

**Biosynthesis of Galactofuranose Containing Glycans  
and Their Relevance for the Pathogenic Fungus  
*Aspergillus fumigatus***

Von der Naturwissenschaftlichen Fakultät  
der Gottfried Wilhelm Leibniz Universität Hannover  
zur Erlangung des Grades  
Doktor der Naturwissenschaften  
*Dr. rer. nat.*

genehmigte Dissertation

von

Dipl.-Biochem. Philipp Sebastian Schmalhorst  
geboren am 29. Juni 1979 in Essen

2009

---

Diese Arbeit wurde angefertigt am Institut für Zelluläre Chemie der Medizinischen Hochschule Hannover.

Referentin: Prof. Dr. Françoise H. Routier  
Institut für Zelluläre Chemie  
Zentrum Biochemie  
Medizinische Hochschule Hannover  
Carl-Neuberg-Straße 1, 30625 Hannover

Korreferenten: PD Dr. Sven Krappmann  
Institut für Molekulare Infektionsbiologie  
Zentrum für Infektionsforschung  
Universität Würzburg  
Röntgenring 11, 97070 Würzburg

Prof. Dr. Peter Valentin-Weigand  
Institut für Mikrobiologie  
Zentrum für Infektionsmedizin  
Stiftung Tierärztliche Hochschule Hannover  
Bischofsholer Damm 15, 30173 Hannover

Tag der Promotion: 13. August 2009

---

---

## Table of Contents

Zusammenfassung .....	2
Summary .....	4
Chapter 1 – General Introduction .....	6
1.1 The pathogen <i>Aspergillus fumigatus</i> .....	6
1.2 Overview .....	7
1.3 The Galactofuranosylation Pathway.....	16
1.4 Objectives.....	19
Chapter 2 – Contribution of Galactofuranose to the Virulence of the Opportunistic Pathogen <i>Aspergillus fumigatus</i> .....	21
Chapter 3 – A single UDP-Galactofuranose transporter is required for galactofuranosylation in <i>Aspergillus fumigatus</i> .....	36
Chapter 4 – General Discussion .....	55
4.1 Importance of galactofuranosylation for growth and morphology of <i>A. fumigatus</i> .....	56
4.2 The <i>Aspergillus fumigatus</i> UDP-Galf transporter .....	57
4.3 The galactofuranose biosynthesis pathway as drug target.....	60
4.4 Supplemental figure .....	62
References .....	63
Abbreviations .....	68
Curriculum vitae.....	69
Danksagung.....	70

---

## Zusammenfassung

Invasive Pilzinfektionen stellen eine ernstzunehmende Bedrohung für immunsupprimierte Patienten dar. Die überwiegende Mehrzahl dieser Infektionen wird durch *Candida*- oder *Aspergillus*-Arten hervorgerufen. Der Hauptverursacher der Invasiven Aspergillose, *Aspergillus fumigatus*, befällt vor allem Stammzelltransplantations- und Organtransplantations-Patienten. Als Risikofaktoren gelten die Gabe von Immunsuppressiva sowie hohe Corticosteroid-Dosen. Die Sterblichkeit ist mit 20 bis 80 % sehr hoch, was auf unzureichende Wirksamkeit der etablierten Antimykotika zurückzuführen ist. Daher ist ein wesentliches Ziel der gegenwärtigen mykologischen Forschung die Identifikation neuer therapeutischer Angriffspunkte (*drug targets*).

Da die Polysaccharid-Zellwand der Pilze essentiell für die Pilzzelle ist und deren Strukturen in menschlichen Zellen nicht vorkommen, stellt deren Biosynthese ein attraktives neues *drug target* dar. Ein Hauptbestandteil der Zellwandkomponenten von *A. fumigatus*, darunter Glykoproteine, Glykolipide und das Polysaccharid Galactomannan, ist der ungewöhnliche Zucker Galactofuranose (Galf). Die Glykane menschlicher Zellen enthalten kein Galf, wohl aber Oberflächenmoleküle vieler pathogener Mikroorganismen, wie z.B. *Mycobacterium tuberculosis* oder *Leishmania major*. Oft sind diese Strukturen bedeutsam für die Virulenz oder sogar Lebensfähigkeit dieser Organismen, was eine möglicherweise ähnliche Bedeutung von Galf für *A. fumigatus* nahelegt.

In dieser Arbeit wurde der Bedeutung der Galactofuranosylierung für *A. fumigatus* nachgegangen. Dazu wurde das UDP-Galf-Biosynthesegen *glfA*, das für die UDP-Galactopyranose-Mutase (UGM) codiert, in *A. fumigatus* entfernt. In mehreren Experimenten konnte gezeigt werden, dass dieser Defekt zu einem vollständigen Verlust von Galf in den Glykanen von *A. fumigatus* führt. Die  $\Delta glfA$ -Mutante ist zudem gekennzeichnet durch einen Wachstumsdefekt und verringerte Sporenbildung, geringere Wärmetoleranz und eine erhöhte Sensitivität gegenüber Antimykotika. In elektronenmikroskopischen Aufnahmen konnte ein Zellwanddefekt als wahrscheinliche Ursache für diesen Phänotyp identifiziert werden. Darüber hinaus erwies sich die  $\Delta glfA$ -Mutante in einem Maus-Infektionsmodell der Invasiven Aspergillose als deutlich weniger virulent als der Wildtyp.

Der zweite Schwerpunkt dieser Arbeit bestand in der Aufklärung weiterer an der Galactofuranosylierung beteiligten Proteine von *A. fumigatus*. In Eukaryonten kann allgemein ein UDP-Galf-Transporter für den Transport von UDP-Galf aus dem Cytosol in das ER bzw. den Golgi-Apparat angenommen werden, allerdings ist eine derartige Aktivität bislang nicht bekannt. Sechzehn Gene aus dem Genom von *A. fumigatus* konnten als mögliche Kandidaten für den UDP-Galf-Transporter identifiziert werden, von denen eines, *glfB*, interessanterweise in unmittelbarer Nähe zu *glfA* gefunden wurde. Transport-Experimente zeigten spezifische Bindung von UDP-Galf an GlfB *in vitro*, was auf eine UDP-Galf-Transportaktivität hinwies. Die Bestätigung lieferte eine  $\Delta glfB$ -Deletionsmutante, deren Analyse wie schon bei der  $\Delta glfA$ -Mutante die völlige Abwesenheit von Galf ergab. Dieser Befund erlaubte den Schluss, dass die gesamte Galactofuranosylierung in *A. fumigatus*

im ER bzw. Golgi-Apparat stattfindet. Dies gilt insbesondere auch für das Zellwand-Polysaccharid Galactomannan, das damit nicht wie andere Zellwand-Polysaccharide an der Plasmamembran synthetisiert wird.

Somit konnte in dieser Arbeit der erste UDP-Galf-Transporter überhaupt identifiziert, sowie dessen Bedeutung für die Galactofuranosylierung in *A. fumigatus* gezeigt werden. Des Weiteren lässt die durch den Verlust von Galf verursachte Verminderung der Virulenz von *A. fumigatus* die UGM als mögliches neues *drug target* in Betracht kommen.

Schlagwörter: *Aspergillus fumigatus*, Galactofuranose, Virulenz

## Summary

Invasive fungal infections pose a serious threat to immunocompromised people. Most of these infections are caused by either *Candida* or *Aspergillus* species, with *A. fumigatus* being the predominant causative agent of Invasive Aspergillosis. Affected people comprise mainly haematopoietic stem cell or solid organ transplant patients who receive either high-dose corticosteroids or immunosuppressants. These risk factors predispose to the development of Invasive Aspergillosis which is lethal in 20 to 80 % of the cases, largely due to insufficient efficacy of current antifungal therapy. Thus one major aim in current mycological research is the identification of new drug targets.

The polysaccharide-based fungal cell wall is both essential to fungi and absent from human cells which makes it appear an attractive new target. Notably, many components of the *A. fumigatus* cell wall, including the polysaccharide galactomannan, glycoproteins, and glycolipids, contain the unusual sugar galactofuranose (Galf). In contrast to the other cell wall monosaccharides, Galf does not occur on human cells but is known as component of cell surface molecules of many pathogenic bacteria and protozoa, such as *Mycobacterium tuberculosis* or *Leishmania major*. These molecules are often essential for virulence or viability of these organisms which suggested a possible role of Galf in the pathogenicity of *A. fumigatus*.

To address the importance of Galf in *A. fumigatus*, the key biosynthesis gene *glfA*, encoding UDP-galactopyranose mutase (UGM), was deleted. In different experimental approaches it was demonstrated that the absence of the *glfA* gene led to a complete loss of Galf-containing glycans. Analysis of the  $\Delta glfA$  phenotype revealed growth and sporulation defects, reduced thermotolerance and an increased susceptibility to antifungal drugs. Electron Microscopy indicated a cell wall defect as a likely cause for the observed impairments. Furthermore, the virulence of the  $\Delta glfA$  mutant was found to be severely attenuated in a murine model of Invasive Aspergillosis.

The second focus of this study was laid on further elucidation of the galactofuranosylation pathway in *A. fumigatus*. In eukaryotes, a UDP-Galf transporter is likely required to transport UDP-Galf from the cytosol into the organelles of the secretory pathway, but no such activity had been described. Sixteen candidate genes were identified in the *A. fumigatus* genome of which one, *glfB*, was found in close proximity to the *glfA* gene. *In vitro* transport assays revealed specificity of GlfB for UDP-Galf suggesting that *glfB* encoded indeed a UDP-Galf transporter. The influence of *glfB* on galactofuranosylation was determined by a  $\Delta glfB$  deletion mutant, which closely recapitulated the  $\Delta glfA$  phenotype and was likewise found to be completely devoid of Galf. It could be concluded that all galactofuranosylation processes in *A. fumigatus* occur in the secretory pathway, including the biosynthesis of the cell wall polysaccharide galactomannan whose subcellular origin was previously disputed.

Thus in the course of this study the first UDP-Galf specific nucleotide sugar transporter was identified and its requirement for galactofuranosylation in *A. fumigatus* demonstrated. Moreover, it was shown that blocking the galactofuranosylation pathway impaired virulence of *A. fumigatus* which suggests the UDP-Galf biosynthesis enzyme UGM as a target for new antifungal drugs.

Keywords: *Aspergillus fumigatus*, galactofuranose, virulence.

## Chapter 1 – General Introduction

### 1.1 The pathogen *Aspergillus fumigatus*

Fungal diseases in healthy individuals comprise harmless superficial infections of skin or mucosa and allergic diseases. Immunocompromised people in contrast, might develop invasive fungal infections that are lethal if not treated rapidly. Most of these infections are caused by either *Aspergillus* or *Candida* species with *Aspergillus fumigatus* being the predominant causative agent of Invasive Aspergillosis (IA). This disease affects in particular patients that undergo chemotherapy or hematopoietic stem cell transplantation (HSCT) due to haematological malignancies (e.g. leukaemia or lymphoma). The incidence of IA in this risk group is currently 2.5-8 % with a mortality of 22-42 % (Pagano et al. 2006, Martino et al. 2002, Neofytos et al. 2009). Solid organ transplantation patients are the second major risk group. The occurrence of IA depends on the transplanted organ and affects 1-15 % of transplant patients with a mortality of 55-80 % (Singh and Paterson 2005, Sanchez and Larsen 2007). The critical factors predisposing to IA have been identified as neutropenia, i.e. blood neutrophil counts  $< 500 \text{ mm}^{-3}$ , and long term therapy with high doses of corticosteroids which impairs innate immune cell functions.

The high mortality makes IA one of the major complications after stem cell or solid organ transplantation, it has been estimated to account for 39 % of the nonrelapse mortality (9 % of the overall mortality) in HSCT patients (Fukuda et al. 2003). These numbers clearly reflect a deficit in effective antifungals.

