCATCCGAATTCC-3'
THA36	5'- TCTGGTTCGAAGAAGAACCC-3'
THA37	5'- TATgttAACTAGGTCTAAACTACCCAG-3'
THA38	5'- TATggccTGAGTggccCTCCGCATATAACGAATTCATG-3'
THA39	5'- TATggccATCTAggccGAAACCCACATGAACCGATC-3'
THA40	5'- TATgttAACTGGCCACCAAGAGCCG-3'

Primer	Sequence
THA41	5’- TGCAGGCATTTATGGTTGCG-3’
THA42	5’- TAAGGAGCAACAGTATTCACC-3’
THA43	5’- CTTCTTACTAATCCGGTCTCC-3’
THA44	5’- TATggccATCTAggccTCTACTGTTACTTCTAGACCG-3’
THA45	5’- TATgttAACTGCAACTGCCACGTCC-3’
THA46	5’- TATggccTGAGTggccTTCGTCGAAGAATTTCGACAG-3’
THA47	5’- TATgttAACGCTCTGGTGTTACTAAATC-3’
THA48	5’- TGACATGCGGAATTGCAACG-3’
THA49	5’- GTACTCCGTACCGATTATGG-3’
THA50	5’- ATTGCATACCTGTTGCTATGG-3’
THA51	5’- TATgtttAAACAGTGCCCTATTCACTCG-3’
THA52	5’- TATggccTGAGTggccATTCCGTGCTTCTGTACAGG-3’
THA53	5’- TATggccATCTAggccAGACTCAGGTATGTCGAGAC-3’
THA54	5’- TATgtttAAACATTGCCAAGTGAGACATTG-3’
THA55	5’- TATGGTGTATTCTGCCCACC-3’
THA56	5’- ACATCCAGTTGTCAATCATGC-3’
THA57	5’- ATAAGCTCGAGACGGAACGC-3’
THA58	5’- TATgttAACGTGCGCTGTGGGAGAC-3’
THA59	5’- TATggccTGAGTggccCACAGCGAGGTACTTGCATC-3’
THA60	5’- TATggccATCTAggccATGCACGGTTGGCGTACATG-3’
THA61	5’- TATgttAACTGCTTTCGCTCGGATCG-3’
THA62	5’- TGGAAGAAAAGCCATGGACC-3’
THA63	5’- TAGTGCCGAACTTGAAGCAG-3’
THA64	5’- TATGTAACGTAGCAGACAAGG-3’
THA65	5’- tatGTTAACGATAGACCGGCCC-3’
THA66	5’- TATggccTGAGTggccGATGGACTCGGATCTCAATC-3’
THA67	5’- TATggccATCTAggccAATGCTACCTTAACCTAGTGG-3’
THA68	5’- TATgttAACACATGGGAGCTATACGAG-3’
THA69	5’- ATCTGCAACATTGGCACAGG-3’
THA70	5’- TCCAAATGCTTCGACGATGC-3’
THA71	5’- TTACCATCCTTGCTGCAGTG-3’
THA72	5’- GCAGAATTGGGACGAATTGG-3’
Sv287	5’- ACCGGAACGGCACTGGTCAAC-3’
Sv288	5’- TAAGATCGGCCGCAGCGATCG-3’
Sv300	5’-CAACTCTAAATTCTACTGTTGTTTC-3’
Sv301	5’-GTTCGAGCCATCCATAGTTCCAGTC-3’
Sv302	5’-ATTCTGGACTGTACCTTATGTAAG-3’
Sv630	5’-TCGGAGAACACCTTGCTGACG-3’
Sv712	5’-ACAACGCCCAAACCTGTTATCC-3’
Sv713	5’-AAGGCTGTGAACAAGCCAAGCC-3’

Tab. 6: Primers used in qRT-PCR experiments

Primer	Sequence
THA73	5’- GGACGTTACCTCACCTGCTC-3’
THA74	5’- CACGCTTGAACAACTCCTGA-3’
THA75	5’- TTCGGGCACTTGGCGAGACA-3’
THA76	5’- AACTGGGCTTTGGGGCTTGC-3’
THA77	5’- TGTGGTTCTGGCTCGCAGGT-3’
THA78	5’- ATCCACCATCCGCGGAAACG-3’
THA79	5’- AATGCCACCTTCTGGGGCCT-3’
THA80	5’- TCGATGCGGTGGATGCGAAG-3’
THA81	5’- CAGCGAGTTCATCGGCGGTT-3’
THA82	5’- TTGCCGAGCATCCAGAGGGT -3’
THA83	5’- TCTGGATGCGAGCGGTGTCA-3’
THA84	5’- AGCGGGAAAGCAGTCTTGCG-3’
THA85	5’- TCCGCCTGCAACAGGGATCA-3’
THA86	5’- TAATGGGCCGGCAGGAAGCA-3’
THA87	5’- TACGGCCTGCATCGTCTCCT-3’
THA88	5’- ATCGAACGCCGCTGCAATCA-3’
THA89	5’- GCGTTGCAACCAACCAGCTC-3’
THA90	5’- TGAGACCAGCCCCGAGCAAA-3’
THA91	5’- TGTCGACGCACAGCACATCG-3’
THA92	5’- CCGGATCGCACTGGCATTGT-3’
THA93	5’- ACCCAACCCAACCGGCAGTA-3’
THA94	5’- AATTTCCGGCCCAGAGGGCT-3’
THA95	5’- CTTGTTGCCTGCCGGGTGTT-3’
THA96	5’- AAGCGTCGGAGAGCAGGGTT-3’
THA97	5’- GCGACTGTGACAAACCGGCA-3’
THA98	5’- AGCGGATCAGTGGACAACGC-3’
THA99	5’- CGAGCATGTGCTGCGTCGAT-3’
THA100	5’- GTTCGTCGGGGTAAGCGCAA-3’
THA101	5’- TGGCAGATCCCGAACACGGT-3’
THA102	5’- ATCAGCCACGGGTCCTTCGT-3’
THA103	5’- TCCGGCAACTTCGTCACCCA-3’
THA104	5’- ATCGCGGCGTACATCATGGC-3’
THA105	5’- ACCTACGTGCTCGGCGGTAT-3’
THA106	5’- AACGCCAGGATCGCCACAAC-3’
THA107	5’- GAGCCCTTTTCCGACCTGAT-3’
THA108	5’- GGAACTCCTCCCGGATCTTG-3’
THA109	5’- CGAGCACAGCTTCTTTGCAG-3’
THA110	5’- CCCATGGTGTCCGTTCTGA-3’

Tab. 7: Primers used in *S. cerevisiae* experiments

Primer	Sequence
THA111	5’- CTTTAAGCATGAGAGCCGATG -3’
THA112	5’- TATgtcgacCTAGAGATGTGGAACATTCC -3’
THA113	5’- TATgtcgacATGCCCCAAATTCTCCTGCT -3’
THA114	5’- TCGTGCAAAGCATTAAAGAGC -3’
THA115	5’- TATgcggccgcTCAAGACCAAGACTTGGGGC-3’
THA116	5’- TATgtcgacATGAAGGACAAGATCGTCGAG -3’
THA117	5’- CCGGAAGAAATTGATACAGTG-3’
THA118	5’- TATgcggccgcTCAATTCCAACTCCGCGGC-3’
THA119	5’- TATctcgagATGGAGTCTAAGAAGGAGTC-3’
THA120	5’- GTAGCAGTGACATACAGGAAG-3’
THA121	5’- TATgcggccgcTCAGGTCCAAGTTCCATTCG -3’
THA122	5’- TATgcggccgcATGGCTTCTAAGGAGCAAAC -3’
THA123	5’- CGAGAGATCCTCCATCCATC-3’
THA124	5’- TATgcggccgcTCATGACCAAGTCCTCTCGC -3’
THA125	5’- TATgtcgacATGGCTCCAATTCAGTCACAG -3’
THA126	5’- GGGACAATATACCAGATAGC-3’
THA127	5’- TATgcggccgcCTATTCCCATTTCCCTTTCG-3’
THA128	5’- TATgtcgacATGTTTCATTGCACATTTCCC-3’
THA129	5’- GGATCATACAACAAGATCTGC-3’
THA130	5’- TATgcggccgcCTAGTCCAAAGCAAAACACC-3’
THA131	5’- TATgtcgacATGCTGGACAAAATGGGCC-3’
THA132	5’- TCAGCTGGGTAGACAATCAG-3’
THA133	5’- TATgcggccgcCTAGATGCAATGATCAGGATTC-3’
THA134	5’- TATctcgagATGGATGACAAGAACCGCTC-3’
THA135	5’- CTCAAAACACCTCCGAAAGC-3’
THA136	5’- TATgcggccgcTTACCAGGAAGATGGCCCAA-3’
THA137	5’- TATgtcgacATGGACAAAATAAGGGCAGTAA-3’
THA138	5’- CATCGGCTCTCATGCTTAAAG-3’
THA139	5’- CTGCGTTAGTTTGCTGATTCC-3’
THA140	5’- TCCGATCTGAATGTTGGTCG-3’
THA141	5’- ATATCACGGCAGTCGACAAC-3’
THA142	5’- TCGCTGAGGACATTGAGCTG-3’
THA143	5’- GATTAGCGACGGCATAGACG-3’
THA144	5’- ATGTTTGCCAGAGGTCGTCC-3’
THA145	5’- CAAGCTGGTAGTTCGAGACG-3’
THA146	5’- CATAGAGGATGCAGGAGAAG-3’
THA147	5’- AACCGACGATGAACTCTGTC-3’
THA148	5’- GACTTCAGGTTGTCTAACTCC-3’
Sv62	5’- ATTTCCTTCGTGTAATACAGGG-3’

3.1.4. Chemicals and equipment

Chemicals, enzymes and equipment used in this study, unless indicated otherwise, were obtained from one of the following suppliers:

Amersham, Applichem, Bio-Rad, Boehringer, Difco, Eppendorf, MBI Fermentas, GE Healthcare, Gibco, Gilson, Greiner, Invitrogen, Merck, New England Biolabs (NEB), MWG-Biotech, Oxoid, Peqlab, Pharmacia, Roth, Serva and Sigma.

Tab. 8: Equipment used in this study

Equipment	**Supplier**
Bioanalyzer 2100	Agilent
Tabletop centrifuge (Biofuge Pico)	Heraeus
Cooled tabletop centrifuge (Fresco 21)	Heraeus
Cooled centrifuge (Biofuge Primo R)	Heraeus
Digital camera Powershot A650 IS	Canon
Electrophoresis Powersupply EV222	Peqlab
Electrophoresis Chamber PerfectBlue Mini M+L	Peqlab
Geldocumentation system	Bio-Rad
Film developer Cawomat 2000 IR	Cawo
Hybridization oven Shake 'n' Stack	Hybaid
37°C Incubator HeraCell	Heraeus
30°C Incubator (Model 400)	Memmert
Shaking incubator KS15 with climate hood TH15 (30°C and 37°C)	Edmund Bühler GmbH
Microflow biological safety cabinet	Nunc GmbH
Microscope Axiolab re	Zeiss
Binocular microscope Stemi 2000-C with KL1500 electronic light source	Zeiss
Nanodrop 1000	Peqlab
Spectrophotometer (BioPhotometer)	Eppendorf
Realtime PCR thermocycler MyiQ iCycler	Bio-Rad
Thermocycler Mastercycler Gradient	Eppendorf
UV Crosslinker Stratalinker 1800	Strategene
Temperature-adjustable Waterbath (Typ 1002)	GFL mbH

3.2. Methods

3.2.1. E. coli culture conditions

Recombinant *E. coli* strains were routinely grown in LB liquid medium (1% Peptone, 0.5% Yeast extract, 0.5% NaCl) normally under selective conditions (100 µg/ml ampicillin or 50 µg/ml kanamycin). For growth on plates, 1.5% agar (Agar agar,

Serva) was added to the medium. The cells in liquid culture were grown at 37°C for 16-18 hours with shaking at 200 rpm and the plates were incubated at 37°C until the colonies appeared.

3.2.2. S. cerevisiae culture conditions

For non-selective culture conditions, *S. cerevisiae* was grown in YPD (1% yeast extract, 2% peptone, 1% glucose, pH 5.8; 1.5% agarose for solid media) medium at 30°C. All selective media were based on 0.17g/l yeast nitrogen base (YNB) without amino acids and ammonium sulfate (Difco), 2% glucose, 0.1% proline and 200µM histidine; this medium base was named YNB(GPH). If needed, one or more of the following supplements were added: 230µM leucine, 200µM lysine, 200µM uracile.

For phenotypic characterization, a single colony of an *S. cerevisiae* strains was used to inoculate 5ml of YNB(GPH) +leu/+lys/+ura liquid medium and was grown at 30°C for 48 hours. After washing twice with sterile water, serial dilutions in sterile water of each strain were plated on selective YNB(GPH) medium that was supplemented with a specific oligopeptide as the sole available leucine or lysine source. For tests of complementation for leucine auxotrophy, the pentapeptide KLLLG (aquired from BioLux GmbH), the tetrapeptide (Des-Tyr^1)-Leu-Enkephalin (GGFL) and the tetrapeptide LWMR were added. These peptides were added as supplements to 100ml YNB(GPH) +lys/-leu/-ura medium, 10mg of KLLLG, 20mg of GGFL and 30mg of LWMR. For tests of complementation of lysine auxotrophy the oligopeptide kentsin (TPRK) was added at 20 mg of per 100ml YNB(GPH) +leu/-lys/-ura medium. GGFL, LWMR and TPRK were aquired from Bachem AG. The plates were incubated at 30°C for 3-4 days.

3.2.3. A. fumigatus culture conditions

As a default, *A. fumigatus* was grown in nitrate based minimal medium (1% glucose, 1x AspA (1:50 dilution of 50x AspA (3.5M $NaNO_3$, 350mM KCl, 550mM KH_2PO_4, pH 5.5)), 0.25mM $MgSO_4$, 1x Trace elements solution; 2% Agar agar (Serva) for solid media) and was incubated at 37°C. For selection of a resistance marker the following chemical agents were used: 50 µg/ml hygromycin B (InvivoGen), 20µg/ml phleomycin (InvivoGen) or 0.1 µg/ml pyrithiamine.

For phenotypic characterization, the minimal medium base (1:50 dilution of the stock solution (26 g/l KCl, 26 g/l $MgSO_4$, 76g/l KH_2PO_4, 50 ml/l Trace elements solution), pH 6.5) was supplemented with appropriate amounts of carbon and nitrogen sources. When glucose was the carbon source, it was added to a final concentration of 1% in the medium. Ammonium was added as ammonium tartrate to a final concentration of 5mM, nitrate was added as sodium nitrate to a final concentration of 10mM. Collagen hydrolysate (Mobiforte, Twardy), with an average fragment size of 3000 kD corresponding to 20-30 amino acid long oligopeptides, was added to a final concentration of 0.5 g/100ml, BSA (Albumin Fraktion V, Roth) was added to a final concentration of 0.5 g/100ml. For solid media agarose was added to a final concentration of 1.5%.

For the oligopeptide growth assays, minimal medium base was supplemented with glucose to a final concentration of 1%. The growth assays were carried out in 12 well incubation plates (Nunc) filled with a final volume of 3 ml solid growth medium. Each well contained the protease inhibitors 2mM AEBSF (Sigma), 5µM Pepstatin A (Sigma) and 1mM EDTA (Sigma). Each well contained 10 mg of a defined oligopeptide as the only available nitrogen source, all but one (KLLLG, purchased from BioLux GmbH), were aquired from Bachem AG.

For lung agar, 5 g of fresh porcine lung was snap-frozen in liquid nitrogen and pulverized using a pre-cooled mortar. The resulting powder was put into a 50 ml reaction tube, filled up with an equal amount of sterile saline and briefly incubated in a 50°C warm waterbath. To this an equal amount of 50°C warm, liquid water agar (1.5% agarose in water) was added, the mix was vortexed for 10 seconds and poured in equal amounts onto two solid water agar plates. After solidification, the agar plates were placed upside down on the UV table of a geldocumentation system and stayed there for 30 minutes, in order to sterilize the medium.

For all growth assays on solid media, the growth medium was inoculated with 1µl of a fresh *A. fumigatus* spore suspension (10^6 conidia/ml in water supplemented with 0.9% NaCl and 0.02% Tween 80) and incubated at 37°C for 2-3 days.

3.2.4. Transformation of E. coli

E. coli competent cells were prepared using the calcium chloride method. A single colony of DH5α was inoculated in 10 ml LB medium and grown at 37°C with shaking at 200 rpm for 16-18 hours. The overnight grown culture was diluted 1:100 in

50 ml fresh LB medium and incubated at 37°C shaker for 3-4 hours, until it reached an OD_{600} of 0.7-0.9. The culture was then transferred to chilled 50 ml tubes and centrifuged at 3,000 rpm for 10 min at 4°C in a chilled rotor. The supernatant was discarded and the cell pellet was resuspended in 20 ml of ice cold 100 mM calcium chloride. The cell mix was incubated on ice for 30 minutes and centrifuged at 4,000 rpm for 10 minutes at 4°C. The pellet was then resuspended gently in 2.5 ml of 100 mM calcium chloride and 86% glycerol was added to a final concentration of 15% (v/v). Aliquotes of 200 µl competent cells were transfered into 1.5 ml reaction tubes and stored at -80°C for later use. For each transformation a frozen aliquot of competent cells was thawed on ice. The DNA to be transformed (20 µl) was added to 200 µl competent *E. coli* cells and incubated for 30 minutes on ice. The cells were subjected to heat shock at 42°C for 90 seconds and then chilled on ice for 1 minute. After adding 1 ml LB medium the cells were allowed to grow for 1h at 37°C. Finally, the cells were spread on LB selection plate (containing 100µg/ml ampicillin or 50µg/ml kanamycin), and the plates were incubated at 37°C until the colonies appeared.

3.2.5. Transformation of S. cerevisiae

A 0.5ml aliquot of a fresh, saturated YPD overnight *S. cerevisiae* culture is pelleted by centrifugation with a tabletop centrifuge at room temperature and 3000 rpm. The supernatant is removed completely and the following solutions are added: 25 µl sterile water and 100 µg of carrier DNA in a 10 µl volume; the solution is mixed well by pipetting up and down. To this 0.5 ml of freshly prepared PEG solution (40% PEG 3350, 0.1M LiOAc, 10mM Tris pH 7.5, 1mM EDTA, 0.1M DTT) is added and mixed by vortexing. The reaction mix is stored overnight at room temperature, followed by a 42°C heatshock for 12 minutes. After this the cells are pelleted by centrifugation at 3000 rpm for 10 minutes in a tabletop centrifuge, the supernatant is removed and the pellet resuspended in 1 ml YPD solution. The cells are incubated at 30°C for 30 minutes, pelleted by centrifugation and washed once with 0.5 ml sterile water. Finally, the cells are resuspended in 0.5 ml sterile water, plated on selective medium and incubated at 30°C until colonies appear.

3.2.6. Transformation of A. fumigatus

An overnight culture of *A. fumigatus* is prepared by inoculating 200 ml minimal medium with 10^8 spores and incubating it at 37°C and 100 rpm for 18-20 hours. The mycelium is filtered through a Miracloth (Merck) filter and washed three times with citrate buffer (150mM KCl, 580mM NaCl, 50mM sodium citrate, pH 5.5). The mycelium is "semi-dried" by rolling it inside a miracloth filter and incubated in 20 ml enzyme mix (400 mg Vinoflow FCS dissolved in 20ml citrate buffer; filter sterilized) for about 1 hour 20 minutes at 30°C and gentle shaking. The mixture is then filtered through a sterile miracloth filter and the flowthrough is collected in a 50 ml reaction tube on ice. Ice cold STC 1700 buffer (1.2M sorbitol, 10mM Tris pH 5.5, 50mM $CaCl_2$, 35mM NaCl) is added to fill up the reaction tube, the mix is incubated on ice for another 5 minutes and then centrifuged in a cooled centrifuge (Biofuge Primo R) for 12 minutes at 3200 rpm at 4°C. The supernatant is discarded, the reaction tube is filled up with ice cold STC 1700 and the centrifugation is repeated. After this the supernatant is discarded again, the the protoplasts at the bottom of the tube are resuspended in 0.5 ml STC 1700. To each 200 µl aliquot of protoplasts 5-10 µg DNA dissolved in 30 µl water is added, gently mixed and incubated in a 15 ml reaction tube over ice for 25 minutes. After this PEG 4000 solution (60% (w/v) PEG 4000, 50mM $CaCl_2$, 10mM Tris pH 7.5) is added in two aliquots a 250 µl and a final aliquot a 850 µl, each time mixing the solution thoroughly. The reaction mix is incubated over ice for 20 minutes, after which 15 ml of ice cold STC 1700 solution is added. The reaction tube is centrifuged for 15 minutes at 3200 rpm and 4°C, the supernatant is discarded and the pellet dissolved in 500 µl STC 1700. This solution is divided into three aliquots and 5 ml liquid, 50°C warm TOP Agar (minimal medium with 1.2M sorbitol) is added. The reaction tube is inverted several times and the contents poured onto selective plates (minimal medium with 1.2M sorbitol and selective agent at appropriate concentration). After solidification of the agarose, the plates are incubated at 37°C for several days until colonies appear.

3.2.7. A. fumigatus liquid mediashifts

The experimental setup was modified after (Narendja *et al.*, 2002). 200 ml minimal medium base with 5mM ammonium tartrate was inoculated with 10^8 freshly harvested, 5 day old *A. fumigatus* ATCC 46645 conidia and grown at 37°C and 150 rpm for 16 hours. This pre-culture was then harvested, washed liberally with sterile saline

and divided into lumps of equal size on a sterile surface. The portions were then added to 100ml of minimal medium base without nitrogen source. When a dedicated carbon source was needed, 1% glucose was added. The flasks were incubated for 20 min at 37°C. Thereafter one of the following sterile nitrogen sources was added: a) ammonium tartrate to a final concentration of 5mM, b) 2 g peptone and 1 g yeast extract suspended in 30ml minimal medium base to create a rich medium, c) 0.5 g collagen hydrolysate (Mobiforte Collagenhydrolysat, Twardy) suspended in 15 ml minimal medium base, d) 1 g proline suspended in 2 ml minimal medium base or e) 0.5 g BSA (Albumin Fraktion V, ROTH), suspended in 15 ml minimal medium base. The cultures were incubated for another 40 – 60 minutes, harvested by filtering through Miracloth filters, snap frozen in liquid nitrogen and ground using a cooled mortar to get a fine powder.

3.2.8. Measuring A. fumigatus extracellular proteolytic activity

The supernatants for measuring the extracellular proteolytic activity were obtained from liquid cultures of *A. fumigatus*. For this 40 ml of a modified minimal medium (minimal medium base, 1% glucose, 0.5% BSA, 0.1mM ammonium tartrate, 100 μg/ml ampicillin) were inoculated with 10^8 freshly harvested conidia, and incubated at 37°C for 60 hours. The supernatant was harvested by filtrating the culture through Miracloth filters, and stored briefly on ice. In order to test for proteolytic activity, 10μl of the culture supernatant were applied on a BioMax MS X-Ray film (Eastman Kodak) and incubated at 37°C for 1 hour, after which the film was rinsed with warm water for 1min.

3.2.9. DNA extraction from A. fumigatus

The mycelia from a liquid culture was filtered through a Miracloth (Merck) filter and washed with saline. The mycelium was dried by pressing it between paper towels and snap-froozen in liquid nitrogen. The froozen mycelium was transfered to a pre-cooled mortar and ground together with a pinch of sand to a fine powder. About 0.8 ml of the powder was then transfered to a 2 ml reaction tube, an equal amount of lysis buffer (50mM Tris-HCl (pH 7.2), 50mM EDTA, 3% SDS, 1% β-Mercaptoethanol) was added, the mixture was vortexed for 10 seconds and incubated at 65°C for 1 hour, mixing it several times during the process. After this, 0.8ml of phenol/chloroform (1:1) was added, the mixture was shaken for a few seconds and centrifuged in a tabletop

centrifuge at room temperature for 15 minutes at maximum speed. The supernatant was transfered into a new 1.5ml reaction tube, 0.8 vol 2-propanol was added and the mixture was shaken several times. The reaction tube was centrifuged at maximum speed for 5 minutes, the supernatant was removed and the pellet was washed with 1 ml 70% ethanol for 5 minutes, violently shaking it the whole time. After centrifuging the reaction tube for 2 minutes, the supernatant was removed completely, and the pellet was dried at 37°C for 15 minutes. The pellet was then dissolved in 150-200 µl 5mM Tris-HCl (pH 8.0) by incubating it at 65°C for about 15 minutes, after which it was stored at 4°C.

3.2.10. Agarose gel electrophoresis

To the DNA solution 0.1 Vol of a 10 x sample buffer (25% (w/v) Ficoll 400, 0,25% (w/v) Bromphenol blue, 0,25% (w/v) Xylenxyanol, 200mM EDTA pH 8) was added and loaded onto a 1% agarose gel (agarose in TAE-buffer (40mM Tris-Acetat, 20mM NaOAc, 2mM EDTA, pH 8,3)). The gel was run in a TAE buffer at 90V for about 1 hour, stained in an ethidium bromide bath for 15 minutes, destained in water for 10 minutes and examined using a geldocumentation system. As a size standard the "GeneRuler 1kb DNA ladder" (Fermentas) was used.

3.2.11. Extraction of DNA fragments from agarose gels

The commercially available NucleoSpin Extract II system (Machery & Nagel) was used according to manufacturer's instructions.

3.2.12. Plasmid DNA extraction from E. coli cultures

For small scale DNA extraction from a 5 ml *E. coli* overnight culture, either the QIAprep Spin Miniprep system (Qiagen) or the NucleoSpin Plasmid system (Machery & Nagel) were used according to the manufacturer's instructions. For large scale DNA extraction from a 50 ml *E. coli* overnight culture, the Nucleobond Xtra Midi system (Machery & Nagel) was used according to the manufacturer's instructions.

3.2.13. Polymerase Chain Reaction (PCR)

As default conditions the PCR was performed in a reaction volume of 20 µl, which contained 10-100 ng DNA template, two times 50 nmol gene specific primers and 1 µl dNTP mix (Fermentas). As DNA polymerases either the Paq5000 polymerase (Stratagene), or the high fidelity Phusion (Finnzymes) polymerase, together with their respective buffers, were used according to the manufacturer's instructions. For the Phusion polymerase the following PCR programm was used: an initial denaturation step of 98°C for 45 seconds, 30 times the PCR cycle (98°C, 10 seconds for denaturation; 55-60°C, 20 seconds annealing, 72°C 30s/kb elongation), and a final 5-10 min elongation step at 72°C. The PCR programm for the Paq5000 polymerase was similar, except the denaturation took place at 95°C. Fusion PCR, using the Phusion polymerase, was carried out as previously described (Szewczyk *et al.*, 2006).

3.2.14. DNA digestion with restriction enzymes

DNA restriction digestions were carried out by using commercially available reagents (NEB, MBI Fermentas), in accordance with the manufacturer's instructions. As default conditions, 30µl reaction volume was used, and digestion was carried out for 6 hours for plasmids, and overnight for PCR products.

3.2.15. Dephosphorylation of DNA 5' and 3' ends

For dephosporylation of DNA ends the calf intestine alkaline phosphatase (MBI Fermentas) was used in accordance with the manufacturer's instructions.

3.2.16. Ligation of DNA fragments

Commercially available T4 DNA ligase was used according to the manufacturer's instructions (NEB, MBI Fermentas). As default conditions, 30 µl reaction volume was used, which contained 5 units of DNA ligase activity. The reaction was carried out overnight at 16°C in a thermocycler, and the ligation products were either used directly in a transformation with 300 µl competent *E. coli* Dh5α, or stored at -20°C for later use.

3.2.17. DNA sequencing

Sequencing of DNA was performed by the commercial supplier SeqLab GmbH Göttingen, and the samples were prepared according to the company's instructions. The sequences were analyzed using the freely available programm 4Peaks (http://mekentosj.com/science/4peaks/).

3.2.18. Southern hybridization

For each sample about 10 µg of genomic DNA was digested by restriction digest overnight, 0.1 vol of sample buffer was added and agarose gel electrophoresis using a 1% agarose gel was performed for about 2 hours at 90V. After this the gel was incubated at room temperature and under gentle shaking in three solutions: first, 15 minutes in 0.25M HCl, then 25 minutes in 0.5M NaOH/1.5M NaCl and last, 30 minutes in 1.5M NaCl/0.5M Tris pH 7.4. After this the DNA was transfered to a "Hybond-N" nylon membrane (Amersham Pharmacia Biotech) using the dryblot method at room temperature for 3 hours. Following this, the membrane was washed for 2 minutes in 2 x SSC (1:5 dilution of 10 x SSC (87,6g NaCl, 44,1g sodium citrate, fill up to 1 litre with nucleasefree water)), dried for 10 minutes at 75°C and the DNA was fixed to the membrane by exposure to UV light using the Stratalinker 1800 (Stratagene).

For labeling and detection the "Gene Images AlkPhos Direct Labelling and Detection System" (GE Healthcare) was used according to the manufacturer's instructions. The probe to be labeled was an, on average 500 basepairs long, PCR product. For final signal detection the membrane was exposed to a "Fuji medical X-ray film" (Fuji) for 30 minutes to 1 hour and the film was developed using the Cawomat 2000 IR developing machine (Cawo).

3.2.19. RNA extraction from A. fumigatus

The mycelia from a liquid culture was filtered through a Miracloth (Merck) filter and washed with saline. The mycelium was quickly dried by pressing it between paper towels and snap-froozen in liquid nitrogen. The froozen mycelium was transfered to a pre-cooled mortar and ground together with a pinch of sand to a fine powder. About 2 vol of cold Trizol (Invitrogen) were added to 1 vol of mycelium powder, and immediately vortexed vigorously until a homogeneous suspension was obtained. The

mix was incubated on ice for another 5 minutes, vortexed shortly and 800 µl of it were transfered into a 2 ml reaction tube with 160 µl chloroform in it. The mixture was shaken immediately and vigorously, and incubated again for 5 minutes on ice. The reaction tube was centrifuged in a cooled tabletop centrifuge for 15 minutes at maximum speed at 4°C, after which 200µl of the supernatant was removed and pipetted into a fresh, RNAse-free 1.5ml reaction tube with 240µl isopropanol in it. The mix was shaken vigorously, and incubated at room temperature for 10 minutes. After this, the reaction tube was centrifuged for 15 minutes at maximum speed at 4°C, the supernatant discarded and the pellet washed twice with 1 ml 70% ethanol (using nucleasefree water) for 5 minutes, violently shaking it the whole time. After a final centrifugation step at maximum speed at 4°C, the supernatant was completely removed and the pellet dried at 37°C for 15 minutes. Finally, the pellet was dissolved in 150 µl nucleasefree water at room temperature, and the RNA containing solution was stored at -80°C.

3.2.20. Northern hybridization

10 µl of a RNA solution was added to 30 µl RNA sample buffer (720 µl Formamid, 260 µl nucleasefree water, 100 µl glycin, 4 µl ethidiumbromid, small amount of bromphenol blue) and incubated at 65°C for 10 minutes. After this, the sample was immediately placed on ice. The sample was then loaded onto a denaturing agarose gel (1.4% agarose, 87 ml nucleasefree water, 10 ml 10xMOPS (48.1 g MOPS, 16.6 ml NaOAc (3M), 20 ml EDTA (0.5M), add nucleasefree water until the solution is 1 litre, set pH to 7.0 and autoclave) und 3ml formaldehyde) and was run at 120 V for about 60 minutes. After this, the RNA was blotted onto a "Hybond-N" nylon membrane (Amersham Pharmacia Biotech) at 4°C overnight, using the semi-dryblot method in 10 x SSC (87.6 g NaCl, 44.1 g sodium citrate, fill up to 1 litre with nucleasefree water).

After this the RNA was fixed to the membrane by exposure to UV light using the Stratalinker 1800 (Stratagene) and incubated in a pre-hybridization buffer (Church-buffer: 7% SDS, 1% BSA, 1mM EDTA, 32.1 g Na_2HPO_4, 9.66g NaH_2PO_4, fill up with water to 1 litre and set pH to 7.2) for 2 hours at 65°C.

The probe was synthesized using the "PrimeIt II Random Primer Labeling Kit" (Stratagene) and radioactive α-P^{32}-dATP (Hartmann Analytic GmbH) according to the manufacturer's instructions, using an average probe length of 500 basepairs, whose DNA template was supplied as a PCR product. The probe together with the membrane was incubated in Church buffer at 65°C overnight. The following day the membrane

was washed twice for 30 minutes using Northern Wash solution (50mM NaCl, 20mM sodium phosphate buffer pH 6.5, 1mM EDTA, 0.5% SDS) at 65°C. After this the membrane was exposed for 3 hours to a "Biomax MS" film (Eastman Kodak), and developed using the Cawomat 2000 IR developing machine (Cawo).

3.2.21. DNAse digest of total RNA

For removal of genomic DNA from extracted RNA, the solution was treated with RNAse free DNAseI (Fermentas) according to the manufacturer's instructions. As a default, the reaction volume was 100 µl, with 10 µl DNAseI enzyme, 10 µl buffer and 10 µl total RNA solution. Incubation time was 30 minutes at 37°C, after which the solution was immediately cleaned using the RNeasy Mini Kit (Qiagen).

3.2.22. Cleanup of RNA

For the cleanup of RNA samples the RNeasy Mini Kit (Qiagen) was used according to the manufacturer's instructions.

3.2.23. Quality control of RNA

Intergrity of RNA was investigated using the Nanodrop 1000 (Peqlab) and the BioAnalyzer 2100 (Agilent) according to manufacturer's instructions.

3.2.24. Reverse transcription of RNA

For reverse transcription of DNAseI digested RNA into cDNA the commercially available "SuperScript III First-Strand Synthesis SuperMix for qRT-PCR" (Invitrogen) was used according to the manufacturer's instructions. For the reaction 1 µg of RNA per 20 µl reaction mix was used as a default.

3.2.25. Realtime PCR

For the quantification of transcript levels "quantitative Realtime PCR" (qRT-PCR) was employed, using the MyiQ iCycler thermocycler (Bio-Rad) and the iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The reactions

were performed using 96-well PCR plates and Microseal 'B' film (Bio-Rad). The standard volume of each individual reaction was 25 µl, which contained a 1:50 dilution of cDNA as a template, two primers at a concentration of 100nM each, generating a 200bp PCR product, and a 1:2 dilution of the SYBR Green supermix. The primers were designed using the freely available Primer3Plus programm (Untergasser *et al.*, 2007) and checked for their quality using the FastPCR programm (available at http://primerdigital.com/fastpcr.html). The PCR programm contained an initial, 3 minute long incubation step at 95°C, and 40 cycles of 15 seconds at 95°C, 15 seconds at 60°C and 15 seconds at 72°C, while the fluorescence was measured after each elongation step. A meltcurve of the final products was generated by first heating up the samples to 95°C, cooling them down to 50°C and re-heating them in 0.5°C intervalls. All reactions were performed at least in technical duplicate. For bioanalytical analysis of the qRT-PCR runs, the iCycler software package (version 5, Bio-Rad) was used, and the quantification was based on the ΔΔCt method (Pfaffl, 2001).

3.2.26. Virulence model of pulmonary aspergillosis

Outbred male mice (strain CD1, 20 to 28 g; Charles Rivers Breeders) were used for animal experiments. Immunosuppression was carried out by subcutaneous injection of 112 mg/kg hydrocortisone acetate and intraperitoneal injection of 150 mg/kg cyclophosphamide following a sequential protocol as described previously (Smith *et al.*, 1994). Bacterial infections were prevented by adding 1 g/liter tetracycline and 64 mg/liter ciproxicin to the drinking water. Inocula of 3 x 10^4 conidiospores in 40 µl of saline were prepared by harvesting spores from 5-day-old slants of solid medium followed by filtration through Miracloth (Merck) and washing with saline. Mice were anesthetized by inhalation of isofluorane and infected by intranasal instillation. The weights of infected mice were monitored for 4 days daily before animals were culled to isolate their lungs. In order to assess fungal burdens as a quantitative virulence criterion, qPCRs were performed on equivalent amounts (100 ng) of genomic DNA that had been extracted from equal amounts (250 mg) of homogenized lung tissue, as described previously (Bowman *et al.*, 2001). Using this DNA as a template, the qRT-PCR was performed employing one fungal-specific primer pair THA107/THA108 targeting the fungal β-tubulin gene and one mouse-specific primer pair THA109/110 targeting the mouse actin gene.

4. RESULTS

4.1. Transcriptional response of *A. fumigatus* to secondary nitrogen sources

The metabolic versatility of *Aspergillus fumigatus* allows it to grow on a large variety of nutrient sources. The basis of this versatility lies in its ability to sense changing environmental conditions and to adapt quickly, making use of a multitude of transporters and metabolic enzymes. One way to gain a deeper insight into an organisms' response to environmental change is transcriptome analysis by employing DNA microarrays, observing the transcript levels of almost all expressed genes at once.

This work concentrates on the nitrogen metabolism of *A. fumigatus*. To this end, the transcriptome of *A. fumigatus* during growth on minimal medium supplemented with one of four nitrogen sources was analyzed: 5 mM ammonium tartrate, 10 mM sodium nitrate, 1% proline or 0.5% BSA. Ammonium and nitrate were chosen as simple, inorganic nitrogen sources with ammonium being the prefered compound of the two (Goldman and Osmani, 2008). Proline represents a simple, organic nitrogen source which was chosen over other amino acids for its relative abundance in the extracellular matrix components of the mammalian lung, especially in collagen (Traub *et al.*, 1969). BSA represents a complex, organic nitrogen source which cannot be taken up directly by the fungus, but has to be digested outside the cell first, so that the degradation products can be transported into the cell cytoplasm. For each condition, the RNA of two independent biological replicates was prepared.