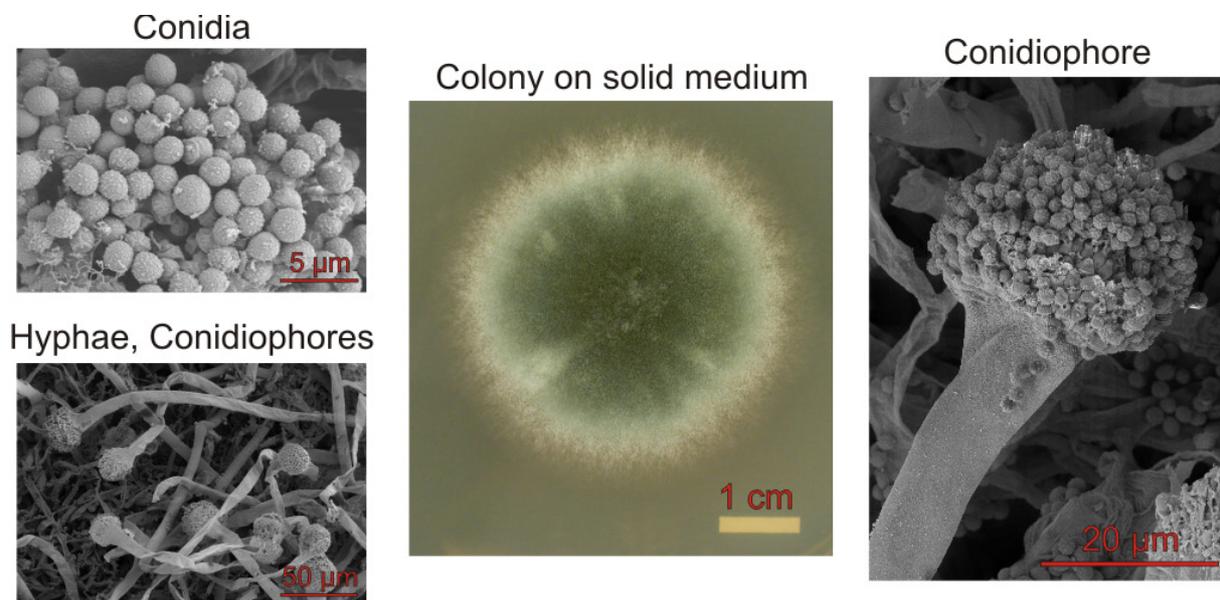
Amphotericin B is the classical drug in the therapy of invasive fungal infections. Its binding to ergosterol, the principal sterol in the fungal cell membrane, disturbs membrane integrity and eventually causes leakage (Odds et al. 2003). Although active against a broad range of fungi, amphotericin B is not very selective and thus associated with severe side effects. New lipid formulations with less toxicity have been developed, but their high cost opposes routine clinical use.

The current standard antifungals, the triazoles fluconazole, itraconazole, voriconazole, and posaconazole, show improved efficacy and less toxicity (Herbrecht et al. 2002). They act as inhibitors of fungal ergosterol biosynthesis. The main drawback is unfavourable interaction with many other drugs, including immunosuppressants. Nevertheless the clinical use of voriconazole has considerably improved the management of IA (Zonios and Bennett 2008). The third and newest class of antifungals comprises the echinocandins caspofungin, micafungin, and anidulafungin, the two latter being approved only for candidiasis. Echinocandins inhibit the biosynthesis of the major cell wall polysaccharide,  $\beta$ 1,3-glucan, and thus introduce a new drug target in *A. fumigatus*. Caspofungin is currently used as salvage therapy for IA and can be effectively combined with established antifungals (Singh et al. 2006, Caillot et al. 2007).

## 1.2 Overview

### 1.2.1 Biology of *Aspergillus fumigatus*

The natural habitat of *Aspergillus fumigatus* is the soil. The saprophytic fungus feeds on degradation products of organic matter by secreting various hydrolytic enzymes. The *A. fumigatus* life cycle starts with a haploid asexual spore (conidium) that measures 2.5-3  $\mu\text{m}$  in diameter and has a gray-green colour due to the presence of melanins in the cell wall. Upon settling on organic substrate that provides water, trace elements and sources of carbon and nitrogen the conidium starts swelling (5 h). Thereby the outermost layer of the conidial cell wall, a dense hydrophobic protein coat, breaks up and exposes the cell wall polysaccharides to the surface. A germ tube eventually emerges from the swollen conidium and by constitutive growth at its tip finally forms hyphae. During elongation the nuclei divide and hyphae are septated by cell wall-like material yielding separate hyphal cells with a single diploid nucleus each. Contact with air induces the formation of conidiophores, i.e. specialized hyphal structures that produce conidia (fig. 1-1). Conidiophores consist of a foot cell which terminates in a large vesicle covered with a layer of conidia-producing cells (phialides). The conidia form by septation of the elongated phialide and remain organized in loosely attached chains. They are easily released by air currents and thus ubiquitously found in the environment (Brakhage and Langfelder 2002).



**Figure 1-1** Microscopic and macroscopic views of *A. fumigatus*. Left and right panels, Scanning electron micrographs of *A. fumigatus* conidia, hyphae, and conidiophores at different magnifications (M. Rohde, HZI Braunschweig). Middle panel, *A. fumigatus* culture after three days at 37 °C on minimal medium agar.

*A. fumigatus* is positioned taxonomically in the pezizomycotina subphylum (formerly euascomycetes) of the ascomycota (Hibbett et al. 2007). The pezizomycotina comprise the hyphae-forming

ascomycota ('filamentous fungi') and harbour most of the species that are pathogenic to humans or plants. They can be clearly separated from two other subphyla, the taphrinomycotina and saccharomycotina which typically live as unicellular organisms ('yeasts', table 1-1). The shared characteristic of the ascomycota is the morphologically distinctive *ascus*, a specialized cell that produces sexual ascospores. However, in *A. fumigatus*, like in many other *Aspergilli*, sexual reproduction is very rare and probably plays a minor role in nature. Indeed, the existence of a sexual stage of *A. fumigatus* was doubted for a long time and was proven only recently (O'Gorman et al. 2009).

Phylum	Species	Pathogenic to
Ascomycota		
	'Yeasts' (Saccharomycotina, Taphrinomycotina)	
	<i>Saccharomyces cerevisiae</i>	Model organism
	<i>Schizosaccharomyces pombe</i>	Model organism
	<i>Candida albicans</i>	Humans
	'Filamentous fungi' (Pezizomycotina)	
	<i>Aspergillus fumigatus</i>	Humans
	<i>Penicillium marneffeii</i>	Humans
	<i>Neurospora crassa</i>	Model organism
	<i>Histoplasma capsulatum</i>	Humans
	<i>Coccidioides immitis</i>	Humans
	<i>Paracoccidioides brasiliensis</i>	Humans
	<i>Blastomyces dermatitidis</i>	Humans
	<i>Trichophyton rubrum</i>	Humans (Dermatophyte)
	<i>Botryotinia fuckeliana</i>	Plants
	<i>Gibberella zeae</i>	Plants
	<i>Magnaporthe grisea</i>	Plants
Basidiomycota		
	<i>Ustilago maydis</i>	Plants
	<i>Cryptococcus neoformans</i>	Humans

**Table 1-1** Taxonomy of common fungi. Most of the pathogenic fungi belong to the subphylum pezizomycotina.

## 1.2.2 Pathobiology of *Aspergillus fumigatus*

### *Immunity in immunocompetent hosts*

It is estimated that humans typically inhale hundreds of *A. fumigatus* conidia per day (Latgé 1999). Because of their small size these spores reach the lung alveoli, but infection in healthy individuals is very rare. Three cellular lines of defence prevent *A. fumigatus* outgrowth, tissue-resident alveolar macrophages and neutrophil granulocytes as part of the innate immune system and *A. fumigatus* specific T<sub>H</sub>1 cells as part of the adaptive immunity.

In the first line, macrophages phagocytise conidia and secrete pro-inflammatory cytokines (importantly TNF- $\alpha$ ) and chemokines such as CXCL-2 (Hohl et al. 2005, Steele et al. 2005, Gersuk et al. 2006). In the pro-inflammatory cytokine environment, macrophages are stimulated to kill conidia in the phagosome by oxidative and non-oxidative mechanisms (Bonnett et al. 2006, Philippe et al. 2003). Neutrophilic granulocytes leave the bloodstream and migrate to the site of infection in response to the chemokines released by alveolar macrophages. They show less phagocytosis capacity, but attach to fungal cells and effectively damage conidia and hyphae by releasing antimicrobial agents (Diamond et al. 1978, Bonnett et al. 2006); reactive oxygen species play a major role in this process (Bonnett et al. 2006).

The cells of the innate immune system are thought to be most important in preventing *Aspergillus* infection, however, as seen by the susceptibility of AIDS patients to Invasive Aspergillosis, T cells are also necessary to provide full resistance. Immature dendritic cells in the lung phagocytise dormant conidia, mature and migrate to the lymph nodes where they prime naive CD4<sup>+</sup> T cells to become T<sub>H</sub>1 cells (Langlois and Legge 2007). These cells protect against *Aspergillus* infection by activating macrophages mainly through Interferon- $\gamma$  and thus increasing their microbicidal potential. The protective effect of *A. fumigatus*-specific T<sub>H</sub>1 cells could be demonstrated in a mouse model of Invasive Aspergillosis (Cenci et al. 2000). Germinated conidia can also be phagocytised by immature DCs, however other receptors are involved in binding and uptake. Interestingly, in this case DCs drive T cell activation towards T<sub>H</sub>2 cells. Besides inducing a B cell mediated antibody response, T<sub>H</sub>2 cells deactivate macrophages which worsens the outcome of *Aspergillus* infection (Langlois and Legge 2007).

### *Impaired immunity under immunosuppressive conditions*

Glucocorticoids, e.g. cortisone, decrease the pro-inflammatory capacity of macrophages and neutrophils in many ways (Lionakis and Kontoyiannis 2003). The expression of pro-inflammatory cytokines and chemokines is downregulated in macrophages and T cells because important signalling pathways such as NF- $\kappa$ B, AP-1 and MAPK/ERK are inhibited. As a result the cytokine profile is shifted towards a T<sub>H</sub>2 type which decreases the phagocytosis potential of macrophages and leads to insufficient fungal clearance (Lasa 2002, Kamberi 2002, Balloy 2005). Moreover, due to the lack of chemokines, the attraction of neutrophils to the site of infection is hindered which further impairs the innate immune response.

Neutropenia or impairment of neutrophil function also predisposes to Invasive Aspergillosis. Neutropenia is an unavoidable effect of chemotherapy in the treatment of haematological malignancies. Alkylating agents such as cyclophosphamide inhibit cell division of malignant cells but also the differentiation of neutrophilic granulocytes which have a short lifetime and thus have to be constantly replenished. Chronic Granulomatous Disease is another risk factor for developing Invasive Aspergillosis (Antachopoulos et al. 2007). The underlying defect in this disease, a defective NADPH

oxidase required to synthesize reactive oxygen species, highlights the importance of oxidative killing mechanisms in neutrophil function against *A. fumigatus*.

### *Recognition of cell wall carbohydrate motifs by innate immune cells*

The molecular recognition of *A. fumigatus* by macrophages is only partly understood, but it is clear that several receptors exploiting independent signalling pathways are involved. Importantly, conidial swelling is a prerequisite for the induction of a cytokine response suggesting that carbohydrate structures of the cell wall serve as ligands for macrophage receptors. This has been shown for dectin-1, a  $\beta$ 1,3-glucan receptor which binds to *A. fumigatus* conidia in the phagosome and contributes to the production of T<sub>H</sub>1 cytokines and chemokines (Hohl et al. 2005, Steele et al. 2005). Toll-like receptor 2 (TLR2) is also required to ensure full capacity of cytokine production, however its ligand on the fungal cell has not been identified (Steele et al. 2005). TLR2 and dectin-1 employ different signalling pathways which result in the activation of NF- $\kappa$ B or AP-1 respectively (Toyotome et al. 2008); moreover the MAPK/ERK pathway has been shown to be of a certain importance for *A. fumigatus* clearance by alveolar macrophages (Dubourdeau et al. 2006).

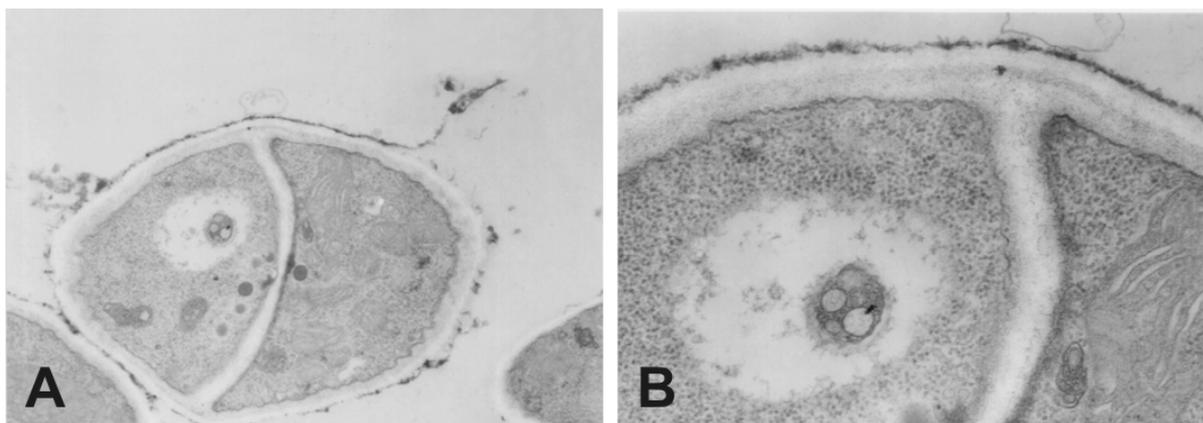
Lung surfactant proteins SP-A and SP-D are lectins that bind *A. fumigatus* cell wall polysaccharides and enhance the phagocytosis and killing capacities of macrophages and neutrophils (Madan et al. 1997). Dendritic cell receptors for *A. fumigatus* comprise dectin-1 and DC-SIGN, the latter being specific for mannose-containing polysaccharides (Serrano-Gómez et al. 2004).

### **1.2.3 The *A. fumigatus* cell wall**

#### *Polysaccharides*

The *A. fumigatus* cell wall mainly consists of a voluminous polysaccharide network, (glyco)proteins, and glycolipids. It serves vital cellular functions, such as mechanical protection and resistance against osmotic stress. In transmission electron micrographs the hyphal cell wall appears as a 50 to 330 nm bi-layered structure (fig. 1-2) (Reijula 1991, Campbell 1970). The massive electron-lucent inner layer contains mostly polysaccharides, while the thin electron-dense outer layer probably represents cell wall-associated proteins (Klis et al. 2007). The conidial cell wall has a stronger outer protein layer that consists of a dense coat of hydrophobins that prevent desiccation and contains melanin which gives conidia their gray-green colour.

The molecular structure of the *A. fumigatus* cell wall is relatively well characterized (Fontaine et al. 2000, Barreto-Bergter et al. 1981). For technical reasons two fractions are commonly distinguished, based on their solubility in hot sodium hydroxide: The alkali-soluble fraction (60 % of the cell wall mass) containing mostly medium molecular weight polysaccharides and a small amount of protein, and the alkali-insoluble fraction (40 %) consisting of a covalent network of high molecular weight polysaccharides (fig. 1-3).

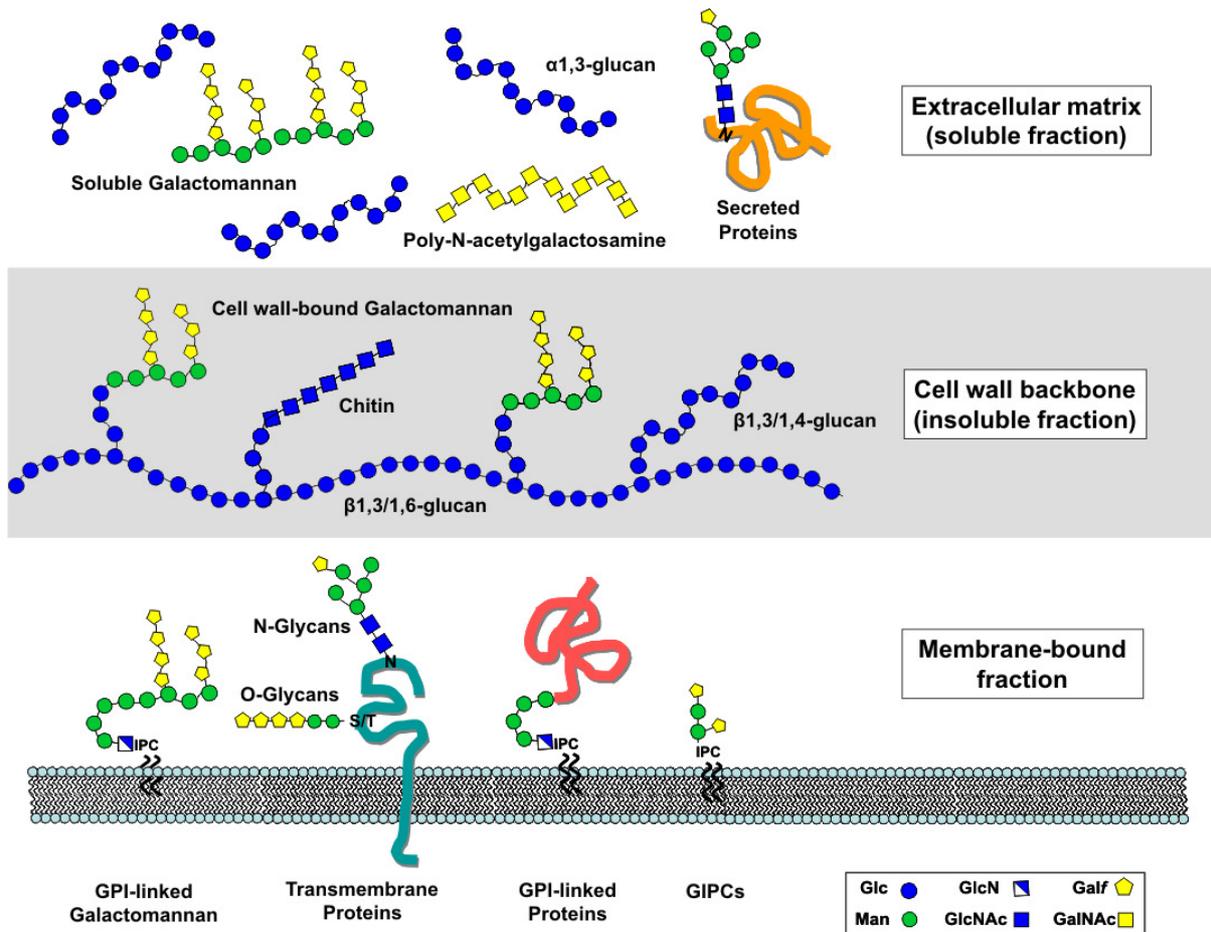


**Figure 1-2** Transmission electron micrographs of *A. fumigatus* hyphae. A thick electron-lucent layer of polysaccharides is covered by a thin electron-dense layer, probably containing proteins. A, 1100x magnification; B, 3100x magnification.

© 2001 Antimicrobial Agents and Chemotherapy, Vol. 45, p. 3310-3321, DOI: 10.1128/AAC.45.12.3310-3321.2001.  
Reproduced with permission from American Society of Microbiology.

The main component (around 45 %) of the alkali-insoluble fraction is  $\beta$ 1,3-glucan, i.e. a  $\beta$ 1,3-linked polymer of glucose. Beta-1,3-glucan is common to all ascomycota and forms the cell wall backbone to which the other polysaccharides of the alkali-insoluble fraction are attached. In *A. fumigatus* approximately 4 % of the glucose residues in the  $\beta$ 1,3-glucan are substituted at the C6 with small  $\beta$ 1,3-glucan side chains. These branches provide the anchoring points for chitin (10-20 %), galactomannan (14-22 %), and a  $\beta$ 1,3/ $\beta$ 1,4-glucan (around 10 %). Chitin, a  $\beta$ 1,4-N-acetylglucosamine polymer, is also a frequent component of fungal cell walls and is thought to confer mechanical rigidity. The  $\beta$ 1,3/1,4-glucan has not yet been described in any other fungi and its function and importance is currently unknown (Fontaine et al. 2000). Galactomannan is a more complex polysaccharide and consists of an  $\alpha$ 1,2- and  $\alpha$ 1,6-linked mannose core to which several side chains of up to five  $\beta$ 1,5-linked galactofuranose (Gal<sub>f</sub>) residues are attached. Since Gal<sub>f</sub> does not occur in humans, galactomannan is highly immunogenic and assumed to be involved in *A. fumigatus* pathogenicity (Bernard and Latgé 2001).

The alkali-soluble cell wall fraction contains  $\alpha$ 1,3-glucan (~ 60 %), free galactomannan (~ 30 %), a poly-N-acetylgalactosamine (~ 5 %) and glycoproteins (< 5 %) (Beauvais et al. 2005b, Fontaine et al. 2000). Due to its missing covalent link to the cell wall backbone this fraction has been described as a kind of extracellular matrix (Beauvais et al. 2007). The main constituent  $\alpha$ 1,3-glucan is a virulence factor in other fungi (*Histoplasma capsulatum*, *Cryptococcus neoformans*, *Paracoccidioides brasiliensis*, *Blastomyces dermatitidis*) but not in *A. fumigatus* (Beauvais et al. 2005b). Galactomannan has the same structure and antigenic properties as the cell wall-bound form. Structure and function of the minor compound poly-N-acetylgalactosamine are unclear (Fontaine et al. 2000). Unlike in yeast, cell wall glycoproteins are not covalently linked to the cell wall but either anchored in the plasma membrane or secreted (fig. 1-3).



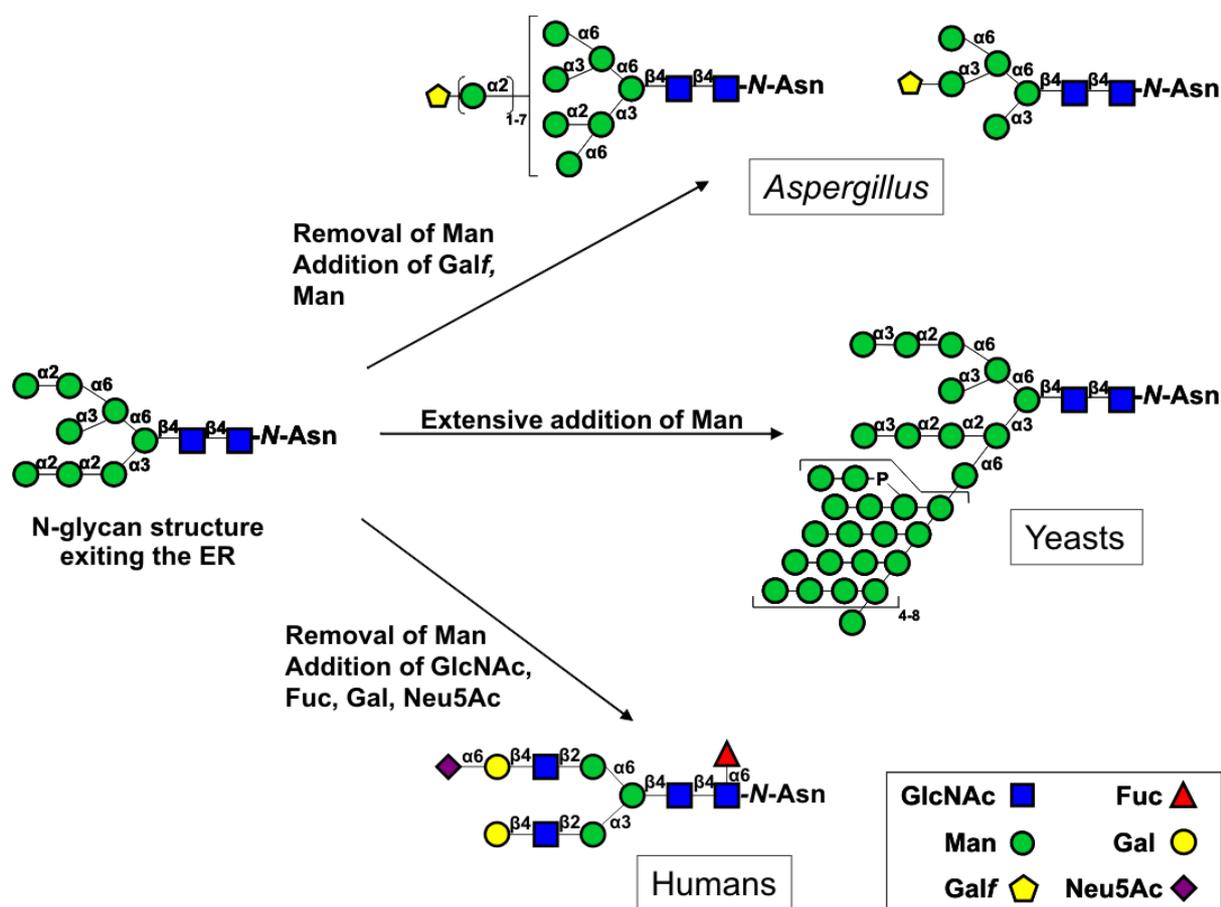
**Figure 1-3** Schematic view of the *A. fumigatus* cell wall constituents. Beta-1,3/1,6-glucan forms a network to which chitin,  $\beta$ 1,3/1,4-glucan and galactomannan are covalently attached. Alpha-1,3-glucan, soluble galactomannan and poly-N-acetylgalactosamine are amorphyously distributed in the cell wall. Glycoproteins are either secreted or embedded in the plasma membrane. GPI, glycosylphosphatidyl; GIPCs, glycoinositolphosphoceramides; Glc, glucose; GlcN, glucosamine; GlcNAc, N-acetylglucosamine; Man, mannose; Galf, galactofuranose; GalNAc, N-acetylgalactosamine.

### Glycoproteins

Glycoproteins, either embedded in the plasma membrane or secreted and associated to the cell wall polysaccharides, also contribute to the cell wall glycan profile. Asparagine-linked (N-glycans) and serine/threonine-linked (O-glycans) glycans are found, but differ from human N- and O-glycans (figure 3). Genome comparison with *Saccharomyces cerevisiae* suggests that the common initiation of N-glycan biosynthesis is also preserved in *A. fumigatus*. Thus, glycoproteins most likely transit from ER to Golgi with the consensus  $\text{Man}_8\text{GlcNAc}_2$  oligosaccharide. The subsequent processing in the Golgi diverges between *Aspergillus*, yeasts, and humans (fig. 1-4). In *Aspergillus niger* one or more galactofuranose residues are added to the N-glycan core while up to three mannose residues are sequentially removed (Takayanagi et al. 1994). In *A. fumigatus* the core structure might be extended by a few mannose residues, before galactofuranose residues are added (Morelle et al. 2005). An often found modification specific for yeasts is the addition of up to 150 additional mannose residues to the

N-glycan core. However this ‘hypermannosylation’ has not been found in *A. fumigatus* or any other filamentous fungus.