Transcriptional analysis of the eight RNA samples was conducted by Febit Biomed GmbH (Heidelberg, Germany) using their proprietary Geniom Biochips platform. For each of the eight samples, the RNA was transcribed into cDNA, labeled and hybridized individually on an oligonucleotide chip. Following signal detection and background subtraction, the raw data were normalized for each individual sample yielding an absolute intensity value for each transcript. The change in the transcriptional profile between two conditions was calculated by directly comparing all the gene intensity values of these two conditions. For this, the intensity values of the two biological replicates for each condition were combined by calculating the median value

for this condition. Thus, three different datasets were generated in which the gene expression on either the nitrate medium, the proline medium or the BSA medium was compared to the reference condition of growth in the presence of ammonium.

The analysis of these datasets focussed on genes that exhibited at least a two-fold regulation compared to the ammonium condition, based on the normalized signal intensity. Additionally, for each gene in all three datasets a p-value was calculated using empirical bayes statistics, to assess the statistical significance of the intensity change between the compared conditions. Only genes that in a specific dataset showed a p-value of 0.05 or lower were taken into consideration. For further functional analysis, we also excluded genes that were annotated to code for hypothetical proteins. The missing functional characterization of these genes does not allow us to place their regulation into a meaningful biological context.

Furthermore, this work will deal primarily with genes that are upregulated compared to the ammonium reference condition. It can be expected that the overwhelming number of genes which are necessary to utilize a new, less-preferred nitrogen source will be upregulated on that condition. These will encompass transport systems necessary for the uptake of the new nitrogen source, or enzymes of newly needed metabolic pathways, which are necessary to feed the substrate into the fungal nitrogen metabolism. Additionally, as the change from ammonium to one of the other nitrogen sources represents a shift from a primary to a secondary nitrogen source, the downregulation of many genes might be simple response to relative nitrogen starvation. An overview of the number of up- and downregulated genes is given below.

Tab. 9: Number of significantly downregulated genes on nitrate, proline and BSA containing medium

condition	downregulated genes including hypothetical proteins	downregulated genes excluding hypothetical proteins
NO_3^-	363	249
Proline	184	125
BSA	716	476

Tab. 10: Number of significantly upregulated genes on nitrate, proline and BSA containing medium

condition	upregulated genes including hypothetical proteins	upregulated genes excluding hypothetical proteins
NO_3^-	289	192
Proline	231	161
BSA	1060	758

It is evident that the number of upregulated genes is comparable between the nitrate and the proline condition, but is almost four times as high under the BSA condition.

Next, a more in-depth analysis was carried out for each of the three data sets with the aim of characterizing the transcriptional response to each nitrogen source in greater detail. To this end, the FungiFun suite (available at https://www.omnifung.hki-jena.de/Rpad/server/FungiFun/FungiFun.pl) was employed, which allows for functional categorization of fungal transcriptome data. For this work, the KEGG (Kyoto Encyclopedia of Genes and Genomes) classification system is used (Kanehisa and Goto, 2000).

The FungiFun suite performs a gene enrichment analysis, which is based on available data on the function of known genes. Each gene had previously been assigned one or more functional categories, which are more specific for the higher levels of categorization. On the lowest (first) level of categorization, each gene is almost always assigned only one functional category, on the higher levels more than one function can be assigned. There are many genes that have not been assigned a functional category yet, so these get excluded from the analysis. When a group of genes are entered into the FungiFun suite, the software tries to find an enrichment of functional categories. The significance of this enrichment process is set by its p-value; the lower the p-value, the more significant the enrichment analysis will be.

For the nitrate condition, the 192 upregulated genes that code for non-hypothetical proteins were analyzed using the KEGG classification system. The results of first level and third level categorization for the nitrate condition are displayed in Fig. 3 and Tab. 11.

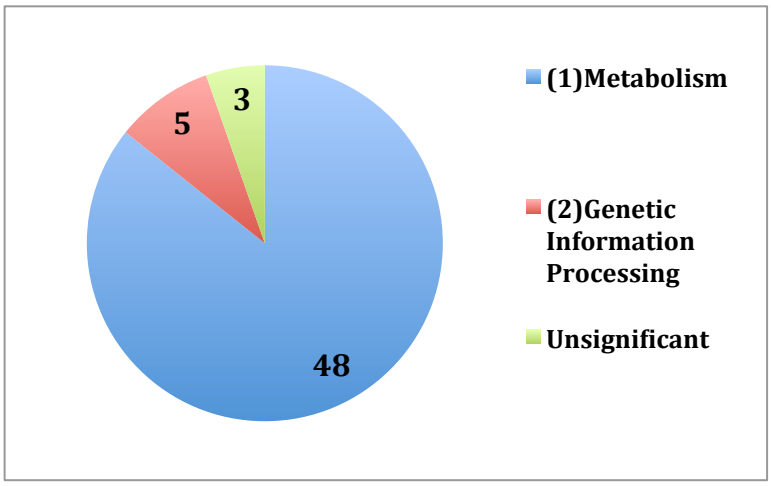

Fig. 3: First level KEGG categorization of the 192 upregulated genes under the nitrate growth condition

Of the 192 upregulated genes under the KEGG categorization, 48 genes can be grouped under Metabolism (KEGG number 1), 5 under Genetic Information Processing (KEGG number 2) and 3 could not be categorized. For enrichment analysis the p-value was set at $p \leq 0.05$.

Tab 11: Third level KEGG categorization of upregulated genes under the nitrate growth condition ($p \leq 0.05$)

KEGG number	Category	hits
1.1.3	Pentose phosphate pathway	4
1.1.13	Butanoate metabolism	7
1.2.8	Nitrogen metabolism	4
1.3.4	Synthesis and degradation of ketone bodies	3
1.5.4	Valine, leucine and isoleucine degradation	4

It can be seen that there is a significant enrichment of metabolic functions among the upregulated genes, with genetic information processing a distant second in the first level KEGG categorization. This becomes even more evident in the third level KEGG categorization where only metabolic functions are present. Here functions of the carbohydrate metabolism (1.1) prevail with 11 functions, followed by energy metabolism (1.2) and amino acid metabolism (1.5) and lastly functions associated with lipid metabolism (1.3). Table 12 takes a detailed look at genes that are associated with nitrate/nitrite metabolism and transport, and which are significantly upregulated in the nitrate condition.

Tab. 12: Transcriptional regulation of nitrate/nitrite metabolizing and transporting proteins depending on the growth medium[a]

Gene name/function	Accession number	NO_3^-	Proline	BSA
nitrate reductase NiaD	Afua_1g12830	41.6	n.s.	4.2
nitrate transporter CrnA	Afua_1g12850	26.4	n.s.	4.6
high affinity nitrate transporter NrtB	Afua_1g17470	23.4	n.s.	2.5
nitrite reductase NiiA	Afua_1g12840	4.4	n.s.	n.s.

[a] the numbers indicate fold up- or downregulation ; n.s. means there is no significant change in transcriptional regulation

It becomes evident that out of these four genes, the first three are dramatically upregulated by nitrate, in fact they constitute the three most upregulated, non-hypothetical genes under the nitrate condition. These three genes are also upregulated to some degree on the BSA condition, but much less so. Proline does not induce any of these four genes. Since the reference condition is the ammonium medium, we also took a look at how ammonium transporters react to the different media (Tab. 13).

Tab. 13: Transcriptional regulation of ammonium transporters depending on the growth medium

Gene name/function	Accession number	NO_3^-	Proline	BSA
ammonium transporter MeaA	Afua_2g05880	3.0	2.5	3.0
ammonium transporter Mep2	Afua_1g10930	2.5	n.s.	n.s.
ammonium transporter MepA	Afua_5g11020	2.2	n.s.	n.s.
plasma membrane ammonium transporter Ato3	Afua_5g01140	- 1.5	n.s.	n.s.

Three out of the four annotated ammonium transporters are upregulated on the nitrate medium, only one is upregulated in the presence of ammonium. The ammonium transporter *meaA* alone is also upregulated in proline medium and BSA medium. In general, it seems as if ammonium transporters are not repressed by the presence of other nitrogen sources.

For the proline condition, the 161 upregulated genes that code for non-hypothetical proteins were analyzed using the KEGG classification system, starting with the first level and third level categorization (Fig. 4 and Tab. 14).

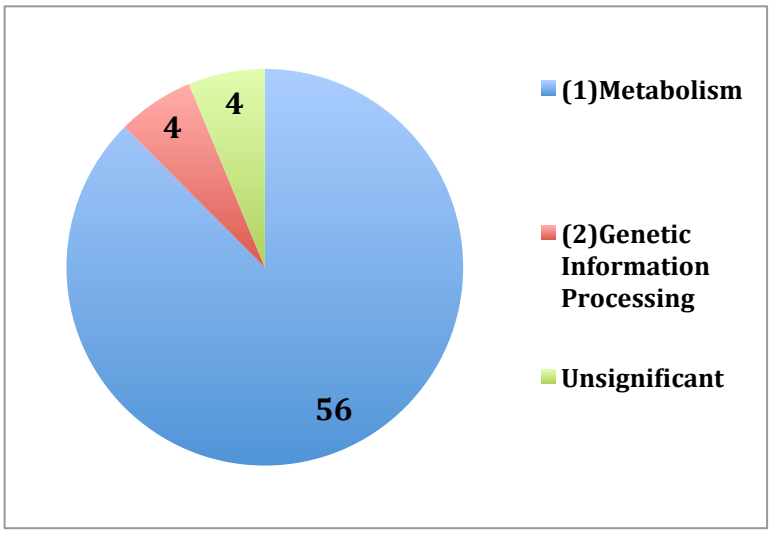

Fig. 4: First level KEGG categorization of the 161 upregulated genes under the proline growth condition

Of the 161 upregulated genes under the KEGG categorization, 56 genes can be grouped under Metabolism (KEGG number 1), 4 under Genetic Information Processing (KEGG number 2) and 4 could not be categorized. For enrichment analysis the p-value was set at $p \leq 0.05$.

Tab 14: Third level KEGG categorization of upregulated genes under the proline growth condition ($p \leq 0.05$)

KEGG number	Category	hits
1.2.8	Nitrogen metabolism	4
1.3.4	Synthesis and degradation of ketone bodies	2
1.5.1	Alanine, aspartate and glutamate metabolism	4
1.5.2	Glycine, serine and threonine metabolism	6
1.5.4	Valine, leucine and isoleucine degradation	7
1.5.5	Valine, leucine and isoleucine biosynthesis	6
1.5.8	Arginine and proline metabolism	8
1.5.10	Tyrosine metabolism	6
1.5.11	Phenylalanine metabolism	4
1.5.13	Phenylalanine, tyrosine and tryptophan biosynthesis	4
1.8.5	Pantothenate and CoA biosynthesis	5

Again, metabolic functions constitute the majority of genes in the first level KEGG categorization with functions of genetic information processing far less enriched. The third level KEGG categorization lists only metabolic functions, the majority of which belong to amino acid metabolism (1.5). Functions of the metabolism of cofactors and vitamins (1.8), energy metabolism (1.2) and lipid metabolism (1.3) are only slightly enriched. Table 15 takes a look at the regulation of the *prn* gene cluster, whose genes have been linked to proline transport and metabolism.

Tab. 15: Transcriptional regulation of the *prn* gene cluster during growth on different nitrogen sources

Gene name/function	Accession number	NO_3^-	Proline	BSA
proline oxidase PrnD	Afua_6g08760	n.s.	521.8	16.6
proline permease PrnB	Afua_7g01090	n.s.	21.7	2.5
proline utilization protein PrnX	Afua_6g08780	n.s.	5.7	2.8
Δ-1-pyrroline-5-carboxylate dehydrogenase PrnC	Afua_6g08750	n.s.	2.2	n.s.
C6 transcription factor PrnA	Afua_6g08790	n.s.	n.s.	n.s.

The transcript of proline oxidase *prnD* is by far the most strongly upregulated gene in the proline medium, the transcript of proline permease *prnB* shows the fifth highest upregulation. The gene *prnC*, which encodes the third structural gene of the *prn* cluster, is more moderately upregulated at 2.2 fold, the transcription factor *prnA* is not upregulated significantly. No other genes associated with amino acid transport are upregulated in proline medium. There are two more genes annotated as proline permeases (Afua_8g02200, Afua_2g11220) and one more proline oxidase (Afua_gG0230) which are not significantly upregulated on the proline condition. The genes *prnD*, *prnB* and *prnX*, that are significantly upregulated by proline are also upregulated in BSA medium, but to a decidedly smaller degree. In contrast to that, nitrate does not lead to any transcriptional upregulation of any of these genes.

Another group of genes that was well represented among the upregulated genes are aminotransferases (Tab. 16, next page). The first five aminotransferases are among the 25 most upregulated genes under the proline condition. Three of ten proline induced aminotransferases are also upregulated in the presence of BSA, while two are downregulated on this medium. One aminotransferase (Afua_1g01680) is induced under all three conditions, while one (Afua_6g04970) is upregulated by proline and repressed in the other two media. In general, aminotransferases seem to play an important role during growth on proline.

Tab. 16: Transcriptional regulation of aminotransferases depending on the growth medium

Gene name/function	Accession number	NO_3^-	Proline	BSA
aromatic aminotransferase Aro8	Afua_2g13630	n.s.	18.1	3.2
branched-chain amino acid aminotransferase	Afua_2g10420	n.s.	9.2	n.s.
alanine aminotransferase	Afua_6g07770	n.s.	8.3	n.s.
aminotransferase	Afua_6g00290	n.s.	8.1	- 3.8
aminotransferase family protein LolT	Afua_2g13295	n.s.	8.0	n.s.
branched-chain amino acid aminotransferase	Afua_4g06160	n.s.	3.8	n.s.
acetylornithine aminotransferase	Afua_2g12470	n.s.	3.4	n.s.
L-ornithine aminotransferase Car2	Afua_4g09140	n.s.	3.3	4.6
branched-chain amino acid aminotransferase	Afua_1g01680	3.1	2.4	5.4
phosphoserine aminotransferase	Afua_6g04970	- 5.4	2.3	- 12.0

Finally, the 758 upregulated genes for the BSA condition that code for non-hypothetical proteins were analyzed using the KEGG classification system, again starting with first level and third level KEGG categorization (Fig. 5 and Tab. 17).

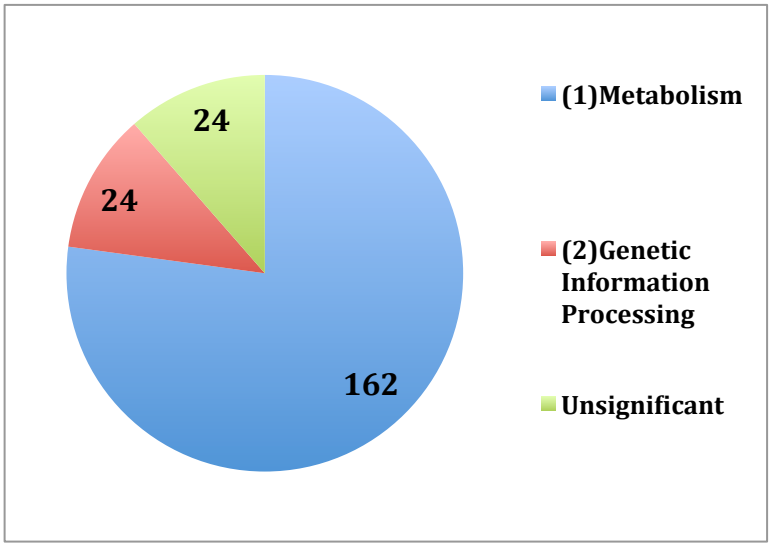

Fig. 5: First level KEGG categorization of the 758 upregulated genes under the BSA growth condition

Of the 758 upregulated genes under the KEGG categorization, 162 genes can be grouped under Metabolism (KEGG number 1), 24 under Genetic Information Processing (KEGG number 2) and 24 could not be categorized. For enrichment analysis the p-value was set at $p \leq 0.05$.

Tab 17: Third level KEGG categorization of upregulated genes under the BSA growth condition ($p \leq 0.05$)

KEGG number	Category	hits
1.1.1	Glycolysis / Gluconeogenesis	11
1.2.1	Oxidative phosphorylation	2
1.2.8	Nitrogen metabolism	10
1.3.1	Fatty acid biosynthesis	4
1.3.3	Fatty acid metabolism	8
1.5.4	Valine, leucine and isoleucine degradation	11
1.5.8	Arginine and proline metabolism	14
1.5.11	Phenylalanine metabolism	10
1.5.12	Tryptophan metabolism	9
1.6.1	beta-Alanine metabolism	7
1.6.5	Cyanoamino acid metabolism	7
1.7.1	N-Glycan biosynthesis	8
1.8.6	Biotin metabolism	3
1.11.23	Metabolism of xenobiotics by cytochrome P450	7
2.1.3	Spliceosome	2
4.1.4	Regulation of autophagy	4

Again, metabolic functions constitute the vast majority in the first level KEGG categorization, again well ahead of functions related to genetic information processing. A look at the third level KEGG categorization reveals an even greater diversity of functions than observed for the other two conditions. Overall, amino acid metabolism (1.5) still constitutes the biggest subgroup with 44 functions all in all, even more when functions of metabolism of other amino acids (1.6) are included. Functions of carbohydrate metabolism (1.1), energy metabolism (1.2) and lipid metabolism (1.3) that appeared in the other conditions as well, are also represented here. Additionally, glycan biosynthesis and metabolism (1.7) and xenobiotics biodegradation and metabolism (1.11) show up in the enrichment analysis. Thus, while the metabolism of amino acids is still the dominant enriched category, more functional complexity seems to be induced by BSA than by proline.

Apart from the quite general analysis by KEGG classification, a more detailed look at which genes are upregulated by BSA can shed a brighter light on *A. fumigatus'* response to BSA as the sole nitrogen source. Table 18 lists the proteases and peptidases that are upregulated on this medium.

Tab. 18: Transcriptional regulation of proteases and peptidases depending on the growth medium; an (*) marks proteins that have been shown or are predicted to be secreted

Gene name/function	Accession number	NO_3^-	Proline	BSA
carboxypeptidase S1	Afua_1g00420	3.8	n.s.	100.8
***secreted dipeptidyl peptidase DppV**	Afua_2g09030	2.0	n.s.	16.8
***extracellular dipeptidyl-peptidase Dpp4**	Afua_4g09320	n.s.	n.s.	15.1
***aminopeptidase Y**	Afua_3g00650	n.s.	n.s.	10.6
vacuolar aspartyl aminopeptidase Lap4	Afua_5g03990	n.s.	n.s.	9.9
prolidase pepP	Afua_2g07500	n.s.	n.s.	8.0
***tripeptidyl-peptidase TppA**	Afua_4g03490	n.s.	- 2.2	6.7
***peptidase**	Afua_6g06800	n.s.	n.s.	4.8
***extracelular serine carboxypeptidase**	Afua_2g17330	n.s.	n.s.	4.5
***aspartic-type endopeptidase**	Afua_3g01220	n.s.	2.1	4.5
***serine peptidase**	Afua_2g01250	n.s.	n.s.	3.9
***carboxypeptidase CpyA/Prc1**	Afua_6g13540	n.s.	n.s.	3.7
***serine carboxypeptidase CpdS**	Afua_6g00310	n.s.	n.s.	3.6
***aspartic endopeptidase Pep2**	Afua_3g11400	n.s.	n.s.	3.3
***aspartic endopeptidase Pep1/aspergillopepsin F**	Afua_5g13300	n.s.	n.s.	3.2
***vacuolar carboxypeptidase Cps1**	Afua_3g07040	n.s.	n.s.	3.2
***aspergillopepsin**	Afua_3g02970	- 2.5	n.s.	3.2
***Peptidase family M28 family**	Afua_1g05960	n.s.	n.s.	3.1
***carboxypeptidase S1**	Afua_5g01200	n.s.	n.s.	2.9
***zinc carboxypeptidase**	Afua_2g08790	n.s.	n.s.	2.7
peptidase D	Afua_1g14920	n.s.	3.2	2.6
metallopeptidase MepB	Afua_7g05930	n.s.	n.s.	2.5
aspartyl aminopeptidase	Afua_3g08290	n.s.	n.s.	2.4
***metalloprotease MEP1**	Afua_1g07730	n.s.	n.s.	2.3
methionine aminopeptidase, type II	Afua_8g00410	2.0	2.1	2.2
dipeptidase	Afua_6g11500	n.s.	n.s.	2.1
***aminopeptidase**	Afua_2g00220	n.s.	n.s.	2.0

All in all, 27 proteases and peptidases are upregulated in BSA medium, the majority of which (n=19) are secreted (Latge and Steinbach, 2009) and five of which were previously shown to be under the control of PrtT: Afua_4g09320, Afua_4g03490, Afua_2g17330, Afua_6g00310 and Afua_5g13300 (Bergmann *et al.*, 2009; Sharon *et*

al., 2009). This is in contrast to the other two growth conditions, where only a very small number of these genes are upregulated, often even to a smaller degree than on BSA. While the proteases and peptidases are significantly upregulated, they are overall not among the most strongly induced genes. Only the carboxypeptidase S1 (Afua_1g00420) is in the top ten most upregulated genes, the two dipeptidyl peptidases are barely in the top 60. Thus, in contrast to the nitrate condition where the nitrate and nitrite associated genes are among the most highly upregulated, or the proline condition where the proline oxidase *prnD* and the proline permease *prnB* are among the strongest induced, the response of proteases and peptidases to BSA is broader, more evenly distributed.

Another group of genes that are especially upregulated by BSA are amidases and aminotransferases (Tab. 19).

Tab. 19: Transcriptional regulation of amidases and aminotransferases depending on the growth medium

Gene name/function	Accession number	NO_3^-	Proline	BSA
general amidase GmdA	Afua_6g08000	n.s.	n.s.	119.2
general amidase	Afua_4g00370	n.s.	n.s.	9.4
general amidase GmdB	Afua_5g00470	2.0	n.s.	6.1
branched-chain amino acid aminotransferase	Afua_1g01680	3.1	2.4	5.4
L-ornithine aminotransferase Car2	Afua_4g09140	n.s.	3.3	4.6
amidase	Afua_5g09140	n.s.	n.s.	4.5
aromatic amino acid aminotransferase	Afua_5g02990	n.s.	n.s.	3.8
aromatic aminotransferase Aro8	Afua_2g13630	n.s.	18.1	3.2
acetamidase	Afua_8g05220	n.s.	n.s.	2.7
aminotransferase	Afua_7g00690	n.s.	n.s.	2.7
aminotransferase, classes I and II	Afua_4g11460	n.s.	n.s.	2.6
general amidase	Afua_1g14530	n.s.	n.s.	2.4

Overall 12 amidases and aminotransferases are upregulated by BSA, among these two genes are also upregulated by nitrate. Comparing table 19 with table 16, it is apparent that there is an overlap of three aminotransferases among the ten aminotransferases induced by proline and the six aminotransferases induced by BSA. It seems that there is a certain substrate specificity in the transcriptional induction of these proteins, which also becomes apparent in the regulation of the aromatic aminotransferase *aro8*, which is much stronger induced by proline than by BSA. Interestingly there is not a single amidase upregulated on proline, compared to six in the

BSA medium. The general amidase *gmdA* is the third most upregulated gene by BSA, the rest of the genes in Tab. 19 are only moderatly upregulated compared to that.

The last focus is on proteins that transport amino acids or peptides (Tab. 20). In total, 11 amino acid or peptide transporters are upregulated by BSA, two of which are also upregulated by nitrate and one by proline. Among the 11 transporters there are five amino acid permeases, two amino acid transporters, two MFS peptide transporters and two oligopeptide transporters. The two MFS peptide transporters, *ptrE* (Afua_1g12240) and *ptrF* (Afua_8g02550), listed in Tab. 20 are not the only MFS transporters among the 758 upregulated genes by BSA, however they are the only two annotated explicitly as peptide transporters. All in all, nine uncharacterized MFS transporters are upregulated in the presence of BSA, some of which might also be implicated in peptide transport. There are two proline permeases upregulated by BSA; it is notable that *prnB* is much stronger upregulated by proline than by BSA, the other proline permease (Afua_1g09120) on the other hand is only upregulated by BSA, but not by proline. Among the two oligopeptide transporters, one (Afua_5g13850) is among the top 20 most upregulated genes in the BSA medium.

Tab. 20: Transcriptional regulation of amino acid and peptide transporters depending on the growth medium

Gene name/function	Accession number	NO_3^-	Proline	BSA
small oligopeptide transporter, OPT family	Afua_5g13850	2.5	n.s.	29.6
proline permease	Afua_8g02200	n.s.	n.s.	17.8
amino acid permease	Afua_1g09120	n.s.	n.s.	15.1
amino acid transporter	Afua_8g00800	n.s.	n.s.	8.8
MFS peptide transporter, putative	Afua_1g12240	n.s.	n.s.	6.8
MFS peptide transporter	Afua_8g02550	n.s.	n.s.	6.5
amino acid permease Gap1	Afua_7g04290	2.8	n.s.	6.4
aromatic amino acid and leucine permease	Afua_8g05860	n.s.	n.s.	6.3
small oligopeptide transporter, OPT family	Afua_2g15240	n.s.	n.s.	5.3
amino acid transporter	Afua_5g09300	n.s.	n.s.	4.9
proline permease PrnB	Afua_7g01090	n.s.	21.7	2.5

Transcription factors deserve special interest when analyzing the transcriptional response to external stimuli, as their regulation and expression can influence a great number of other genes. A number of transcription factors were differentially regulated under each nutrient condition (Tab. 21).

Tab. 21: Transcriptional regulation of transcription factors on nitrate, proline and BSA containing medium

condition	upregulated transcription factors	downregulated transcription factors
NO_3^-	8	12
Proline	2	5
BSA	18	30

The number of regulated transcription factors corresponds loosely with the overall number of up- and downregulated genes under each condition. While many were not further characterized and just annotated as transcription factors, the annotation of some other genes was more detailed (Tab. 22).

Tab. 22: Transcriptional regulation of transcription factors with a more detailed annotation under all three nitrogen source conditions

	Upregulated		Downregulated	
Condition	**Description**	**Accession No.**	**Description**	**Accession No.**
NO_3^-	bZIP TF (Atf7)	Afua_6g12150	AflR-like C6 TF	Afua_2g15340
	C2H2 TF (PacC)	Afua_3g11970	C6 TF (AflR)	Afua_4g14540
	C6 TF (FacB/Cat8)	Afua_1g13510	Zn cluster TF (Rds2)	Afua_2g12330
	HLH TF (GlcDγ)	Afua_1g09670	GATA TF (AreA)	Afua_6g01970
			C6 TF (Ctf1B)	Afua_8g04130
Proline			C6 TF (Ctf1B)	Afua_8g04130
			TF (Snd1/p100)	Afua_5g09250
			Zn cluster TF (Rds2)	Afua_2g12330
BSA	HLH TF (GlcDγ)	Afua_1g09670	PHD TF (Rum1)	Afua_5g03430
	C6 TF (OefC)	Afua_3g09670	TF (AbaA)	Afua_1g04830
	C6 TF (Leu3)	Afua_2g15260	C2H2 conidiation TF (FlbC)	Afua_2g13770
	C6 TF (AlcR)	Afua_5g07510	C2H2 TF (Crz1)	Afua_1g06900
	HLH TF (Hpa3)	Afua_2g14800	transcript. repressor (TupA/RocA)	Afua_6g05150
	GATA TF (AreB)	Afua_2g13380	siderophore TF (SreA)	Afua_5g11260
	bZIP TF (Atf7)	Afua_6g12150	bZIP TF (CpcA)	Afua_4g12470
	C6 TF (PrtT)	Afua_4g10120	bZIP TF (MeaB)	Afua_3g10930
			C6 TF (Ctf1B)	Afua_8g04130
			Zn cluster TF (Rds2)	Afua_2g12330

It becomes evident, that transcription factors from a wide range of families, such as zinc cluster transcription factors, C6 transcription factors or bZIP transcription factors are present among the transcriptionally regulated genes.

There are a number of transcription factors, that are known to be involved in nitrogen metabolism in aspergilli, whose regulation is of particular interest (Krappmann and Braus, 2005)(Tab. 23).

Tab. 23: Transcriptional regulation of known transcription factors involved in nitrogen metabolism on each nitrogen source condition

Gene name	Accession number	NO_3^-	Proline	BSA
prtT	Afua_4g10120	n.s.	n.s.	6.7
cpcA	Afua_4g12470	n.s.	n.s.	- 3.2
areA	Afua_6g01970	- 2.9	n.s.	n.s.
prnA	Afua_6g08790	n.s.	n.s.	n.s.
nirA	Afua_5g12020	n.s.	n.s.	n.s.
meaB	Afua_3g10930	n.s.	n.s.	- 3.2

It can be seen that, with the exception of *prtT* on the BSA medium, none of these transcription factors are significantly upregulated on any of the three secondary nitrogen sources. There is no regulation either way on the proline medium, on the nitrate medium only *areA* is downregulated and two (*cpcA* and *meaB*) out of six transcription factors are downregulated by BSA.

After looking at each dataset individually we decided to also investigate to what degree the three datasets show an overlap in their upregulated genes. The Venn diagram in figure 6 gives an idea about that.

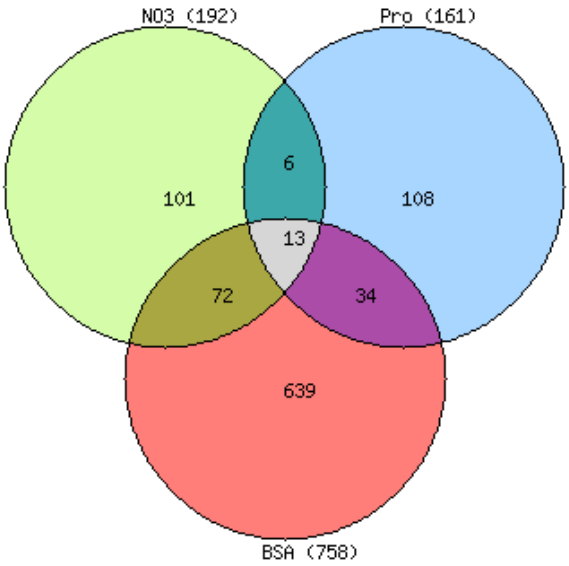

Fig. 6: Venn diagram showing the overlap of transcriptionally upregulated genes between the growth conditions: (NO3) nitrate as sole nitrogen source, (Pro) proline as sole nitrogen source and BSA as sole nitrogen source.
A. fumigatus, when grown on nitrate as the sole nitrogen source, transcriptionally upregulates 192 genes that code for non-hypothetical proteins. On proline as sole nitrogen source 161 such genes are upregulated, on BSA 758 genes coding for non-hypothetical proteins are upregulated. There are six genes that are upregulated on both nitrate and proline medium, 34 genes are upregulated on both proline and BSA medium and 72 genes are upregulated on both BSA and nitrate medium. 13 genes are upregulated under all three conditions.
The transcriptional profile *of A. fumigatus* grown on ammonium medium served as the reference condition for all three conditions.

The overlap between the nitrate and the BSA condition is biggest with 72 shared genes, followed by the overlap between proline and BSA with 34 shared genes. The overlap between nitrate and the proline condition is only six genes. Thirteen genes are upregulated on all three conditions, which are listed in Tab. 24 below.

Tab. 24: Transcriptional upregulation of the 13 genes that are upregulated under all three nitrogen source conditions

Gene name/function	Accession number	NO_3^-	Proline	BSA
branched-chain amino acid aminotransferase	Afua_1g01680	3.1	2.4	5.4
F-box domain protein	Afua_1g02200	3.4	2.1	2.7
urease Ure	Afua_1g04560	2.8	2.3	7.6
conserved histidine-rich protein	Afua_1g11910	2.8	2.8	3.0
uridine permease Fui1	Afua_1g13210	12.2	4.8	55.9
MFS transporter	Afua_1g17160	2.9	2.9	2.5
ammonium transporter MeaA	Afua_2g05880	3.0	2.5	3.0
hydroxymethylglutaryl-CoA lyase	Afua_2g12450	7.2	3.0	21.0
phosphoglycerate mutase family protein	Afua_6g02600	3.2	3.1	16.1
C-5 cytosine methyltransferase DmtA	Afua_6g03740	2.1	2.8	2.6
sugar isomerase, KpsF/GutQ	Afua_6g08860	2.7	2.1	10.3
glutamine-serine-proline rich protein	Afua_7g04870	2.0	2.3	2.4
methionine aminopeptidase, type II	Afua_8g00410	2.0	2.1	2.2

In ten out of thirteen genes is the BSA condition the one with the highest upregulation among the three conditions. There is no obvious enrichment of any molecular function, except for the phosphoglycerate mutase (Afua_6g02600) and the sugar isomerase (Afua_6g08860) which might both play a role in gluconeogenesis.

The 34 overlapping genes between the proline and the BSA condition are of particular interest, since both conditions contain amino acids as the sole nitrogen source, either in the soluble form or as building blocks of a larger molecular structure. As before, the genes were categorized using FungiFun and the KEGG classification. It was not possible to obtain any meaningful first level categorization under KEGG, on the second level 6 genes could be grouped under amino acid metabolism (1.5). The results of the third level KEGG categorization are displayed in Tab. 25 below.

Tab 25: Third level KEGG categorization of the 34 overlapping upregulated genes between the proline growth condition and the BSA growth condition ($p \leq 0.1$)

KEGG number	Category	hits
1.5.4	Valine, leucine and isoleucine degradation	2
1.5.6	Lysine biosynthesis	1
1.5.8	Arginine and proline metabolism	2
1.8.6	Biotin metabolism	1
2.3.3	Ubiquitin mediated proteolysis	2

Five of the six genes grouped under amino acid metabolism could be categorized down to the third KEGG level. However, for this dataset of 34 genes, the descriptive power of the KEGG classification is rather poor. For instance, it completely fails to categorize the proline oxidase *prnD*, which is clearly involved in proline metabolism and whose transcriptional upregulation had been found to be shared by the proline medium and the BSA medium before (Tab. 15).

Thus, FungiFun was used again to analyze the 34 genes, this time using the FunCat (Functional Catalogue) classification (Ruepp *et al.*, 2004), concentrating on the results of first and second level categorization (Fig. 7 and Tab. 26).

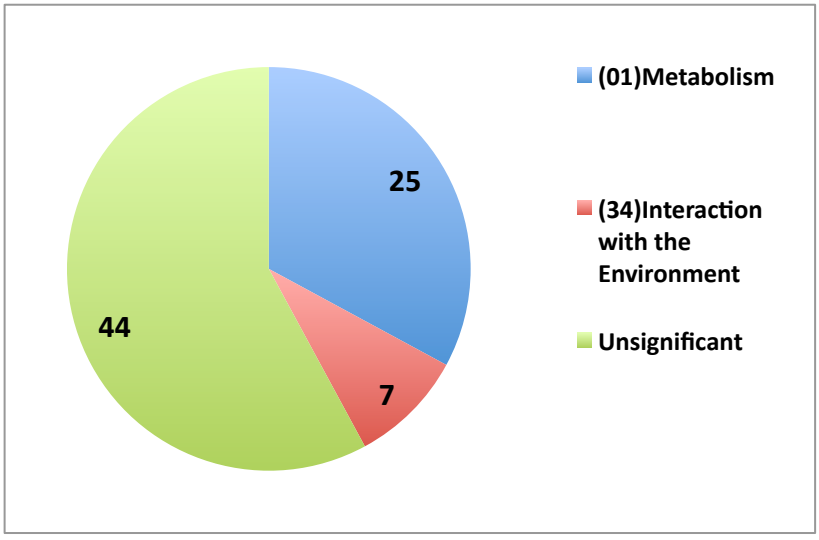

Fig. 7: First level FunCat (Functional Catalogue) categorization of the 34 overlapping upregulated genes between the proline growth condition and the BSA growth condition
Among the 34 upregulated genes, 76 first level molecular functions could be identified. 25 functions could be grouped under the functional category "metabolism" (FunCat ID 01), 7 under the category "interaction with the environment" (FunCat ID 34) and 44 functions could not be significantly grouped into a category. For enrichment analysis the p-value was set at $p \leq 0.1$.

Tab. 26: Second level FunCat categorization of the 34 overlapping upregulated genes between the proline growth condition and the BSA growth condition ($p \leq 0.1$)

FunCat ID	Category	hits
01.01	amino acid metabolism	12
01.02	nitrogen, sulfur and selenium metabolism	5
01.05	C-compound and carbohydrate metabolism	11
01.20	secondary metabolism	8
14.13	protein/peptide degradation	4
34.11	cellular sensing and response to external stimulus	6

It can be seen that the FunCat classification is much more promiscuous than KEGG in categorizing genes, since in the first level FunCat categorization, each gene is assigned two molecular functions on average. There are also many more hits in table 26 than under the KEGG second level categorization or the third level in table 25.

Among the 12 hits under amino acid metabolism (FunCat ID 01.01), the most significant genes are those that have already appeared before: the peptidase D (Afua_1g14920), the aromatic aminotransferase *aro8* (Afua_2g13630), the L-ornithine aminotransferase *car2* (Afua_4g09140), the proline oxidase *prnD* (Afua_6g08760) and the proline utilization protein *prnX* (Afua_6g08780). The peptidase D does also appear under protein/peptide degradation (FunCat ID 14.13), the other interesting hit under this category is an aspartic-type endopeptidase (Afua_3g01220). Under the category secondary metabolism (FunCat ID 01.20) there are three members of the cytochrome P450 superfamily, two genes annotated as monooxygenases (Afua_4g09470, Afua_6g02210), which were also categorized under amino acid metabolism, and one annotated as an alkane hydroxylase (Afua_6g08460). The category cellular sensing and response to external stimulus (FunCat ID 34.11) interestingly contains two transporters, one annotated as an MFS sugar transporter (Afua_1g02530) and the proline permease *prnB* (Afua_7g01090). There is also a MAP kinase kinase *pbs2* (Afua_1g15950) in that category. The genes grouped under C-compound and carbohydrate metabolism (FunCat ID 01.05) come from a great variety of metabolic processes.

From this transcriptome analysis it becomes evident, that growth on a proteinaceous nitrogen source such as BSA, demands from *A. fumigatus* the ability to digest the extracellular protein and take up the breakdown products. While the action of secreted proteases is relatively well understood in this fungus, little is known about the transport systems necessary to import the nutrients into the cell. Thus, we focussed our further investigation on this aspect, concentrating on oligopeptide transport.

4.2. A first *in silico* characterization of the eight member *opt* genefamily in *Aspergillus fumigatus*

The *opt* gene family had not been characterized at all in *Aspergillus fumigatus*, thus it was not even known how many genes there are. In order to identify possible members of the *opt* gene family in *Aspergillus fumigatus*, a BLASTP search in an *A. fumigatus* gene database (http://www.cadre-genomes.org.uk/Aspergillus_fumigatus) using standard parameters was performed. A positive hit was defined as having an E-value lower than 1 x e^{-60} across the entire length of the amino acid sequence, compared to the reference protein. Using the *Candida albicans* Opt1p protein sequence (available at http://www.candidagenome.org), seven open reading frames (ORFs) encoding putative proteins fullfilling this criterion could be identified. The proteins following these first seven genes all showed E-values of 0.4 or higher and where thus insignificant hits. In addition to that, a search using the *C. albicans* Opt8p protein yielded an eighth candidate gene.

The identified genes are listed in Tab. 27, the database accession numbers refer to the Central *Aspergillus* Data REpository (C*A*DRE) database (http://www.cadre-genomes.org.uk/), which also predicted the length of the ORFs in amino acids. The number of transmembrane domains was predicted using a web-based prediction program (http://www.cbs.dtu.dk/services/TMHMM/).

Tab. 27: Putative members of the *opt* gene family in *A. fumigatus*

Gene name	Database Accession Number	Length in amino acids	Number of transmembrane domains
optA	Afua_1g13620	843	14
optB	Afua_2g15240	792	14
optC	Afua_3g12200	757	14
optD	Afua_5g13850	766	11
optE	Afua_6g10050	751	12
optF	Afua_6g10220	750	15
optG	Afua_6g03140	787	12
optH	Afua_7g00910	757	13

As can also be seen in Fig. 8, the genes hardly cluster on the *A. fumigatus* genome, instead they spread on six of the eight chromosomes. With the exception of *optA*, the proteins encoded by the *opt* genes are between 750 and 800 amino acids in length and they have between 11 and 14 transmembrane domains. A representative

example of the membrane topology of OPT proteins is shown in Fig. 9. The even distribution of the transmembrane domains, which suggests the formation of a central pore, is in accordance with the proposed function of the protein to mediate substrate transport across membranes (Saier, 2003).

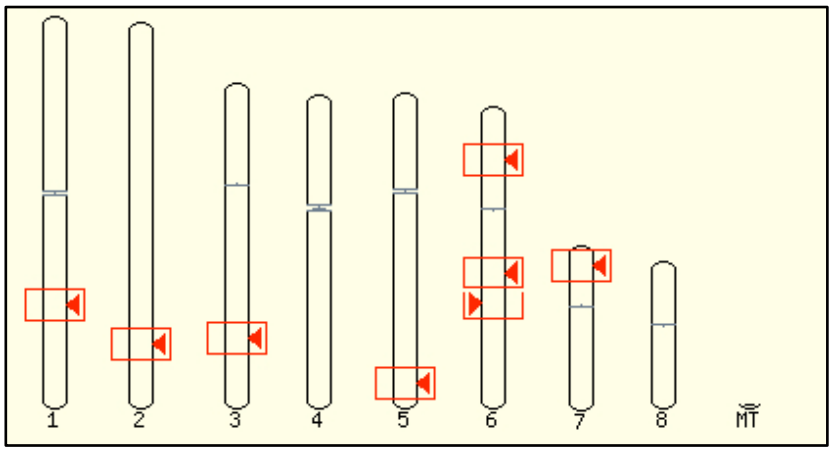

Fig. 8: Distribution of the putative *opt* genes on the *A. fumigatus* genome
The eight chromosomes that comprise the genome of *A. fumigatus* are shown, as well as the mitochondrial genome (MT). The position of each *opt* gene is indicated by a boxed, red arrow. The eight members of the *opt* gene family are spread over six of the eight chromosomes. Chromosome six harbours three *opt* genes two of which, *optE* and *optF* are localized in close proximity to each other, about 48.5kb apart.

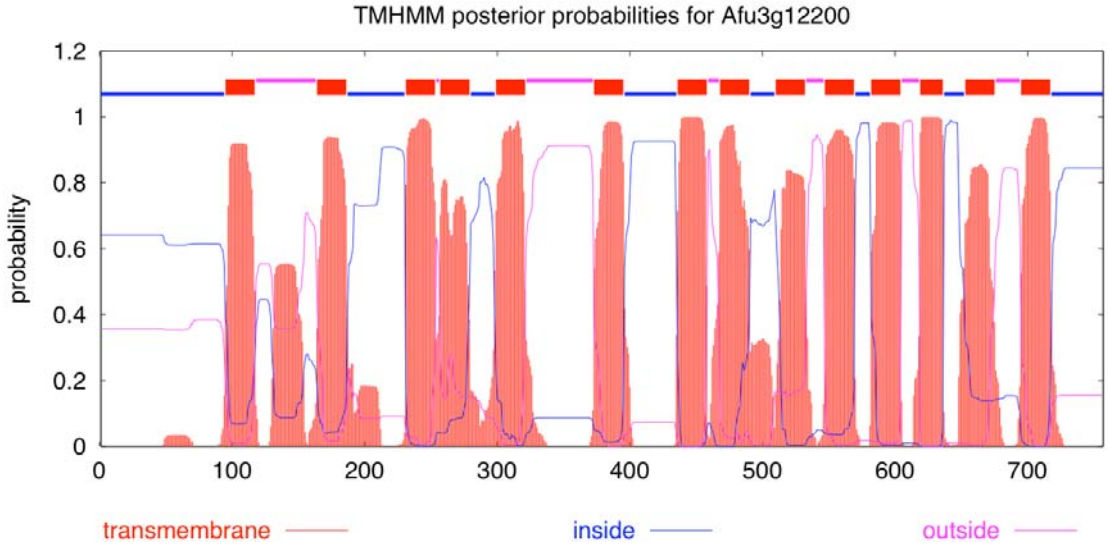

Fig. 9: Membrane topology of the OptC protein
On the x-axis the OptC amino acid sequence is displayed, ranging from the N-terminus at position one to the C-terminus at position 757. The y-axis displays the probability of a stretch of sequence being located inside a membrane. After a short N-terminal leader sequence, the transmembrane domains are spread evenly across the full length of the protein. The topology of the proteins was predicted by a web-based prediction program (http://www.cbs.dtu.dk/services/TMHMM/).

In an effort to analyze the putative *opt* gene family in greater detail, protein sequence alignments were carried out, in which the amino acid sequences of the eight *opt* genes were compared amongst each other and against the *C. albicans OPT1* gene product (Tab. 28, Fig. 10).

Table 28: Per cent identity (upper right) and similarity (lower left) among the proteins of the *A. fumigatus opt* gene family and *C. albicans* Opt1p. [a]

	OptA	OptB	OptC	OptD	OptE	OptF	OptG	OptH	CaOPT1
OptA		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
OptB	n.s.		37	40	41	41	25	25	48
OptC	n.s.	58		38	38	39	25	24	39
OptD	n.s.	59	58		49	51	26	27	42
OptE	n.s.	61	58	67		48	26	25	41
OptF	n.s.	60	57	70	68		23	24	43
OptG	n.s.	40	42	45	45	41		25	25
OptH	n.s.	43	43	44	45	42	43		24
CaOPT1	n.s.	64	57	61	59	61	44	42	

[a] **n.s. refers to a non-significant similarity/identity. The sequences of the *A. fumigatus* proteins were based on cDNA information, which was aquired in the course of this work. The *C. albicans* Opt1p protein sequence was retrieved from http://www.candidagenome.org.**

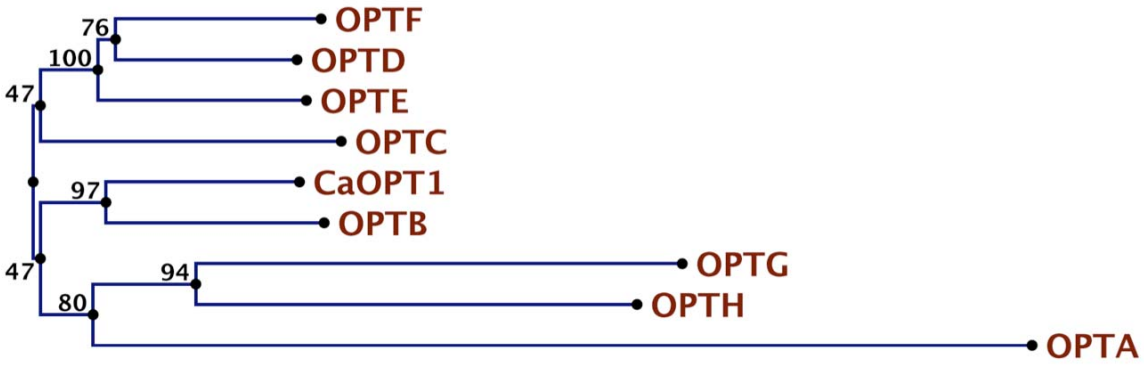

Fig. 10: Dendrogram using the bootstrap method of the eight *A. fumigatus opt* genes and *C. albicans* Opt1p based on their amino acid sequence
The dendrogram was created using the program CLC Free Workbench 4.5.1 using the default parameters. The *A. fumigatus* protein sequences are based on the cDNA information aquired during this work. The *C. albicans* Opt1p protein sequence was aquired from http://www.candidagenome.org.

Both Tab. 28 and Fig. 10 clearly show that OptA occupies a special position within the *opt* gene family. There is no significant homology of OptA to any of the other seven OPTs, and in the dendrogram it constitutes a very isolated branch of the tree. There is however a high homology to *C. albicans* Opt8p, with 40% sequence

identity and 59% amino acid similarity. Almost identical values of identity (38%) and similarity (55%) are displayed when compared to the *S. cerevisiae* YGL114wp. This suggests that OptA is the one member of the yellow stripe (YS) clade of oligo peptide transporters in *A. fumigatus* (Lubkowitz, 2006).

OptB through OptF show a high sequence homology among themselves, with OptD to OptF forming a particularly tight cluster with sequence identity of around 50%. Overall this group of five transporters are most closely related to the *C. albicans* Opt1p, and constitute the core group within the *A. fumigatus opt* gene family.

OptG and OptH show a markedly lower homology to the other OPTs, with sequence identities of around 25%. However, they also show only 25% identity to each other, which means they do not form a distinct subgroup within the *opt* gene family, but are rather two independently separate family members. While OptH is only moderately similar to the other OPTs in *A. fumigatus*, it shows a striking resemblance to MTD1 in *Schizophyllum commune* of 49% sequence identity on the protein level (Lengeler and Kothe, 1999). Accordingly, OptB to OptH form the peptide transport (PT) clade of oligo peptide transporters in *A. fumigatus*.

In order to assess the significance of the *opt* gene family during the *in vivo* infection process of a mammalian host, the data of a trancriptome study conducted by another group were analyzed (McDonagh *et al.*, 2008). In that study the transcriptional profile of *A. fumigatus* during the early stages of infection of a mouse was analyzed, comparing the mRNA expression of genes *in vivo* with mRNA levels of a culture grown on YPD medium. The results concerning the *opt* gene family are displayed in Fig. 11. The *in vivo* data suggest, that at least three OPTs might play a role in the infection process, as their genes are significantly higher expressed inside the murine lung.

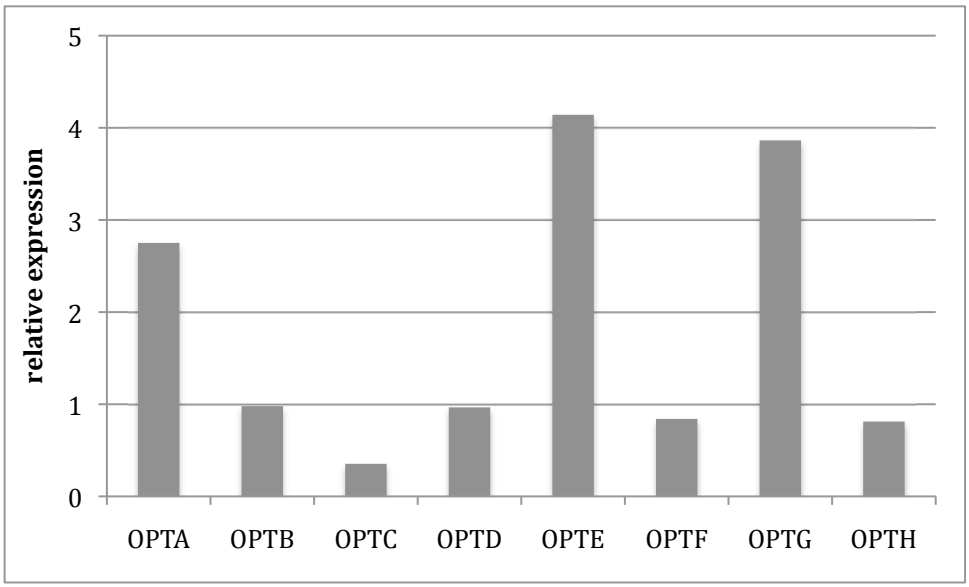

Fig. 11: Expression of *A. fumigatus opt* genes during the early mammalian infection process
Relative expression of the *opt* gene family *in vivo* compared to growth on YPD medium. Three *opt* genes, *optA*, *optE* and *optG* are significantly upregulated. The *optC* gene is downregulated, the remaining four *opt* genes show no significant differences in expression. (based on data in (McDonagh *et al.*, 2008))

However, looking purely at the fold up- or down-regulation of genes might not be sufficient. Apart from the fact that one or more of the *opt* genes might be expressed constitutively high irrespective of the growth conditions, the YPD medium which acts as the baseline for comparison against *in vivo* expression, also contains significant amounts of oligopeptides. YPD medium consists, among other things, of peptone which is an enzymatic digest of proteins. This procedure leads to a mixture of free amino acids, and a large number of different peptides (according to Difco Laboratories, MD, USA). Thus, it is conceivable that some *opt* genes might be expressed highly on YPD already, so that these highly expressed genes could be major oligopeptide transporters inside the mouse as well, without being further upregulated.

This is why an attempt was made to estimate the absolute expression of the genes. Using the available raw data to approximate the absolute mRNA levels. The spot intensity on the microarrays as a measure of mRNA abundance was used. This approach assumes a positive correlation between the brightness of a spot, and the absolute mRNA level of a given gene. This approximation is very rough, as it neglects effects such as different reverse transcription efficiency of mRNAs, dye labeling efficiency or binding efficiency of the labelled cDNA to the target sequence on the chip. Figure 12 shows the absolute spot intensities of the *opt* genes during the mouse infection.

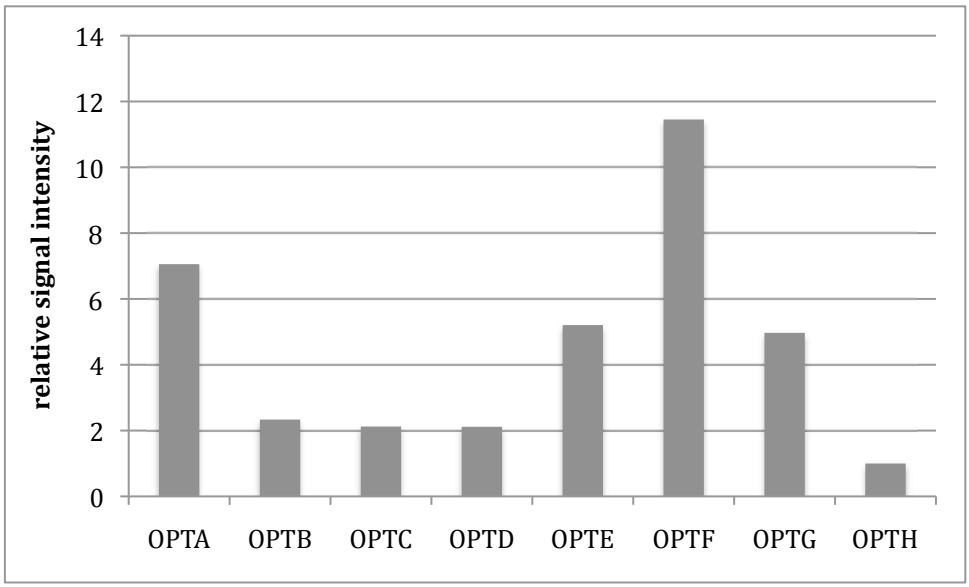

Fig. 12: Approximation of absolute mRNA levels of *opt* genes during infection of a mammalian host based on microarray spot intensities
For each gene the absolute signal intensity of the array spot was taken, and compared to the intensity of the darkest spot, which was *optH*. The value of *optH* was set as the baseline value one. Four spots, *optA*, *optE*, *optF* and *optG*, were significantly brighter than *optH*, with the *optF* signal being more than eleven times higher. (E. Bignell, personal communication)

The comparison of signal intensities shows, that the three *opt* genes that had been transcriptionally upregulated *in vivo*, *optA*, *optE* and *optG*, are also among those with the brightest array spots. Additionally, the *optF* signal appears exceptionally bright. Assuming a correlation between spot intensity and mRNA abundance, and assuming that higher mRNA abundance leads to higher protein levels, this might give a hint to a possible role for OptF during the infection process.

4.3. Heterologous expression of *A. fumigatus opt* genes in *S. cerevisiae*

In order to assess the function of the individual oligopeptide transporters of *A. fumigatus*, we turned to *Saccharomyces cerevisiae* as a model organism for heterologous expression of the proteins. The experimental setup was adapted from the previously described expression of *Arabidopsis thaliana* oligopeptide transporters in yeast (Koh *et al.*, 2002).

The basis of these complementation experiments is an *S. cerevisiae* strain that is auxotrophic for one particular amino acid. This strain is grown on a synthetic dropout medium that lacks this amino acid, but which is supplemented with an oligopeptide that contains this specific amino acid. Since *S. cerevisiae* does not hydrolyze oligopeptides extracellularly (Hauser *et al.*, 2000), the yeast can only grow if it is able to transport sufficient amounts of the oligopeptide into the cell cytoplasm. If the auxotrophic *S. cerevisiae* strain by itself is not able to transport enough of this oligopeptide, then there is no or only little growth on the medium. If however, a functional oligopeptide transporter is expressed which is able to transport that particular oligopeptide in sufficient amounts, then it is possible for the auxotrophic *S. cerevisiae* strain to grow on a medium, where this oligopeptide is the only available source of the amino acid the yeast is auxotrophic for. This finding would support the hypothesis, that the expressed oligopeptide transporter is functional and can mediate the transport of this particular oligopeptide.

We chose the *S. cerevisiae* strain BY4742 as the starting point of the experiments, which carries auxotrophies for histidine, leucine, lysine and uracil. We aimed to express all eight *A. fumigatus opt* genes in BY4742, as well as the *C. albicans OPT1* gene as a positive control (Lubkowitz *et al.*, 1997). As the expression vector we chose pSK51, a high copy plasmid which carries the *URA3* gene as a marker and the methionine-repressible *MET25* promotor for expression of a chosen gene cloned behind it (Funk *et al.*, 2002; Krappmann *et al.*, 2006).

First, we amplified via PCR the full-length cDNA of all eight *A. fumigatus* oligopeptide transporters from already available cDNA which we had used in qRT-PCR experiments (described in 4.4). The cDNA was based on extracted RNA from those samples, where either collagen or BSA was the only nitrogen source, since these were the conditions under which the *opt* gene family was most highly expressed. For the

C. albicans, OPT1 template cDNA based on RNA from a BSA culture was kindly provided by the group of Joachim Morschhäuser (IMIB, Universität Würzburg).

Amplification was achieved by PCR using a gene-specific primer pair for each oligopeptide transporter. Each primer was flanked by restriction sites, that would enable a directed insertion into pSK51. *A. fumigatus optA* was amplified using the primer pair THA112/113, *optB* was amplified using the primer pair THA115/116, *optC* was amplified using the primer pair THA118/119, *optD* was amplified using the primer pair THA121/122, *optE* was amplified using the primer pair THA124/125, *optF* was amplified using the primer pair THA127/128, *optG* was amplified using the primer pair THA130/131, *optH* was amplified using the primer pair THA133/134 and *C. albicans OPT1* was amplified using the primer pair THA136/137. The amplified fragments then underwent a restriction enzyme digest, which led to "sticky ends" that made insertion into digested pSK51 possible.

Thus, the PCR fragments of *optB, optE, optF, optG* and *OPT1* underwent a NotI/SalI double digestion and were cloned into a NotI/SalI double digested pSK51 via ligation. The fragments of *optC* and *optH* were double digested with NotI and XhoI and also cloned into NotI/SalI digested pSK51. The *optD* fragment was digested with NotI only and inserted into a NotI digested pSK51 vector backbone that had undergone an additional dephosphorylation step. For *optA* it was necessary to first insert the fragment into the TOPO cloning vector forming pSK527, and excise it via a SalI restriction digest, which was then inserted into a SalI digested and dephosphorylated pSK51 vector backbone. After transformation of the ligation reactions into *E. coli* DH5α, the correct clones were identified by colony PCR using the pSK51 specific primer Sv62 and a gene specific primer, which was THA111 for *optA*, THA114 for *optB*, THA117 for *optC*, THA120 for *optD*, THA123 for *optE*, THA126 for *optF*, THA129 for *optG*, THA132 for *optH* and THA135 for *CaOPT1*. Positive plasmids were extracted from *E. coli*, and sequenced using Sv62 and THA148 as pSK51 specific primers and gene specific primers for each *opt* gene, THA138 and THA139 for *optA*, THA140 for *optB*, THA141 for *optC*, THA142 for *optD*, THA143 for *optE*, THA144 for *optF*, THA145 for *optG*, THA146 for *optH* and THA147 for *OPT1*. For each *opt* gene at least two plasmids were sequenced in order to account for possible mutations of the cDNA compared to the genomic sequence of *A. fumigatus*. It was possible to obtain a pSK51 plasmid carrying the correct cDNA sequence for each of the eight *A. fumigatus opt* genes and for *C. albicans OPT1*, which were named pSK528 to pSK536 as listed in Tab. 2.

The plasmids pSK528 to pSK536 and pSK51 were transformed into *S. cerevisiae* BY4742, and the transformants were plated onto selective YNB(GPH) +leu/+lys/-ura medium (described in detail in materials and methods), which selects for the presence of the *URA3*-carrying pSK51 vector backbone. The resulting plasmid-carrying yeast strains were named SKY133 to SKY142 as listed in Tab. 4.

For growth assays, a single colony of each yeast strain was grown in liquid YNB(GPH) +leu/+lys/-ura medium for 48 hours. After washing twice with sterile water, serial dilutions of each strain were plated on selective YNB(GPH) medium that was supplemented with a specific oligopeptide as the sole available leucine or lysine source. As leucine containing oligopeptides, we chose the pentapeptide KLLLG, the tetrapeptide (Des-Tyr^1)-Leu-Enkephalin (GGFL) and the tetrapeptide LWMR. These peptides were added as supplements to 100ml YNB(GPH) +lys/-leu/-ura medium, 10mg of KLLLG, 20mg of GGFL and 30mg of LWMR. Since BY4742 is also lysine auxotroph we included the lysine containing kentsin (TPRK), 20mg of which was added to 100ml YNB(GPH) +leu/-lys/-ura medium. As a growth control, to make sure equal amounts of viable cells were plated, YNB(GPH) +leu/+lys/-ura medium was included.

The plates were incubated for 96 hours, after which growth was assessed (Fig. 13). The growth tests on oligopeptide containing media show the functionality of several *A. fumigatus opt* gene products as oligopeptide transporters. OptB seems to be the most versatile transporter, as it has the widest substrate spectrum, enabling growth on each of the four oligopeptides, and the highest transport efficiency, as it is the protein that promotes the best growth on all media. In these traits, it is most similar to the *C. albicans* Opt1p transporter. Second in versatility is OptG, which promotes growth on KLLLG, GGFL and LWMR. However, OptG is not able to support as vigorous growth as OptB, especially on GGFL and LWMR, barely enabling BY4742 to grow better than the negative control. OptC seems best suited to transport GGFL, almost enabling growth as well as OptB. On LWMR, OptC seems to support BY4742 growth only to a small degree, but still better than the negative control. The substrate spectrum of OptH is the same as that of OptC, however, especially on GGFL it seems not as efficient a transporter as OptC. OptE is the protein with the most narrow substrate specificity. It only promotes better growth on LWMR and the difference to the negative control is very small. The remaining oligopeptide transporters OptA, OptD and OptF do not seem to transport any of the oligopeptides employed in this assay.

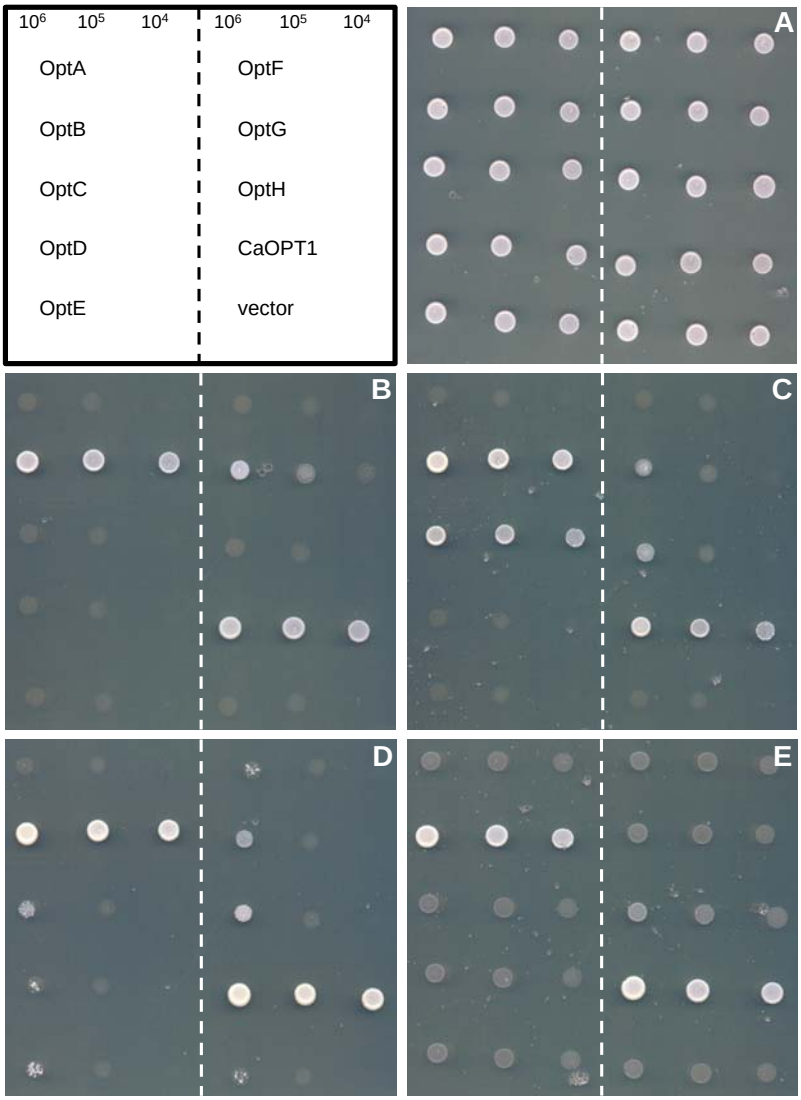

Fig 13: Growth of *S. cerevisiae* BY4742, carrying heterologously expressed *A. fumigatus* oligopeptide transporter genes, on medium with oligopeptides as sole source for leucine or lysine

The schematic panel at the top-left illustrates the plate setup that is identical on each plate. The strains are identified by the oligopeptide transporter gene they carry on the pSK51 plasmid, i.e. OptA refers to strain SKY133 carrying the *optA* gene; "vector" refers to SKY142, which harbours the pSK51 plasmid itself and which is the negative control. The number of cells plated on each spot is indicated at the top, i.e. from left to right 10^6, 10^5 and 10^4 cells were plated.

Panel A shows the growth control for each strain on YNB(GPH) +leu/+lys/-ura medium; all strains grow equally well for all dilutions. Panel B shows growth on medium with the pentapeptide KLLLG, the *optB* carrying strain grows very well, the *optG* carrying strain still grows significantly better than the negative control. The plates on panel C contain the tetrapeptide GGFL; the *optB* carrying strain grows very well, the *optC* carrying strain grows only slightly worse and the two strains carrying *optG* and *optH* grow visibly better than the negative control only for the 10^6 spot. On panel D growth on medium with the tetrapeptide LWMR is shown. Again, the *optB* carrying strain grows very well, among the other strains there is visibly better growth than the negative control for the 10^6 spot for *optH* and *optG*, and somewhat better growth for *optC* and *optE*. On panel E growth on medium with the tetrapeptide TPRK is shown. Only the *optB* carrying strain grows very well.

On all four oligopeptide plates, the *C. albicans OPT1* carrying strain grew very well, the strain carrying the empty pSK51 vector grew very little (D, E) or not at all (B,C).

4.4. The transcriptional response of peptide uptake systems to different nitrogen sources

In order to elucidate the role the *opt* gene family plays in *A. fumigatus*, the transcriptional response of the *opt* genes to growth on different media was investigated. Especially the transcriptional reaction to differing nitrogen sources was of interest. We concentrated on four nitrogen sources: ammonium as the prefered nitrogen source in mineral medium, YPD as a very diverse nutrient-rich medium, collagen as one of the main components of the extracellular matrix in the lung and BSA as an undigested proteinaceous source of carbon and nitrogen.

The experimental setup was modified after Narendja *et al.* (Narendja *et al.*, 2002), and using qRT-PCR the transcriptional profile of the *opt* gene family was analysed. As the internal control we chose the housekeeping gene encoding the tubulin β subunit (Afua_1g10910), for which the primer pair THA73/74 was used. For each of the *opt* genes a primer pair was designed and used in the qRT-PCR: THA75/76 for *optA*, THA77/78 for *optB*, THA79/80 for *optC*, THA81/82 for *optD*, THA83/84 for *optE*, THA85/86 for *optF*, THA87/88 for *optG* and THA89/90 for *optH*.

Figure 14 shows the results of the qRT-PCR of the *opt* gene family when confronted with the different nitrogen sources. On each medium, the *opt* gene family shows a different expression profile. On YPD only *optB* and *optE* show an about 4-fold upregulation, while the transcript levels of the other *opt* genes are comparable to the NH_4^+ medium. On collagen hydrolysate based medium, it is *optB* and *optH* that are upregulated, but to a vastly different degree. While *optH* is upregulated only about 3.5-fold, *optB* shows a more than 100-fold upregulation. The *opt* gene family as a whole reacts most to the presence of BSA, where almost the entire gene family is upregulated significantly. The individual upregulation varies between 2-fold for *optF* and more than 100-fold for *optB*.

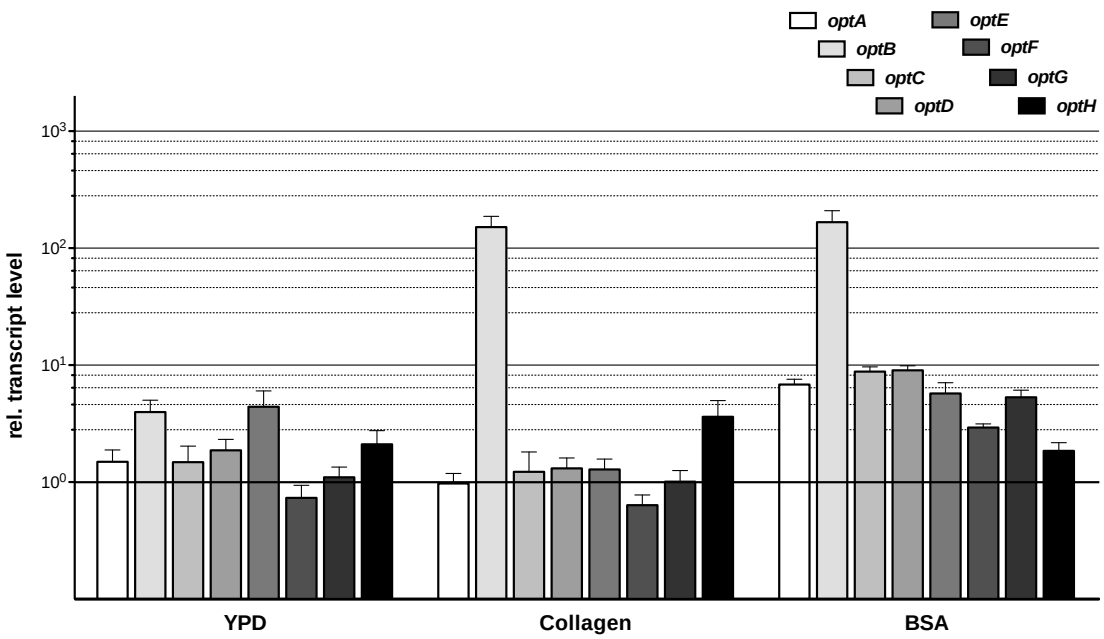

Fig. 14: Transcriptional profile of the *opt* gene family during growth on different nitrogen sources
For each of the eight *opt* genes the transcript level on the NH_4^+ -based medium was set as the baseline value of one, to which the transcriptional level on YPD, collagen hydrolysate and BSA was compared. There is no or only minor induction of the *opt* gene family on YPD. On collagen hydrolysate *optB* is induced dramatically and *optH* shows a much lower but still significant upregulation, while the other six *opt* genes show no difference. On BSA all *opt* genes, except *optH*, are significantly upregulated, with *optB* again showing the most drastic induction.
Experiments for collagen hydrolysate were performed in biological and technical duplicate, experiments for the other conditions were done in biological triplicate and technical duplicate.

From these experiments it appears as if there is a substrate-specific expression pattern for the *opt* gene family. Whereas YPD medium does not significantly stimulate transcription of the *opt* gene family, on substrates where large peptides or proteins act as both nitrogen and carbon source, the oligopeptide transporters do seem to play a role. On collagen hydrolysate, the importance seems to be confined to *optB* and to some degree *optH*. On BSA, the *opt* gene family as a whole is transcriptionally upregulated and transcription of *optB* reacts especially strongly.

Since BSA is the medium which has the broadest effect on the transcription of the *opt* gene family, we conducted further experiments with this condition. We concentrated on two aspects: one, what difference does it make if BSA acts as the sole nitrogen source only, as opposed to being the only carbon and nitrogen source? To this end, we conducted an experiment where the BSA medium was supplemented with 1% glucose.

The second aspect we chose to investigate is, does the expression profile change over time, depending on the length of incubation in BSA medium? In order to adress

this point we repeated the previous experiments, harvesting the mycelia two hours and four hours after the media shift (Fig. 15).

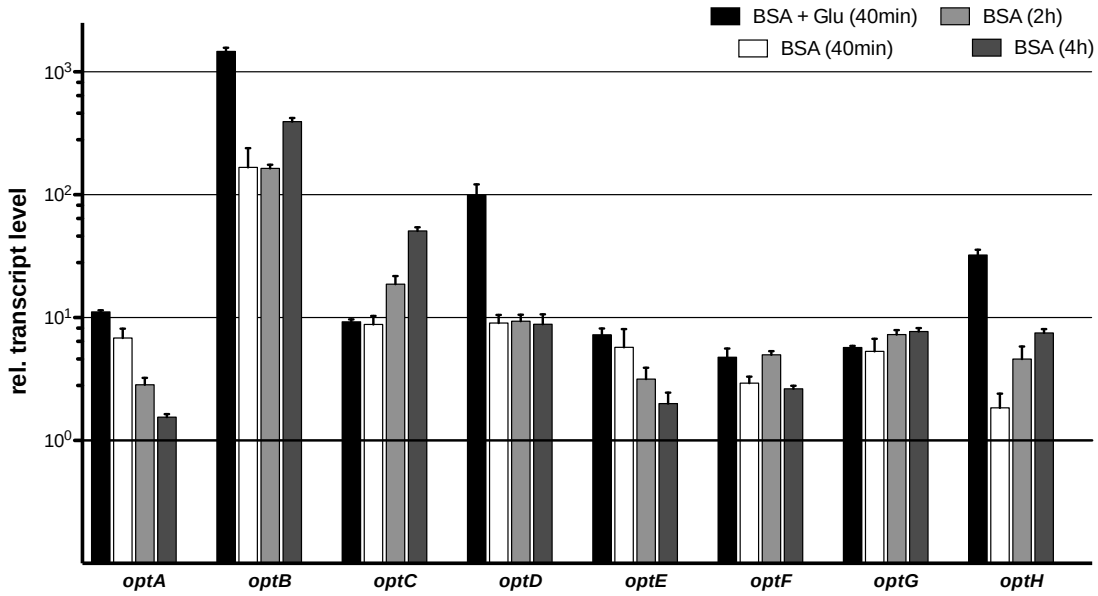

Fig. 15: Transcriptional response of each *opt* gene during growth on BSA as nitrogen source depending on available carbon source or length of incubation
For each of the eight *opt* genes the transcript level on the NH_4^+ -based medium was set as the baseline value of one, to which the transcriptional level of the other conditions was compared. The presence of glucose in BSA medium led to a significantly stronger induction for *optB*, *optD* and *optH*, compared to BSA as both nitrogen and carbon source. The transcript level on BSA over the course of four hours went up for *optB*, *optC* and *optH*, and decreased for *optA* and *optE*. For the other *opt* genes the transcript level stayed the same.
With the exception of BSA medium after 40min of incubation and NH_4^+ medium, which were done in biological triplicate and technical duplicate, experiments for all other conditions were performed in biological and technical duplicate.

It can be seen, that the presence of glucose does have an effect on transcription levels on some, but not all members of the *opt* gene family. The genes whose transcriptional expression changes, do so to quite a high degree. For *optB*, the induction compared to NH_4^+ medium increases from more than 100-fold on BSA medium to more than 1000-fold on the BSA/glucose medium. For *optD*, the numbers are 10-fold induction on BSA medium and 100-fold induction on BSA/glucose, for *optH* from no significant induction on BSA medium to a 30-fold induction on BSA/glucose medium.

The length of incubation also affects a number of *opt* genes, while others do not react. Again, it is *optB* and *optH* whose transcript levels rise, for *optB* quite abruptly between two and four hours, for *optH* the level rises gradually to an 8-fold induction after four hours. The same gradual rise can be seen for *optC*, which shows a 50-fold induction after four hours. In contrast to that, *optA* and *optE* both gradually decrease in their transcript levels.