In contrast to the N-glycan remodelling in fungi, human N-glycans are often transformed into complex N-glycans containing little mannose, but instead N-acetylglucosamine, galactose<sup>1</sup>, fucose and sialic acid, all of which are not found in *A. fumigatus* N-glycans. Notably, galactofuranose is not present, neither in yeast nor in human N-glycans.



**Figure 1-4** Different N-glycan processing in *Aspergillus*, yeasts and humans. Glycoproteins transit from the ER to the Golgi where the ER-derived  $\text{Man}_8\text{GlcNAc}_2$  precursor (left) is extensively modified. In *Aspergillus*, Galf is added after partial removal of Man. Yeasts add 50-150 Man residues without removing Man, and in humans GlcNAc, Fuc, Gal, and Neu5Ac are sequentially added after Man has been removed. The addition of Galf is specific for *Aspergillus*. Depicted N-glycan structures were suggested for *A. fumigatus* (Morelle et al. 2005) (top left) and *A. niger* (Takayanagi et al. 1994) (top right). Fuc, fucose; Gal, galactose; Neu5Ac, N-acetyl neuraminic acid (sialic acid).

While the N-glycans of *A. fumigatus* and humans share a common precursor whose structure is partly conserved in the mature structures (fig. 1-4), the O-glycans of *A. fumigatus* and humans are totally different. In the fungus, O-glycans are based on a mannose disaccharide to which a chain of up to four

<sup>1</sup> Galactose (Gal) and UDP-galactose (UDP-Gal), whenever used in this text without  $-f$  suffix, denote the pyranose form of (UDP-)galactose (cf. also 3.2.4).

galactofuranose residues is added (fig. 1-3). In turn, human O-glycans contain N-acetylgalactosamine (GalNAc), galactose, sialic acid, fucose and xylose, which are absent in *A. fumigatus*.

### *Glycolipids*

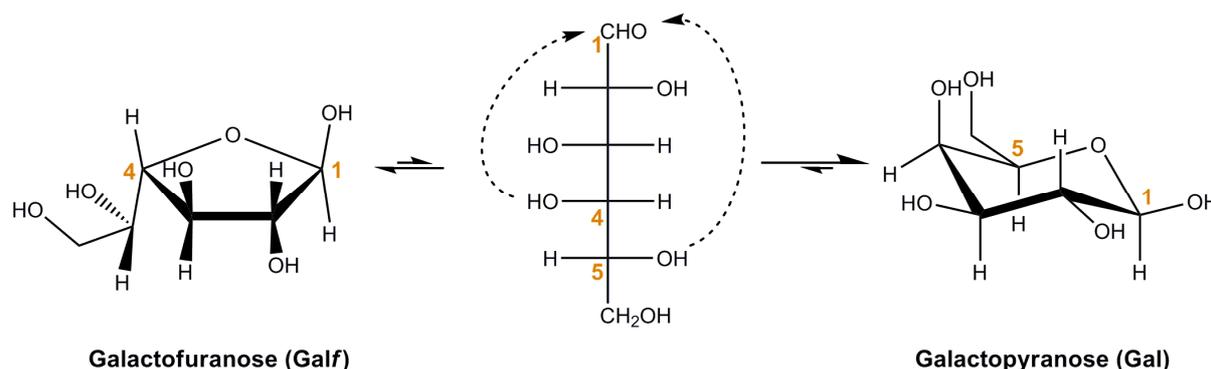
*A. fumigatus* synthesises inositolphosphoceramide (IPC) based glycolipids called GIPCs that have no equivalent in human cells. Like in N- and O-glycans and galactomannan, the glycan moiety consists of mannose and galactofuranose (Simenel et al. 2008, Toledo et al. 2007). The function of these structures is not known, however in the pathogenic fungus *Candida albicans* transfer of the first mannose residue to IPC is required for survival in macrophages and full virulence (Mille et al. 2004). Furthermore, *A. fumigatus* synthesizes glycosylphosphatidylinositol (GPI) anchors. Like in other eukaryotes, GPI anchors are fused to the C-terminus of certain proteins and attach them to the plasma membrane (fig. 1-3). The *A. fumigatus* GPI biosynthesis follows the consensus biosynthetic pathway of most eukaryotes and is surprisingly not essential for *A. fumigatus* (Fontaine et al. 2003, Li et al. 2007). Recently, a galactomannan with a structure identical to the cell wall-bound and soluble form was found to be GPI-linked (Costachel et al. 2005). This lipogalactomannan represents a peculiar finding, since GPI-based glycoconjugates have been known only from trypanosomatid parasites.

In summary, the glycan structures of *A. fumigatus* and humans are highly divergent. The complex glycans of *A. fumigatus* are based on a mannose core and are often decorated with one or more galactofuranose residues. In contrast, human glycans contain little mannose but N-acetylglucosamine, galactose, fucose and sialic acid. These structural differences are based on different biosynthetic pathways which provide potential new drug targets given that there is a certain implication of these structures in the virulence of *A. fumigatus*. It is however difficult to predict specific functions for certain glycan structures. While cell wall polysaccharides including galactomannan might play a role for cell wall stability, the importance of N- and O-glycans depends on the underlying protein for whose activity glycosylation might be relevant or not. An intriguing feature of *A. fumigatus* glycans is the frequent occurrence of galactofuranose, because this isoform of galactose is not present in humans, but in many other pathogenic microorganisms.

### *1.2.4 Galactofuranose and its occurrence in microorganisms*

#### *Galactofuranose*

For thermodynamic reasons, 6-carbon aldehyde sugars (aldohexoses) like glucose, galactose or mannose appear in nature predominantly as six-membered rings formed by a link between the C5 hydroxyl group and the C1 carbon atom; this conformation is referred to as the pyranose form.



**Figure 1-5** The different conformations of galactose. Galactopyranose (Gal) is the most stable and the most often encountered conformation in glycan structures while galactofuranose is much rarer. The open chain conformation is an unstable intermediate in aqueous solution and does not occur in glycans. The orange numbers designate carbon atoms involved in ring formation.

Galactose is exceptionally found to occur in the furanose form, i.e. a five-membered ring through the C4 hydroxyl group and C1, designated galactofuranose (*Galf*) (fig. 1-5). Its prevalence is mainly restricted to microorganisms, such as bacteria, protozoa, fungi, and algae but might also be present in certain ancient multicellular organisms.

Already in the 1930's a polysaccharide containing *Galf* was isolated from *Penicillium charlesii* (Haworth et al. 1937), later *Galf* containing glycans were isolated from bacteria (Bourne et al. 1961) and parasitic trypanosomatids like *Trypanosoma* and *Leishmania* (Turco et al. 1989, de Lederkremer et al. 1980).

#### *Galf* in bacteria

Bacterial cell wall glycans often contain sugars that are not present in multicellular organisms, among them galactofuranose. A well-known example is the mycobacterial arabinogalactan which connects the mycolic acids of the outer mycobacterial cell wall layer covalently to the peptidoglycan through a chain of galactofuranose and arabinofuranose. A *Mycobacterium smegmatis* mutant that is unable to synthesize *Galf* is not viable, probably due to the loss of mycolic acids (Pan et al. 2001). In other gram-positive bacteria, *Galf* occurs for example in teichoic acids of certain serotypes of *Streptococcus pneumoniae* (Beynon et al. 1997), however the precise role of these polymers remains to be clarified. *Galf* is occasionally found in the lipopolysaccharide (LPS) O-antigen of gram-negative bacteria. Being the predominant component of the outer membrane, LPS mediates adhesion, evokes immune reactions and provides resistance against bacteriophages. The O-antigen part of the molecule consists of numerous repeats of an oligosaccharide unit that varies within a species and defines strain-specific antigenicity ("O serotypes"). Once in the host, pathogenic bacteria quickly adapt size and composition of the LPS O-antigen to escape immune responses, therefore it is recognized as a virulence factor. Among the bacterial species that contain *Galf* in the LPS of certain serotypes are *Escherichia coli*,

*Shigella dysenteriae*, *Klebsiella pneumoniae*, *Salmonella enterica*, and *Actinobacillus pleuropneumoniae*.

### *Galf in protozoa*

Parasitic protozoa like *Trypanosoma cruzi* or *Leishmania* spp. synthesize cell surface glycolipids that contain *Galf*. An important example is the *Leishmania* lipophosphoglycan (LPG) which consists of a phosphodisaccharide repeat unit attached to a *Galf* containing glycan core. LPG is essential for establishment of infection in mammals and thus considered as an important virulence factor of *Leishmania*. Loss of the putative glycosyltransferase required for *Galf* incorporation into LPG leads to the absence of LPG molecules and strongly reduced virulence (Späth et al. 2000). In contrast, the terminal *Galf* residues of glycoinositolphospholipids (GIPLs) do not contribute to *Leishmania major* pathogenesis, however in *Trypanosoma cruzi* they are required for adherence to the midgut epithelium of the insect vector (Kleczka et al. 2007, Nogueira et al. 2007).

Similar to *A. fumigatus*, *Galf* also occurs as a terminal unit in N- and O-glycans of trypanosomatid glycoproteins, however the importance of these modifications has not been investigated yet.

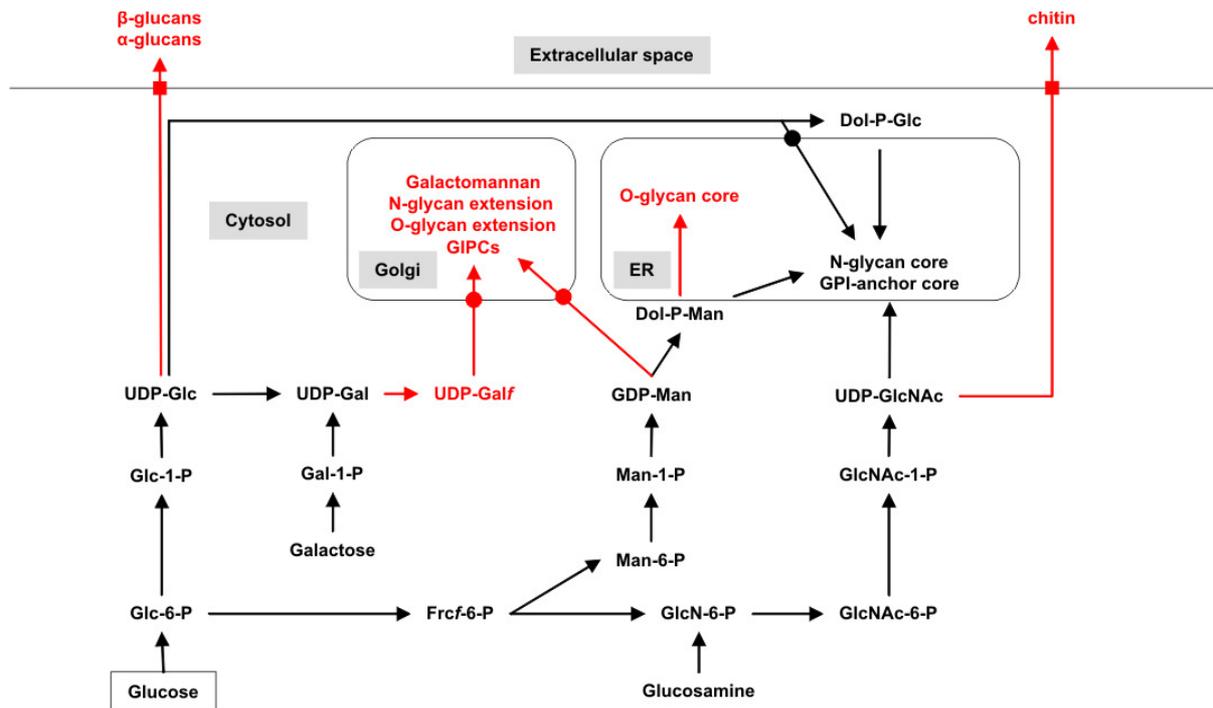
### *Galf in other organisms*

Although *Galf* is often regarded as a ‘microbial sugar’ a few reports on *Galf* in invertebrates and plants exist. Glycosides containing *Galf* have been isolated from flowers and algae (Maksyutina 1967, Igarashi et al. 1999) and O-glycans of the alga *Chlamydomonas reinhardtii* also contain *Galf* (Bollig et al. 2007). The only animal known to produce *Galf* is starfish (Riccio et al. 1987), besides, genetic and biochemical evidence suggests *Galf* biosynthesis potential also for *Caenorhabditis elegans* (Bakker et al. 2005a, Beverley et al. 2005). *Galf* has never been found in any vertebrate.