Overall, the transcriptional response of the *opt* gene family is quite dynamic being substrate-specific regarding available nitrogen and carbon sources, and changing with time of incubation.

Besides the *opt* gene family, there are other peptide transport systems that are able to transport protein degradation products across the cell membrane into the cell. The *ptr* gene family is of particular interest here, as it is conceivable that it acts in concert with the oligopeptide transporters in taking up the peptide fragments of a extracellular proteolytic digest. In order to investigate this, the cDNA of the earlier media shift experiments with the *A. fumigatus* wildtype was examined for the transcriptional profile of the *ptr* family. As growth conditions, the NH_4^+ based medium was chosen to establish a baseline expression, YPD and BSA were used as complex media (Fig. 16, next page).

For the qRT-PCR for each *ptr* gene a specific primer pair was used: THA103/104 for *ptrA* (Afua_7g01490), THA95/96 for *ptrB* (Afua_1g01480), THA101/102 for *ptrC* (Afua_4g00830), THA99/100 for *ptrD* (Afua_3g00540), THA97/98 for *ptrE* (Afua_1g12240) and THA105/106 for *ptrF* (Afua_8g02550).

It becomes evident, that in the wildtype the *ptr* gene family does not seem to play a prominent role during growth on YPD medium, as there is hardly any difference in transcription compared to minimal medium. During growth on BSA medium, there is a general rise in transcript levels across the entire *ptr* gene family, with two *ptr* genes, *ptrA* and *ptrF* being particularly prominent. Thus, the induction of the *ptr* gene family in the presence of BSA suggests, that dipeptide transport is used to ensure sufficient nutrient supply.

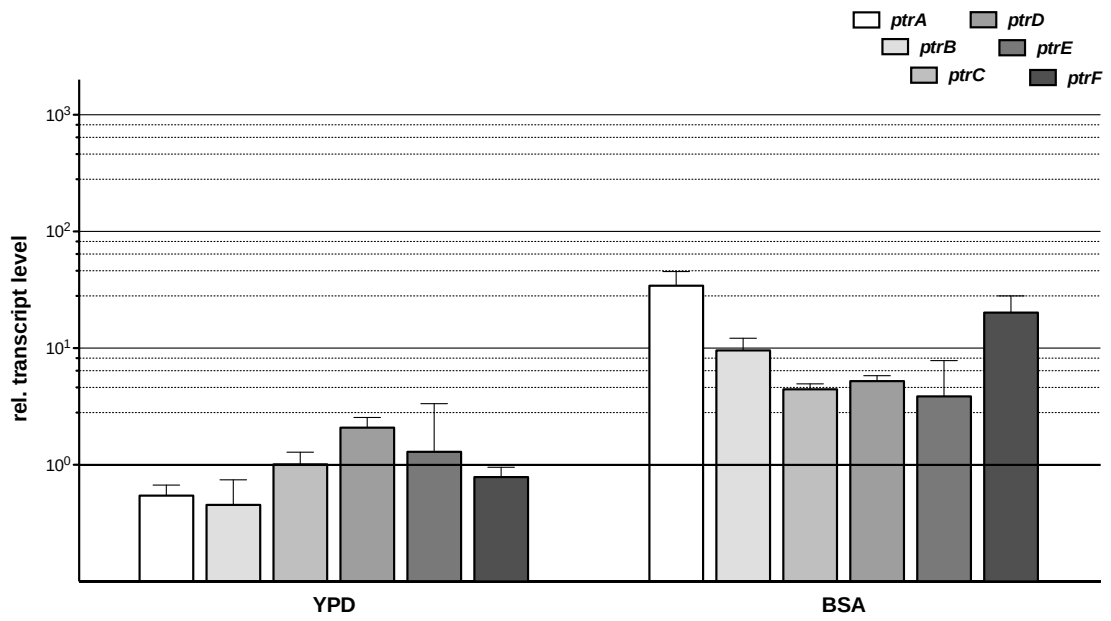

Fig 16: Transcriptional profile of the *ptr* gene family in the wildtype on different nitrogen sources
For each of the six *ptr* genes the transcript level on the NH_4^+ -based medium was set as the baseline value of one, to which the transcriptional level on YPD medium and BSA medium were compared. The *ptr* genes show no significant up- or down-regulation on YPD medium. All *ptr* genes show a significant increase in their transcription when grown on BSA medium, with *ptrA* and *ptrF* having the strongest increase.
All experiments were performed in biological and technical duplicate.

For the ability to grow on proteins and similar macromolecular nitrogen sources, *A. fumigatus* needs not only uptake systems such as the oligopeptide transporters, but also proteases that make the nitrogen source accessible for transport into the cell. Thus, it is of interest to understand the regulation of these enzymes as well.

Here we concentrated on the dipeptidyl peptidases *dpp4* and *dpp5*. Using the cDNA from the aforementioned *opt* gene family experiments, similar qRT-PCR experiments were carried out for the dipeptidyl peptidases, using the primer pair THA91/92 for *dpp4* and THA93/94 for *dpp5* (Fig. 17, next page).

While on the nutrient rich YPD medium the two dipeptidyl peptidases are not upregulated at all, collagen hydrolysate and BSA lead to a very strong induction of transcription. For both *dpp4* and *dpp5* the rise in transcript levels is equally strong on collagen hydrolysate at around 400-fold, which is the highest of all media. On BSA medium, *dpp4* is transcriptionally more activated than *dpp5*, and for both genes the addition of glucose to the medium leads to a further increase in transcript levels.

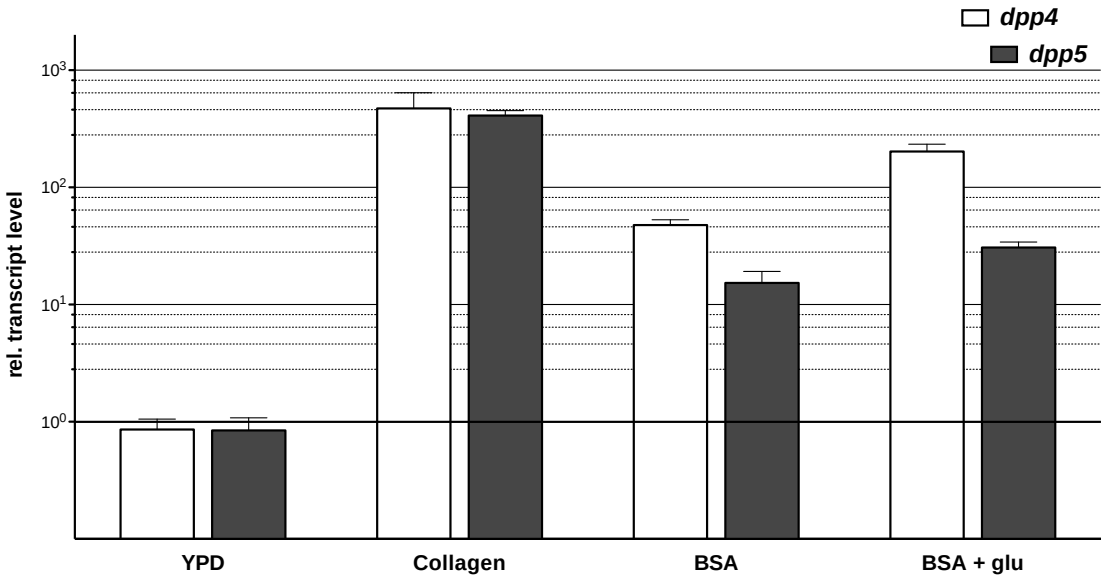

Fig. 17: Transcriptional response of *dpp4* and *dpp5* to growth on different nitrogen sources
For *dpp4* and *dpp5* the transcript level on the NH_4^+ -based medium was set as the baseline value of one, to which the transcriptional level of the other conditions was compared. The transcription of *dpp4* and *dpp5* does not change on YPD medium. On collagen hydrolysate medium, the transcript levels for both genes go up several hundred fold. On BSA medium, both genes are strongly upregulated, the presence of glucose leads to a further increase compared to BSA alone. On both BSA based media, *dpp4* transcript levels are higher than those of *dpp5*. Experiments for all conditions were performed in biological and technical duplicate.

Overall, the dipeptidyl peptidases are only upregulated on media where the sole available nitrogen source are peptide chains (collagen hydrolysate) or entire proteins (BSA), which need further proteolytic breakdown to be utilized by *A. fumigatus*. There is some substrate specificity of trancription in the fact, that while the two *dpp* genes are almost equally upregulated on collagen hydrolysate, *dpp4* is significantly more upregulated on the BSA media.

4.5. Generation and characterization of *opt* gene deletion mutants

One way to decipher the function of a gene or an entire gene family is to create deletion mutants, strains in which the gene(s) of interest are replaced by a selectable marker gene. Thus, it was our goal to delete the entire *opt* gene family in *A. fumigatus* in the hopes that the resulting deletion strain would display phenotypic features, which would shed light on the specific cellular role of this transporter family.

Additionally, we deemed it interesting to investigate the role of the *dpp* family as well, since these enzymes are able to cleave dipeptides off certain oligopeptides, thereby potentially affecting the need for oligopeptide transporters. Also, both *dpp* genes were upregulated in the *in vivo* transcriptome of the mouse infection model, *dpp4* 40-fold and *dpp5* 2.5-fold (McDonagh *et al.*, 2008). This is why a deletion of these two genes, in a addition to the *opt* gene deletion, was also a goal of this work.

In *A. fumigatus*, multiple gene deletions have been made possible by employing one of several dominant markers, that confer resistance to an antifungal agent (Punt and van den Hondel, 1992; Kubodera *et al.*, 2002). Additionally, the introduction of recyclable marker cassettes have made multiple gene deletions possible. One such system is the Cre/*loxP* system, in which a first transformation replaces the gene of interest with a marker cassette, that is then excised via the Cre recombinase during a second transformation step (Krappmann *et al.*, 2005). In this work, we attempted to delete the entire *opt* gene family in *A. fumigatus* using the Cre/*loxP* system. The details of how to delete a gene using this system are illustrated in Fig. 18 below.

Two different marker cassettes were employed, one carrying the phleomycin (*ble*) resistance gene, the other one carrying a resistance gene against hygromycin B (*hph*). In order to integrate the marker cassette at the right locus in the *A. fumigatus* genome by homologous recombination, it has to be flanked on both sides by the one to two kb long genomic region neighbouring the gene of interest (Krappmann, 2006). For each *opt* gene, these regions were amplified via PCR and two sets of primer pairs, which introduced restriction sites at the ends of the PCR fragment. On the one side, an SfiI site was introduced, that allowed the directed fusion of the flanking regions to the SfiI sites of the marker cassette. The SfiI - sites of the 5' and 3' flanking regions were different at the five variable nucleotides in the middle, to make sure that the fragments could only be assembled in the correct order when ligated later (Kämper, 2004). On the other side of the PCR fragment, HpaI or PmeI halfsites were set, which later allowed for

excision of the cassette from the cloning plasmid and precise introduction of the replacement cassette at the target locus.

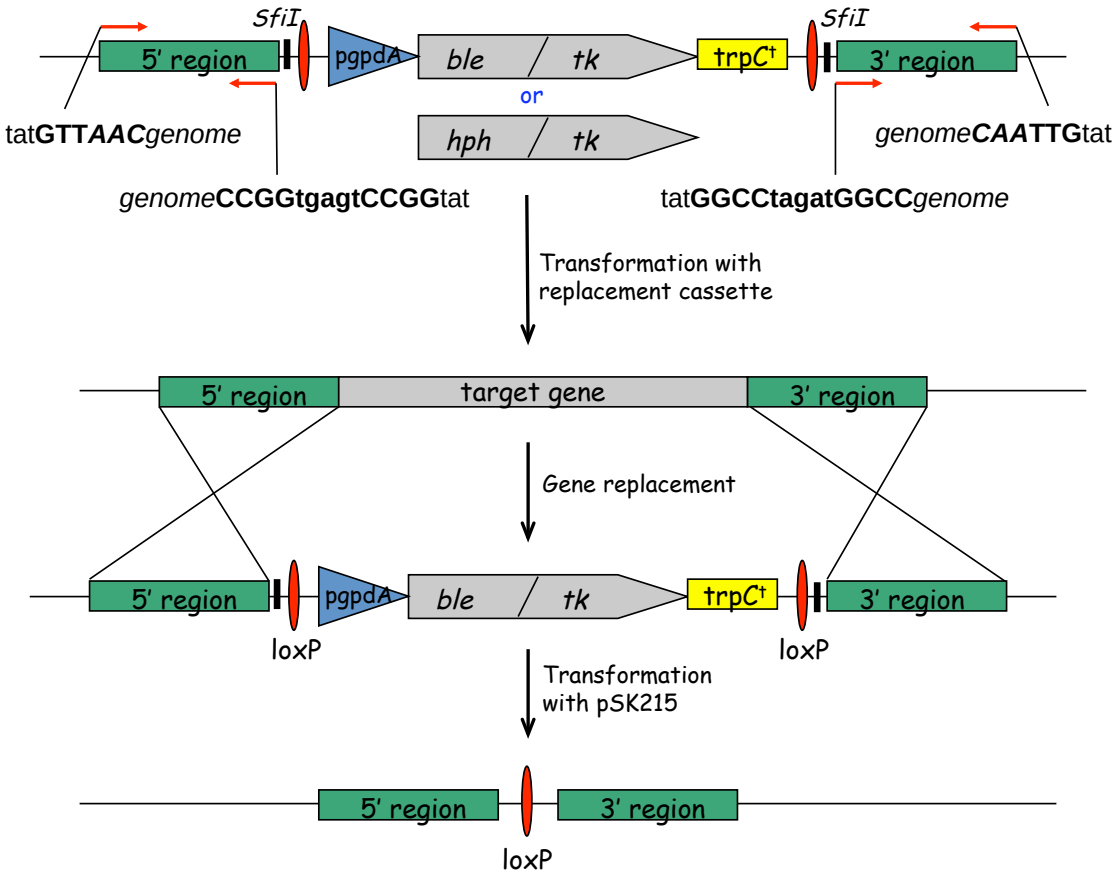

Fig. 18: General principle of replacement cassette construction, gene replacement and marker excision using the Cre/*loxP* system in *A. fumigatus*
The 5' flanking region and the 3' flanking region of a target gene are fused via ligation, using two non-identical SfiI sites, to one of two marker cassettes: one cassette carries the phleomycin resistance gene (*ble*), one carries the hygromycin B resistance gene (*hph*). The linear cassette is transformed into *A. fumigatus*, where homologous recombination at the target gene locus leads to the replacement of the target gene by the marker cassette. In a second round of transformation the Cre-recombinase carrying plasmid pSK215 is introduced into the transformed strain, which leads to the transient expression of the Cre-recombinase and excision of the marker cassette via the two *loxP* sites, leaving only a *loxP* behind.

Thus for *optA*, the 3' flanking region was amplified using the primer pair THA1/THA2, the 5' flanking region using the primer pair THA3/THA4. The resulting PCR fragments were digested with SfiI, and then ligated with a marker cassette that had been removed by SfiI digestion from a donor plasmid, pSK341 being the source of the phleomycin resistance cassette, or pSK397 the source for the hygromycin resistance cassette. The ligation product was recovered and inserted into the pCR-Blunt II-TOPO vector, from which the entire replacement cassette could be liberated via an HpaI

restriction digest. For *optA* only one replacement cassette harbouring the phleomycin resistance gene was created, which was named pSK512.

For all the other seven *opt* genes and for the two *dpp* genes, replacement cassettes were created in a similar fashion. For the PCR of the 3' flanking region of *optB* the primer pair THA8/THA9 was used, for the 5' flanking region THA10/THA11. The restriction enzyme digested PCR fragments were ligated with the phleomycin resistance cassette or the hygromycin resistance cassette, the ligation products inserted into the TOPO vector and named pSK512 and pSK517 respectively.

For the PCR of the 3' flanking region of *optC* the primer pair THA15/THA16 was used, for the 5' flanking region THA17/THA18. The restriction enzyme digested PCR fragments were ligated with the phleomycin resistance cassette or the hygromycin resistance cassette, the ligation products inserted into the TOPO vector and named pSK513 and pSK518 respectively.

For the PCR of the 3' flanking region of *optD* the primer pair THA24/THA26 was used, for the 5' flanking region THA22/THA23. The restriction enzyme digested PCR fragments were ligated with the phleomycin resistance cassette or the hygromycin resistance cassette, the ligation products inserted into the TOPO vector and named pSK514 and pSK519 respectively.

For the PCR of the 3' flanking region of *optE* the primer pair THA32/THA33 was used, for the 5' flanking region THA30/THA31. The restriction enzyme digested PCR fragments were ligated with the phleomycin resistance cassette or the hygromycin resistance cassette, the ligation products inserted into the TOPO vector and named pSK515 and pSK520 respectively.

For the PCR of the 3' flanking region of *optF* the primer pair THA39/THA40 was used, for the 5' flanking region THA37/THA38. The restriction enzyme digested PCR fragments were ligated with the phleomycin resistance cassette or the hygromycin resistance cassette, the ligation products inserted into the TOPO vector and named pSK516 and pSK521 respectively.

For the PCR of the 3' flanking region of *optG* the primer pair THA44/THA45 was used, for the 5' flanking region THA46/THA47. The restriction enzyme digested PCR fragments were ligated with the hygromycin resistance cassette, the ligation product inserted into the pJET vector and named pSK522.

For the PCR of the 3' flanking region of *optH* the primer pair THA53/THA54 was used, for the 5' flanking region THA51/THA52. The restriction enzyme digested

PCR fragments were ligated with the hygromycin resistance cassette, the ligation product inserted into the pJET vector and named pSK523.

For the PCR of the 3' flanking region of *dpp4* the primer pair THA60/THA61 was used, for the 5' flanking region THA58/THA59. The restriction enzyme digested PCR fragments were ligated with the phleomycin resistance cassette, the ligation product inserted into the TOPO vector and named pSK524.

For the PCR of the 3' flanking region of *dpp5* the primer pair THA67/THA68 was used, for the 5' flanking region THA65/THA66. The restriction enzyme digested PCR fragments were ligated with the hygromycin resistance cassette, the ligation products inserted into the TOPO vector and named pSK525.

The order of *opt* gene deletion was determined with the help of the previously available *in vivo* trancriptome data (McDonagh *et al.*, 2008). Accordingly, the *opt* genes that might be important for infection were deleted first, *optA*, *optE*, *optF* and *optG*, and then the rest of the gene family. Additionally, we chose to create a mutant in which the entire *opt* gene family and the two dipeptidyl peptidases *dpp4* and *dpp5* were deleted. In that mutant the *dpp* genes were deleted first, and after that the *opt* genes in the same order as before. Last, each of the ten genes was deleted individually, as to get single deletion mutants as well.

As outlined in Fig. 18, for each gene deletion the replacement cassette was excised from the donor plasmid and transformed into the *A. fumigatus* recipient strain. The wildtype strain *A. fumigatus* ATCC 46645 was the basis for all subsequent deletion strains. Thus, the project was started with the *optA* replacement cassette taken from pSK511 and the *dpp4* replacement cassette taken from pSK524. After every round of transformation with a replacement cassette, the clones were pre-screened by conidia PCR using a primer pair that gave a product only in case of homologous recombination at the target locus, as illustrated in Fig. 19. For *optA*, the primer pair was THA7/Sv287, for *dpp4* the primer pair was THA62/Sv287.

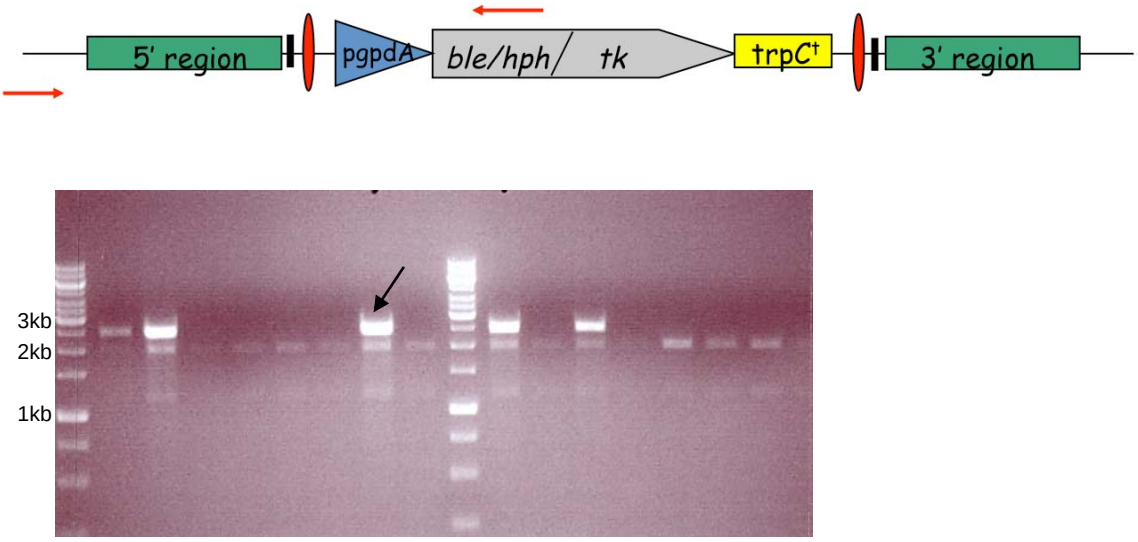

Fig. 19: General principle of the pre-screen PCR
The PCR uses two primers; one primer binding just upstream of the 5' flanking region site of integration, the other primer, Sv287 for the phleomycin resistance marker, Sv288 for the hygromycin resistance marker, is binding inside the marker cassette. Only in case of a correct integration event of the replacement cassette at the target locus does the PCR amplify a product. This gel photo shows the result of the pre-screen PCR on primary transformants of *A. fumigatus* ATCC 46645 with the *optA* replacement cassette. The arrow indicates the PCR product of the right size, other signals are unspecific.

The positive pre-screened clones were verified by Southern hybridization using at least three different restriction enzymes on the genomic DNA, in order to make sure that a) the replacement cassette had been integrated at the right locus and b) that there were no other, ectopic insertions of the cassette into the genome. The correct clones were named AfS100 for the *optA* deletion strain and AfS122 for the *dpp4* deletion strain.

AfS100 and AfS122 then underwent a second transformation using the pSK215 plasmid, which when transformed into *A. fumigatus*, enabled the fungus to grow on pyrithiamine containing plates using the *ptrA* gene on the plasmid. More importantly, this second transformation lead to the possibility of marker excision at the two *loxP* sites by the Cre recombinase gene pSK215 carries. A successful marker excision was verified two-fold: first, the inability of the strain to grow on phleomycin/hygromycin, and second, by a PCR using a primer pair that led to different product sizes, depending on the presence or absence of the marker cassette, as illustrated in Fig. 20. For the *optA* gene this primer pair was THA5/THA6, for *dpp4* it was THA63/THA64.

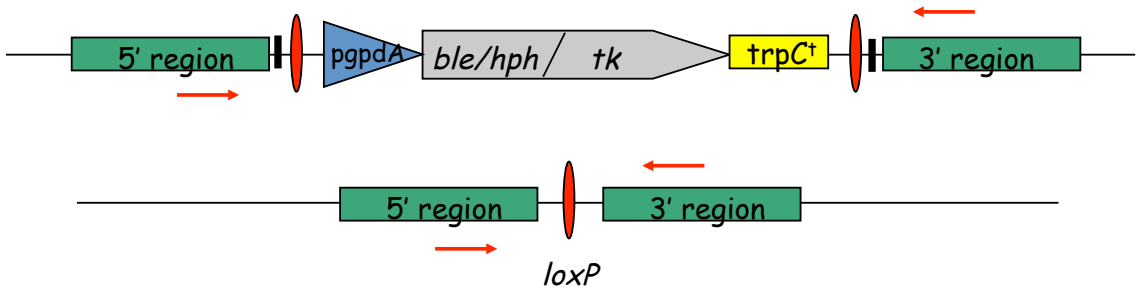

Fig. 20: General principle of the diagnostic PCR for successful marker excision by the Cre/*loxP* system
The PCR uses two primers: one primer in the 5' flanking region, the other in the 3' flanking region. In the case of a successful marker excision the resulting PCR product will be significantly smaller than in the presence of the marker cassette.

In this fashion AfS100 was turned into AfS101, where *optA* was replaced by a single *loxP* site, and AfS122 was turned into AfS123, where *dpp4* was replaced by a single *loxP* site.

This procedure was repeated with all the other gene replacement cassettes, both for the single gene deletion mutants and for the multiple deletion mutants that would eventually result in a complete *opt* gene family deletion strain, and a strain that had the two dipeptityl peptidases deleted on top of that. For the *opt* octuple knockout strain, the order of gene deletion was first *optA*, then *optE*, *optG*, *optF*, *optB*, *optC*, *optD* and finally *optH*. For the 10-tuple knockout strain, first *dpp4* and *dpp5* were deleted in that order, and then the *opt* gene family in the same order as the *opt* octuple knockout strain, except for *optG* that was deleted before *optE*. For the PCR pre-screen, the primer pairs were as follows: Sv287 for the phleomycin marker or Sv288 for the hygromycin marker; the gene specific primers just outside the 5' flanking regions were THA14 for *optB*, THA21 for *optC*, THA29 for *optD*, THA36 for *optE*, THA43 for *optF*, THA50 for *optG*, THA55 for *optH* and THA69 for *dpp5*. For the diagnostic PCR to show successful marker excision the following primer pairs were used: THA12/THA13 for *optB*, THA19/THA20 for *optC*, THA27/THA28 for *optD*, THA34/THA35 for *optE*, THA41/THA42 for *optF*, THA48/THA49 for *optG*, THA56/THA57 for *optH* and THA70/THA71 for *dpp5*. Figure 21 displays diagnostic PCRs targeting all 10 deleted genes in the strain AfS139.

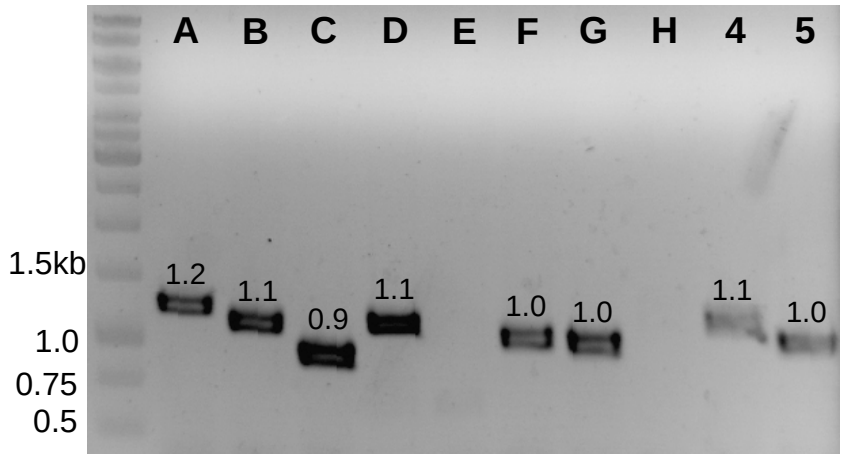

Fig. 21: *A. fumigatus* strain AfS139 in a diagnostic PCR for successful marker excision by the Cre/*loxP* system
The PCR was performed on AfS139 genomic DNA using the appropriate primer pairs for each gene. The letters and numbers at the top are the shorthands for the genes i.e. "A" refers to *optA* and "4" refers to *dpp4*. Successful marker excision can be seen for the two *dpp* genes and for six *opt* genes. For *optE* and *optH* there is no signal, because the size of the marker cassettes at these loci did not permit sufficient amplification in this PCR setup. At the *optE* locus the phleomycin marker cassette stayed in place, the hygromycin marker is still located at the *optH* locus.

Initially, it was planned to make full use of the Cre/*loxP* system and the two available dominant markers by first deleting one gene with a phleomycin marker cassette, right after that the next gene using a hygromycin marker cassette and excise both markers at the same time using the Cre recombinase. While this is possible, as evident in the conversion of AfS126 to AfS127, we were not able to proceed like this once *optE* had been deleted, which was very early on in the sequential deletion process. Despite numerous efforts it seems impossible to excise the marker cassette at the *optE* locus. The *optE* replacement cassettes of both pSK515 and pSK520 were successfully used to delete *optE,* and the *loxP* sites of both constructs were sequenced and found to be intact. Still, once the replacement cassettes were at the *optE* locus, the marker cassette stayed there even after 6 rounds of transformation with pSK215. Thus all deletion strains from AfS109 to AfS121 and from AfS129 to AfS140 carry the phleomycin marker cassette at the *optE* locus.

The final strains of this project are on the one hand AfS121, in which all eight *opt* genes were deleted, the *optE* locus carrying the phleomycin resistance marker and the other seven *opt* genes being replaced by a single *loxP* site. The other final strain is AfS140 which is similar to AfS121 with the two *dpp* genes replaced by a *loxP* site additionally. These two strains were created indepedently from each other, both starting from the parental isolate *A. fumigatus* ATCC 46645.

One caveat in the use of the Cre/*loxP* system is the possibility of trans-recombination between *loxP* sites of different replacement cassettes. This could lead to massive chromosome re-arrangements or deletions of large parts of a chromosome. In order to elucidate this question, Southern hybridizations of AfS121 and AfS140 were performed, in which the loci of all *opt* genes were investigated again (Fig. 23). Additionally, Fig. 22 illustrates the clean deletion of the two *dpp* genes in AfS125, a predecessor strain of AfS140.

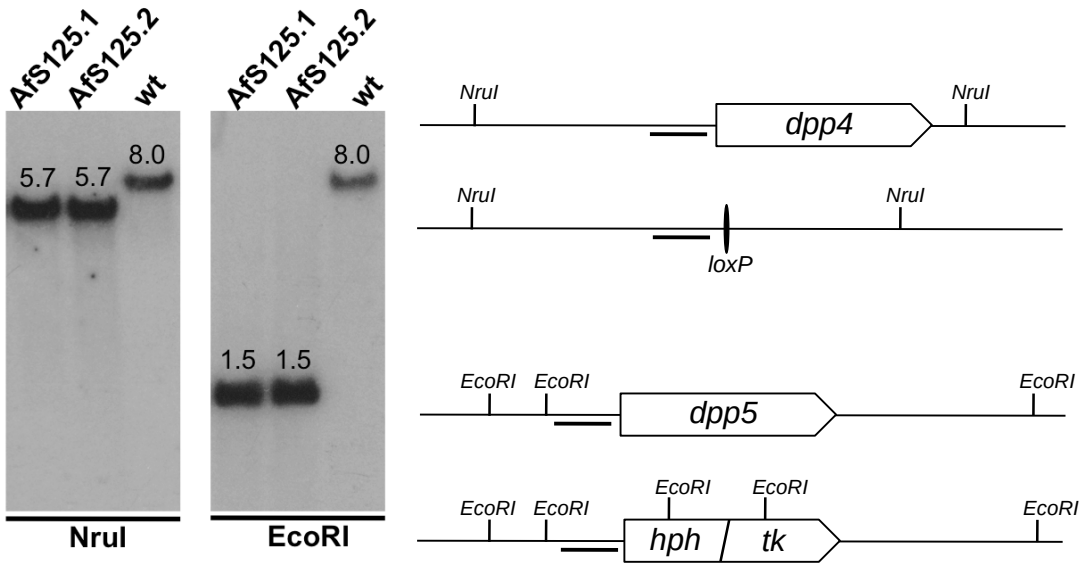

Fig. 22: Southern hybridizations of strain AfS125 showing the clean deletion of *dpp4* (left) and *dpp5* (right)
Southern hybridization of two different clones of AfS125 are shown. The genomic DNA was digested with the restriction enzyme NruI for the hybridization targeting the *dpp4* locus, and with EcoRI for the hybridization targeting the *dpp5* locus. The Southern probes (black bars) bind in the 5' flanking region of the respective gene locus. Both Southern hybridizations show the successful deletion of *dpp4* and *dpp5* respectively.

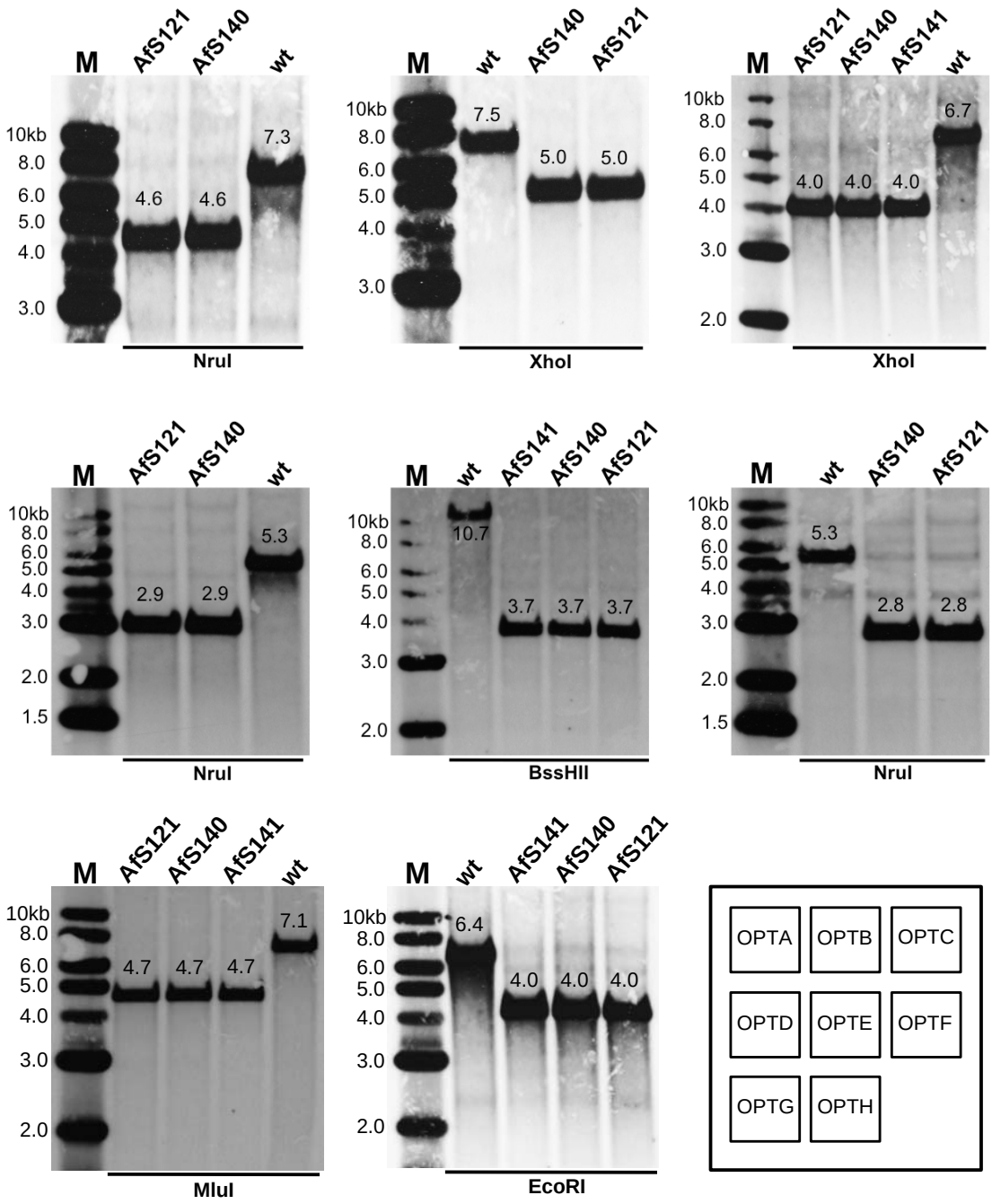

Fig. 23: Southern hybridizations of strains AfS121, AfS140 and AfS141 showing the complete *opt* gene family deletion
The scheme in the lower righthand corner depicts the order of Southern hybridizations, i.e. in panel OPTA the Southern probe binds in the 5' flanking region at the *optA* locus. At the bottom of each panel the restriction enzyme with which the genomic DNA was digested is shown. In the Southern hybridizations of OPTC, OPTE, OPTG and OPTH the strain AfS141 was also included, which will be described in detail further below. The chromosomal surroundings at all former *opt* gene loci are still intact. wt refers to ATCC 46645.

The final result of these efforts was thus the creation of two strains AfS121 and AfS140, the first being the *opt* gene family octuple knockout strain, the second being the 10-tuple knockout strain that had the two *dpp* genes deleted as well.

Phenotypic studies with all available strains were conducted in an effort to investigate the cellular role of the *opt* gene family. To this end, we performed growth experiments on different media, in order to find conditions under which an *A. fumigatus* strain lacking the *opt* gene family might suffer negative effects. The heterologous expression of *A. fumigatus opt* genes in *S. cerevisiae* had shown the functionality of the *opt* gene products as oligopeptide transporters. Thus, we tried to investigate, if the complete *opt* deletion mutant in *A. fumigatus* would result in diminished growth in medium which contained oligopeptides as the sole nitrogen source. For this, minimal medium without a nitrogen source was prepared and supplemented with a defined oligopeptide. Additionally, protease inhibitors were added in order to limit possible degradation of the oligopeptides by secreted proteases. We added AEBSF, an inhibitor of serine proteases, Pepstatin A as an aspartic protease inhibitor and EDTA a divalent cation chelator which acts as a metalloprotease inhibitor (Aoyagi *et al.*, 1971; Walsmann *et al.*, 1972). Figure 24 shows the growth of the complete *opt* deletion mutant on selected oligopeptides.

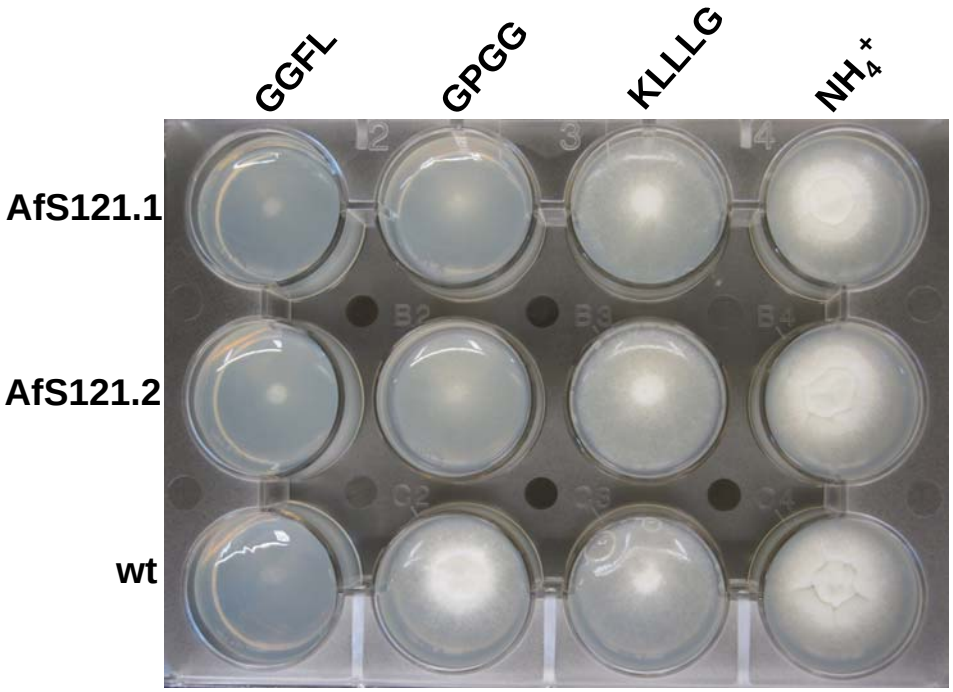

Fig. 24: Growth of *opt* deletion mutant AfS121 on medium with oligopeptides as sole nitrogen source
AfS121 refers to the complete *opt* deletion strain of which two different clones were tested. The wildtype control is ATCC 46645. On top the supplemented oligopeptides are shown in the one-letter amino acid code, on the very right the control medium with ammonium as nitrogen source is shown. On each spot 2 µl of a 10^6 conidia/ml spore suspension was applied.
On three media, those supplemented with the oligopeptides GGFL and KLLLG and the medium with ammonium as nitrogen source, all strains grow equally well. On the GPGG medium AfS121 shows significantly worse growth than the wildtype.

It can be seen that the absence of oligopeptide transporters can indeed lead to diminished growth on oligopeptide containing medium. However, this diminished growth depends on the nature of the oligopeptide added. GGFL for instance seems to be a very poor source of nitrogen even for the wildtype, KLLLG is a somewhat better nitrogen source, but here too the presence of oligopeptide transporters does not facilitate growth. In contrast to that on medium containing GPGG, *A. fumigatus* grows markedly worse in the absence of oligopeptide transporters, as seen in the more vigorous wildtype growth compared to the *opt* deletion mutant. Thus, GPGG appears to be a substrate for at least one oligopeptide transporter. These experiments were repeated using other oligopeptides (LSKL, GGKAA, PGKAR, LWL, GGGGG, LWMR, APYA, YGGFM, TPRK and glutathione) but GPGG remained the only oligopeptide on which AfS121 grew consistently worse than the wildtype (not shown).

Next, the question was if *opt* gene deletion would result in a growth defect on other media as well. For these growth assays, AspA minimal medium served as the growth control medium, and medium with BSA served as a medium where proteins are the sole available nitrogen source. Since the primary site of an *Aspergillus* infection in humans is the lung, we also included growth experiments on porcine lung agar, which consists of water, agarose and homogenized lung tissue. Figure 25 displays the results of a first set of experiments using the *opt* deletion mutants.

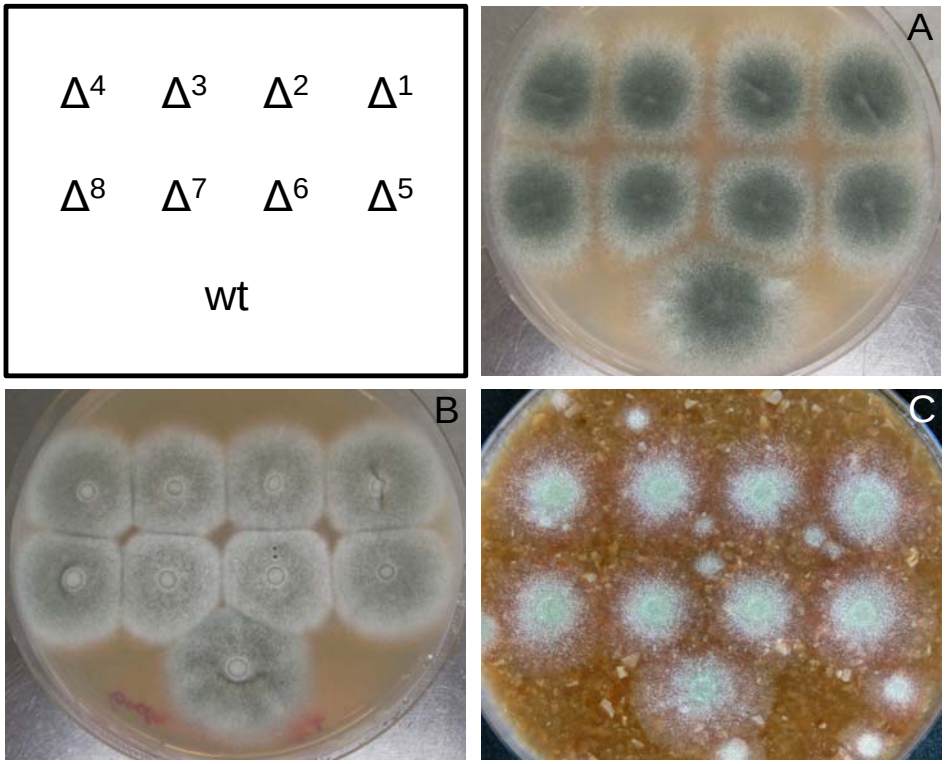

Fig. 25: Growth of sequential *opt* deletion mutants on different media
The scheme in the upper lefthand corner displays the arrangement of the *opt* deletion mutants. Δ^1 is the single *opt* gene knockout, i.e. strain AfS101 which is missing *optA* only, Δ^2 is the double knockout AfS109 missing *optA* and *optE*, and so on. The wildtype is ATCC 46645. On each spot 2 µl of a 10^6 conidia/ml spore suspension was applied.
Panel A shows growth on minimal medium, Panel B shows growth on BSA/glucose medium, Panel C shows growth on the porcine lung medium. All strains grow equally well on all three media.

It is apparent that all *opt* deletion mutants grow equally well on minimal medium, which shows that the OPTs are not necessary for growth on minimal medium with nitrate as the sole nitrogen source. The same is true for growth on medium where BSA is the sole nitrogen source and on the porcine lung medium, where the lung tissue is the sole source of any nutrients. Apparently, *A. fumigatus* can take up the degradation products of the proteolytic digest of BSA and of lung tissue without possessing oligopeptide transporters. These experiments also suggest, that *A. fumigatus* did not suffer any major defects from the successive gene deletions using the Cre/*loxP* system, since even the octuple knockout strain, which had undergone eight rounds of gene deletion and marker excision, grew just as well as the wildtype strain. When the experiments in Fig. 25 were carried out using any of the single *opt* deletion mutants similar results were obtained (not shown). Similar experiments were conducted with the strains missing both the *opt* genes and the *dpp* genes (Fig. 26).

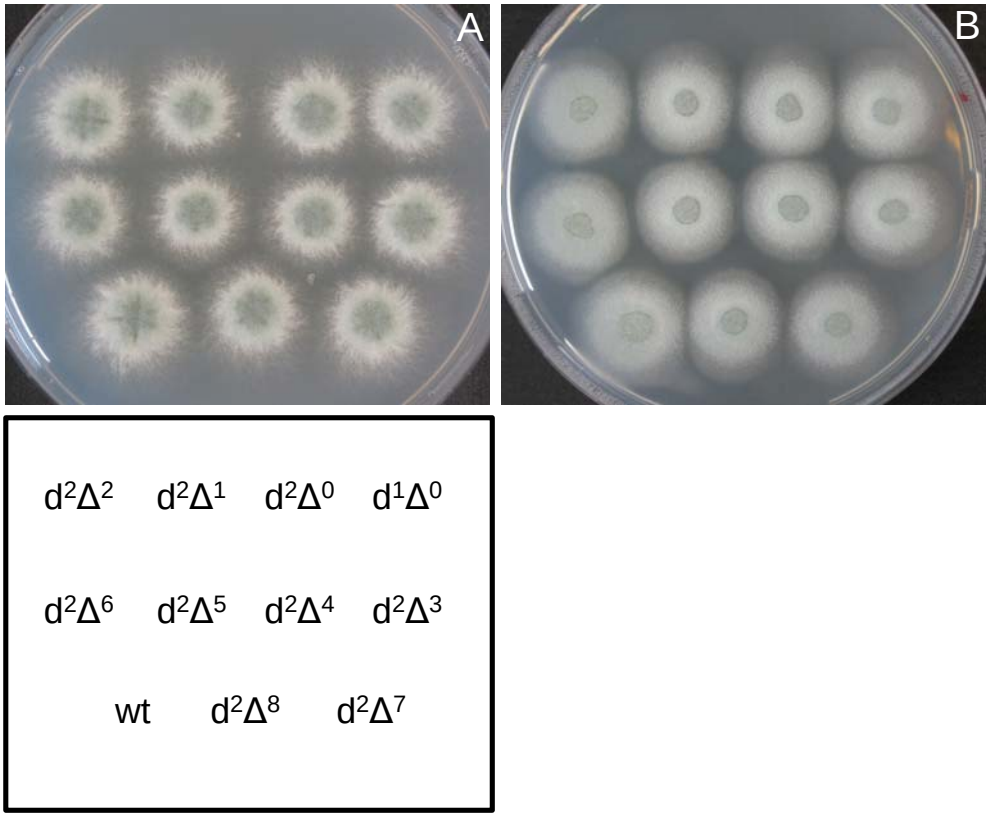

Fig. 26: Growth of sequential *opt* deletion mutants in the *Δdpp4/Δdpp5* background on different media
The scheme at the bottom displays the arrangement of the deletion mutants. Like in Fig. 25, Δ^X refers to the number of *opt* genes that had been deleted in the strain, d^X refers to deletions in the *dpp* gene family, d^1 being the *dpp4* deletion, d^2 being the *dpp4/dpp5* double deletion. Thus, $d^2\Delta^1$ refers to strain AfS127 missing both *dpp* genes and *optA*. The wildtype is ATCC 46645. On each spot 2 µl of a 10^6 conidia/ml spore suspension was applied.
Panel A shows growth on minimal medium, Panel B shows growth on BSA/glucose medium. All strains grow equally well on both media.

It becomes evident, that on both, minimal medium and BSA medium, neither oligopeptide transporters nor dipeptidyl peptidases are necessary for growth. The previously conducted qRT-PCR experiments suggested a role of at least two oligopeptide transporters, OptB and OptH, and of both dipeptidyl peptidases during growth on collagen hydrolysate. The results of growth experiments of a selected deletion mutants on collagen hydrolysate medium are shown in Fig. 27.

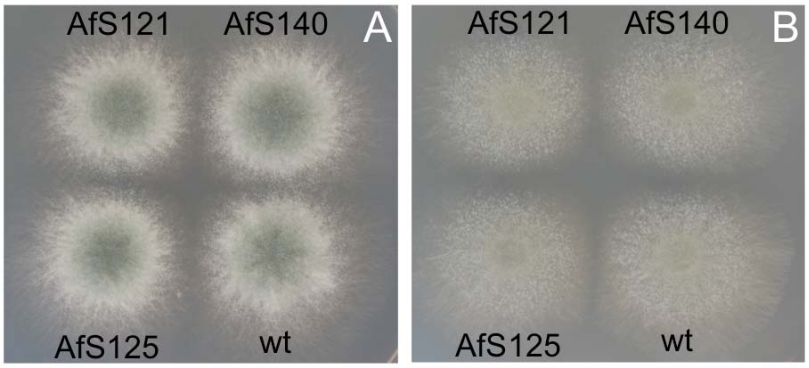

Fig. 27: Growth of strains AfS121, AfS140, AfS125 and ATCC 46645 on medium with collagen hydrolysate as sole nitrogen and carbon source
AfS121 refers to the complete *opt* deletion strain, in strain AfS140 all *opt* genes and the two *dpp* genes are deleted, in strain AfS125 both *dpp* genes are deleted, the wildtype is ATCC 46645. On each spot 2 µl of a 10^6 conidia/ml spore suspension was applied.
Panel A shows growth on minimal medium, Panel B shows growth on medium with collagen as the sole nitrogen and carbon source. All strains grow equally well on both media.

It can be seen that neither oligopeptide transporters nor dipeptidyl peptidases are necessary for growth on collagen hydrolysate.

Figure 28 shows growth on lung agar using strains with the *opt* gene family deletion and/or the two *dpp* gene deletions.

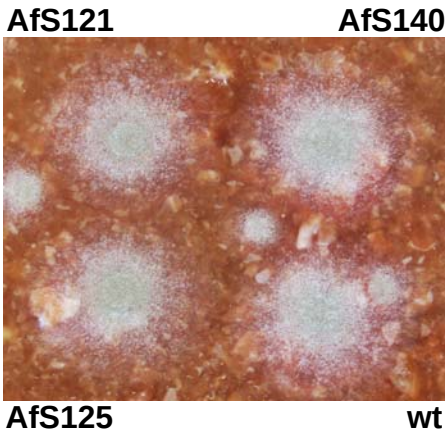

Fig. 28: Growth of strains AfS121, AfS140 and AfS125 on porcine lung medium
Strain AfS121 missing all eight *opt* genes, strain AfS140 is missing both *dpp* genes and all *opt* genes, strain AfS125 is missing both *dpp* genes. The wildtype is ATCC 46645. On each spot 2 µl of a 10^6 conidia/ml spore suspension was applied.
All strains grow equally well.

Again, neither oligopeptide transporters nor dipeptidyl peptidases are necessary to utilize the lung tissue.

Strains AfS121 and AfS140 together with the ATCC 46645 wildtype control were also propagated in liquid BSA medium. This was to find out two things, a) if the strains behaved differently immersed in liquid media compared to growth on an agarose surface and b) if the absence of oligopeptide transporters as possible sensors for

proteolytic degradation products might have an effect on the secretion of extracellular proteolytic activity. The strains were grown for 60 hours in medium which contained BSA as the sole nitrogen source. At the end of the incubation time, the biomass was separated from the supernatant by filtration, dried for 12 hours at 70°C and weighed. The biomass after 60 hours of incubation did not differ between the strains (data not shown), suggesting that also in liquid culture with BSA as the sole nitrogen source, the OPTs and DPPs are dispensible. The supernatants of these experiments were used for a simple assay testing proteolytic activity using the gelatin in an undeveloped X-Ray film (Bouchara *et al.*, 1993) (Fig. 29).

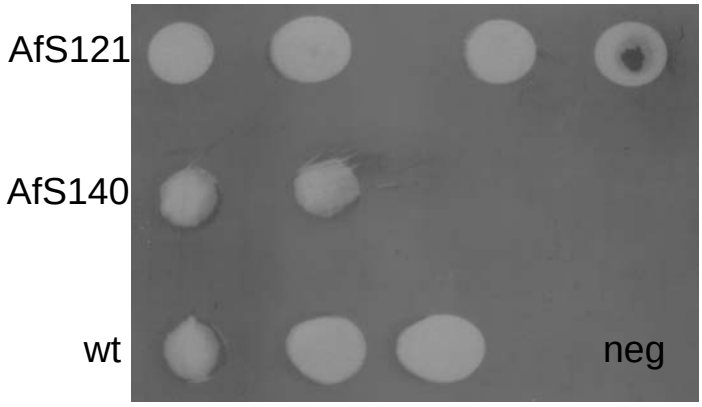

Fig. 29: Proteolytic activity of BSA culture supernatants of strains AfS121 and AfS140 compared to the wildtype.
Strain AfS121 is missing all eight *opt* genes, two independent clones were used. Strain AfS140 is missing both *dpp* genes and all *opt* genes. The wildtype is ATCC 46645, the negative control was BSA culture medium.
10 µl of culture supernatant of a 60h liquid BSA culture were applied on an X-Ray film and incubated at 37°C for 1 hour, after which the film was rinsed with warm water for 1min; experiments for each strain were done at least in biological duplicate.
There is no significant difference in proteolytic activity between the strains.

Thus it can be concluded that at least on BSA, the oligopeptide transporters do not act in a significant way as sensors for extracellular proteolytic degradation products, since their absence does not affect the expression of secreted extracellular proteases.

Because of the lack of a phenotype with the existing strains on medium where protein is the only nitrogen source, we chose to target one further gene, *prtT,* the transcriptional regulator of extracellular proteolytic activity (Bergmann *et al.*, 2009) in the *opt* gene deletion background AfS121. The rationale behind this was, that a *prtT* deletion and the resulting downregulation of extracellular protease expression might alter the makeup of the protein degradation products in the growth medium. The lack of

a substantial number of proteases might result in an accumulation of longer degradation products such as oligopeptides, which would make the need for oligopeptide transporters more urgent for the fungus.

For the construction of such strain, the phleomycin resistance cassette in plasmid pSK462 was excised by SfiI. The remaining linearized plasmid was ligated with the SfiI digested hygromycin marker cassette taken from pSK397, resulting in plasmid pSK526. From pSK526, the *prtT* gene replacement cassette was excised by a PmeI digest and transformed into the AfS121 strain, and the resulting strain was named AfS141. Primary transformants were pre-screened by colony PCR using the primer pair THA72/Sv288 to check for transformants were the *prtT* gene replacement cassette had been inserted at the *prtT* locus. Following that, Southern hybridization using two different restriction enzymes to digest the genomic DNA was performed in order to verify the correct recombination event at the *prtT* locus. For simplicity, Fig. 30 shows only one of the two digests in a Southern hybridization, the two clones displayed were also correct using the second restriction enzyme KpnI.

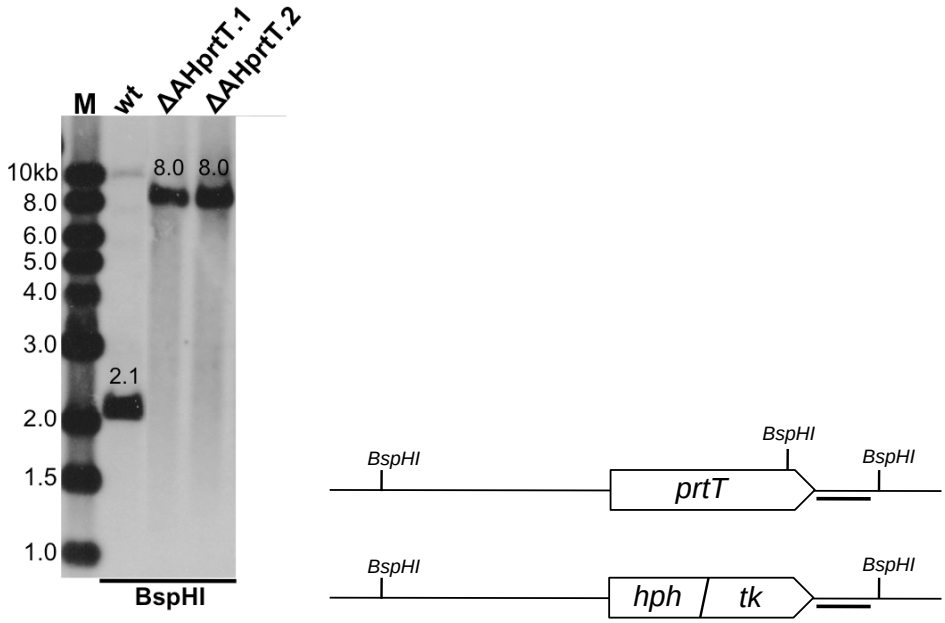

Fig. 30: Southern hybridization of strain AfS141 showing the *prtT* gene deletion in the AfS121 background

Southern hybridization of two different clones of AfS141 are shown. The genomic DNA was digested with the restriction enzyme BspHI, the Southern probe (black bar) binds in the 3' flanking region of the *prtT* locus. The single insertion of the *prtT* gene replacement cassette at the *prtT* locus was successful for both strains.

Following this procedure, *prtT* was reintroduced into AfS141 by transforming that strain with the *prtT* reconstitution cassette which had been excised via PmeI digest from the plasmid pSK463, resulting in strain AfS142. Using these new strains the aforementioned growth experiments were repeated (Fig. 31).

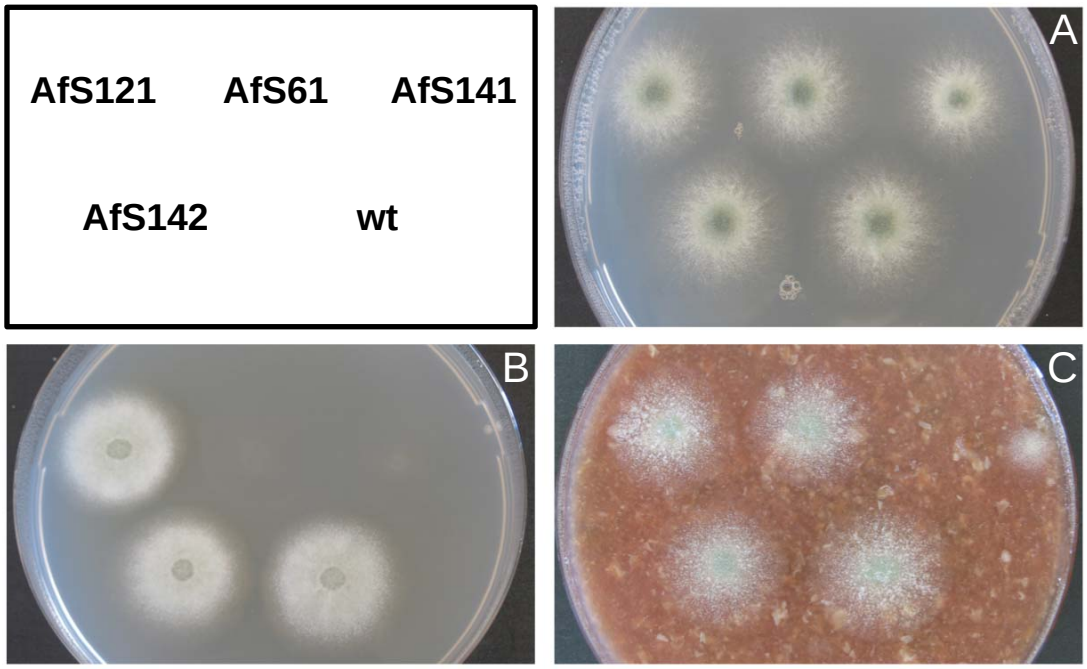

Fig. 31: Growth of the complete *opt* deletion mutant with and without a copy of *prtT* in the genome on different media
The scheme in the upper lefthand corner displays the arrangement of the deletion mutants. AfS121 refers to the complete *opt* deletion strain, AfS61 refers to the *prtT* deletion strain, strain AfS141 is the *prtT* deletion in the AfS121 background, in strain AfS142 the *prtT* gene was reintroduced into AfS141.The wildtype is ATCC 46645. On each spot 2 µl of a 10^6 conidia/ml spore suspension was applied.
Panel A shows growth on minimal medium, Panel B shows growth on BSA/glucose medium, Panel C shows growth on the porcine lung medium. All strains grow equally well on minimal medium, on the BSA/glucose medium AfS61 and AfS141 grow very little, AfS121, AfS142 and the wildtype grow equally well. On the porcine lung agar, AfS141 doesn't grow at all, the other strains grow equally well.

The combined deletion of the *opt* gene family and *prtT,* produced a strain with a drastic growth defect on porcine lung agar, while strains in which only the *opt* gene family or the *prtT* gene had been deleted grew like wildtype. Re-introduction of *prtT* led to wildtype growth again, indicating that the growth defect is indeed the result of a synergistic effect of the *prtT* deletion in the *opt* gene deletion background.

In summary it could be demonstrated, that it is possible to delete the entire *opt* gene family in *A. fumigatus*. Its deletion produces a growth phenotype only on medium which contains the oligopeptide GPGG as the sole nitrogen source. However, there is a synthetic growth phenotype on porcine lung medium, when the transcriptional regulator for extracellular proteolytic activity *prtT* is deleted in as well.

4.6. The effect of *opt* gene deletions on the transcriptional regulation of peptide uptake systems on different nitrogen sources

Using the generated *A. fumigatus* deletion mutants, further qRT-PCR studies were conducted in order to investigate the role of the *opt* gene family in the context of nutrient utilization, and to understand the interplay between different metabolic components in this process.

Since in previous experiments *optB* was the most strongly regulated gene of the family, the expression profile of an *optB* deletion mutant on different nitrogen sources is of particular interest. Thus, the media shift experiments on NH_4^+ medium, YPD medium and BSA medium were repeated with this strain (Fig. 32).

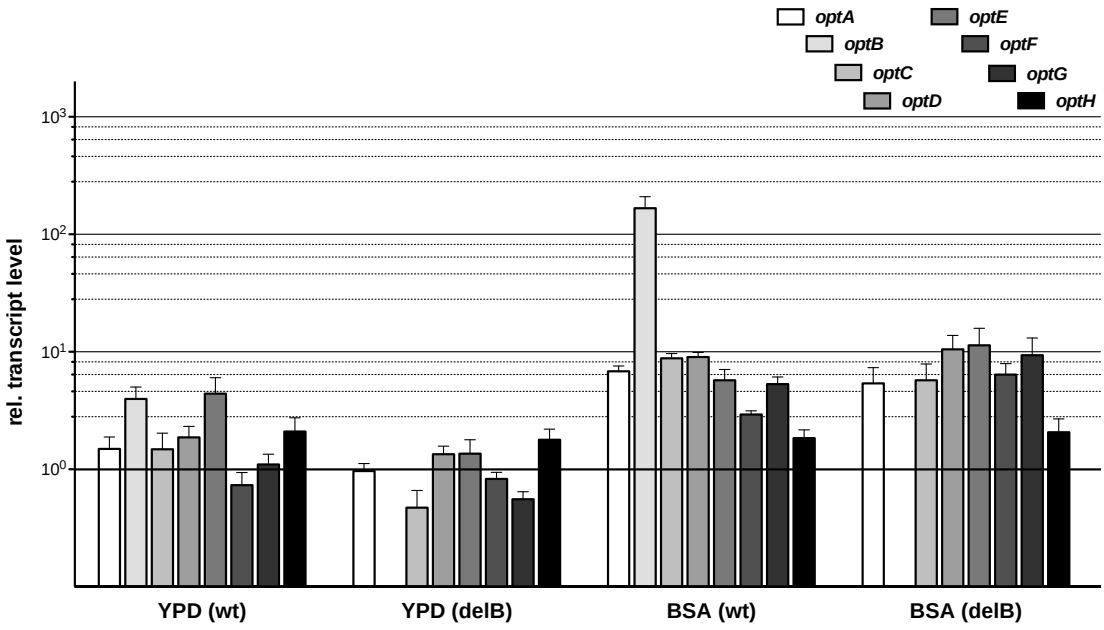

Fig 32: Transcriptional profile of the *opt* gene family in the wildtype and the *optB* deletion mutant AfS102 on different nitrogen sources
For each of the eight *opt* genes, the transcript level on the NH_4^+ -based medium was set as the baseline value of one, to which the transcriptional level on YPD and BSA was compared.
On YPD only *optC* and *optE* react to the absence of *optB* with a minor reduction in transcription, the remaining *opt* genes show similar transcript levels in both strain backgrounds. On BSA only *optE*, *optF* and *optG* show an increase in transcription in the *optB* deletion mutant, roughly doubling their transcript levels compared to the wildtype. The other *opt* genes do not exhibit a significant transcriptional change in the two genetic backgrounds.
Experiments in the wildtype strain were done in biological triplicate and technical duplicate, the experiments in the *optB* deletion mutant were performed in biological and technical duplicate.

Overall, there is only little change in the transcriptional profile of the *opt* gene family when *optB* is deleted. On YPD medium, *optC* and *optG* show a minor reduction in transcript levels, while the rest stay the same. On BSA medium, there is a significant, but still rather moderate increase in transcription for three *opt* genes, *optE*, *optF* and

optG. Taken together, these results suggest, that in the absence of *optB* the remaining seven *opt* genes do not compensate for its loss by being strongly upregulated in their transcription.

As seen before, the *ptr* family is transcriptionally upregulated during growth on BSA medium. Thus, it is of particular interest, how the transcriptional regulation of this gene family will react to the absence of oligopeptide transporters. To this end, the transcriptional profile of the *ptr* gene family was investigated in the complete *opt* gene family deletion strain AfS121 (Fig. 33).

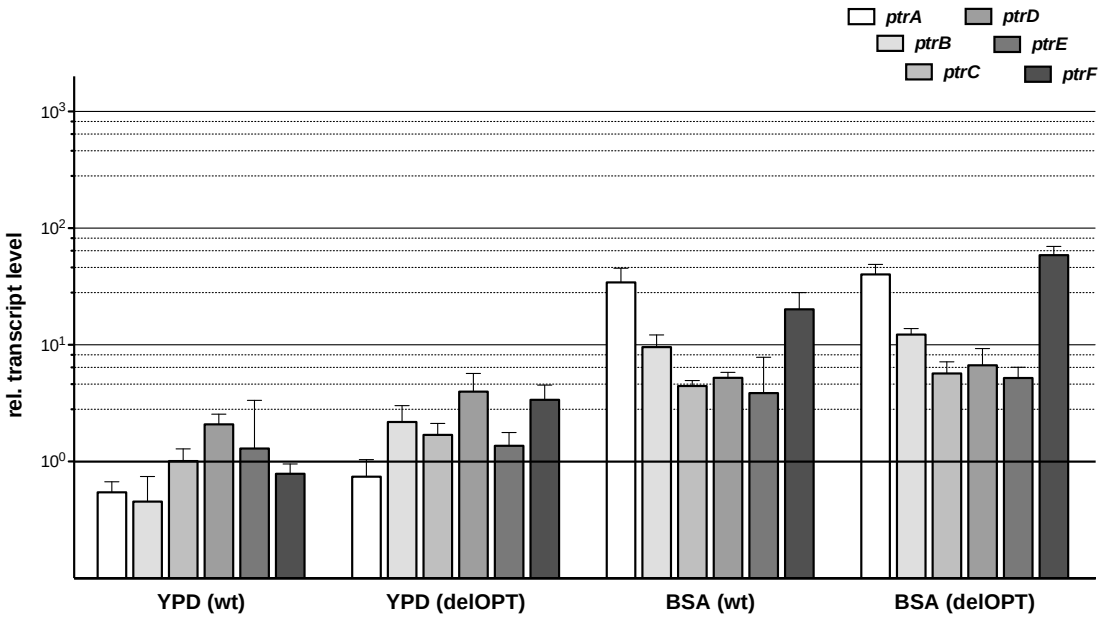

Fig 33: Transcriptional profile of the *ptr* gene family in the wildtype and the complete *opt* deletion mutant AfS121 on different nitrogen sources
For each of the six *ptr* genes the transcript level on the NH_4^+ -based medium was set as the baseline value of one, to which the transcriptional level on YPD and BSA medium was compared. In the wildtype, the *ptr* genes show no significant up- or down-regulation on YPD medium. In the *opt* deletion background, *ptrD* and *ptrF* are upregulated slightly in their transcription. In the wildtype, all *ptr* genes show a significant increase in their transcription when grown on BSA medium, with *ptrA* and *ptrF* having the strongest increase. In the *opt* deletion mutant, only *ptrF* exhibits a further increase in transcript levels over the wildtype, the other *ptr* genes stay the same.
All experiments were performed in biological and technical duplicate.

It can be seen that in the wildtype the *ptr* gene family does not seem to play a prominent role during growth on YPD medium, and this does not change much in the *opt* deletion mutant, where there is a slight upregulation only in *ptrD* and *ptrF*, which is still rather small at about 2-fold up. In the *A. fumigatus* wildtype grown on BSA medium, there is a general rise in transcript levels across the entire *ptr* gene family, with two *ptr* genes, *ptrA* and *ptrF* being particularly prominent. In the absence of the *opt*

gene family, the transcription levels stay essentially the same, with only *ptrF* going from a 20-fold induction over NH_4^+ medium in the wildtype to a 60-fold induction in the deletion mutant.

In summary, judging from the transcript levels, the *ptr* family does play a role for growth on BSA medium as the sole nitrogen and carbon source, however the *ptr* system does not seem to compensate for the lack of oligopeptide transporters by being broadly upregulated in their absence.

Finally, we were interested in the effect the full *opt* gene family deletion might have on the expression of the dipeptidyl peptidases, as it is conceivable that the absence of oligopeptide transporters might make the breakdown of the oligopeptides into even smaller degradation products more important. To this end, we used the cDNA from previous media shift experiments and examined the expression of *dpp4* and *dpp5* (Fig. 34).

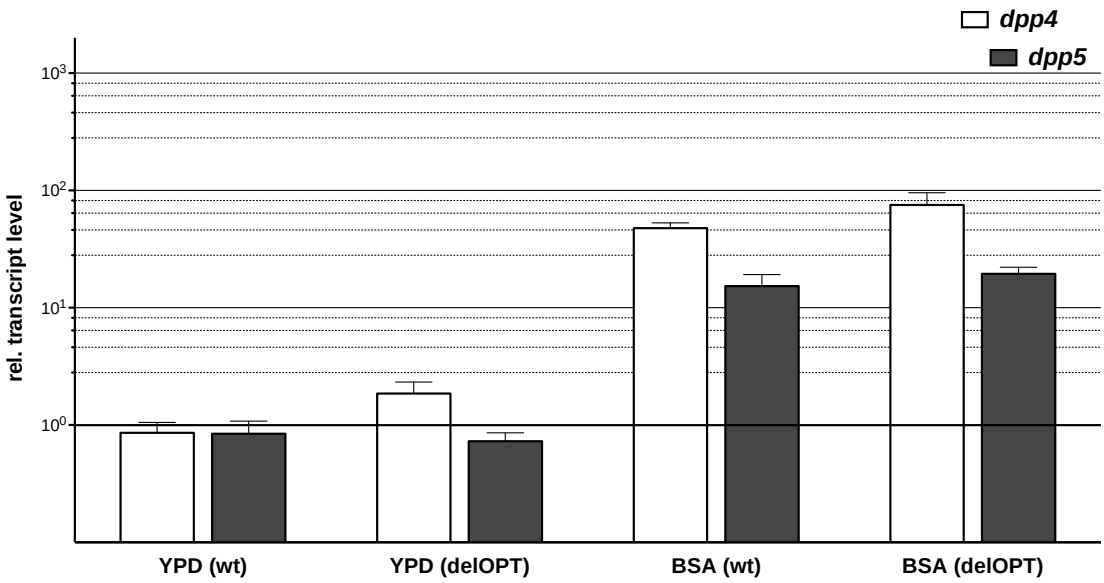

Fig 34: Transcriptional profile of *dpp4* and *dpp5* in the wildtype and the complete *opt* deletion mutant AfS121 on different nitrogen sources
For each of the six *ptr* genes, the transcript level on the NH_4^+ -based medium was set as the baseline value of one, to which the transcriptional level on YPD and BSA medium was compared. On YPD medium there is no significant change in transcript levels of the two *dpp*, irrespective of genetic background. The same holds true for growth on BSA medium.
All experiments were performed in biological and technical duplicate.

It becomes evident, that the transcriptional regulation of the two dipeptidyl peptidases does not differ significantly between wildtype and the complete *opt* deletion mutant AfS121, neither on YPD nor on BSA medium.

4.7. The effect of deleting the *opt* gene family on virulence in a murine model of pulmonary aspergillosis

When working with a human pathogen such as *Aspergillus fumigatus*, one of the most pressing questions for a gene deletion mutant is whether or not the deletion has an effect on the virulence of that pathogen. This question was addressed for some of the deletion strains generated in this work.

In order to investigate the virulence of our *A. fumigatus* deletion strains, we modified an already established murine mouse model (Smith *et al.*, 1994; Bergmann *et al.*, 2009). Using a smaller number of mice, compared to a classical survival assay, this approach allows for screening a larger number of mutants sacrificing the same number of animals (Sheppard *et al.*, 2006). The virulence of a strain is assessed on two levels: the weight loss of the infected mice as a measure of overall fitness of the animals, and the fungal burden of the infected lungs as determined by qRT-PCR. For this the animals were sacrificed at day four post infection (or when they have lost 20% of their weight), the lungs were extracted and homogenized and the DNA isolated. Using this DNA as a template, the qRT-PCR is performed employing one fungal-specific primer pair THA107/THA108 targeting the fungal β-tubulin gene and one mouse-specific primer pair THA109/110 targeting the mouse actin gene. The principle of how this qRT-PCR data is translated into fungal burden is illustrated in Fig. 35.

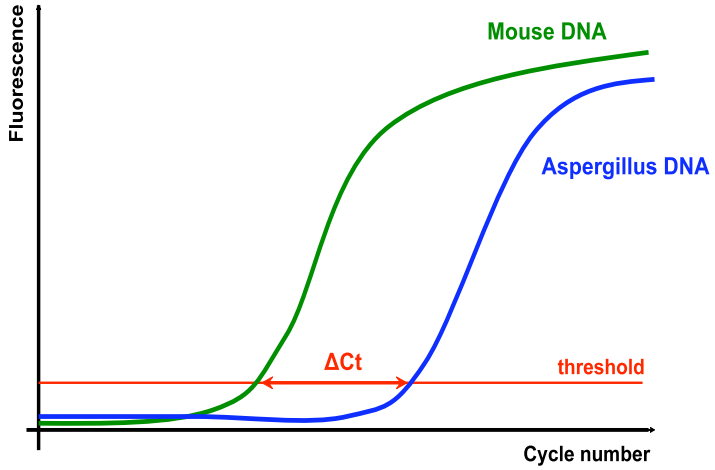

Fig. 35: General principle of using qRT-PCR to determine fungal burden in a mouse infection model
The extracted DNA from the mouse lung is subjected to qRT-PCR using two different primer pairs: a mouse-specific primer pair and a fungal-specific primer pair. The difference ΔCt in the two Ct values corresponds with the fungal burden of the mouse lung - the smaller the ΔCt, the higher the fungal burden.