## 1.3 The Galactofuranosylation Pathway

The biosynthesis of complex glycans in eukaryotes occurs in the ER and Golgi and follows three consecutive steps: i) biosynthesis of nucleotide sugars, i.e. sugars substituted with a nucleoside phosphate (UDP, GDP) at the C1, generally in the cytosol; ii) translocation of nucleotide sugars into the ER or Golgi lumen by special transport proteins, the nucleotide sugar transporters (NST; see below); iii) transfer of sugar residues onto the acceptor by glycosyltransferases.

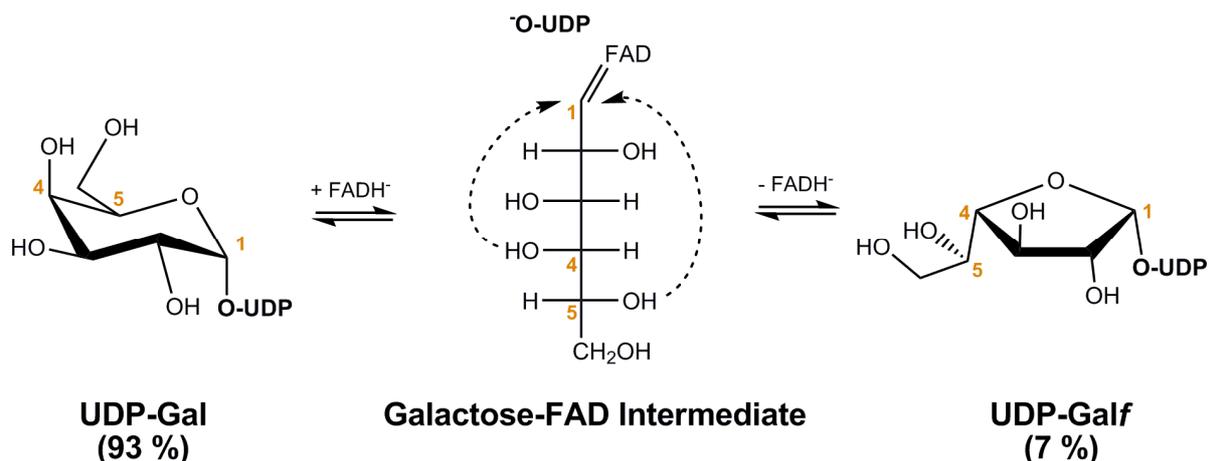
The biosynthesis of the nucleotide sugars UDP-Glc, UDP-Gal, UDP-GlcNAc, and GDP-Man is conserved between *Aspergillus* and humans (fig. 1-6). On the level of nucleotide sugar biosynthesis only the conversion of UDP-Gal into UDP-*Galf* is specific for *A. fumigatus*.



**Figure 1-6** Glycan biosynthesis pathways in *A. fumigatus*. Pathway elements are depicted as either specific for *A. fumigatus* (red) or shared with humans (black). Arrows denote enzyme reactions or metabolite transport, solid circles represent nucleotide sugar transporters, and solid squares glucan or chitin synthase complexes. Human and *A. fumigatus* enzyme homologs were identified from the KEGG database ([www.genome.jp/kegg/](http://www.genome.jp/kegg/)).

### 1.3.1 UDP-galactopyranose mutase

The enzymatic conversion of UDP-Gal to UDP-Galf was postulated early in fungi (Trejo et al. 1971) but the enzyme, UDP-galactopyranose mutase (UGM), was cloned only much later from *E. coli* (Nassau et al. 1996). The reaction follows a unique catalytic mechanism employing an FADH<sup>-</sup> cofactor which facilitates transient ring opening (Soltero-Higgin et al. 2004) (fig. 1-7). The mycobacterial UGM is required for arabinogalactan biosynthesis and survival and thus recognized as a promising new target in tuberculosis therapy. Recently, inhibitors against mycobacterial UGM showed *in vitro* activity against *Mycobacterium smegmatis* that was comparable to current antimycobacterial drugs (Dykhuizen et al. 2008). The first eukaryotic homologs were identified only recently in *A. fumigatus* and *Leishmania major* (Beverley et al. 2005, Bakker et al. 2005a). The conservation of the UGM gene allowed identification of homologs in many other eukaryotes, including nematodes, algae and tunicates, suggesting a more widespread occurrence of Galf than previously thought (Beverley et al. 2005). *L. major* is however the only eukaryote in which the requirement of UGM was studied. Despite absence of a detectable growth phenotype, the *L. major* UGM deletion mutant displayed attenuated virulence in a mouse infection model, probably due to the loss of surface lipophosphoglycan (Kleczka et al. 2007).



**Figure 1-7** Reaction mechanism of UDP-galactopyranose mutase. First an  $\text{FADH}^-$  molecule attacks the C1 of UDP-Gal and displaces UDP. A second bond to the C1 opens the ring and yields the depicted galactose-FAD intermediate. The ring is closed again by nucleophilic attack of the C4 or C5 hydroxyl group at the C1 resulting in either furanose (right) or pyranose (left) formation. Finally, UDP displaces  $\text{FADH}^-$  at the C1 which reforms the nucleotide sugar. The reaction is fully reversible and the relative equilibrium amounts are given in parentheses.

### 1.3.2 Galactofuranosyltransferases

Galactofuranosylation has mainly been studied in bacteria and besides UGM, a few galactofuranosyltransferases involved in the biosynthesis of bacterial polysaccharides have been characterized. The *Mycobacterium tuberculosis* enzymes *glfT* (Rv3808c) and *glfT2* (Rv3782) catalyze the formation of  $\beta$ 1,5- and  $\beta$ 1,6-Galf linkages in arabinogalactan (Kremer et al. 2001, Mikušová et al. 2006) and *WbbI* (*E. coli*) and *WbbO* (*Klebsiella pneumoniae*) are  $\beta$ -galactofuranosyltransferases involved in LPS O-antigen biosynthesis (Wing et al. 2006, Guan et al. 2001). All of them utilize UDP-Galf as a donor but no homologs could be found in the genome of *A. fumigatus* or any other eukaryote.

In contrast to bacteria, only one putative galactofuranosyltransferase of eukaryotic origin has been described. The *Leishmania* protein LPG1 presumably catalyzes the transfer of Galf to the nascent LPG chain forming a Galf- $\beta$ 1,3-Man linkage. LPG1 is localized in the Golgi and shows a typical glycosyltransferase topology with a short cytoplasmic N-terminus, a single transmembrane domain and a large C-terminal part in the Golgi lumen (Ryan et al. 1993, Sean Ha et al. 1996). Homologs were identified in other trypanosomatid species but not in any other eukaryote (Zhang et al. 2004).

### 1.3.3 Nucleotide sugar transporters

In all eukaryotes studied so far the processing of the N-glycan precursor, O-glycan biosynthesis and putatively also GIPC biosynthesis occur in the Golgi. It is thus reasonable to assume that the secretory pathway, most likely the Golgi apparatus, is the subcellular compartment of galactofuranosylation in

*Aspergillus*. As UGM is a cytosolic enzyme, a transport mechanism is required to translocate UDP-Galf from the cytosol into the lumen of the secretory pathway organelle. The negative charge of nucleotide sugars prevents passive diffusion through lipid membranes and thus transport is accomplished by a special class of transport proteins named nucleotide sugar transporters (NSTs). These are very hydrophobic type III transmembrane proteins with 8 or 10 membrane-spanning  $\alpha$ -helices and can be classified into currently six different families ([www.tcdb.org](http://www.tcdb.org)) (Jack et al. 2001). NSTs show weak primary sequence conservation and little is known on amino acid residues involved in substrate binding (Handford et al. 2006). Nevertheless it is still possible to extract candidate NST genes from annotated genomes, however in most cases their function cannot be predicted.

NSTs work as antiporters, i.e. they exchange one nucleotide sugar molecule for one equally charged nucleotide which is often the corresponding nucleoside monophosphate (e.g. UDP-Gal/UMP, GDP-Man/GMP) (Aoki et al. 2003, Segawa et al. 2005). Often NSTs are found to be monospecific with little functional redundancy between different NSTs. Consequently, the loss of an NST severely impairs glycosylation, as exemplified by the lethal GDP-Man transporter deficiency in several yeasts (Dean et al. 1997, Nishikawa et al. 2002a, Nishikawa et al. 2002b). Disease due to NST deficiency is also known in humans. For instance, a defective GDP-fucose transporter has been identified to cause type II leukocyte adhesion deficiency. This rare inherited disorder is characterized by reduced N-glycan fucosylation and results in recurrent bacterial infections and severe developmental abnormalities (Lübke et al. 2001).

### 1.4 Objectives

Bacterial infections can be easily treated with a range of antibiotics since vital cellular functions such as protein biosynthesis differ largely from the corresponding processes in human cells. Conversely, eukaryotic pathogens, comprising fungi and protozoan parasites, present a particularly challenging problem for drug design because of the similarity between the host cell and the pathogen cell. Therefore we only dispose of a limited number of drugs for the therapy of fungal and parasitic infections. Besides, the available drugs present low to moderate efficacy and are often toxic to human cells resulting in severe side effects during therapy.

Although fungal cells share most of the essential biosynthesis pathways with human cells, a few pathways exist that could possibly provide new drug targets. The fungal cell wall is an essential feature of fungal cells and consists mainly of glycans that are absent from human cells. Thus the corresponding glycan biosynthesis pathways appear as attractive source of new antifungal drugs. Indeed, the newest antifungals, the echinocandins, interfere with the synthesis of the main cell wall polysaccharide  $\beta$ 1,3-glucan and show good efficacy with only mild side effects.

Galactomannan is the second-most abundant polysaccharide in the *A. fumigatus* cell wall. It consists in roughly equal proportions of mannose and galactofuranose, the latter being entirely absent from

human cells. *Galf* has been known as component of cell surface molecules implicated in virulence or even viability of pathogenic microorganisms, such as mycobacterial arabinogalactan or lipophosphoglycan of *Leishmania major*. This suggested that *Galf* could likewise play a role in pathogenicity or viability of *A. fumigatus*, not least because *Galf* is also present in *A. fumigatus* glycoproteins and glycolipids.

To test this hypothesis was the main objective of this study. The recent identification of the UDP-galactopyranose mutase gene, *glfA*, in *A. fumigatus* allowed the generation of a targeted deletion mutant ( $\Delta glfA$ ) devoid of *Galf*. Various techniques were employed to prove absence of *Galf* on the surface glycans of the  $\Delta glfA$  strain. The consequences of the loss of *Galf* on the *in vitro* phenotype were studied and the virulence of the  $\Delta glfA$  mutant was assessed in a murine infection model of Invasive Aspergillosis.

Moreover, it was attempted to further define the galactofuranosylation pathway in eukaryotes. Unlike bacteria, eukaryotes likely need a UDP-*Galf* transporter for the biosynthesis of *Galf*-containing glycans. A suitable candidate gene, *glfB*, was selected from the *A. fumigatus* genome and its function analyzed by *in vitro* transport assays. The implication of *glfB* in galactofuranosylation of *A. fumigatus* was determined by analyzing the glycan structures and the phenotype of a  $\Delta glfB$  deletion mutant.

The results presented in this study allow to determine the potential of galactofuranose biosynthesis as a new antifungal drug target and furthermore help delineating the galactofuranosylation pathway in eukaryotes.

## Chapter 2 – Contribution of Galactofuranose to the Virulence of the Opportunistic Pathogen *Aspergillus fumigatus*

- Manuscript originally published in Eukaryotic Cell -

Philipp S. Schmalhorst,<sup>1</sup> Sven Krappmann,<sup>†2</sup> Wouter Verwecken,<sup>3</sup> Manfred Rohde,<sup>4</sup> Meike Müller,<sup>5</sup> Gerhard H. Braus,<sup>2</sup> Roland Contreras,<sup>3</sup> Armin Braun,<sup>5</sup> Hans Bakker,<sup>1</sup> and Françoise H. Routier<sup>1\*</sup>

Department of Cellular Chemistry, Hannover Medical School, Hannover, Germany<sup>1</sup>;

Department of Molecular Microbiology and Genetics, Georg August University, Göttingen, Germany<sup>2</sup>;

Department of Molecular Biology, Ghent University, and Department for Molecular Biomedical Research, VIB, Ghent, Belgium<sup>3</sup>;

Department of Microbial Pathogenicity, Helmholtz Centre for Infection Research, Braunschweig, Germany<sup>4</sup>; and Department of Immunology, Allergology and Immunotoxicology, Fraunhofer Institute of Toxicology and Experimental Medicine, Hannover, Germany<sup>5</sup>

Received 27 March 2008 / Accepted 9 June 2008

EUKARYOTIC CELL, Aug. 2008, p. 1268–1277 Vol. 7, No. 8

doi:10.1128/EC.00109-08

Copyright © 2008, American Society for Microbiology.