Virulence studies were conducted for four strains: *A. fumigatus* ATCC 46645 as the wildtype, the complete *opt* gene deletion strain AfS120, strain AfS139 - the *dpp4/dpp5* deletion in the AfS120 background, and AfS125 which is the *dpp4/dpp5* deletion strain. For each mutant, five immnocompromised, neutropenic mice were to be infected intranasaly with an inoculum of $3x10^4$ spores. During the process of inoculum application, one mouse each died for strains ATCC 46645 and AfS139, leaving four mice for each of these two strains. The remaining mice were monitored over four days and weighed daily at the same time, the results of which are displayed in Fig. 36.

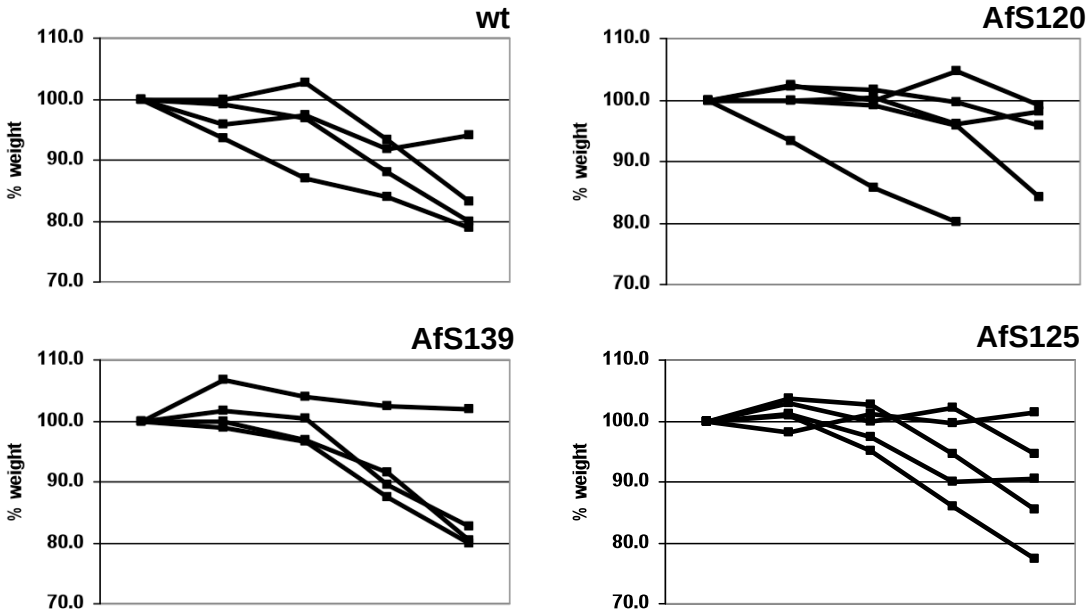

Fig. 36: Weight loss profiles of immunocompromised mice infected with *A. fumigatus* ATCC 46645, AfS120, AfS139 and AfS125 over the course of four days
The notation of the strains is as follows: the wildtype is ATCC 46645, AfS120 represents the complete *opt* gene deletion strain, AfS139 represents the complete *opt* and *dpp* gene deletion strain and AfS125 represents the *dpp* double knockout strain.
All the mice infected with the wildtype ATCC 46645 lost weight steadily over the course of the four days. Four of the five mice infected with AfS120 had lost weight by day four post infection, two of them dramatically, one mouse kept its weight throughout the experiment. Three of the four mice infected with AfS139 had lost almost 20% of their weight by day four post infection, one mouse kept its weight throughout the experiment. Of the five mice infected with AfS125, four had lost weight by day four post infection, one mouse kept its weight throughout the experiment.

Despite obvious variations between individuals within the same group, there is an overall tendency of significant weight loss for all strains visible by day four post infection, suggesting unaltered virulence of the deletion strains.

At the end of day four, the animals were sacrificed and the lungs processed to yield the template DNA for qRT-PCR. The resulting fungal burden for each strain is displayed in Fig. 37.

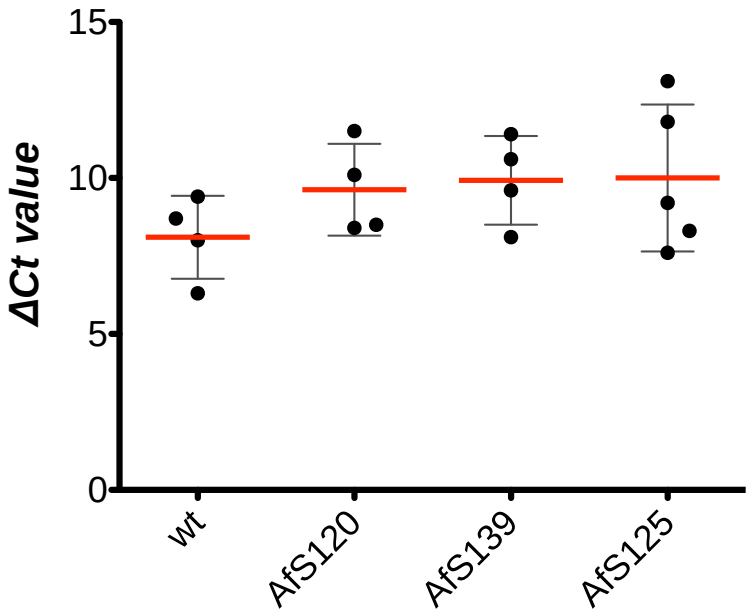

Fig. 37: Fungal burden of *A. fumigatus* ATCC 46645, AfS120, AfS139 and AfS125 in a mouse infection model
The notation of the strains is as follows: the wildtype is ATCC 46645, AfS120 represents the complete *opt* gene deletion strain, AfS139 represents the complete *opt* and *dpp* gene deletion strain and AfS125 represents the *dpp* double knockout strain.
The DNA extracted from the lungs of infected mice was subjected to qRT-PCR using mouse-specific and fungal-specific primer pairs, and the fungal burden was determined as the ΔCt value. While the fungal burden of the mutant strains is slightly lower than that of the wildtype strain, apparent in a ΔCt value that is two cycles higher, the difference is not statistically significant.

The fungal burden is comparable among the strains. It is almost exactly the same among the three deletion mutants, and only slightly but not significantly lower than that of the wildtype strain.

Taken together, the data suggest, that neither a complete deletion of the *opt* gene family, nor a deletion of the two *dpp* genes, nor a combination of these gene deletions lead to an attenuation of *A. fumigatus* virulence in a neutropenic mouse model for pulmonary aspergillosis.

4.8. A novel system for gene targeting in *A. fumigatus* employing the β-Rec/*six* site-specific recombination system

As demonstrated before, the Cre/*loxP* system allows for the deletion of up to 10 genes in *A. fumigatus*. For each of these 10 gene deletions, two transformations had to be performed: the first transformation replaced the target gene with a marker cassette, the second transformation transiently introduced the Cre recombinase for marker excision. There are alternative systems for sequential gene targeting in other fungal organisms, such as the Flp/*FRT* system in *C. albicans*, which do not need a second transformation step for marker excision. Here we try to adapt the β-Rec/*six* site-specific recombination system to *A. fumigatus* (Canosa *et al.*, 1996).

For this, in between two identical, asymmetric β recombinase binding sites (*six* sites), a synthetic, codon-optimized *β-rec* allele in front of the *A. nidulans trpC* transcriptional terminator, was placed into the cloning vector pMA-RQ and named pSK479 (custom-made and purchased from GeneArt, Germany). The pyrithiamine resistance gene *ptrA*, as a dominant marker, was inserted via BglII sites into this plasmid.

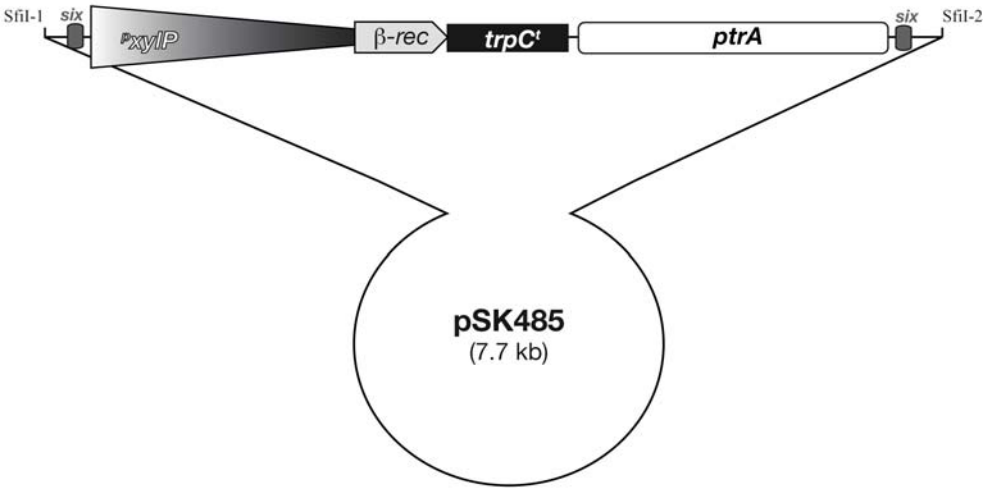

Fig. 38: The β-Rec/*six* marker cassette in detail
The central element of the marker cassette is the codon-optimized β recombinase gene (β-*rec*), which is under the control of the xylose-inducible *xylP* promoter from *P. chrysogenum*, and the *trpC* terminator from *A. nidulans*. The *ptrA* gene from *A. oryzae* confers resistance to pyrithiamine as a means of stabilizing the plasmid in *A. fumigatus*. The whole marker cassette is flanked by two *six* sites, which are the recognition sites of the β recombinase. The two incompatible SfiI restriction sites aide in the assembly process of gene specific deletion cassettes.

As a promoter the xylose inducible *Penicillium chrysogenum xylP* promoter region (Zadra *et al.*, 2000) was inserted as a 1.7 kb PCR fragment via PstI, so that the marker cassette was finally assembled in plasmid pSK485 (Fig. 38). In order to show the functionality of this novel marker cassette, gene deletion of the *abr2* gene was attempted, whose deletion leads to a brown conidia phenotype (Tsai *et al.*, 1999). For this, the marker cassette was excised from pSK485 by SfiI digestion and ligated to the SfiI backbone of pSK346, which contains the 5' and 3' flanking region of the *abr2* gene, resulting in the plasmid pSK509. The newly generated *abr2* gene replacement cassette was liberated from this plasmid by a HindIII digest and transformed into AfS77, which is an *akuA::loxP* derivative of *A. fumigatus* ATCC 46645 (Krappmann *et al.*, 2006).

Successful *abr2* deletion manifested itself in brown conidia. Removal of the marker cassette from the genome of the derived strain was achieved by streaking its conidia on xylose containing minimal medium, leaving a single *six* site at the original *abr2* locus (Fig. 39).

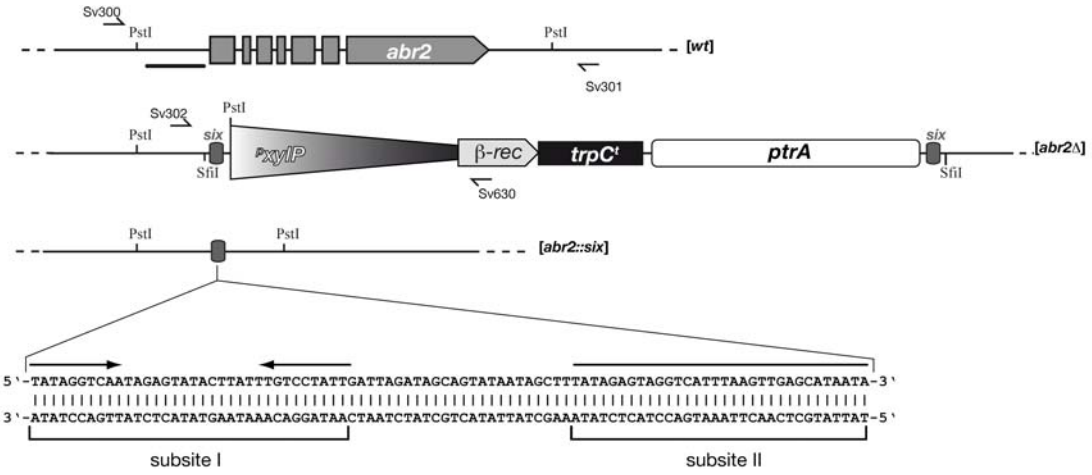

Fig. 39: Workflow of the *abr2* gene deletion and marker excision using the β-Rec/*six* marker cassette
On the top, a schematic overview of the *abr2* gene locus; the black bar indicates the position of the Southern hybridization probe, relevant restriction sites are indicated. In the middle, the *abr2* locus after gene replacement with the marker cassette, below the locus after marker excision. At the very bottom, the recombined *six* site with subsites I and II showing the β recombinase binding sites and the arrows detailing the inverted repeats.

Thus, this experiment was performed, resulting in two new strains: the *abr2Δ* strain in which the *abr2* gene was replaced by the marker cassette, and the *abr2::six* strain, where there would be a single *six* site at the former site of the *abr2* gene. Three independent clones of each strain were purified on minimal medium containing glucose

and verified by diagnostic PCR and Southern hybridization. Figure 40 visualizes typical results of these experiments, which were identical for all clonal isolates.

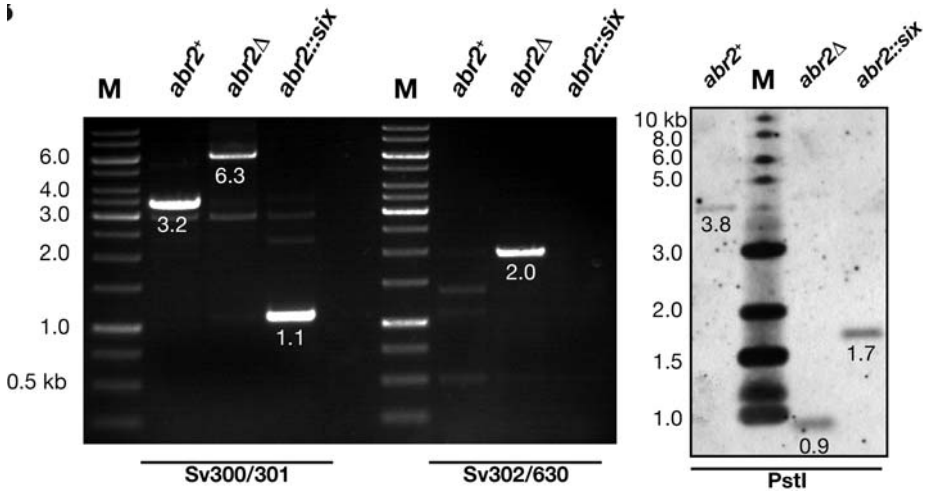

Fig. 40: Diagnostic PCRs and Southern hybridization of the wildtype and the *abr2* deletion strains at the *abr2* locus

Using the primer pairs Sv300/Sv301 and Sv302/Sv630 at the *abr2* locus, a diagnostic PCR on genomic DNA of each strain was performed. The signals appear at the correct size, indicating a successful gene deletion and marker excision of the *abr2* gene. For the Southern hybridization, genomic DNA was digested with PstI and a Southern hybridization using a probe at the *abr2* locus was performed. All three strains show signals of the expected size, indicating a successful gene deletion and marker excision.

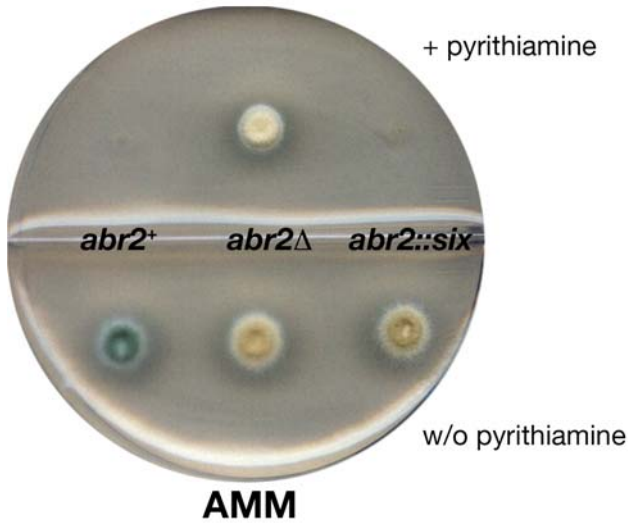

Fig. 41: Growth of wildtype and *abr2* deletion strains on minimal medium with and without pyrithiamine

The upper half shows growth of the strains on minimal medium with pyrithiamine. Only the *abr2Δ* deletion strain still harbouring the marker cassette is able to grow, the excised *abr2::six* deletion strain is unable to do so. The lower half shows growth on minimal medium. The wildtype strain shows typical green conidia, the conidia of the *abr2* deletion strains are characteristically brown.

To confirm these genotypes, growth phenotypes of the two new strains were assessed on medium with and without pyrithiamine (Fig. 41). It can be seen that the strains resulting from the transformation with the new marker cassette yield the expected results on the molecular and phenotypic level. The *abr2Δ* strains stably maintained the *six* - ^{P}*xylP::β-rec::trpC*^{t} - *ptrA* - *six* marker module, which got removed after passage in the presence of xylose leaving a single *six* site at the original locus of *abr2*.

If this new system is to be used for multiple, sequential gene deletions one has to be sure that recombination by the β recombinase is strictly in *cis*, meaning not between the *six* sites of the replacement cassette and leftover six sites from previous, marker excision events. In order to validate this, a second gene was targeted: *pksP*, which like *abr2* is part of the same pigment-encoding gene cluster. These two genes are about 8 kb apart, and the deletion of pksP results in white conidia (Langfelder *et al.*, 1998; Tsai *et al.*, 1998).

A *pksP* gene replacement cassette was generated by fusion PCR (Szewczyk *et al.*, 2006), where the 1.8 kb long 5' region and the 1.6 kb long 3' region of the *pksP* gene were fused to the marker module. The assembled *pksP* gene replacement cassette was ligated into the pCR-Blunt II-TOPO vector, generating the plasmid pSK510. From here the cassette was liberated via FspI digest and used for the transformation of the *abr2::six* strain. Like before with the *abr2* deletion, primary transformants of the *abr2::six/pksPΔ* deletion strain with white conidia were purified on minimal medium with glucose, verified by PCR and streaked on xylose-containing medium for marker excision, which eventually resulted in the *abr2::six/pksP::six* deletion strain. In a final diagnostic PCR, for three clonal isolates the former loci of *abr2* and *pksP* were interrogated to validate the correct excision of the marker cassette from the *pksP* locus. Figure 42 demonstrates, that correct marker excision could be achieved without compromising genomic integrity.

As before, the genotype of the deletion mutants was verified on minimal medium with and without pyrithiamine, which revealed the expected result of the *abr2::six/pksPΔ* deletion strain being resistant to pyrithiamine, and the *abr2::six/pksP::six* deletion strain being sensitive again (Fig. 43).

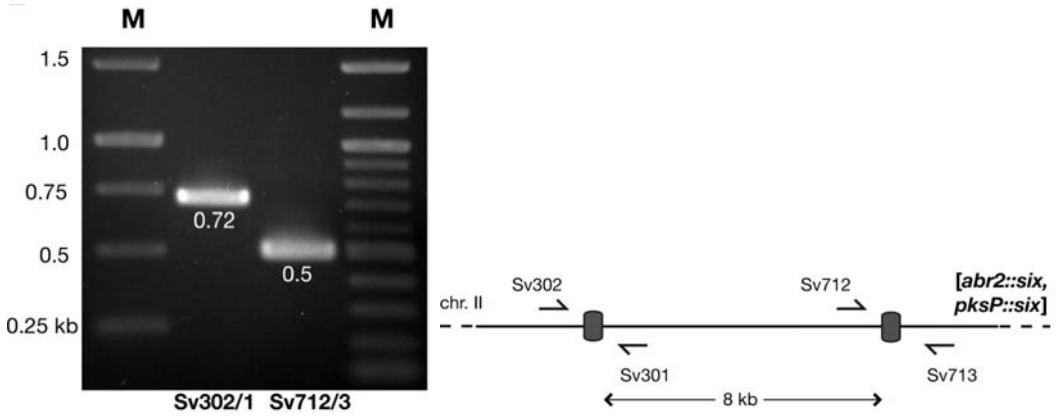

Fig. 42: Diagnostic PCRs of the excised *abr2::six/pksP::six* deletion strain at the *pksP* and the *abr2* locus
In the scheme, the former loci of *abr2* on the left and *pksP* on the right are occupied by *six* sites. The arrows indicate the position of the primer pairs for the diagnostic PCR. Using the primer pair Sv301/Sv302 for the *abr2* locus and Sv712/Sv713 for the *pksP* locus, a diagnostic PCR on genomic DNA of the excised *abr2::six/pksP::six* deletion strain was performed. The signals appear at the correct size, indicating a successful gene deletion and marker excision of both genes.

Fig. 43: Growth of the *abr2* deletion strain and the *abr2/pksP* deletion strains on minimal medium with and without pyrithiamine
The upper half shows growth of the strains on minimal medium with pyrithiamine. Only the *abr2::six/pksPΔ* deletion strain still harbouring the marker cassette at the *pksP* locus is able to grow, the excised *abr2::six/pksP*$^{+}$ deletion strain and the *abr2::six/pksP::six* deletion strain are unable to do so. The lower half shows growth on minimal medium. The conidia of the *abr2::six/pksP*$^{+}$ deletion strain are characteristically brown, the conidia of the *abr2/pksP* deletion strains are white.

In summary, it could be demonstrated that the β-Rec/*six* site-specific recombination system was successfully adopted for *A. fumigatus*, and that it could be employed for sequential deletion of two genes.

5. DISCUSSION

5.1. Transcriptional profile of *A. fumigatus* during growth on different secondary nitrogen sources

The transcriptional response of *A. fumigatus* to three different secondary nitrogen sources was investigated in this work: nitrate, proline and BSA. For the interpretation of the transcriptional profiling data, one has to keep in mind that many genes are still poorly characterized in *A. fumigatus*, and in large parts annotation of the open reading frames (ORF) was performed by algorithms, based on sequence homology to known genes from other organisms.

On nitrate medium, 192 genes encoding non-hypothetical proteins were upregulated. Using the KEGG categorization for gene enrichment analysis, there was no significant enrichment of any particular functional category among these 192 genes, other than an enrichment of general metabolic functions. Compared to the other two nitrogen sources, the transcriptional response on nitrate seems less focused on certain, specific metabolic functions, such as the vast overrepresentation of functions associated with amino acid metabolism on proline medium (discussed below). This could indicate, that nitrate induces primarily a general starvation response. There are some hallmark genes of nitrate metabolism, which are strongly induced by nitrate, in particular the genes *niaD*, *niiA* and *crnA*, which were shown in *A. nidulans* to be part of a gene cluster that is regulated by the transcription factors AreA and NirA (Krappmann and Braus, 2005, and References therein). Additionally, the nitrate transporter NrtB, a transporter that had previously been described in *A. nidulans*, was strongly upregulated (Wang *et al.*, 2008). All this is not surprising, given the fact that these proteins are essential for nitrate utilization.

At the same time, the genes of three ammonium transporters were upregulated by nitrate. One of these, *meaA*, which codes for the main ammonium transporter in *A. nidulans*, is upregulated on all three secondary nitrogen sources (Monahan *et al.*, 2002). A likely explanation for this is the fact, that after an initial downregulation of ammonium transporter genes following the nitrogen starvation and the shift to a different nitrogen source, the metabolism of the secondary nitrogen sources led to the

production of significant amounts of ammonium, which easily converts into ammonia and diffuses through the cell membrane into the medium. This accumulation of extracellular ammonia/ammonium then induced ammonium uptake systems. This hypothesis could also explain, why nitrate leads to the induction of more ammonium transporters than the other two nitrogen sources. Nitrate, through the action of NiaD and NiiA is converted directly into ammonium, which is then incorporated into glutamate and glutamine (Krappmann and Braus, 2005, and References therein). Thus, there will be a significant concentration of free ammonium in the cytoplasm, which can convert into ammonia. This is different for the utilization of proline and BSA, where nitrogen is more likely to be transfered from an amino acid to an α-keto acid through the action of transaminases, leading to much less free ammonium inside the cell.

Among the 161 genes coding for non-hypothetical proteins that were upregulated by proline, the KEGG categorization showed a high enrichment of metabolic functions associated with amino acid metabolism. This is expected, as proline as the only nitrogen source needs to be utilized and its nitrogen fed into various metabolic pathways. As with nitrate, the genes of a particular cluster necessary for proline utilization, the *prn* gene cluster, were strongly upregulated (Arst and MacDonald, 1975). The high number of upregulated aminotransferases underlines the need of *A. fumigatus* to use the nitrogen supplied by proline to synthesize other amino acids.

In BSA medium, a much higher number of genes was upregulated than on the other two secondary nitrogen sources. This shows that BSA, as a much more complex source of nitrogen warrants a broader metabolic response than simple molecules such as nitrate or proline. Functions associated with amino acid metabolism are enriched in the KEGG categorization. This is unsuprising since BSA is made up of amino acids, which eventually, after processing of BSA and its peptide degradation products, constitute the sole nitrogen source. However, there is also an enrichment of functions associated with carbon metabolism, such as glycolysis/gluconeogenesis, fatty acid metabolism or *N*-glycan biosynthesis. A possible reason for this might be, that the metabolism of the taken up amino acids leads to an accumulation of the carbohydrates in the cell. In the BSA medium there is again an upregulation of numerous aminotransferases and amidases, which remove the nitrogen from the amino acids in order to make it available for various cellular needs. The remaining carbohydrate backbones must also be metabolized to avoid accumulation of potentially toxic substances, hence the induction

of metabolic pathways that utilize carbohydrates. This might also explain the enrichment of cytochrome P450 proteins, which are widely used to metabolize various organic substances (Voet *et al.*, 2002).

This leaves the question of why does proline not induce similar pathways, as aminotransferases play an even greater role in proline medium than in BSA medium? The answer to this might be, that proline is metabolized in two steps by PrnD and PrnC to glutamate, which, after another aminotransferase step to remove the amino group, is fed into the TCA cycle (Goldman and Osmani, 2008). The TCA cycle is very active already, since glucose is the carbon source in the proline medium, so a further induction by proline does not happen (Gonzalez *et al.*, 1997). Thus, the carbon backbones of proline are much more easily metabolized than those generated by feeding on BSA, and there is no need to upregulate further carbohydrate metabolic pathways.

Six aminotransferases were upregulated in BSA medium, compared to proline medium, where it was ten. Also, aminotransferases were not as strongly upregulated by BSA as they were by proline. This suggests, that while aminotransferases do play a role in BSA medium, it is smaller than during growth in proline. An explanation for this might be, that during growth in BSA medium, the amino acids needed to synthesize proteins are all present already, and just the ratios need to be adjusted by aminotransferase reactions. In proline medium, every amino acid except proline needs to by synthesized, creating a much higher need for aminotransferase activity. In BSA medium, six amidases were significantly upregulated, compared to none under the proline condition. Amidases turn amides into carboxylic acids and ammonia, and as such play an important role in the metabolism of amino acids (Bray *et al.*, 1949; Bray *et al.*, 1950). Nitrogen is not just an important component of amino acids, but is also needed for the de novo biosynthesis of nucleobases, the building blocks of DNA and RNA. The synthesis of all five nucleobases consumes large amounts of glutamine, which is readily synthesized using two glutamate molecules (Voet *et al.*, 2002). Thus, it is possible, that in proline medium the nitrogen necessary for nucleobase biosynthesis comes from abundant glutamate, which as seen before is created in large amounts by the action of PrnC and PrnD, and is channeled from there into the appropriate pathways. In BSA medium, glutamate is much less readily available, so the nitrogen necessary for nucleobase biosynthetic pathways has to be mobilized from a more diverse pool of amino acids, explaining the greater need for amidases.

Not surprisingly, a large number of genes encoding (secreted) proteases was upregulated on BSA medium. The secreted proteases are needed to digest BSA into small enough pieces, so that *A. fumigatus* can take them up, either as free amino acids or as peptides. These peptides might be broken down further by intracellular peptidases. In accordance with this hypothesis, a number of peptide and amino acid uptake systems were upregulated by BSA. Two oligopeptide transporters, *optB* (Afua_2g15240) and *optD* (Afua_5g13850), and two transporters of the *ptr* gene family, *ptrE* (Afua_1g12240) and *ptrF* (Afua_8g02550), were transcriptionally upregulated in BSA medium. This is in partial agreement with the qRT-PCR data, which suggested a much broader and stronger upregulation of both uptake systems in BSA medium. This might be primarily a technical difference between qRT-PCR and the DNA hybridization on a DNA microarray, since the culture conditions were identical in both experimental setups.

The fact that seven amino acid transporters were upregulated in BSA medium indicates, that the extracellular proteolytic digest of BSA results in the generation of significant amounts of free amino acids in the medium. It is interesting to see that the proline permease *prnB* was much more strongly induced in proline medium than in the presence of BSA, while another putative proline permease (Afua_8g02200) was strongly induced by BSA and not at all upregulated by proline. While the data for *prnB* can be easily explained by the much higher concentration of free proline, the behaviour of the yet uncharacterized Afua_8g02200 is less clear. Either this ORF encodes a proline permease that is only transcribed at low proline concentrations and repressed at high concentrations of proline, or Afua_8g02200 was misannotated. Overall, from the upregulation of these uptake systems it can be concluded, that the extracellular proteolytic digest of BSA results in the generation of both, free amino acids and peptides of varying lengths.

There are 13 genes that are upregulated under all three nitrogen conditions. However, it is difficult to put these very diverse genes into a meaningful biological context. The reason for this might be, that proline and BSA, as an amino acid and a protein made up from amino acids, are quite related. On the other hand, nitrate as a simple inorganic nitrogen source is very different from these two. Thus, it is more insightful to look at the genes upregulated by proline and by BSA. A significant functional overlap can be observed in the induction of aminotransferases, which are necessary for growth in both media, in order to move amino groups between

carbohydrate backbones, resulting in the synthesis of the different amino acids. Also, the induction of genes of the *prn* gene cluster by both nitrogen sources, can be explained with the presence of proline in both media. Three genes encoding cytochrome P450 proteins are induced by proline and BSA. The reason for this might be, that during growth on proline and BSA a larger number of carbohydrates (in the form of amino acids or peptides) is entering the cell, than during growth on simple, inorganic nitrogen sources like ammonium and nitrate. As cytochrome P450 proteins play a large role in metabolizing many organic substances, there might be a greater need for this class of enzymes during growth on amino acids and proteins, in order to deal with the carbohydrate backbones of amino acids (Voet *et al.*, 2002).

On each medium there are a number of differentially regulated transcription factors, but most of them are poorly, or not at all, characterized in aspergilli. When looking at transcription factors, which have been associated with nitrogen metabolism in *A. nidulans*, it becomes apparent, that there is almost no transcriptional regulation on nitrate and proline medium. The slight downregulation of *areA* transcription on nitrate medium is in disagreement with data from *A. nidulans* (Morozov *et al.*, 2001; Caddick *et al.*, 2006). This could either be a genuine difference between *A. fumigatus* and *A. nidulans*, or a possible false result. This would need to be verified by Northern hybridization or qRT-PCR. Besides this, the missing transcriptional regulation of *nirA* and *meaB* on nitrate (Narendja *et al.*, 2002; Wong *et al.*, 2007), and of *areA* and *prnA* on proline are in agreement with published results (Gomez *et al.*, 2002; Caddick *et al.*, 2006).

The transcriptional regulation of transcription factors on BSA medium shows some unexpected results. The upregulation of the positive regulator of extracellular proteolytic activity *prtT*, while understandable from a teleological point of view, runs counter to published results, which did not show any upregulation of *prtT* on proteinaceous nitrogen sources (Bergmann *et al.*, 2009). The same is true for *cpcA*, the transcriptional regulator of the Cross-Pathway Control. It was shown that *cpcA* transcript levels increase under amino acid starvation conditions (Hoffmann *et al.*, 2001; Krappmann *et al.*, 2004), but downregulation in response to amino acid abundance has not been observed. Both results need to be verified by other experimental methods, in order to ensure their validity.

The second downregulated gene, *meaB*, whose gene product controls *nmrA* gene expression and thus indirectly influences the NCR, has been described in *A. nidulans*

(Wong *et al.*, 2007). Here *meaB* expression was not regulated in response to any nitrogen source (ammonium, glutamine, alanine, glutamate) or nitrogen starvation condition. Thus, this result needs to be verified by Northern hybridization or qRT-PCR and if true, would constitute the first condition under which *meaB* transcription is regulated. As with the proline and nitrate condition, *areA*, *prnA* and *nirA* did not respond to changing nitrogen sources by altering their mRNA levels. For the first time this work demonstrated, that this is also the case for a complex nitrogen source, such as BSA.

The mammalian lung can also be considered a complex nutrient source, so how does the transcriptional response of *A. fumigatus in vivo* (McDonagh *et al.*, 2008) compare to the BSA growth condition? Among the genes induced *in vivo* there is also an overrepresentation of functions associated with amino acid catabolism and fatty acid metabolism, a characteristic that is shared with the response to BSA. However, *in vivo* a much greater diversity of molecular functions is upregulated, especially the transport of metal ions and carbohydrates. This illustrates that the lung is a much more complex and challenging environment than a BSA/glucose medium. On the level of individual genes, 5 of the 19 secreted proteases, 3 of the 6 amidases, 2 of the 6 aminotransferases and 5 of the 11 transporters (2 proline permeases, 2 amino acid permeases and 1 PTR transporter) which were upregulated in BSA medium, were also induced *in vivo*. Thus, on the level of mobilization of protein as an extracellular nutrient source, there appears to be a certain similarity between growth in BSA medium and growth inside the mammalian lung, a hypothesis that had been raised before (Ibrahim-Granet *et al.*, 2008).

In sum, the profiling of the overall transcriptional response to three secondary nitrogen sources provided a broad insight into the physiology of *A. fumigatus*, and the data could serve as a starting point for further experiments trying to elucidate nitrogen metabolism of this fungus.

5.2. The *opt* gene family encodes functional oligopeptide transporters

During this work, a great number of *Aspergillus fumigatus* gene deletion mutants were generated, culminating in a strain lacking the entire *opt* gene family, a strain lacking the *opt* genes and two dipeptidyl peptidases and a strain lacking the *opt* genes and the transcriptional regulator *prtT*, all of which were subsequently characterized *in vitro*.

Additionally, the *A. fumigatus opt* genes were expressed and functionally characterized in *S. cerevisiae*.

It appears that even though the *opt* gene family is transcriptionally upregulated during growth on collagen hydrolysate and BSA as the sole nitrogen source, it is dispensible for growth on many complex nutrient sources. A strain lacking all eight *opt* genes is able to grow like the wildtype on media as complex as BSA or porcine lung. Thus, uptake of degradation products of a proteolytic digest of extracellular protein is not dependent on oligopeptide transporters to ensure sufficient nutrient supply for *A. fumigatus*. Other uptake systems, such as amino acid permeases or peptide transporters, seem to transport enough degradation products to allow for unimpeded growth.

It could be shown that the deletion of the *opt* gene family can impair growth, when one particular oligopeptide (GPGG) is the only available source of nitrogen. This suggests that the oligopeptide GPGG can only be taken up by one or more members of the *opt* gene family, not by any other uptake system. For other oligopeptides there was no such effect. Either they are transported into the cell by other uptake systems, or they are degraded by extracellular proteases and taken up as single amino acids or small peptides. While the first option cannot be excluded, it seems the less likely of the two explanations, as up to now in fungi only members of the *opt* gene family are known to transport oligopeptides of 4-5 amino acids in length. It is more probable that the other oligopeptides were degraded by extracellular proteases, which were not inhibited by the protease inhibitors added into the medium. The reason for this lack of inhibition might be that either these proteases are not inhibited at all by one of the three added protease inhibitors, or that one or more of the protease inhibitors became unstable and non-functional over the course of the incubation period, allowing the previously inhibited proteases to function again. This demonstrates, that characterizing the substrate specificity of oligopeptide transporters in *A. fumigatus* itself is not trivial.

The question of transport activity and substrate specificity was better answered when using *S. cerevisiae* as a host for heterologous gene expression. It could be shown that four oligopeptide transporters from *A. fumigatus*, OptB, OptC, OptG and OptH, could mediate oligopeptide transport in *S. cerevisiae*. The transported oligopeptides KLLLG, LWMR and GGFL had previously been described as oligopeptide transporter substrates, the oligopeptide TPRK was newly described as a substrate in this work (Koh *et al.*, 2002; Reuss and Morschhauser, 2006; Tsay *et al.*, 2007). It appears as if OptB is

the general oligopeptide transporter in *A. fumigatus*, as it has an exceptional substrate range and transport activity. Not only does it transport all four investigated oligopeptides, it also conferred the most vigorous growth to the yeast, indicating a generally high transport capacity at least in *S. cerevisiae*. It remains to be determined if this also applies to transport in *A. fumigatus* itself. The substrate specificity was much narrower for OptC, OptG and OptH, and there are distinct differences in substrate preference between these transporters. Overall, compared to OptB, the transport capacity of these three proteins was lower for all substrates. Whether this is also the case in *A. fumigatus* remains to be investigated, however the available data from expression in *S. cerevisiae* suggests a more specific role in oligopeptide transport for OptC, OptG and OptH.

There are two possible reasons why the other four oligopeptide transporters did not transport any of the oligopeptides used in our experiments. Either these transporters have a different substrate specificity, which none of the used oligopeptides complied with, or these transporters are not functionally expressed in our *S. cerevisiae* expression system. For further studies, a greater number of oligopeptides as substrate could be used, or a different expression system such as *Pichia pastoris* could be employed in the hopes of better functionality in a new host.

In the literature, one possible case of an oligopeptide transporter acting as a sensor for extracellular protein degradation products was described (Gonzalez-Lopez *et al.*, 2002). While that work investigated the transcriptional expression of two particular secreted proteases, we took a look at the overall extracellular proteolytic activity of culture supernatant in a simple activity test. We could not detect a difference in that activity in the absence of the *opt* gene family. This does not exclude the possibility that one or more particular proteases are differentially expressed in response to a sensing event by an oligopeptide transporter, but from this work it cannot be concluded that the *opt* gene family is involved in sensing.

A deletion of the *opt* gene family alone did not result in a growth defect on medium, which contains only a complex nitrogen source. However, there is an interesting phenotype when the transcriptional regulator of extracellular proteolytic activity *prtT* is deleted in an *opt* deficient background. On medium where porcine lung is the only available nutrient source, there is a synergistic effect of the deletion of *prtT* and the *opt* gene family. This suggests that in the wildtype, secreted proteases digest protein and oligopeptide transporters, among other uptake systems, import the

degradation products into the cytosol. When the oligopeptide transporters are missing, *A. fumigatus* is still able to fulfill its nutritional needs by just taking up the lower molecular breakdown products, such as single amino acids and di- or tripeptides. When *prtT* is deleted, and consequently some proteases are not expressed any more (Bergmann *et al.*, 2009; Sharon *et al.*, 2009), the remaining transport capacity, including the oligopeptide transporters, is sufficient enough to support wildtype-like growth on porcine lung agar. The composition of the degradation products might change though, as the missing proteases lead to an accumulation of longer peptides. This accumulation of longer peptides then causes the growth defect in a mutant strain that is missing both *prtT* and oligopeptide transporters. The loss of oligopeptide transport activity, which cannot be compensated by the remaining uptake systems, and a shortage of smaller degradation products as a result of missing extracellular proteolytic activity, lead to a synthetic growth defect on porcine lung medium. Whether this hypothetical scenario is true, remains to be investigated. It would be especially interesting, if there is indeed a shift away from short peptides and single amino acids towards longer oligopeptides in the *prtT* deletion mutant. And conversely, if the loss of the *opt* gene family leads to an increased activity of certain proteases, which are responsible for the generation of shorter breakdown products.

A similar rationale was followed when the two *dpp* genes where deleted in an *opt* gene family deletion background. However, in all conducted experiments the combined deletion mutant behaved the same way as the *opt* gene family deletion mutant alone. This was the case even for growth on medium where collagen hydrolysate was the sole nitrogen source, which is somewhat unexpected since collagen with its very high proline content is an ideal substrate especially for Dpp4 (Beauvais *et al.*, 1997). From the growth experiments it seems that *A. fumigatus* is still able to digest the collagen hydrolysate into smaller pieces, utilizing the remaining extracellular proteolytic activity. Thus it can be concluded, that the loss of two major dipeptidyl peptidase activities did not significantly increase the need for oligopeptide uptake systems.

5.3. Transcriptional response of peptide uptake systems during growth in the presence of different nitrogen sources

One of the main approaches for understanding the significance of oligopeptide transporters in *A. fumigatus*, was profiling the transcriptional response of the *opt* gene family to growth on different nitrogen sources. Expression studies that initially focused on the *opt* genes, were later supplemented with an investigation of other genes, such as the *ptr* gene family or two dipeptidyl peptidases, to gain a more comprehensive picture.

When *A. fumigatus* grows on BSA medium, where proteins are the only nitrogen and carbon source, almost the entire *opt* gene family is transcriptionally upregulated. This suggests that on this medium the fungus does employ the function of this transporter family, most likely to take up oligopeptide degradation products into the cell. The transcriptional profile of the *opt* gene family does change slightly over time, which might be in response to the changing composition of the proteolytic breakdown products during the course of incubation. For some *opt* genes, the addition of glucose as the carbon source increases the transcriptional upregulation. One possible explanation for this is, that the fungus can grow much more vigorously on glucose containing medium, which results in an even greater need for sufficient nitrogen supply leading to an enhanced upregulation of relevant transport systems.

The transcriptional response of the *opt* gene family to collagen hydrolysate is limited to two genes, *optB* and *optH*. This change suggests, that different substrates could lead to distinct *opt* expression patterns, depending on the composition of the resulting oligopeptides and the substrate specificity of each oligopeptide transporter. While this idea is appealing, more experiments on different complex substrates are needed to support this hypothesis. On both, BSA and collagen hydrolysate, *optB* is by far the most strongly upregulated *opt* gene. This correlates with the data from the heterologous expression in yeast, where OptB was the oligopeptide transporter conferring the broadest transport activity. Taken together this data indicates, that OptB is the main oligopeptide transporter in *A. fumigatus* in the presence of oligopeptides, while the other transporters might be reserved for only a limited number of nutritional conditions. When *optB* was deleted, three other *opt* genes were transcriptionally upregulated, while transcription levels of the rest remained unchanged. This might indicate that the upregulated oligopeptide transporters compensate for the loss of OptB.

The *ptr* gene family reacted to growth on BSA, in similar fashion as the *opt* genes, with a broad transcriptional upregulation. This suggests that *A. fumigatus*, when grown on substrates that are proteolytically digested, utilizes dipeptide transporters as well. In the absence of the *opt* gene family, only a single *ptr* gene was significantly upregulated during growth on BSA, suggesting that the *ptr* gene family as a whole does not greatly compensate for the loss of oligopeptide transport function. Thus, either the contribution of oligopeptide transport during growth on BSA is so small, that it does not need to be compensated, or nutrient uptake systems other than the *ptr* system increase their transport activity. Most likely these would be amino acid transporters, but this hypothesis would need to be experimentally supported.

When *A. fumigatus* is grown on a rich medium like YPD, the *opt* gene family was neither transcriptionally induced, nor repressed significantly. The same was observed for the *ptr* gene family. This indicates that these transporter systems are not needed for growth on rich medium, at least not during the first 40 minutes of incubation. YPD is rich in free amino acids, which would act as the preferred nitrogen source until they get exhausted. The second conclusion one can draw from the data is, that neither the *opt* genes, nor the *ptr* genes are broadly repressed in the presence of free amino acids. On rich medium such as YPD, general mechanisms like the nitrogen catabolite repression do not seem to play an inhibitory role on the transcriptional expression of these two peptide uptake systems.

The two dipeptidyl peptidases Dpp4 and Dpp5 were investigated for their potential role in breaking down oligopeptides into smaller peptides, thus decreasing the substrate pool for the oligopeptide transporters. On the YPD medium, the *dpp* genes were neither induced nor repressed, suggesting that on a medium rich in free amino acids there is no need for dipeptidyl peptidase activity. On both, BSA and collagen hydrolysate, these two genes were upregulated, significantly stronger on collagen than on BSA. The reason for this might be, that collagen hydrolysate contains many more free N-termini on which these two enzymes act, than BSA which largely is digested by endoproteases first. During growth in BSA medium, there was no change in the transcriptional upregulation of the two *dpp* genes, in a strain where the entire *opt* gene family had been deleted, when compared to the wildtype. This is surprising, as it could be expected that in such a strain there would be an increased need to break down oligopeptides into shorter degradation products. One likely explanation for this is, that the enzymatic activity of Dpp4 and Dpp5 is already enough to satisfy the nutritional

demands of *A. fumigatus* even in the absence of oligopeptide transporters, making any further upregulation unnecessary.

5.4. The *opt* gene family is dispensable for virulence in a murine model of pulmonary aspergillosis

Several strains generated in this work were tested for their virulence in a neutropenic mouse model for pulmonary aspergillosis. The complete *opt* deletion strain and the *dpp4/dpp5* deletion in that strain background are of particular interest for understanding the significance of oligopeptide transport in the infection of a mammalian host. Neither of these two strains showed a significant change in virulence, which means that both the oligopeptide transporters and the two dipeptidyl peptidases are dispensible for the successful colonization of the lungs in a neutropenic mouse. This is also in accordance with the fact, that neither of these two strains show any growth defect on the porcine lung medium. When using a mammalian lung as a growth substrate, *in vitro* or *in vivo*, the presence of any member of the *opt* gene family is not necessary. As explained before, *in vitro A. fumigatus* most likely breaks down the protein of the lung into small enough pieces, so that transport systems other than oligopeptide transporters can cover the nutritional needs. The situation inside the mammalian lung is more complex. Either, just like on the porcine lung agar, *A. fumigatus* also digests the murine lung thoroughly enough to rely solely on amino acid permeases and/or dipeptide transporters to support its growth. Or other nutrient sources are available *in vivo*, which make the need to feed on proteolytic digestion products less urgent. One possible source could be blood, which would be available after a vessel had been penetrated by mechanical force or through the action of elastinolytic enzymes. Another convenient source for a great number of nutrients are the epithelial cells that are coating the alveoli, whose lysis would liberate their cytosolic contents.

As one strain, the *prtT* deletion in the *opt* gene family deletion background, did exhibit a pronounced growth defect on the porcine lung agar, it will be most interesting to see if this also leads to an attenuated virulence in a murine infection model. If it did, that would strengthen the case for the importance *in vivo* of extracellular proteases, the products of their activity and the respective uptake systems. If there was still no diminished virulence, this would support the hypothesis put forward by other scientists, that proteases are not instrumental in the successful infection of the lungs of a

mammalian host (Monod *et al.*, 2002; Latge and Steinbach, 2009), and neither are oligopeptide uptake systems.

5.5. The β-Rec/*six* site-specific recombination system: a tool for efficient genetic manipulation of *A. fumigatus*

In this work, the Cre/*loxP* system was successfuly employed for the sequential deletion of up to 10 genes in *A. fumigatus*. The resulting strains did not show any negative effects from this procedure, neither for growth *in vitro* on any of the tested media, nor in their virulence in a murine model of pulmonary invasive aspergillosis. Additionally, no case of *trans* recombination between the *loxP* sites at different gene loci was observed. Thus, the Cre/*loxP* system has proven itself to be a reliable and robust system for sequential gene targeting in *A. fumigatus*.

This work also established a novel tool for genetic manipulation of *A. fumigatus*, the prokaryotic β-Rec/*six* site-specific recombination system. The functionality of this system was validated by the successful deletion of two genes, *abr2* and *pksP*, of the melanin pigment-encoding gene cluster. This demonstrates, that *A. fumigatus* provides the cellular components necessary for the β recombinase to be functional. In plants, for which a β recombinase system had also been developed, an essential component that had to be provided by the host are HMG domain proteins (Ritt *et al.*, 1998; Stemmer *et al.*, 2002). Appearantly, *A. fumigatus* also provides such a host factor, and it can be speculated that other fungi will also be able to support a β recombinase based system, be it aspergilli or even fungi in general. The successful deletion of *abr2* and *pksP* also demonstrated another important feature of the β recombinase, which has a strict *cis* action, i.e. it does not act on unlinked recombination sites (Rojo *et al.*, 1993; Alonso *et al.*, 1995; Rojo and Alonso, 1995). The two genes are just 8 kb apart, still we could not observe recombination events between the *six* sites of the two different marker modules.

The adoption of the β-Rec/*six* site-specific recombination system for *A. fumigatus* was supported by two things, the inducible *xylP* promoter and the availability of a codon optimized β recombinase gene. The *xylP* promoter seems to be sufficiently repressed in the presence of glucose, which is essential for the stable integration of the replacement cassette into the genome. On xylose, on the other hand, that promoter is induced strongly enough, to guarantee an adequate recombinase expression level for the function of the system. Codon-optimization of the β

recombinase gene facilitates its expression in the *A. fumigatus* host system, as the effects of negative codon bias are circumvented. This strategy has proven necessary in other fungal species, increasing gene expression levels or making it at all possible (Bayram *et al.*, 2008; Gooch *et al.*, 2008).

In summary, the β-Rec/*six* site-specific recombination system is a valuable addition to the genetic toolkit for *A. fumigatus*, and it should prove very useful for future large-scale gene deletion studies in this fungus, and potentially other fungal organisms as well.

5.6. Conclusions and Perspectives

This work investigated different aspects of the nitrogen metabolism of *Aspergillus fumigatus*. The insights gained from the transcriptional profiling of the response to growth on three different secondary nitrogen sources can be used as the starting point for future, in-depth studies of the metabolic and regulatory pathways involved. Additionally, it might be interesting to examine the transcriptional response to other complex nitrogen sources, especially media that closely resemble the nutritional situation inside the human lung. A transcriptome study using some form of lung medium would therefore be most illuminating.

This study represents the first characterization of the oligopeptide transporter (OPT) gene family in *A. fumigatus*. It could be shown that the gene family encodes functional oligopeptide transporters, whose gene transcription is differentially regulated, especially in response to proteinaceous nitrogen sources. Despite this, upon complete deletion of all *opt* genes in *A. fumigatus*, the fungus grew well on almost all tested media, with the exception of a medium with the oligopeptide GPGG as the sole nitrogen source. Future experiments might focus on finding more growth conditions, such as different oligopeptides as the only source of nitrogen, where the absence of the *opt* gene family leads to a growth phenotype or a decrease in fitness. Functional characterization of the OPTs in *A. fumigatus* could also be expanded by take-up experiments, using toxic or fluorophore labelled oligopeptides.

The synthetic growth defect on porcine lung agar when both, oligopeptide transport and extracellular proteolytic activity were genetically impaired, suggests an interdependence of these two processes. A more detailed investigation would try to characterize this phenomenon by one of two approaches: first, by trying to elucidate

possible combinations of oligopeptide transporters and secreted proteases, whose deletion leads to a growth defect on porcine lung medium. Second, by finding a more clearly defined medium, that also leads to a growth phenotype, when the mutant strain is propagated on it. Lastly, it could be shown that the *opt* gene family is dispensable for virulence in a murine model of pulmonary aspergillosis. However, as the combined deletion of *prtT* and the *opt* genes led to impaired growth on the porcine lung medium, it would be most interesting to find out, whether this also leads to attenuation of virulence inside the mammalian lung.

Finally, the introduction of the β-Rec/*six* site-specific recombination system will make gene deletion studies, such as the one undertaken in this work, simpler and faster. It remains to be seen, whether this system is as reliable and robust as the Cre/*loxP* based system used for the *opt* gene family, but the ease with which *abr2* and *pksP* could be deleted suggests, that this novel genetic tool will be a useful addition to the fungal genetic toolkit.

6. REFERENCES

Alagramam, K., F. Naider, and J. M. Becker. 1995. A recognition component of the ubiquitin system is required for peptide transport in *Saccharomyces cerevisiae*. *Mol Microbiol* **15:**225-234.

Alonso, J. C., C. Gutierrez, and F. Rojo. 1995. The role of chromatin-associated protein Hbsu in beta-mediated DNA recombination is to facilitate the joining of distant recombination sites. *Mol Microbiol* **18:**471-478.

Andre, B. 1995. An overview of membrane transport proteins in *Saccharomyces cerevisiae*. *Yeast* **11:**1575-1611.

Andreasson, C., and P. O. Ljungdahl. 2002. Receptor-mediated endoproteolytic activation of two transcription factors in yeast. *Genes Dev* **16:**3158-3172.

Andreasson, C., and P. O. Ljungdahl. 2004. The N-terminal regulatory domain of Stp1p is modular and, fused to an artificial transcription factor, confers full Ssy1p-Ptr3p-Ssy5p sensor control. *Mol Cell Biol* **24:**7503-7513.

Andrianopoulos, A., S. Kourambas, J. A. Sharp, M. A. Davis, and M. J. Hynes. 1998. Characterization of the *Aspergillus nidulans* nmrA gene involved in nitrogen metabolite repression. *J Bacteriol* **180:**1973-1977.

Aouida, M., A. Khodami-Pour, and D. Ramotar. 2009. Novel role for the *Saccharomyces cerevisiae* oligopeptide transporter Opt2 in drug detoxification. *Biochem Cell Biol* **87:**653-661.

Aoyagi, T., S. Kunimoto, H. Morishima, T. Takeuchi, and H. Umezawa. 1971. Effect of pepstatin on acid proteases. *J Antibiot (Tokyo)* **24:**687-694.

Apostolaki, A., Z. Erpapazoglou, L. Harispe, M. Billini, P. Kafasla, D. Kizis, M. A. Penalva, C. Scazzocchio, and V. Sophianopoulou. 2009. AgtA, the dicarboxylic amino acid transporter of *Aspergillus nidulans*, is concertedly down-regulated by exquisite sensitivity to nitrogen metabolite repression and ammonium-elicited endocytosis. *Eukaryot Cell* **8:**339-352.

Arst, H. N., Jr., and D. J. Cove. 1973. Nitrogen metabolite repression in *Aspergillus nidulans*. *Mol Gen Genet* **126:**111-141.

Arst, H. N., Jr., and D. W. MacDonald. 1975. A gene cluster in *Aspergillus nidulans* with an internally located cis-acting regulatory region. *Nature* **254:**26-31.

Askew, D. S. 2008. *Aspergillus fumigatus*: virulence genes in a street-smart mold. *Curr Opin Microbiol* **11:**331-337.

Bartel, B., I. Wunning, and A. Varshavsky. 1990. The recognition component of the N-end rule pathway. *EMBO J* **9:**3179-3189.

Basrai, M. A., M. A. Lubkowitz, J. R. Perry, D. Miller, E. Krainer, F. Naider, and J. M. Becker. 1995. Cloning of a *Candida albicans* peptide transport gene. *Microbiology* **141 (Pt 5):**1147-1156.

Bayram, Ö., S. Krappmann, M. Ni, J. W. Bok, K. Helmstaedt, O. Valerius, S. Braus-Stromeyer, N. J. Kwon, N. P. Keller, J. H. Yu, and G. H. Braus. 2008. VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. *Science* **320:**1504-1506.

Beauvais, A., M. Monod, J. P. Debeaupuis, M. Diaquin, H. Kobayashi, and J. P. Latge. 1997. Biochemical and antigenic characterization of a new dipeptidyl-peptidase isolated from *Aspergillus fumigatus*. *J Biol Chem* **272:**6238-6244.

Beauvais, A., M. Monod, J. Wyniger, J. P. Debeaupuis, E. Grouzmann, N. Brakch, J. Svab, A. G. Hovanessian, and J. P. Latge. 1997. Dipeptidyl-peptidase IV secreted by *Aspergillus fumigatus*, a fungus pathogenic to humans. *Infect Immun* **65:**3042-3047.

Beck, T., and M. N. Hall. 1999. The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. *Nature* **402:**689-692.

Behnsen, J., F. Lessing, S. Schindler, D. Wartenberg, I. D. Jacobsen, M. Thoen, P. F. Zipfel, and A. A. Brakhage. 2010. Secreted *Aspergillus fumigatus* protease Alp1 degrades human complement proteins C3, C4, and C5. *Infect Immun* **78:**3585-3594.

Bergmann, A., T. Hartmann, T. Cairns, E. M. Bignell, and S. Krappmann. 2009. A regulator of *Aspergillus fumigatus* extracellular proteolytic activity is dispensable for virulence. *Infect Immun* **77:**4041-4050.

Berka, R. M., M. Ward, L. J. Wilson, K. J. Hayenga, K. H. Kodama, L. P. Carlomagno, and S. A. Thompson. 1990. Molecular cloning and deletion of the gene encoding aspergillopepsin A from *Aspergillus awamori*. *Gene* **86:**153-162.

Bernard, F., and B. Andre. 2001. Genetic analysis of the signalling pathway activated by external amino acids in *Saccharomyces cerevisiae*. *Mol Microbiol* **41:**489-502.

Bhabhra, R., M. D. Miley, E. Mylonakis, D. Boettner, J. Fortwendel, J. C. Panepinto, M. Postow, J. C. Rhodes, and D. S. Askew. 2004. Disruption of the *Aspergillus fumigatus* gene encoding nucleolar protein CgrA impairs thermotolerant growth and reduces virulence. *Infect Immun* **72:**4731-4740.

Biguet, J., P. Tran Van Ky, J. Fruit, and S. Andrieu. 1967. [Identification of chymotryptic activity in remarkable fractions of an antigenic extract of *Aspergillus fumigatus*. Significance for the immunologic diagnosis of aspergillosis]. *Rev Immunol Ther Antimicrob* **31:**317-328.

Bogs, J., Bourbouloux, A., Cagnac, O., Wachter, A., Rausch, T., Delrot, S. 2003. Functional characterization and expression analysis of a glutathione transporter, BjGT1, from *Brassica juncea*: evidence for regulation by heavy metal exposure *Plant Cell Environment* **26:**1703-1711.

Bok, J. W., S. A. Balajee, K. A. Marr, D. Andes, K. F. Nielsen, J. C. Frisvad, and N. P. Keller. 2005. LaeA, a regulator of morphogenetic fungal virulence factors. *Eukaryot Cell* **4:**1574-1582.

Bouchara, J. P., G. Larcher, F. Joubaud, P. Penn, G. Tronchin, and D. Chabasse. 1993. Extracellular fibrinogenolytic enzyme of *Aspergillus fumigatus*: substrate-dependent variations in the proteinase synthesis and characterization of the enzyme. *FEMS Immunol Med Microbiol* **7:**81-91.

Bourbouloux, A., P. Shahi, A. Chakladar, S. Delrot, and A. K. Bachhawat. 2000. Hgt1p, a high affinity glutathione transporter from the yeast *Saccharomyces cerevisiae*. *J Biol Chem* **275:**13259-13265.

Bowman, J. C., G. K. Abruzzo, J. W. Anderson, A. M. Flattery, C. J. Gill, V. B. Pikounis, D. M. Schmatz, P. A. Liberator, and C. M. Douglas. 2001. Quantitative PCR assay to measure *Aspergillus fumigatus* burden in a murine model of disseminated aspergillosis: demonstration of efficacy of caspofungin acetate. *Antimicrob Agents Chemother* **45:**3474-3481.

Brajtburg, J., and J. Bolard. 1996. Carrier effects on biological activity of amphotericin B. *Clin Microbiol Rev* **9:**512-531.

Brakhage, A. A., and K. Langfelder. 2002. Menacing mold: the molecular biology of *Aspergillus fumigatus*. *Annu Rev Microbiol* **56:**433-455.

Bray, H. G., S. P. James, I. M. Raffan, B. E. Ryman, and W. V. Thorpe. 1949. The fate of certain organic acids and amides in the rabbit. 7. An amidase of rabbit liver. *Biochem J* **44:**618-625.

Bray, H. G., S. P. James, J. W. Thorpe, and M. R. Wasdell. 1950. The fate of certain organic acids and amides in the rabbit; further observations on the hydrolysis of amides by tissue extracts. *Biochem J* **47:**294-299.

Brega, E., R. Zufferey, and C. B. Mamoun. 2004. *Candida albicans* Csy1p is a nutrient sensor important for activation of amino acid uptake and hyphal morphogenesis. *Eukaryot Cell* **3:**135-143.

Burger, G., J. Tilburn, and C. Scazzocchio. 1991. Molecular cloning and functional characterization of the pathway-specific regulatory gene *nirA*, which controls nitrate assimilation in *Aspergillus nidulans*. *Mol Cell Biol* **11:**795-802.

Byrd, C., G. C. Turner, and A. Varshavsky. 1998. The N-end rule pathway controls the import of peptides through degradation of a transcriptional repressor. *EMBO J* **17:**269-277.

Caddick, M. X., M. G. Jones, J. M. van Tonder, H. Le Cordier, F. Narendja, J. Strauss, and I. Y. Morozov. 2006. Opposing signals differentially regulate transcript stability in *Aspergillus nidulans*. *Mol Microbiol* **62:**509-519.

Cagnac, O., A. Bourbouloux, D. Chakrabarty, M. Y. Zhang, and S. Delrot. 2004. AtOPT6 transports glutathione derivatives and is induced by primisulfuron. *Plant Physiol* **135:**1378-1387.

Cai, H., M. Hauser, F. Naider, and J. M. Becker. 2007. Differential regulation and substrate preferences in two peptide transporters of *Saccharomyces cerevisiae*. *Eukaryot Cell* **6:**1805-1813.

Canosa, I., F. Rojo, and J. C. Alonso. 1996. Site-specific recombination by the β protein from the streptococcal plasmid pSM19035: minimal recombination sequences and crossing over site. *Nucleic Acids Res* **24:**2712-2717.

Chague, V., R. Maor, and A. Sharon. 2009. CgOpt1, a putative oligopeptide transporter from *Colletotrichum gloeosporioides* that is involved in responses to auxin and pathogenicity. *BMC Microbiol* **9:**173.

Chang, Y. C., H. F. Tsai, M. Karos, and K. J. Kwon-Chung. 2004. THTA, a thermotolerance gene of *Aspergillus fumigatus*. *Fungal Genet Biol* **41:**888-896.

Chu, S., J. DeRisi, M. Eisen, J. Mulholland, D. Botstein, P. O. Brown, and I. Herskowitz. 1998. The transcriptional program of sporulation in budding yeast. *Science* **282:**699-705.

Clements, J. S., Jr., and J. E. Peacock, Jr. 1990. Amphotericin B revisited: reassessment of toxicity. *Am J Med* **88:**22N-27N.

Clemons, K. V., W. J. Steinbach, D. W. Denning, and D. A. Stevens. 2005. Advances against aspergillosis (September 9-11, 2004, San Francisco, California). *Mycopathologia* **159:**315-317.

Coffman, J. A., R. Rai, D. M. Loprete, T. Cunningham, V. Svetlov, and T. G. Cooper. 1997. Cross regulation of four GATA factors that control nitrogen catabolic gene expression in *Saccharomyces cerevisiae*. *J Bacteriol* **179:**3416-3429.

Curie, C., Z. Panaviene, C. Loulergue, S. L. Dellaporta, J. F. Briat, and E. L. Walker. 2001. Maize yellow stripe1 encodes a membrane protein directly involved in Fe(III) uptake. *Nature* **409:**346-349.

d'Enfert, C. 1996. Selection of multiple disruption events in *Aspergillus fumigatus* using the orotidine-5'-decarboxylase gene, *pyrG*, as a unique transformation marker. *Curr Genet* **30:**76-82.

da Silva Ferreira, M. E., M. R. Kress, M. Savoldi, M. H. Goldman, A. Hartl, T. Heinekamp, A. A. Brakhage, and G. H. Goldman. 2006. The *akuB(KU80)* mutant deficient for nonhomologous end joining is a powerful tool for analyzing pathogenicity in *Aspergillus fumigatus*. *Eukaryot Cell* **5:**207-211.

de Boer, M., P. S. Nielsen, J. P. Bebelman, H. Heerikhuizen, H. A. Andersen, and R. J. Planta. 2000. Stp1p, Stp2p and Abf1p are involved in regulation of expression of the amino acid transporter gene BAP3 of *Saccharomyces cerevisiae*. *Nucleic Acids Res* **28:**974-981.

Denning, D. W., K. Venkateswarlu, K. L. Oakley, M. J. Anderson, N. J. Manning, D. A. Stevens, D. W. Warnock, and S. L. Kelly. 1997. Itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob Agents Chemother* **41:**1364-1368.

Denning, D. W., P. N. Ward, L. E. Fenelon, and E. W. Benbow. 1992. Lack of vessel wall elastolysis in human invasive pulmonary aspergillosis. *Infect Immun* **60:**5153-5156.

Diaz, V., F. Rojo, A. C. Martinez, J. C. Alonso, and A. Bernad. 1999. The prokaryotic β-recombinase catalyzes site-specific recombination in mammalian cells. *J Biol Chem* **274:**6634-6640.

Didion, T., B. Regenberg, M. U. Jorgensen, M. C. Kielland-Brandt, and H. A. Andersen. 1998. The permease homologue Ssy1p controls the expression of amino acid and peptide transporter genes in *Saccharomyces cerevisiae*. *Mol Microbiol* **27:**643-650.

Ding, J., D. Y. Takamoto, A. von Nahmen, M. M. Lipp, K. Y. Lee, A. J. Waring, and J. A. Zasadzinski. 2001. Effects of lung surfactant proteins, SP-B and SP-C, and palmitic acid on monolayer stability. *Biophys J* **80:**2262-2272.

Dohmen, R. J., K. Madura, B. Bartel, and A. Varshavsky. 1991. The N-end rule is mediated by the UBC2(RAD6) ubiquitin-conjugating enzyme. *Proc Natl Acad Sci U S A* **88:**7351-7355.

Donaton, M. C., I. Holsbeeks, O. Lagatie, G. Van Zeebroeck, M. Crauwels, J. Winderickx, and J. M. Thevelein. 2003. The Gap1 general amino acid permease acts as an amino acid sensor for activation of protein kinase A targets in the yeast *Saccharomyces cerevisiae*. *Mol Microbiol* **50:**911-929.

Dowzer, C. E., and J. M. Kelly. 1991. Analysis of the *creA* gene, a regulator of carbon catabolite repression in *Aspergillus nidulans*. *Mol Cell Biol* **11:**5701-5709.

Du, F., F. Navarro-Garcia, Z. Xia, T. Tasaki, and A. Varshavsky. 2002. Pairs of dipeptides synergistically activate the binding of substrate by ubiquitin ligase through dissociation of its autoinhibitory domain. *Proc Natl Acad Sci U S A* **99:**14110-14115.

Dworeck, T., K. Wolf, and M. Zimmermann. 2009. SpOPT1, a member of the oligopeptide family (OPT) of the fission yeast *Schizosaccharomyces pombe*, is involved in the transport of glutathione through the outer membrane of the cell. *Yeast* **26:**67-73.

Ebina, K., S. Ichinowatari, and K. Yokota. 1985. Studies on toxin of *Aspergillus fumigatus*. XXII. Fashion of binding of Asp-hemolysin to human erythrocytes and Asp-hemolysin-binding proteins of erythrocyte membranes. *Microbiol Immunol* **29:**91-101.

Ellis, M. 1999. Therapy of *Aspergillus fumigatus*-related diseases. *Contrib Microbiol* **2:**105-129.

Espinel-Ingroff, A., A. Fothergill, M. Ghannoum, E. Manavathu, L. Ostrosky-Zeichner, M. Pfaller, M. Rinaldi, W. Schell, and T. Walsh. 2005. Quality control and reference guidelines for CLSI broth microdilution susceptibility method (M 38-A document) for amphotericin B, itraconazole, posaconazole, and voriconazole. *J Clin Microbiol* **43:**5243-5246.

Fei, Y. J., Y. Kanai, S. Nussberger, V. Ganapathy, F. H. Leibach, M. F. Romero, S. K. Singh, W. F. Boron, and M. A. Hediger. 1994. Expression cloning of a mammalian proton-coupled oligopeptide transporter. *Nature* **368:**563-566.

Forment, J. V., D. Ramon, and A. P. MacCabe. 2006. Consecutive gene deletions in *Aspergillus nidulans*: application of the Cre/loxP system. *Curr Genet* **50:**217-224.

Forsberg, H., and P. O. Ljungdahl. 2001. Genetic and biochemical analysis of the yeast plasma membrane Ssy1p-Ptr3p-Ssy5p sensor of extracellular amino acids. *Mol Cell Biol* **21:**814-826.

Forsberg, H., and P. O. Ljungdahl. 2001. Sensors of extracellular nutrients in *Saccharomyces cerevisiae*. *Curr Genet* **40:**91-109.

Frosco, M., T. Chase, Jr., and J. D. Macmillan. 1992. Purification and properties of the elastase from *Aspergillus fumigatus*. *Infect Immun* **60:**728-734.

Funk, M., R. Niedenthal, D. Mumberg, K. Brinkmann, V. Ronicke, and T. Henkel. 2002. Vector systems for heterologous expression of proteins in *Saccharomyces cerevisiae*. *Methods Enzymol* **350:**248-257.

Gifford, A. H., J. R. Klippenstein, and M. M. Moore. 2002. Serum stimulates growth of and proteinase secretion by *Aspergillus fumigatus*. *Infect Immun* **70:**19-26.

Goldman, G. H., and S. A. Osmani, Eds. 2008. The Aspergilli: Genomics, Medical Aspects, Biotechnology and Reseach Methods, CRC Press.

Gomez, D., B. Cubero, G. Cecchetto, and C. Scazzocchio. 2002. PrnA, a Zn2Cys6 activator with a unique DNA recognition mode, requires inducer for in vivo binding. *Mol Microbiol* **44:**585-597.

Gomez, D., I. Garcia, C. Scazzocchio, and B. Cubero. 2003. Multiple GATA sites: protein binding and physiological relevance for the regulation of the proline transporter gene of *Aspergillus nidulans*. *Mol Microbiol* **50:**277-289.

Gonzalez, R., V. Gavrias, D. Gomez, C. Scazzocchio, and B. Cubero. 1997. The integration of nitrogen and carbon catabolite repression in *Aspergillus nidulans* requires the GATA factor AreA and an additional positive-acting element, ADA. *EMBO J* **16:**2937-2944.

Gonzalez-Lopez, C. I., R. Szabo, S. Blanchin-Roland, and C. Gaillardin. 2002. Genetic control of extracellular protease synthesis in the yeast *Yarrowia lipolytica*. *Genetics* **160:**417-427.

Gooch, V. D., A. Mehra, L. F. Larrondo, J. Fox, M. Touroutoutoudis, J. J. Loros, and J. C. Dunlap. 2008. Fully codon-optimized luciferase uncovers novel temperature characteristics of the *Neurospora* clock. *Eukaryot Cell* **7:**28-37.

Greenberger, P. A. 2002. Allergic bronchopulmonary aspergillosis. *J Allergy Clin Immunol* **110:**685-692.

Grønlund, J. T., C. Stemmer, J. Lichota, T. Merkle, and K. D. Grasser. 2007. Functionality of the β/six site-specific recombination system in tobacco and *Arabidopsis*: a novel tool for genetic engineering of plant genomes. *Plant Mol Biol* **63:**545-556.

Grouzmann, E., M. Monod, B. Landis, S. Wilk, N. Brakch, K. Nicoucar, R. Giger, D. Malis, I. Szalay-Quinodoz, C. Cavadas, D. R. Morel, and J. S. Lacroix. 2002. Loss of dipeptidylpeptidase IV activity in chronic rhinosinusitis contributes to the neurogenic inflammation induced by substance P in the nasal mucosa. *Faseb J* **16:**1132-1134.

Güldener, U., S. Heck, T. Fielder, J. Beinhauer, and J. H. Hegemann. 1996. A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res* **24:**2519-2524.

Güldener, U., J. Heinisch, G. J. Köhler, D. Voss, and J. H. Hegemann. 2002. A second set of *loxP* marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. *Nucleic Acids Res* **30:**e23.

Hagting, A., E. R. Kunji, K. J. Leenhouts, B. Poolman, and W. N. Konings. 1994. The di- and tripeptide transport protein of *Lactococcus lactis*. A new type of bacterial peptide transporter. *J Biol Chem* **269:**11391-11399.

Hamilton, D. L., and K. Abremski. 1984. Site-specific recombination by the bacteriophage P1 *lox*-Cre system. Cre-mediated synapsis of two *lox* sites. *J Mol Biol* **178:**481-486.

Harder, D., J. Stolz, F. Casagrande, P. Obrdlik, D. Weitz, D. Fotiadis, and H. Daniel. 2008. DtpB (YhiP) and DtpA (TppB, YdgR) are prototypical proton-dependent peptide transporters of *Escherichia coli*. *FEBS J* **275:**3290-3298.

Hauser, M., A. M. Donhardt, D. Barnes, F. Naider, and J. M. Becker. 2000. Enkephalins are transported by a novel eukaryotic peptide uptake system. *J Biol Chem* **275:**3037-3041.

Hauser, M., V. Narita, A. M. Donhardt, F. Naider, and J. M. Becker. 2001. Multiplicity and regulation of genes encoding peptide transporters in *Saccharomyces cerevisiae*. *Mol Membr Biol* **18:**105-112.

Hegemann, J. H., U. Güldener, and G. J. Köhler. 2006. Gene disruption in the budding yeast *Saccharomyces cerevisiae*. *Methods Mol Biol* **313:**129-144.

Hensel, M., H. N. Arst, Jr., A. Aufauvre-Brown, and D. W. Holden. 1998. The role of the *Aspergillus fumigatus areA* gene in invasive pulmonary aspergillosis. *Mol Gen Genet* **258:**553-557.

Hoffmann, B., O. Valerius, M. Andermann, and G. H. Braus. 2001. Transcriptional autoregulation and inhibition of mRNA translation of amino acid regulator gene *cpcA* of filamentous fungus *Aspergillus nidulans*. *Mol Biol Cell* **12:**2846-2857.

Hofman-Bang, J. 1999. Nitrogen catabolite repression in *Saccharomyces cerevisiae*. *Mol Biotechnol* **12:**35-73.

Hope, W. W., T. J. Walsh, and D. W. Denning. 2005. The invasive and saprophytic syndromes due to *Aspergillus spp*. *Med Mycol* **43 Suppl 1:**S207-238.

Ibrahim-Granet, O., M. Dubourdeau, J. P. Latge, P. Ave, M. Huerre, A. A. Brakhage, and M. Brock. 2008. Methylcitrate synthase from *Aspergillus fumigatus* is essential for manifestation of invasive aspergillosis. *Cell Microbiol* **10:**134-148.

Iraqui, I., S. Vissers, F. Bernard, J. O. de Craene, E. Boles, A. Urrestarazu, and B. Andre. 1999. Amino acid signaling in *Saccharomyces cerevisiae*: a permease-like sensor of external amino acids and F-Box protein Grr1p are required for transcriptional induction of the AGP1 gene, which encodes a broad-specificity amino acid permease. *Mol Cell Biol* **19:**989-1001.

Jack, D. L., I. T. Paulsen, and M. H. Saier. 2000. The amino acid/polyamine/organocation (APC) superfamily of transporters specific for amino acids, polyamines and organocations. *Microbiology* **146 (Pt 8):**1797-1814.

Jahn, B., F. Boukhallouk, J. Lotz, K. Langfelder, G. Wanner, and A. A. Brakhage. 2000. Interaction of human phagocytes with pigmentless *Aspergillus* conidia. *Infect Immun* **68:**3736-3739.

Jaton-Ogay, K., S. Paris, M. Huerre, M. Quadroni, R. Falchetto, G. Togni, J. P. Latge, and M. Monod. 1994. Cloning and disruption of the gene encoding an extracellular metalloprotease of *Aspergillus fumigatus*. *Mol Microbiol* **14:**917-928.