## Preface – About this manuscript

Prior to this study the *glfA* gene encoding a homolog of bacterial UDP-galactopyranose mutase (UGM) was identified in *A. fumigatus* by our research group and others (Bakker et al. 2005a, Beverley et al. 2005). The following part of my work aimed at generating an *A. fumigatus glfA* deletion mutant ( $\Delta glfA$ ) to determine the consequences of the loss of *Galf* for viability and virulence of this fungus.

For this purpose, two *A. fumigatus* mutant strains were generated: the  $\Delta glfA$  strain, in which *glfA* of wild type *A. fumigatus* was replaced by a bifunctional selection marker, and *glfA\**, in which the selection marker from  $\Delta glfA$  was replaced again with a modified wild type *glfA* allele. Subsequently, the two mutant strains were analyzed for the presence of *Galf* by a combination of immunochemical, chromatographic and electrophoretic techniques. Several viability parameters were recorded and quantified, including growth, sporulation, and thermotolerance. Further analyses included an antifungal susceptibility assay and electron microscopy studies of the cell wall. Finally, the virulence of the mutant *A. fumigatus* strains was determined in a experimental mouse infection model and correlated to the *in vivo* growth rate by quantitative PCR.

My contributions to this manuscript comprised the generation of the two *A. fumigatus* mutant strains  $\Delta glfA$  and *glfA\**, the characterization of the macroscopic phenotype, the animal studies, and a part of the experiments concerning the molecular phenotype. Prof. Routier and I wrote the paper.

### ABSTRACT

The filamentous fungus *Aspergillus fumigatus* is responsible for a lethal disease called invasive aspergillosis that affects immunocompromised patients. This disease, like other human fungal diseases, is generally treated by compounds targeting the primary fungal cell membrane sterol. Recently, glucan synthesis inhibitors were added to the limited antifungal arsenal and encouraged the search for novel targets in cell wall biosynthesis. Although galactomannan is a major component of the *A. fumigatus* cell wall and extracellular matrix, the biosynthesis and role of galactomannan are currently unknown. By a targeted gene deletion approach, we demonstrate that UDP-galactopyranose mutase, a key enzyme of galactofuranose metabolism, controls the biosynthesis of galactomannan and galactofuranose containing glycoconjugates. The *glfA* deletion mutant generated in this study is devoid of galactofuranose and displays attenuated virulence in a low-dose mouse model of invasive aspergillosis that likely reflects the impaired growth of the mutant at mammalian body temperature. Furthermore, the absence of galactofuranose results in a thinner cell wall that correlates with an increased susceptibility to several antifungal agents. The UDP-galactopyranose mutase thus appears to be an appealing adjunct therapeutic target in combination with other drugs against *A. fumigatus*. Its absence from mammalian cells indeed offers a considerable advantage to achieve therapeutic selectivity.

### INTRODUCTION

The filamentous fungus *Aspergillus fumigatus* is the primary cause of invasive aspergillosis, an often fatal condition affecting people with a weakened immune system. Along with the immunocompromised population, the incidence of invasive aspergillosis is constantly growing but therapy remains problematic. The sterol binding polyene amphotericin B and ergosterol biosynthesis inhibitor itraconazole have long been the drugs of choice for treatment of this infection. But because of their higher efficacy and lower toxicity, new triazoles such as voriconazole or posaconazole are supplanting these drugs (28,33).

Additionally, a novel class of antifungal agents called the echinocandins provides further options for treatment. These compounds inhibit the synthesis of  $\beta$ 1,3-glucan, a major cell wall component with resultant osmotic instability and lysis (12). Their minimal toxicity and synergistic activity with voriconazole and amphotericin B make them particularly attractive for combination therapy although clinical validation is still awaited (35,33). Despite these advances in therapy, invasive aspergillosis is often associated with significant morbidity and mortality emphasizing the need for novel therapeutic strategies based on the fundamental knowledge of *A. fumigatus* pathogenesis.

The development of echinocandins illustrates the viability of targeting enzymes involved in cell wall biosynthesis and encourages the development of chitin synthesis inhibitors. Like glucan and chitin, galactomannan is an abundant component of *A. fumigatus* cell wall (4). This polysaccharide composed of a linear mannan core branched with short  $\beta$ 1,5-linked galactofuranose (Galf) chains (22) is covalently bound to the cell wall  $\beta$ 1,3-glucan, anchored to the lipid membrane by a glycosylphosphatidylinositol (GPI), or released in the environment during tissue invasion or growth in

culture (3,9,14). Besides being an abundant component of the extracellular matrix, secreted galactomannans are used for serological diagnostic of invasive aspergillosis (1). The monosaccharide Galf has also been reported in the N- and O-glycans of some glycoproteins as well as the glycosphingolipids of *A. fumigatus* (47,29,23,41) and represents thus an important constituent of this fungus cell wall. Galf is otherwise infrequent in natural compounds but prevalent in pathogens. Moreover since it is absent from higher eukaryotes and involved in the survival or virulence of various bacteria, the enzymes involved in the biosynthesis of Galf are considered as attractive drug targets (32,34).

Our understanding of Galf metabolism in eukaryotes is limited. Galf is most likely incorporated into cell surface components by specific galactofuranosyltransferases using UDP-Galf as donor. The work of Trejo and colleagues in early 1970s already suggested the existence of an enzyme converting UDP-galactopyranose into UDP-galactofuranose involved in the biosynthesis of fungal cell wall (48). This enzyme named UDP-galactopyranose mutase (UGM) and encoded by the *glf* gene was first described in bacteria (17,50,30) and lately in several eukaryotic pathogens including *A. fumigatus* (2,5). UGM is to date the only characterised enzyme involved in the biosynthesis of galactofuranose containing molecules in eukaryotes whereas several galactofuranosyl-transferases have been described in bacteria (15,19,51,27). The identification of this enzyme, highly conserved amongst lower eukaryotes and present in many fungi, enables studies on the biological role of galactofuranose in these organisms. The present report highlights the role of galactofuranose in *Aspergillus fumigatus* growth and virulence.

**Table 1.** DNA oligonucleotides used in this study.

Oligonucleotide	Sequence (5'->3'; restriction site underlined)	Description (Restriction site)
PS1	ATAAGCGGCCGCAAGCTGGGAACCGGATTCAA	5' flanking region pΔglfA reverse (NotI)
PS12	TATACCGCGGCTGCCAAGCTATCAGTTTCC	5' flanking region pΔglfA sense (SacII)
PS3	<u>ATCCGGT</u> GCTCAGGTATTCCGCA	3' flanking region pΔglfA sense (EcoRV)
PS4	ATCC <u>ATCGAT</u> CATATCCTATGCGGTCTCAG	3' flanking region pΔglfA reverse (ClaI)
PS66A	TTACGCATTCCCAGCAGTTG	Southern Blot probe 1 sense
PS67A	TGCGCTGTGATGAATGGTGT	Southern Blot probe 1 reverse
PS68A	TCCACAATACGTCCCCTACA	Southern Blot probe 2 sense
PS69A	GTATGAACCCTCTCCCAATG	Southern Blot probe 2 reverse
PS20	AAGTTCGTTGCGTCAGTCCA	Southern Blot probe 3 sense
PS21	TCGATGTGTCTGTCTCTCC	Southern Blot probe 3 reverse
PS23s	ATGCCGCTCTCGAGGCTCGT	Site-directed mutagenesis <i>glfA</i> * sense (XhoI)
PS23r	CACGAGCCTCGAGAGCGGCA	Site-directed mutagenesis <i>glfA</i> * reverse (XhoI)
PS28	ATAT <u>GCGGCCG</u> CAAACAGGAGCGAAGTAGT	5' flanking region pglfA* sense (NotI)
PS31	ATAT <u>CCCGGG</u> AGTTTGGTGTGTGGTAGGT	3' flanking region pglfA* reverse (XmaI)
PS78	CGTGCTATCGTACCTTGTGCTT	18S rRNA gene fragment sense
PS79	AACTCAGACTGCATACTTTCAGAACAG	18S rRNA gene fragment reverse
Probe	FAM-CCCGCCGAAGACCCCAACATG-TAMRA <sup>a</sup>	qPCR hybridization probe

<sup>a</sup>FAM, carboxyfluorescein; TAMRA, carboxytetramethylrhodamine.

## MATERIAL AND METHODS

### Strains, media and growth conditions.

*Aspergillus fumigatus* clinical isolate D141 (38) was used as wild type strain in this study. All strains were grown at 37 °C on *Aspergillus* minimal medium (AMM) containing 1 % D-glucose as carbon source and 70 mM NaNO<sub>3</sub> as nitrogen source (36) unless otherwise stated. Phleomycin or 5-fluoro-2'-deoxyuridine (FUDR) were added for selection purposes at 30 µg/mL and 100 µM respectively.

**Generation of *A. fumigatus* mutant strains.** The 5' and 3' flanking regions (1.5 and 2 kb respectively) of *A. fumigatus glfA* coding sequence were amplified from genomic DNA by PCR with primers PS12/PS1 and PS3/PS4 (Table 1) respectively and cloned into the pBluescript II SK(-) vector (Stratagene) using the restriction sites SacII/NotI and EcoRV/ClaI. A SpeI/NotI fragment released from pSK269 containing the phleo/tk blaster (18) was then inserted between the two fragments to obtain the disruption plasmid pΔglfA. For reconstitution of the *glfA* gene locus, the plasmid pglfA\* was constructed as follows. The phleo/tk blaster of pΔglfA was first replaced with the original *A. fumigatus glfA* gene by homologous recombination in *E. coli* strain YZ2000 (Gene

Bridges, Leimen, Germany). A single point mutation was introduced by site-directed mutagenesis. Briefly, non-methylated plasmid DNA was generated from a methylated parent plasmid by Phusion DNA-Polymerase (NEB) using complementary primers that both carried the desired mutation (PS23s/PS23r, Table 1). Prior to transformation the parental, methylated DNA strand was specifically cleaved by DpnI to selectively obtain transformants that harbored the mutated plasmid. Thus, codon 130 of *glfA* coding sequence (GenBank Accession number AJ871145) was changed from CTT to CTC which generated a new XhoI restriction site. Since gene reconstitution by homologous recombination could not be obtained with this construct, 5' and 3' flanking regions were extended to 5 kb by replacement with re-cloned PCR fragments (primer pairs PS28/PS1 and PS3/PS31) to obtain the final pglfA\* construct.

The pΔglfA and pglfA\* plasmids were linearised (KpnI/SacII) before polyethylene glycol-mediated fusion of protoplasts as described in (37). Transformants were grown on AMM plates containing 1.2 M sorbitol as osmotic stabiliser under appropriate selection conditions and singled out twice before further analysis. Accurate gene deletion and reconstitution were confirmed by southern hybridisation. Southern probes were amplified from genomic DNA using primer pairs PS66A/PS67A, PS68A/PS69A and PS20/PS21. All primer sequences are provided in Table 1.

**Western Blots.** Cell wall glycoproteins and soluble polysaccharides were extracted from 30 mg ground *A. fumigatus* mycelium by incubation in 1 mL sample buffer (15 % glycerol, 100 mM Tris/HCl pH 6.8, 1.5 % SDS, 0.25 %  $\beta$ -mercaptoethanol, 0.025 % bromophenol blue) for 12 min at 95 °C. 20  $\mu$ l of the supernatant were separated on a 10 % SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The monoclonal antibody EB-A2 (42) conjugated to horseradish peroxidase (HRP) from the Platelia *Aspergillus* Test (Bio-Rad, Hercules, CA, USA) or HRP-coupled lectin Concanavalin A (ConA, Sigma-Aldrich) were used in a 1:50 dilution or at 0.2  $\mu$ g/mL respectively. HRP activity was visualised by an enhanced chemiluminescence system (Pierce, Rockford, IL, USA).

**N-glycan analysis.** N-glycans of secreted glycoproteins in the supernatant of an *A. fumigatus* liquid culture were analyzed after Peptide N-Glycosidase F (PNGase F) mediated release and 8-amino-1,3,6-pyrenetrisulfonic acid (APTS) labeling by capillary electrophoresis as recently described (20). Separation was carried out on a 4-capillary electrophoresis DNA Sequencer (3130 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). Oligomaltose and bovine RNase B N-glycans (Prozyme, San Leandro, CA, USA) served as reference oligosaccharides.

**Purification and analysis of glycosylinositol-phosphoceramides (GIPCs).** Mycelia (0.5 g) ground in liquid nitrogen with a mortar and pestle were disrupted by sonication in 6 mL of  $\text{CHCl}_3/\text{MeOH}$  1:1. After addition of 3 mL  $\text{CHCl}_3$  (to obtain a  $\text{CHCl}_3/\text{MeOH}$  ratio of 2:1), GIPCs were extracted at room temperature for at least 15 min on a rotating shaker. 3 mL MeOH were then added to lower the density and the mixture centrifuged for 10 min at 2000 g to remove insoluble material. Chloroform and  $\text{H}_2\text{O}$  were then added to the supernatant to obtain a biphasic system with an 8/4/3 ratio of  $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ . After centrifugation for 10 min at 2000 g, GIPCs contained in the upper phase were collected and applied to a C18/SepPak cartridge (Waters, Eschborn, Germany) pre-equilibrated with 5 mL  $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$  3/48/47. After washing of the column with 20 mL  $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$  3/48/47, glycolipids were eluted with 5 mL methanol and dried under a stream of nitrogen. High performance thin layer chromatography and immunostaining with the monoclonal antibody MEST-1 were carried out as previously described (47).

**Growth assay.** For radial growth measurement, a 10  $\mu$ l drop containing 10,000 *A. fumigatus* conidia was placed in the centre of an agar plate containing either minimal (AMM) or complete medium (potato dextrose agar, Becton Dickinson Difco, Heidelberg, Germany). Plates were incubated at various

temperatures and colony diameters were measured twice daily.

**Antifungal susceptibility testing.** The reference broth microdilution test was applied for *A. fumigatus* antifungal susceptibility testing (21). Each antifungal stock was diluted in 200  $\mu$ l double-strength RPMI 2%G (RPMI 1640 liquid medium buffered with 165 mM 4-Morpholinepropane-sulfonic acid (MOPS) to pH 7.0 and supplemented with 2 % glucose) to obtain the highest concentration to be tested. Nine serial 1:2 dilutions in double-strength RPMI 2%G were made and to each dilution an equal volume (100  $\mu$ l) of an *A. fumigatus* spore solution ( $2.5 \cdot 10^5$ /mL in water) was added. Microtitre plates were incubated at 35 °C and fungal growth in each well was read out visually after three days and compared to control wells that contained no antifungal.

**Field emission scanning electron microscopy.** For morphological studies and measurements of the cell wall thickness *A. fumigatus* wild type and  $\Delta$ *glfA* mutant mycelium were fixed in 5 % formaldehyde and 2 % glutaraldehyde in cacodylate buffer (0.1 M cacodylate, 0.01 M  $\text{CaCl}_2$ , 0.01 M  $\text{MgCl}_2$ , 0.09 M sucrose, pH 6.9) for 1 h on ice. Samples were washed several times with cacodylate buffer and subsequently with TE-buffer (20 mM Tris/HCl, 1 mM EDTA, pH 6.9) before dehydration in a graded series of acetone (10, 30, 50, 70, 90, 100 %) on ice for 15 min per step. Samples in the 100 % acetone step were allowed to reach room temperature before another change in 100 % acetone. Samples were then subjected to critical-point drying with liquid  $\text{CO}_2$  (CPD 30, Balzers, Liechtenstein). Dried samples were then mounted onto conductive carbon adhesive tabs on an aluminium stub and sputter coated with a thin gold film (SCD 40, Balzers Union, Liechtenstein). For cell wall thickness measurements mycelium was fractured by pressing another conductive carbon adhesive tab covered stub onto the sample and separating both stubs immediately thereafter. Fractured hyphae were also made conductive by sputter coating with a gold film before examination in a field emission scanning electron microscope (Zeiss DSM 982 Gemini) using the Everhart Thornley SE-detector and the inlens SE-detector in a 50:50 ratio at an acceleration voltage of 5 kV and at calibrated magnifications.

**Mouse infection model.** A low-dose mouse infection model of Invasive Aspergillosis for Balb/c mice which had been established previously (25) was essentially used. Immunosuppressive state was established by intraperitoneal injections of 100 mg/kg cyclophosphamide (Endoxan, Baxter Chemicals) on days -4, -1, 0, 2, 5, 8 and 11 and a single subcutaneous dose (200 mg/kg) of a cortisone acetate suspension (Sigma) on day -1. Groups of 20 mice were infected intranasally with 20,000 conidia of wt,  $\Delta$ *glfA* or *glfA*\* strain on day 0. The control group received PBS only. Survival was monitored for 13 days after infection and

moribund animals were sacrificed. Coincidence of severely reduced mobility, low body temperature and breathing problems was defined as moribundity criterion. Statistical analysis of survival data was carried out using the logrank test implemented in Prism 4 (GraphPad Software, San Diego, CA, USA). For quantification experiments, groups of three to five animals were killed two, four and six days after infection and lungs were removed for further analysis.

**Lung histology.** Female Balb/c mice were immunosuppressed and infected as described above. The animals were killed after 5 days and their lungs removed and fixed in 4 % PBS-buffered paraformaldehyde over night. Tissue samples were dehydrated through a series of graded alcohols, cleared with xylene and embedded in paraffin. Tissue sections (5  $\mu$ m) were stained with either hematoxylin/eosin or by the Periodic Acid Schiff (PAS) method for visualization of fungal cell walls. Photomicrographs were taken with an Axiovert 200 M microscope (Zeiss, Germany) at 10x and 20x magnification.

### Preparation of genomic DNA from mouse lungs.

Tissue homogenisation was modified according to (7). Immediately after removal, mouse lungs were transferred to a 2 mL screw-cap containing 1.4 mm ceramic beads (Lysing matrix D, Qbiogene, Irvine, CA, USA) and 20 % glycerol/PBS. Tissue was disrupted using a FastPrep FP120 instrument (Qbiogene) for 3 times 30 seconds at speed 5 with intermediate cooling on ice. The disrupted tissues were further homogenised with approx. 250 mg acid-washed glass beads (0.45-0.5 mm, Sigma-Aldrich) by vortexing three times for 30 s with intermediate cooling on ice. The DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) was used to extract genomic DNA from an equivalent of 8 % of the starting tissue material of this homogenate. DNA was finally recovered in 200  $\mu$ l elution buffer.

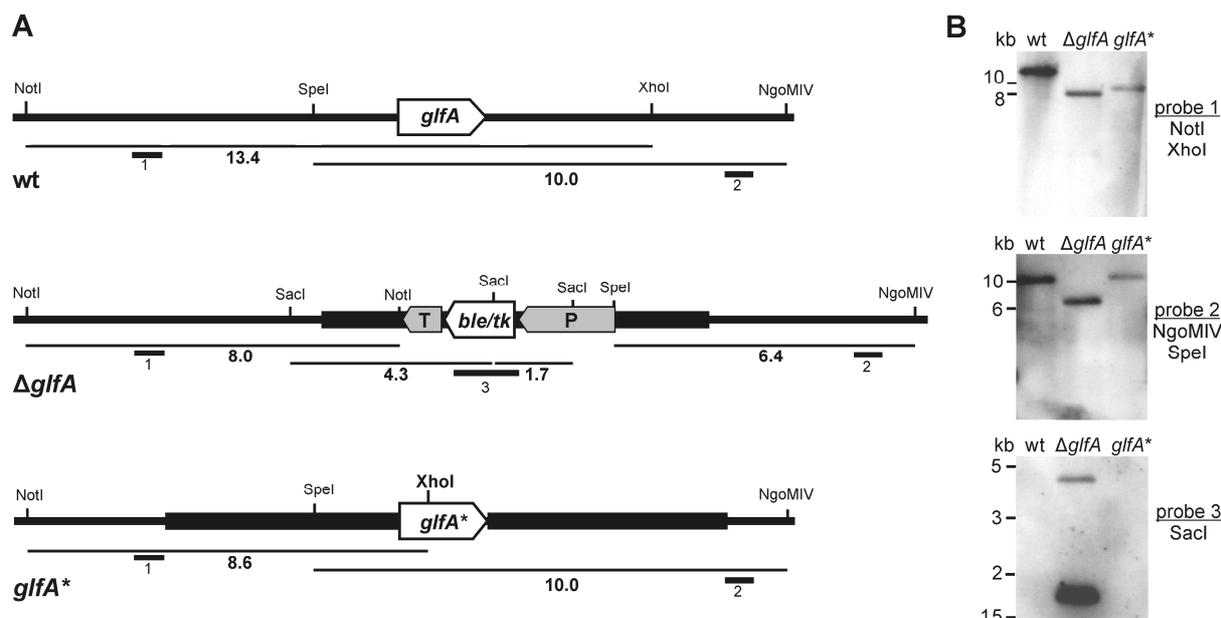
**Quantitative PCR (qPCR).** Quantitative PCR was carried out essentially as described previously (7). Primers for amplification of a 18S rRNA gene (GenBank Accession number AB008401) fragment specific for *A. fumigatus* and a hybridisation probe labeled with carboxyfluorescein (FAM; 5' end) and carboxytetramethylrhodamine (TAMRA; 3' end) were designed using Primer Express software version 3.0 (Applied Biosystems; Table 1). qPCR reactions were performed in a 7500 Fast Real-Time PCR System instrument (Applied Biosystems) loaded with MicroAmp optical 96-well plates sealed with Optical Adhesive Cover (Applied Biosystems). Each qPCR reaction (20  $\mu$ l) contained 5  $\mu$ l sample DNA, 250 nM dual-labeled hybridisation probe, 500 nM primers, 250  $\mu$ g/mL BSA and TaqMan Fast Universal Master Mix (Applied Biosystems) containing dNTPs, buffer and the fluorescent dye carboxyrhodamine (ROX) as

a passive reference. Real-time PCR data was acquired using Sequence Detection Software v1.3.1. FAM/ROX fluorescence ratio was recorded at every cycle and a  $C_T$  value was assigned to each reaction, defining the cycle number at which the FAM/ROX signal surpassed an automatically defined threshold.  $C_T$  values were corrected for differences in yield of genomic DNA by normalization to DNA concentration of a control sample using the formula  $C_{T, \text{norm}} = C_{T, \text{measured}} + \log_2([\text{DNA}]_{\text{sample}} / [\text{DNA}]_{\text{control}})$  (7). Translation of sample  $C_{T, \text{norm}}$  values into rDNA gene copy numbers was done as follows:  $C_T$  values of serial 1:10 dilutions containing  $N = 300$  to 300,000 molecules (calculated from  $M_w$  and DNA concentration determined by  $\text{OD}_{260}$ ) of a plasmid bearing the cloned *A. fumigatus* 18S rDNA gene were plotted against  $N$  to generate a calibration curve which was then used to assign a rDNA copy number to a given sample  $C_{T, \text{norm}}$  value. Conidial equivalents were calculated from gene copy numbers by means of uninfected tissue samples that were spiked with defined numbers of conidia before tissue homogenisation (7). Samples, controls and standards were analyzed in triplicates.

## RESULTS

**Deletion and reconstitution of the *glfA* gene in *A. fumigatus*.** To begin investigating the role of *Galf* in *Aspergillus fumigatus* biology, we deleted the gene encoding UGM (GenBank locus tag AJ871145) and named it *glfA* following the recommendations for gene naming in *Aspergillus*. To do this, we generated a deletion plasmid containing the regions flanking the *glfA* coding sequence separated by the bifunctional selection cassette *phleo/tk* that confers both resistance to phleomycin and sensitivity to 5-fluoro-2'-deoxyuridine (FUDR) (18). This construct was used to transform protoplasts of *A. fumigatus* clinical strain D141 which served as wild type (wt) and phleomycin resistant transformants were analyzed by Southern Blot using several digoxigenin-labeled probes (Fig. 1). One of the clones that had undergone the desired gene replacement (Fig. 1) was selected for further analysis and named  $\Delta glfA$ .

The selected disruptant was further subjected to protoplast transformation with a large DNA fragment encompassing the *glfA* coding sequence which contained a single translationally silent nucleotide exchange that generated an *XhoI* restriction site. Gene replacement in the transformants resulted in the reconstitution of the *glfA* locus (Fig. 1) as detected by FUDR resistance and proven by Southern Blot analysis for a selected clone named *glfA\** (Fig. 1B). The silent mutation introduced in the reconstituted strain allowed differentiating between wild type and *glfA\** as demonstrated in figure 1B (top) and thus enabled us to rule out contamination by the wt strain. The reconstitution of the *glfA* locus ensures that any phenotype observed in the  $\Delta glfA$  strain can be reverted



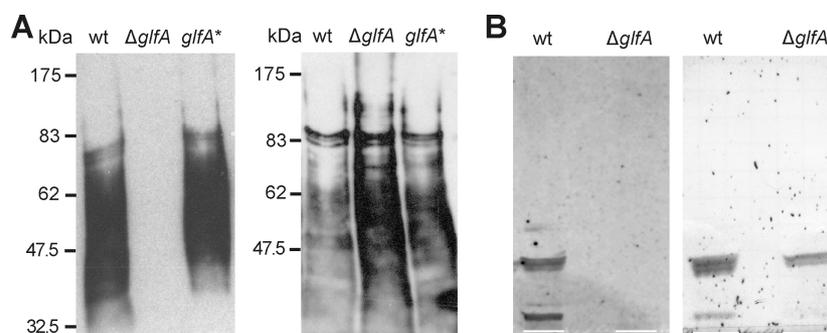
**Figure 1** A) Schematic representation of the chromosomal *glfA* locus in wild type,  $\Delta glfA$  and reconstituted wild type *glfA\**. Thick black bars show flanking regions used for homologous recombination. The positions of probes (1-3) used for Southern Blot along with respective restriction fragments (size in kb) are indicated. B) Southern Blots of genomic DNA digested with indicated restriction enzymes and hybridised to different digoxigenin-labeled probes. wt, wild type; *ble/tk*, phleomycin resistance/thymidine kinase fusion gene; P, promoter; T, terminator.

and hence be securely attributed to the loss of the *glfA* gene.