Kamei, K., and A. Watanabe. 2005. *Aspergillus* mycotoxins and their effect on the host. *Med Mycol* **43 Suppl 1:**S95-99.

Kämper, J. 2004. A PCR-based system for highly efficient generation of gene replacement mutants in *Ustilago maydis*. *Mol Genet Genomics* **271:**103-110.

Kanehisa, M., and S. Goto. 2000. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* **28:**27-30.

Karim, S., K. O. Holmstrom, A. Mandal, P. Dahl, S. Hohmann, G. Brader, E. T. Palva, and M. Pirhonen. 2007. AtPTR3, a wound-induced peptide transporter needed for defence against virulent bacterial pathogens in *Arabidopsis*. *Planta* **225:**1431-1445.

Karim, S., D. Lundh, K. O. Holmstrom, A. Mandal, and M. Pirhonen. 2005. Structural and functional characterization of AtPTR3, a stress-induced peptide transporter of *Arabidopsis*. *J Mol Model* **11:**226-236.

Kilby, N. J., M. R. Snaith, and J. A. Murray. 1993. Site-specific recombinases: tools for genome engineering. *Trends Genet* **9:**413-421.

Klasson, H., G. R. Fink, and P. O. Ljungdahl. 1999. Ssy1p and Ptr3p are plasma membrane components of a yeast system that senses extracellular amino acids. *Mol Cell Biol* **19:**5405-5416.

Koh, S., A. M. Wiles, J. S. Sharp, F. R. Naider, J. M. Becker, and G. Stacey. 2002. An oligopeptide transporter gene family in *Arabidopsis*. *Plant Physiol* **128:**21-29.

Komarova, N. Y., K. Thor, A. Gubler, S. Meier, D. Dietrich, A. Weichert, M. Suter Grotemeyer, M. Tegeder, and D. Rentsch. 2008. AtPTR1 and AtPTR5 transport dipeptides in planta. *Plant Physiol* **148:**856-869.

Kopke, K., B. Hoff, and U. Kuck. 2010. Application of the *Saccharomyces cerevisiae* FLP/*FRT* recombination system in filamentous fungi for marker recycling and construction of knockout strains devoid of heterologous genes. *Appl Environ Microbiol* **76:**4664-4674.

Kota, J., and P. O. Ljungdahl. 2005. Specialized membrane-localized chaperones prevent aggregation of polytopic proteins in the ER. *J Cell Biol* **168:**79-88.

Kothary, M. H., T. Chase, Jr., and J. D. Macmillan. 1984. Correlation of elastase production by some strains of *Aspergillus fumigatus* with ability to cause pulmonary invasive aspergillosis in mice. *Infect Immun* **43:**320-325.

Krappmann, S. 2006. Tools to study molecular mechanisms of *Aspergillus* pathogenicity. *Trends Microbiol* **14:**356-364.

Krappmann, S., O. Bayram, and G. H. Braus. 2005. Deletion and allelic exchange of the *Aspergillus fumigatus veA* locus via a novel recyclable marker module. *Eukaryot Cell* **4:**1298-1307.

Krappmann, S., E. M. Bignell, U. Reichard, T. Rogers, K. Haynes, and G. H. Braus. 2004. The *Aspergillus fumigatus* transcriptional activator CpcA contributes significantly to the virulence of this fungal pathogen. *Mol Microbiol* **52:**785-799.

Krappmann, S., and G. H. Braus. 2003. Deletion of *Aspergillus nidulans aroC* using a novel blaster module that combines ET cloning and marker rescue. *Mol Genet Genomics* **268:**675-683.

Krappmann, S., and G. H. Braus. 2005. Nitrogen metabolism of *Aspergillus* and its role in pathogenicity. *Med Mycol* **43 Suppl 1:**S31-40.

Krappmann, S., N. Jung, B. Medic, S. Busch, R. A. Prade, and G. H. Braus. 2006. The Aspergillus nidulans F-box protein GrrA links SCF activity to meiosis. *Mol Microbiol* **61:**76-88.

Krappmann, S., R. Pries, G. Gellissen, M. Hiller, and G. H. Braus. 2000. *HARO7* encodes chorismate mutase of the methylotrophic yeast *Hansenula polymorpha* and is derepressed upon methanol utilization. *J Bacteriol* **182:**4188-4197.

Krappmann, S., C. Sasse, and G. H. Braus. 2006. Gene targeting in *Aspergillus fumigatus* by homologous recombination is facilitated in a nonhomologous end-joining-deficient genetic background. *Eukaryot Cell* **5:**212-215.

Kubodera, T., N. Yamashita, and A. Nishimura. 2002. Transformation of *Aspergillus sp*. and *Trichoderma reesei* using the pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae*. *Biosci Biotechnol Biochem* **66:**404-406.

Kudla, B., M. X. Caddick, T. Langdon, N. M. Martinez-Rossi, C. F. Bennett, S. Sibley, R. W. Davies, and H. N. Arst, Jr. 1990. The regulatory gene *areA* mediating nitrogen metabolite repression in *Aspergillus nidulans*. Mutations affecting specificity of gene activation alter a loop residue of a putative zinc finger. *EMBO J* **9:**1355-1364.

Kupfahl, C., T. Heinekamp, G. Geginat, T. Ruppert, A. Hartl, H. Hof, and A. A. Brakhage. 2006. Deletion of the *gliP* gene of *Aspergillus fumigatus* results in loss of gliotoxin production but has no effect on virulence of the fungus in a low-dose mouse infection model. *Mol Microbiol* **62:**292-302.

Lambou, K., C. Lamarre, R. Beau, N. Dufour, and J. P. Latge. 2010. Functional analysis of the superoxide dismutase family in *Aspergillus fumigatus*. *Mol Microbiol* **75:**910-923.

Langdon, T., A. Sheerins, A. Ravagnani, M. Gielkens, M. X. Caddick, and H. N. Arst, Jr. 1995. Mutational analysis reveals dispensability of the N-terminal region of the *Aspergillus* transcription factor mediating nitrogen metabolite repression. *Mol Microbiol* **17:**877-888.

Langfelder, K., B. Jahn, H. Gehringer, A. Schmidt, G. Wanner, and A. A. Brakhage. 1998. Identification of a polyketide synthase gene (*pksP*) of *Aspergillus fumigatus* involved in conidial pigment biosynthesis and virulence. *Med Microbiol Immunol* **187:**79-89.

Langfelder, K., B. Philippe, B. Jahn, J. P. Latge, and A. A. Brakhage. 2001. Differential expression of the *Aspergillus fumigatus pksP* gene detected in vitro and in vivo with green fluorescent protein. *Infect Immun* **69:**6411-6418.

Langfelder, K., M. Streibel, B. Jahn, G. Haase, and A. A. Brakhage. 2003. Biosynthesis of fungal melanins and their importance for human pathogenic fungi. *Fungal Genet Biol* **38:**143-158.

Latge, J.-P., and W. J. Steinbach, Eds. 2009. *Aspergillus fumigatus* and Aspergillosis, ASM Press.

Latge, J. P. 1999. *Aspergillus fumigatus* and aspergillosis. *Clin Microbiol Rev* **12:**310-350.

Lengeler, K. B., and E. Kothe. 1999. Mated: a putative peptide transporter of *Schizophyllum commune* expressed in dikaryons. *Curr Genet* **36:**159-164.

Lessing, F., O. Kniemeyer, I. Wozniok, J. Loeffler, O. Kurzai, A. Haertl, and A. A. Brakhage. 2007. The *Aspergillus fumigatus* transcriptional regulator *AfYap1* represents the major regulator for defense against reactive oxygen intermediates but is dispensable for pathogenicity in an intranasal mouse infection model. *Eukaryot Cell* **6:**2290-2302.

Lewis, R. E., N. P. Wiederhold, J. Chi, X. Y. Han, K. V. Komanduri, D. P. Kontoyiannis, and R. A. Prince. 2005. Detection of gliotoxin in experimental and human aspergillosis. *Infect Immun* **73:**635-637.

Liebmann, B., T. W. Muhleisen, M. Muller, M. Hecht, G. Weidner, A. Braun, M. Brock, and A. A. Brakhage. 2004. Deletion of the *Aspergillus fumigatus* lysine biosynthesis gene *lysF* encoding homoaconitase leads to attenuated virulence in a low-dose mouse infection model of invasive aspergillosis. *Arch Microbiol* **181:**378-383.

Liu, Z., J. Thornton, M. Spirek, and R. A. Butow. 2008. Activation of the SPS amino acid-sensing pathway in *Saccharomyces cerevisiae* correlates with the phosphorylation state of a sensor component, Ptr3. *Mol Cell Biol* **28:**551-563.

Lorenz, M. C., and G. R. Fink. 2001. The glyoxylate cycle is required for fungal virulence. *Nature* **412:**83-86.

Lorenz, M. C., and G. R. Fink. 2002. Life and death in a macrophage: role of the glyoxylate cycle in virulence. *Eukaryot Cell* **1:**657-662.

Lubkowitz, M. 2006. The OPT family functions in long-distance peptide and metal transport in plants. *Genet Eng (N Y)* **27:**35-55.

Lubkowitz, M. A., D. Barnes, M. Breslav, A. Burchfield, F. Naider, and J. M. Becker. 1998. *Schizosaccharomyces pombe isp4* encodes a transporter representing a novel family of oligopeptide transporters. *Mol Microbiol* **28:**729-741.

Lubkowitz, M. A., L. Hauser, M. Breslav, F. Naider, and J. M. Becker. 1997. An oligopeptide transport gene from *Candida albicans*. *Microbiology* **143 (Pt 2):**387-396.

Maerker, C., M. Rohde, A. A. Brakhage, and M. Brock. 2005. Methylcitrate synthase from *Aspergillus fumigatus*. Propionyl-CoA affects polyketide synthesis, growth and morphology of conidia. *FEBS J* **272:**3615-3630.

Magasanik, B., and C. A. Kaiser. 2002. Nitrogen regulation in *Saccharomyces cerevisiae*. *Gene* **290:**1-18.

Maheshwari, R., G. Bharadwaj, and M. K. Bhat. 2000. Thermophilic fungi: their physiology and enzymes. *Microbiol Mol Biol Rev* **64:**461-488.

Malicev, E., H. H. Chowdhury, P. Macek, and K. Sepcic. 2007. Effect of ostreolysin, an Asp-hemolysin isoform, on human chondrocytes and osteoblasts, and possible role of Asp-hemolysin in pathogenesis. *Med Mycol* **45:**123-130.

Margolis-Clark, E., I. Hunt, S. Espinosa, and B. J. Bowman. 2001. Identification of the gene at the pmg locus, encoding system II, the general amino acid transporter in *Neurospora crassa*. *Fungal Genet Biol* **33:**127-135.

Markaryan, A., I. Morozova, H. Yu, and P. E. Kolattukudy. 1994. Purification and characterization of an elastinolytic metalloprotease from *Aspergillus fumigatus* and immunoelectron microscopic evidence of secretion of this enzyme by the fungus invading the murine lung. *Infect Immun* **62:**2149-2157.

Martinez, P., and P. O. Ljungdahl. 2004. An ER packaging chaperone determines the amino acid uptake capacity and virulence of *Candida albicans*. *Mol Microbiol* **51:**371-384.

Martinez, P., and P. O. Ljungdahl. 2005. Divergence of Stp1 and Stp2 transcription factors in *Candida albicans* places virulence factors required for proper nutrient acquisition under amino acid control. *Mol Cell Biol* **25:**9435-9446.

Marzluf, G. A. 1997. Genetic regulation of nitrogen metabolism in the fungi. *Microbiol Mol Biol Rev* **61:**17-32.

McDonagh, A., N. D. Fedorova, J. Crabtree, Y. Yu, S. Kim, D. Chen, O. Loss, T. Cairns, G. Goldman, D. Armstrong-James, K. Haynes, H. Haas, M. Schrettl, G. May, W. C. Nierman, and E. Bignell. 2008. Sub-telomere directed gene expression during initiation of invasive aspergillosis. *PLoS Pathog* **4:**e1000154.

McNeil, M. M., S. L. Nash, R. A. Hajjeh, M. A. Phelan, L. A. Conn, B. D. Plikaytis, and D. W. Warnock. 2001. Trends in mortality due to invasive mycotic diseases in the United States, 1980-1997. *Clin Infect Dis* **33:**641-647.

Meletiadis, J., C. Antachopoulos, T. Stergiopoulou, S. Pournaras, E. Roilides, and T. J. Walsh. 2007. Differential fungicidal activities of amphotericin B and voriconazole against *Aspergillus* species determined by microbroth methodology. *Antimicrob Agents Chemother* **51:**3329-3337.

Miyake, T., M. Kanayama, H. Sammoto, and B. Ono. 2002. A novel cis-acting cysteine-responsive regulatory element of the gene for the high-affinity glutathione transporter of *Saccharomyces cerevisiae*. *Mol Genet Genomics* **266:**1004-1011.

Monahan, B. J., J. A. Fraser, M. J. Hynes, and M. A. Davis. 2002. Isolation and characterization of two ammonium permease genes, *meaA* and *mepA*, from *Aspergillus nidulans*. *Eukaryot Cell* **1:**85-94.

Monod, M., S. Capoccia, B. Lechenne, C. Zaugg, M. Holdom, and O. Jousson. 2002. Secreted proteases from pathogenic fungi. *Int J Med Microbiol* **292:**405-419.

Monod, M., B. Lechenne, O. Jousson, D. Grand, C. Zaugg, R. Stocklin, and E. Grouzmann. 2005. Aminopeptidases and dipeptidyl-peptidases secreted by the dermatophyte *Trichophyton rubrum*. *Microbiology* **151:**145-155.

Monod, M., S. Paris, D. Sanglard, K. Jaton-Ogay, J. Bille, and J. P. Latge. 1993. Isolation and characterization of a secreted metalloprotease of *Aspergillus fumigatus*. *Infect Immun* **61:**4099-4104.

Monod, M., S. Paris, J. Sarfati, K. Jaton-Ogay, P. Ave, and J. P. Latge. 1993. Virulence of alkaline protease-deficient mutants of *Aspergillus fumigatus*. *FEMS Microbiol Lett* **106:**39-46.

Monod, M., G. Togni, L. Rahalison, and E. Frenk. 1991. Isolation and characterisation of an extracellular alkaline protease of *Aspergillus fumigatus*. *J Med Microbiol* **35:**23-28.

Moreno, M. A., O. Ibrahim-Granet, R. Vicentefranqueira, J. Amich, P. Ave, F. Leal, J. P. Latge, and J. A. Calera. 2007. The regulation of zinc homeostasis by the ZafA transcriptional activator is essential for *Aspergillus fumigatus* virulence. *Mol Microbiol* **64:**1182-1197.

Morozov, I. Y., M. Galbis-Martinez, M. G. Jones, and M. X. Caddick. 2001. Characterization of nitrogen metabolite signalling in *Aspergillus* via the regulated degradation of *areA* mRNA. *Mol Microbiol* **42:**269-277.

Morschhäuser, J., S. Michel, and P. Staib. 1999. Sequential gene disruption in *Candida albicans* by *FLP*-mediated site-specific recombination. *Mol Microbiol* **32:**547-556.

Morschhäuser, J., P. Staib, and G. Kohler. 2005. Targeted gene deletion in *Candida albicans* wild-type strains by MPAR flipping. *Methods Mol Med* **118:**35-44.

Moutaouakil, M., M. Monod, M. C. Prevost, J. P. Bouchara, S. Paris, and J. P. Latge. 1993. Identification of the 33-kDa alkaline protease of *Aspergillus fumigatus* in vitro and in vivo. *J Med Microbiol* **39:**393-399.

Mullins, J., R. Harvey, and A. Seaton. 1976. Sources and incidence of airborne *Aspergillus fumigatus* (Fres). *Clin Allergy* **6:**209-217.

Narendja, F., S. P. Goller, M. Wolschek, and J. Strauss. 2002. Nitrate and the GATA factor AreA are necessary for in vivo binding of NirA, the pathway-specific transcriptional activator of *Aspergillus nidulans*. *Mol Microbiol* **44:**573-583.

O'Gorman, C. M., H. T. Fuller, and P. S. Dyer. 2009. Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*. *Nature* **457:**471-474.

Orciuolo, E., M. Stanzani, M. Canestraro, S. Galimberti, G. Carulli, R. Lewis, M. Petrini, and K. V. Komanduri. 2007. Effects of *Aspergillus fumigatus* gliotoxin and methylprednisolone on human neutrophils: implications for the pathogenesis of invasive aspergillosis. *J Leukoc Biol* **82:**839-848.

Osawa, H., G. Stacey, and W. Gassmann. 2006. ScOPT1 and AtOPT4 function as proton-coupled oligopeptide transporters with broad but distinct substrate specificities. *Biochem J* **393:**267-275.

Paananen, R., A. D. Postle, G. Clark, V. Glumoff, and M. Hallman. 2002. Eustachian tube surfactant is different from alveolar surfactant: determination of phospholipid composition of porcine eustachian tube lavage fluid. *J Lipid Res* **43:**99-106.

Panepinto, J. C., B. G. Oliver, J. R. Fortwendel, D. L. Smith, D. S. Askew, and J. C. Rhodes. 2003. Deletion of the *Aspergillus fumigatus* gene encoding the Ras-related protein RhbA reduces virulence in a model of Invasive pulmonary aspergillosis. *Infect Immun* **71:**2819-2826.

Pao, S. S., I. T. Paulsen, and M. H. Saier, Jr. 1998. Major facilitator superfamily. *Microbiol Mol Biol Rev* **62:**1-34.

Paris, S., D. Wysong, J. P. Debeaupuis, K. Shibuya, B. Philippe, R. D. Diamond, and J. P. Latge. 2003. Catalases of *Aspergillus fumigatus*. *Infect Immun* **71:**3551-3562.

Perrin, R. M., N. D. Fedorova, J. W. Bok, R. A. Cramer, J. R. Wortman, H. S. Kim, W. C. Nierman, and N. P. Keller. 2007. Transcriptional regulation of chemical diversity in *Aspergillus fumigatus* by LaeA. *PLoS Pathog* **3:**e50.

Perry, J. R., M. A. Basrai, H. Y. Steiner, F. Naider, and J. M. Becker. 1994. Isolation and characterization of a *Saccharomyces cerevisiae* peptide transport gene. *Mol Cell Biol* **14:**104-115.

Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29:**e45.

Pfaller, M. A., S. A. Messer, R. J. Hollis, and R. N. Jones. 2002. Antifungal activities of posaconazole, ravuconazole, and voriconazole compared to those of itraconazole and amphotericin B against 239 clinical isolates of *Aspergillus spp*. and other filamentous fungi: report from SENTRY Antimicrobial Surveillance Program, 2000. *Antimicrob Agents Chemother* **46:**1032-1037.

Pike, S., A. Patel, G. Stacey, and W. Gassmann. 2009. *Arabidopsis* OPT6 is an oligopeptide transporter with exceptionally broad substrate specificity. *Plant Cell Physiol* **50:**1923-1932.

Poulsen, P., B. Wu, R. F. Gaber, K. Ottow, H. A. Andersen, and M. C. Kielland-Brandt. 2005. Amino acid sensing by Ssy1. *Biochem Soc Trans* **33:**261-264.

Punt, P. J., and C. A. van den Hondel. 1992. Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. *Methods Enzymol* **216:**447-457.

Regenberg, B., L. During-Olsen, M. C. Kielland-Brandt, and S. Holmberg. 1999. Substrate specificity and gene expression of the amino-acid permeases in *Saccharomyces cerevisiae*. *Curr Genet* **36:**317-328.

Reichard, U., S. Buttner, H. Eiffert, F. Staib, and R. Ruchel. 1990. Purification and characterisation of an extracellular serine proteinase from *Aspergillus fumigatus* and its detection in tissue. *J Med Microbiol* **33:**243-251.

Reichard, U., G. T. Cole, T. W. Hill, R. Ruchel, and M. Monod. 2000. Molecular characterization and influence on fungal development of ALP2, a novel serine proteinase from *Aspergillus fumigatus*. *Int J Med Microbiol* **290:**549-558.

Reichard, U., H. Eiffert, and R. Ruchel. 1994. Purification and characterization of an extracellular aspartic proteinase from *Aspergillus fumigatus*. *J Med Vet Mycol* **32:**427-436.

Reichard, U., B. Lechenne, A. R. Asif, F. Streit, E. Grouzmann, O. Jousson, and M. Monod. 2006. Sedolisins, a new class of secreted proteases from *Aspergillus fumigatus* with endoprotease or tripeptidyl-peptidase activity at acidic pHs. *Appl Environ Microbiol* **72:**1739-1748.

Reichard, U., M. Monod, F. Odds, and R. Ruchel. 1997. Virulence of an aspergillopepsin-deficient mutant of *Aspergillus fumigatus* and evidence for another aspartic proteinase linked to the fungal cell wall. *J Med Vet Mycol* **35:**189-196.

Reuss, O., and J. Morschhauser. 2006. A family of oligopeptide transporters is required for growth of *Candida albicans* on proteins. *Mol Microbiol* **60:**795-812.

Reuss, O., A. Vik, R. Kolter, and J. Morschhäuser. 2004. The SAT1 flipper, an optimized tool for gene disruption in *Candida albicans*. *Gene* **341:**119-127.

Ritt, C., R. Grimm, S. Fernandez, J. C. Alonso, and K. D. Grasser. 1998. Four differently chromatin-associated maize HMG domain proteins modulate DNA structure and act as architectural elements in nucleoprotein complexes. *Plant J* **14:**623-631.

Rojo, F., and J. C. Alonso. 1994. A novel site-specific recombinase encoded by the *Streptococcus pyogenes* plasmid pSM19035. *J Mol Biol* **238:**159-172.

Rojo, F., and J. C. Alonso. 1995. The β recombinase of plasmid pSM19035 binds to two adjacent sites, making different contacts at each of them. *Nucleic Acids Res* **23:**3181-3188.

Rojo, F., F. Weise, and J. C. Alonso. 1993. Purification of the β product encoded by the *Streptococcus pyogenes* plasmid pSM19035. A putative DNA recombinase required to resolve plasmid oligomers. *FEBS Lett* **328:**169-173.

Ruepp, A., A. Zollner, D. Maier, K. Albermann, J. Hani, M. Mokrejs, I. Tetko, U. Guldener, G. Mannhaupt, M. Munsterkotter, and H. W. Mewes. 2004. The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. *Nucleic Acids Res* **32:**5539-5545.

Sadowski, P. 1986. Site-specific recombinases: changing partners and doing the twist. *J Bacteriol* **165:**341-347.

Saier, M. H., Jr. 2000. Families of transmembrane transporters selective for amino acids and their derivatives. *Microbiology* **146 (Pt 8):**1775-1795.

Saier, M. H., Jr. 2000. A functional-phylogenetic classification system for transmembrane solute transporters. *Microbiol Mol Biol Rev* **64:**354-411.

Saier, M. H., Jr. 2003. Tracing pathways of transport protein evolution. *Mol Microbiol* **48:**1145-1156.

Sarfati, J., M. Monod, P. Recco, A. Sulahian, C. Pinel, E. Candolfi, T. Fontaine, J. P. Debeaupuis, M. Tabouret, and J. P. Latge. 2006. Recombinant antigens as diagnostic markers for aspergillosis. *Diagn Microbiol Infect Dis* **55:**279-291.

Sasse, C., E. M. Bignell, M. Hasenberg, K. Haynes, M. Gunzer, G. H. Braus, and S. Krappmann. 2008. Basal expression of the *Aspergillus fumigatus* transcriptional activator CpcA is sufficient to support pulmonary aspergillosis. *Fungal Genet Biol* **45:**693-704.

Schobel, F., O. Ibrahim-Granet, P. Ave, J. P. Latge, A. A. Brakhage, and M. Brock. 2007. *Aspergillus fumigatus* does not require fatty acid metabolism via isocitrate lyase for development of invasive aspergillosis. *Infect Immun* **75:**1237-1244.

Schobel, F., I. D. Jacobsen, and M. Brock. 2010. Evaluation of lysine biosynthesis as an antifungal drug target: biochemical characterization of *Aspergillus fumigatus* homocitrate synthase and virulence studies. *Eukaryot Cell* **9:**878-893.

Schrettl, M., E. Bignell, C. Kragl, C. Joechl, T. Rogers, H. N. Arst, Jr., K. Haynes, and H. Haas. 2004. Siderophore biosynthesis but not reductive iron assimilation is essential for *Aspergillus fumigatus* virulence. *J Exp Med* **200:**1213-1219.

Schrettl, M., E. Bignell, C. Kragl, Y. Sabiha, O. Loss, M. Eisendle, A. Wallner, H. N. Arst, Jr., K. Haynes, and H. Haas. 2007. Distinct roles for intra- and extracellular siderophores during *Aspergillus fumigatus* infection. *PLoS Pathog* **3:**1195-1207.

Sharma, K. K., and H. N. Arst, Jr. 1985. The product of the regulatory gene of the proline catabolism gene cluster of *Aspergillus nidulans* is a positive-acting protein. *Curr Genet* **9:**299-304.

Sharon, H., S. Hagag, and N. Osherov. 2009. Transcription factor PrtT controls expression of multiple secreted proteases in the human pathogenic mold *Aspergillus fumigatus*. *Infect Immun* **77:**4051-4060.

Sheppard, D. C., K. A. Marr, D. N. Fredricks, L. Y. Chiang, T. Doedt, and S. G. Filler. 2006. Comparison of three methodologies for the determination of pulmonary fungal burden in experimental murine aspergillosis. *Clin Microbiol Infect* **12:**376-380.

Singh, N., and D. L. Paterson. 2005. *Aspergillus infections* in transplant recipients. *Clin Microbiol Rev* **18:**44-69.

Smith, J. M., C. M. Tang, S. Van Noorden, and D. W. Holden. 1994. Virulence of *Aspergillus fumigatus* double mutants lacking restriction and an alkaline protease in a low-dose model of invasive pulmonary aspergillosis. *Infect Immun* **62:**5247-5254.

Sophianopoulou, V., T. Suarez, G. Diallinas, and C. Scazzocchio. 1993. Operator derepressed mutations in the proline utilisation gene cluster of *Aspergillus nidulans*. *Mol Gen Genet* **236:**209-213.

Sriranganadane, D., P. Waridel, K. Salamin, U. Reichard, E. Grouzmann, J. M. Neuhaus, M. Quadroni, and M. Monod. 2010. *Aspergillus* protein degradation pathways with different secreted protease sets at neutral and acidic pH. *J Proteome Res* **9:**3511-3519.

Stacey, M. G., H. Osawa, A. Patel, W. Gassmann, and G. Stacey. 2006. Expression analyses of *Arabidopsis* oligopeptide transporters during seed germination, vegetative growth and reproduction. *Planta* **223:**291-305.

Stacey, M. G., A. Patel, W. E. McClain, M. Mathieu, M. Remley, E. E. Rogers, W. Gassmann, D. G. Blevins, and G. Stacey. 2008. The *Arabidopsis* AtOPT3 protein functions in metal homeostasis and movement of iron to developing seeds. *Plant Physiol* **146:**589-601.

Stanbrough, M., and B. Magasanik. 1996. Two transcription factors, Gln3p and Nil1p, use the same GATAAG sites to activate the expression of GAP1 of *Saccharomyces cerevisiae*. *J Bacteriol* **178:**2465-2468.

Steiner, H. Y., F. Naider, and J. M. Becker. 1995. The PTR family: a new group of peptide transporters. *Mol Microbiol* **16:**825-834.

Steiner, H. Y., W. Song, L. Zhang, F. Naider, J. M. Becker, and G. Stacey. 1994. An *Arabidopsis* peptide transporter is a member of a new class of membrane transport proteins. *Plant Cell* **6:**1289-1299.

Stemmer, C., S. Fernandez, G. Lopez, J. C. Alonso, and K. D. Grasser. 2002. Plant chromosomal HMGB proteins efficiently promote the bacterial site-specific β-mediated recombination *in vitro* and *in vivo*. *Biochemistry* **41:**7763-7770.

Stevens, D. A., V. L. Kan, M. A. Judson, V. A. Morrison, S. Dummer, D. W. Denning, J. E. Bennett, T. J. Walsh, T. F. Patterson, and G. A. Pankey. 2000. Practice guidelines for diseases caused by *Aspergillus*. Infectious Diseases Society of America. *Clin Infect Dis* **30:**696-709.

Stuart, W. D., K. Koo, and S. J. Vollmer. 1988. Cloning of mtr, an amino acid transport gene of *Neurospora crassa*. *Genome* **30:**198-203.

Sturtevant, J. E., and J. P. Latge. 1992. Interactions between conidia of *Aspergillus fumigatus* and human complement component C3. *Infect Immun* **60:**1913-1918.

Sugareva, V., A. Hartl, M. Brock, K. Hubner, M. Rohde, T. Heinekamp, and A. A. Brakhage. 2006. Characterisation of the laccase-encoding gene *abr2* of the dihydroxynaphthalene-like melanin gene cluster of *Aspergillus fumigatus*. *Arch Microbiol* **186:**345-355.

Sugui, J. A., J. Pardo, Y. C. Chang, K. A. Zarember, G. Nardone, E. M. Galvez, A. Mullbacher, J. I. Gallin, M. M. Simon, and K. J. Kwon-Chung. 2007. Gliotoxin is a virulence factor of *Aspergillus fumigatus: gliP* deletion attenuates virulence in mice immunosuppressed with hydrocortisone. *Eukaryot Cell* **6:**1562-1569.

Szewczyk, E., T. Nayak, C. E. Oakley, H. Edgerton, Y. Xiong, N. Taheri-Talesh, S. A. Osmani, and B. R. Oakley. 2006. Fusion PCR and gene targeting in *Aspergillus nidulans*. *Nat Protoc* **1:**3111-3120.

Tang, C. M., J. Cohen, T. Krausz, S. Van Noorden, and D. W. Holden. 1993. The alkaline protease of *Aspergillus fumigatus* is not a virulence determinant in two murine models of invasive pulmonary aspergillosis. *Infect Immun* **61:**1650-1656.

Tekaia, F., and J. P. Latge. 2005. *Aspergillus fumigatus*: saprophyte or pathogen? *Curr Opin Microbiol* **8:**385-392.

Todd, R. B., J. A. Fraser, K. H. Wong, M. A. Davis, and M. J. Hynes. 2005. Nuclear accumulation of the GATA factor AreA in response to complete nitrogen starvation by regulation of nuclear export. *Eukaryot Cell* **4:**1646-1653.

Tong, K. B., C. J. Lau, K. Murtagh, A. J. Layton, and R. Seifeldin. 2009. The economic impact of aspergillosis: analysis of hospital expenditures across patient subgroups. *Int J Infect Dis* **13:**24-36.

Traub, W., A. Yonath, and D. M. Segal. 1969. On the molecular structure of collagen. *Nature* **221:**914-917.

Trip, H., M. E. Evers, and A. J. Driessen. 2004. *PcMtr*, an aromatic and neutral aliphatic amino acid permease of *Penicillium chrysogenum*. *Biochim Biophys Acta* **1667:**167-173.

Tsai, H. F., Y. C. Chang, R. G. Washburn, M. H. Wheeler, and K. J. Kwon-Chung. 1998. The developmentally regulated *alb1* gene of *Aspergillus fumigatus*: its role in modulation of conidial morphology and virulence. *J Bacteriol* **180:**3031-3038.

Tsai, H. F., M. H. Wheeler, Y. C. Chang, and K. J. Kwon-Chung. 1999. A developmentally regulated gene cluster involved in conidial pigment biosynthesis in *Aspergillus fumigatus*. *J Bacteriol* **181:**6469-6477.

Tsay, Y. F., C. C. Chiu, C. B. Tsai, C. H. Ho, and P. K. Hsu. 2007. Nitrate transporters and peptide transporters. *FEBS Lett* **581:**2290-2300.

Turner, G. C., F. Du, and A. Varshavsky. 2000. Peptides accelerate their uptake by activating a ubiquitin-dependent proteolytic pathway. *Nature* **405:**579-583.

Untergasser, A., H. Nijveen, X. Rao, T. Bisseling, R. Geurts, and J. A. Leunissen. 2007. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res* **35:**W71-74.

Varshavsky, A. 1996. The N-end rule: functions, mysteries, uses. *Proc Natl Acad Sci U S A* **93:**12142-12149.

Velculescu, V. E., L. Zhang, W. Zhou, J. Vogelstein, M. A. Basrai, D. E. Bassett, Jr., P. Hieter, B. Vogelstein, and K. W. Kinzler. 1997. Characterization of the yeast transcriptome. *Cell* **88:**243-251.

Vickers, I., E. P. Reeves, K. A. Kavanagh, and S. Doyle. 2007. Isolation, activity and immunological characterisation of a secreted aspartic protease, CtsD, from Aspergillus fumigatus. *Protein Expr Purif* **53:**216-224.

Voet, D. J., J. G. Voet, and C. W. Pratt 2002. Lehrbuch der Biochemie, Wiley-VCH Verlag GmbH & Co. KGaA.

Walsmann, P., M. Richter, and F. Markwardt. 1972. [Inactivation of trypsin and thrombin by 4-amidinobenzolsulfofluoride and 4-(2-aminoethyl)-benzolsulfofluoride]. *Acta Biol Med Ger* **28:**577-585.

Wang, Y., W. Li, Y. Siddiqi, V. F. Symington, J. R. Kinghorn, S. E. Unkles, and A. D. Glass. 2008. Nitrite transport is mediated by the nitrite-specific high-affinity NitA transporter and by nitrate transporters NrtA, NrtB in *Aspergillus nidulans*. *Fungal Genet Biol* **45:**94-102.

Waterworth, W. M., and C. M. Bray. 2006. Enigma variations for peptides and their transporters in higher plants. *Ann Bot* **98:**1-8.

Wezensky, S. J., and R. A. Cramer. 2010. Implications of hypoxic microenvironments during invasive aspergillosis. *Med Mycol*.

Wiame, J. M., M. Grenson, and H. N. Arst, Jr. 1985. Nitrogen catabolite repression in yeasts and filamentous fungi. *Adv Microb Physiol* **26:**1-88.

Wieczorke, R., S. Krampe, T. Weierstall, K. Freidel, C. P. Hollenberg, and E. Boles. 1999. Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. *FEBS Lett* **464:**123-128.

Wiederhold, E., T. Gandhi, H. P. Permentier, R. Breitling, B. Poolman, and D. J. Slotboom. 2009. The yeast vacuolar membrane proteome. *Mol Cell Proteomics* **8:**380-392.

Wiles, A. M., H. Cai, F. Naider, and J. M. Becker. 2006. Nutrient regulation of oligopeptide transport in *Saccharomyces cerevisiae*. *Microbiology* **152:**3133-3145.

Wiles, A. M., F. Naider, and J. M. Becker. 2006. Transmembrane domain prediction and consensus sequence identification of the oligopeptide transport family. *Res Microbiol* **157:**395-406.

Willger, S. D., S. Puttikamonkul, K. H. Kim, J. B. Burritt, N. Grahl, L. J. Metzler, R. Barbuch, M. Bard, C. B. Lawrence, and R. A. Cramer, Jr. 2008. A sterol-regulatory element binding protein is required for cell polarity, hypoxia adaptation, azole drug resistance, and virulence in *Aspergillus fumigatus*. *PLoS Pathog* **4:**e1000200.

Wilson, R. A., and H. N. Arst, Jr. 1998. Mutational analysis of AREA, a transcriptional activator mediating nitrogen metabolite repression in *Aspergillus nidulans* and a member of the "streetwise" GATA family of transcription factors. *Microbiol Mol Biol Rev* **62:**586-596.

Wintz, H., T. Fox, Y. Y. Wu, V. Feng, W. Chen, H. S. Chang, T. Zhu, and C. Vulpe. 2003. Expression profiles of *Arabidopsis thaliana* in mineral deficiencies reveal novel transporters involved in metal homeostasis. *J Biol Chem* **278:**47644-47653.

Wong, K. H., M. J. Hynes, R. B. Todd, and M. A. Davis. 2007. Transcriptional control of *nmrA* by the bZIP transcription factor MeaB reveals a new level of nitrogen regulation in *Aspergillus nidulans*. *Mol Microbiol* **66:**534-551.

Xu, S., D. A. Falvey, and M. C. Brandriss. 1995. Roles of URE2 and GLN3 in the proline utilization pathway in *Saccharomyces cerevisiae*. *Mol Cell Biol* **15:**2321-2330.

Yen, M. R., Y. H. Tseng, and M. H. Saier, Jr. 2001. Maize Yellow Stripe1, an iron-phytosiderophore uptake transporter, is a member of the oligopeptide transporter (OPT) family. *Microbiology* **147:**2881-2883.

Young, G. B., D. L. Jack, D. W. Smith, and M. H. Saier, Jr. 1999. The amino acid/auxin:proton symport permease family. *Biochim Biophys Acta* **1415:**306-322.

Zadra, I., B. Abt, W. Parson, and H. Haas. 2000. *xylP* promoter-based expression system and its use for antisense downregulation of the *Penicillium chrysogenum* nitrogen regulator NRE. *Appl Environ Microbiol* **66:**4810-4816.

Zaugg, C., O. Jousson, B. Lechenne, P. Staib, and M. Monod. 2008. *Trichophyton rubrum* secreted and membrane-associated carboxypeptidases. *Int J Med Microbiol* **298:**669-682.

Zhao, W., J. C. Panepinto, J. R. Fortwendel, L. Fox, B. G. Oliver, D. S. Askew, and J. C. Rhodes. 2006. Deletion of the regulatory subunit of protein kinase A in *Aspergillus fumigatus* alters morphology, sensitivity to oxidative damage, and virulence. *Infect Immun* **74:**4865-4874.

Zhou, H., H. Hu, L. Zhang, R. Li, H. Ouyang, J. Ming, and C. Jin. 2007. O-Mannosyltransferase 1 in *Aspergillus fumigatus* (AfPmt1p) is crucial for cell wall integrity and conidium morphology, especially at an elevated temperature. *Eukaryot Cell* **6:**2260-2268.

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AFFIDAVIT

(Eidesstattliche Erklärung)

I hereby declare that my thesis entitled:

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is the result of my own work.
I did not receive any help or support from commercial consultants.
All sources and materials applied are listed and specified in this thesis.

Furthermore, I verify that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg,.....