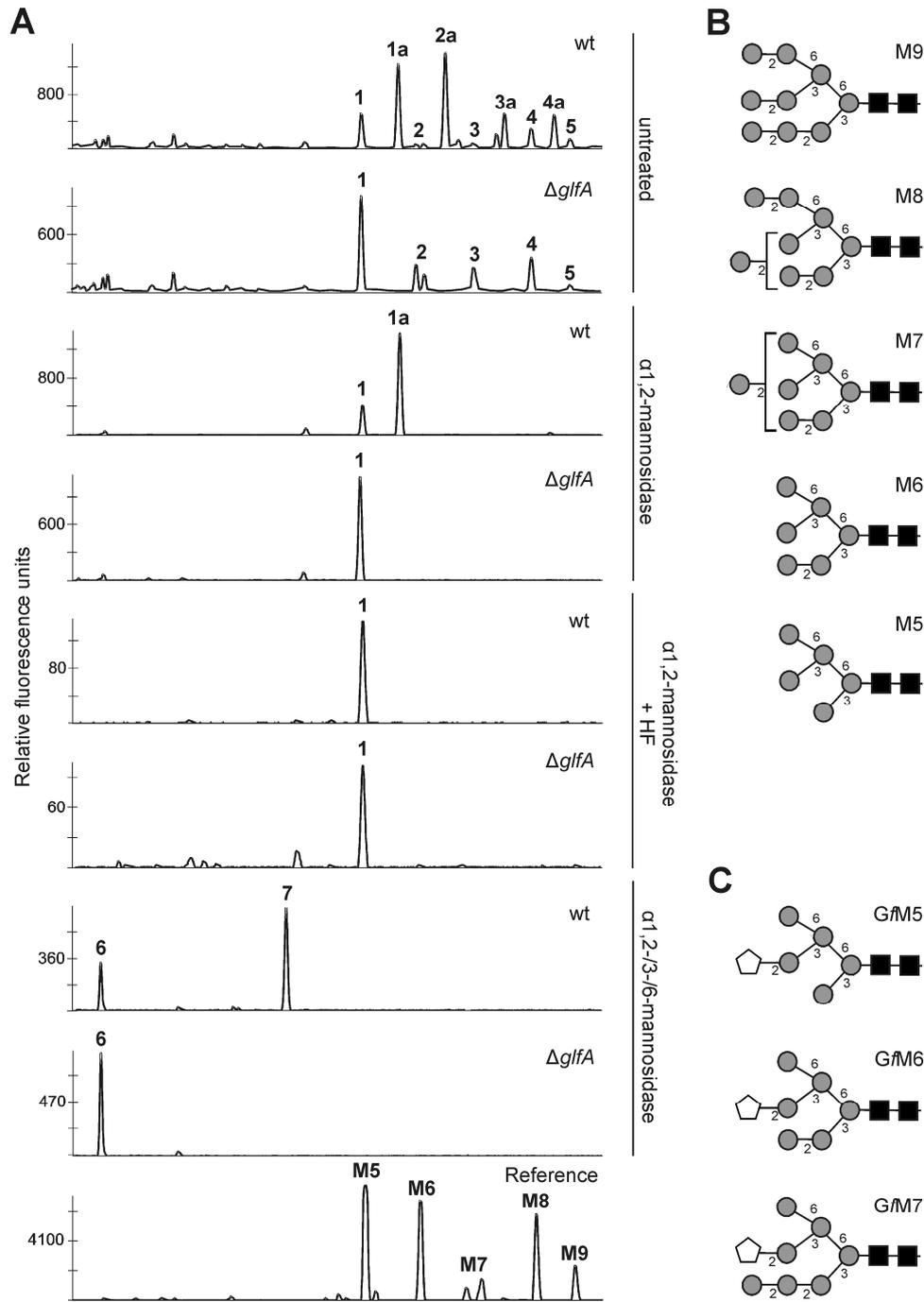
**Galf is absent from the *A. fumigatus*  $\Delta glfA$  mutant.** To confirm that deletion of *glfA* indeed altered the expression of Galf containing glycoconjugates, aqueous mycelial extracts were tested for reactivity to the Galf-specific monoclonal antibody (mAb) EB-A2. This antibody recognizes preferably  $\beta$ 1,5-linked Galf-residues that are present in all forms of galactomannan (cell-wall bound, membrane bound or secreted) (42) as well as in some O-glycans (23). Moreover a second binding epitope, Galf( $\beta$ 1,2)Man, which is part of galactofuranosylated N-glycans has been postulated (29). Thus, EB-A2 can be used to simultaneously detect galactomannan and galactofuranosylated glycoproteins. Western blot

analysis of wt and *glfA\** total mycelial extracts labeled with horseradish peroxidase (HRP) conjugated EB-A2 revealed a smear migrating around 40 to 80 kDa in accordance with previous findings (42). In contrast, the  $\Delta glfA$  mycelial extract was not stained at all, indicating absence of Galf in the galactomannan and glycoproteins of this mutant (Fig. 2A, left). In contrast, Concanavalin A (ConA) used as loading control bound slightly better to the  $\Delta glfA$  extract than to those of wt and *glfA\** (Fig. 2A, right). The lack of Galf in the  $\Delta glfA$  mutant might increase the accessibility of the mannan for ConA and thus could explain this finding.

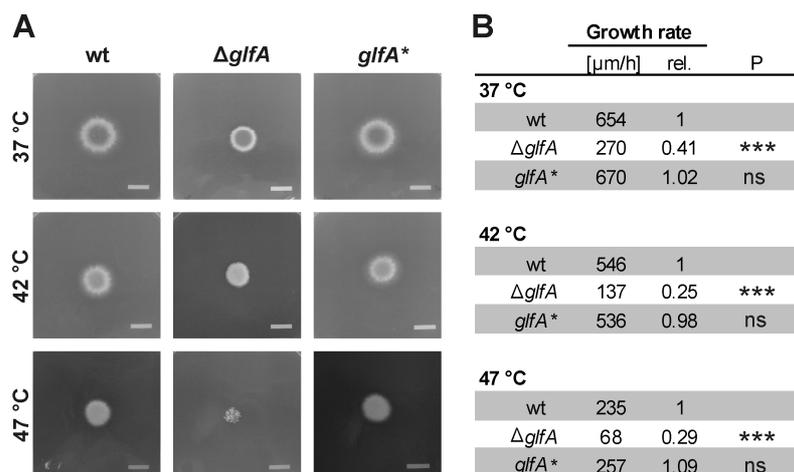
Similarly, the absence of Galf in  $\Delta glfA$  glycolipids was shown by the absence of reactivity to the monoclonal antibody MEST-1. This antibody that recognizes  $\beta$ 1,3- and  $\beta$ 1,6-linked Galf residues (43)



**Figure 2** A) Western Blots of *A. fumigatus* mycelial extracts containing glycoproteins and cell wall polysaccharides stained with horseradish peroxidase conjugates of either Galf-specific mAb EB-A2 (left) or  $\alpha$ -mannose binding lectin Concanavalin A (right). B) *A. fumigatus* GIPCs separated by High Performance Thin Layer Chromatography and stained with Galf-specific mAb MEST-1 (left) or orcinol (right). White bars indicate the origin.



**Figure 3** A) Electropherograms of fluorescently labeled N-glycans enzymatically released from secreted *A. fumigatus* glycoproteins. Oligosaccharides from wt and  $\Delta glfA$  were either untreated (panels 1 and 2), digested with *T. reesei*  $\alpha 1,2$ -exomannosidase with or without hydrofluoric acid treatment (panels 3 to 6) or digested with Jack Bean  $\alpha$ -mannosidase (panels 7 and 8). Bovine RNase B N-glycans served as reference (panel 9). B) Structures of bovine RNase B reference N-glycans. C) Major N-glycans found on *A. niger*  $\alpha$ -galactosidase and  $\alpha$ -glucosidase (44,45). Black squares, N-acetylglucosamine; grey circles, mannose; white pentagon, galactofuranose.



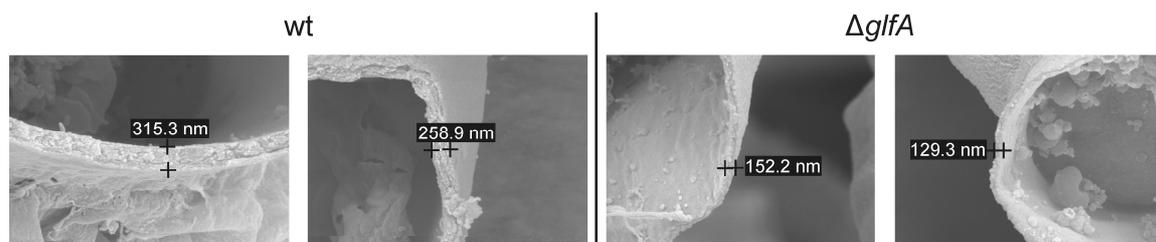
**Figure 4** A) Colony morphology of *A. fumigatus* on minimal agar after two days. White bars represent 1 cm. B) Absolute and relative (compared to wild type) growth rates derived from three independent experiments. P value from a t test indicates statistical significance (\*\*\*,  $P < 0.001$ ; ns, not significant).

labeled several *A. fumigatus* glycosylinositolphosphoceramides (GIPCs) after separation by high-performance thin layer chromatography as previously shown (47) but did not label glycosphingolipids extracted from the  $\Delta glfA$  mutant (Fig. 2B, left). The upper bands observed in this panel might be attributed to GIPCs containing 1 or 2 Galf and 2 or 3 mannose residues as recently described (41,47). In addition, Simenel et al. reported an unusual GIPC containing a Galf residue substituted by a choline phosphate. The lower band present in the wt chromatogram could correspond to a similar GIPC. Staining of glycolipids by orcinol was used as loading control (Fig. 2B, right). The simpler  $\Delta glfA$  chromatogram is compatible with the absence of Galf containing GIPCs. The uppermost band observed in the chromatogram most probably correspond to  $\text{Man}(\alpha 1,3)\text{Man}(\alpha 1,2)\text{Ins-P-Cer}$  while the band just beneath could be attributed to  $\text{Man}(\alpha 1,2)\text{Man}(\alpha 1,3)\text{Man}(\alpha 1,2)\text{Ins-P-Cer}$  (47). The chromatograms obtained from the reconstituted mutant *glfA*\* and wt were undistinguishable (data not shown).

Additionally, N-glycans enzymatically released from *A. fumigatus* secreted proteins were analyzed by capillary electrophoresis after fluorescent labeling (20,8). The profiles obtained are presented in figure 3A (panels 1 and 2). The peaks labeled 1, 2, 3, 4 and 5 present in both electropherograms co-migrated with reference oligosaccharides M5 to M9 (Fig. 3A; panel 9 and Fig. 3B). Moreover, digestion of these N-glycans by *Trichoderma reesei*  $\alpha 1,2$ -mannosidase indicates that 2, 3, 4 and 5 arise from substitution of oligosaccharide 1 with one to four mannose residues linked in  $\alpha 1,2$  (Fig. 3A; panels 3 and 4). The profile obtained with wt N-glycans (Fig. 3A; panel 1) presents four additional peaks labeled 1a, 2a, 3a and

4a that were absent from *glfA* N-glycans. The retention times of these peaks suggest that they arise from substitution of oligosaccharides 1-4 with a single Galf residue. The presence of a terminal non-reducing Galf residue in *A. fumigatus* N-glycans has been previously reported (9) and was demonstrated by hydrofluoric acid (HF) treatment of the N-glycans after *Trichoderma reesei*  $\alpha 1,2$ -mannosidase digestion (Fig. 3A, panels 5 and 6). This mild acid treatment, known to release Galf, entirely converted oligosaccharide 1a into oligosaccharide 1 (Fig. 3A; panels 3 and 5). In contrast, HF treatment did not change the profile of  $\Delta glfA$  N-glycans digested with  $\alpha 1,2$  mannosidase (Fig. 3A, panels 4 and 6).

Interestingly, the comparison of wt and  $\Delta glfA$  N-glycans digested with *T. reesei*  $\alpha 1,2$  mannosidase or Jack Bean mannosidase helps positioning the Galf residue. Alpha1,2-mannosidase treatment converted the oligosaccharides 2a, 3a and 4a into 1a while the oligosaccharides 2, 3 and 4 generated 1 (Fig. 3A; compare panels 1 and 2 with 3 and 4). This indicates that the Galf residue does not protect any mannose residues from the exomannosidase digestion and thus does not substitute an  $\alpha 1,2$ -linked mannose (Fig. 3A; panels 3 and 4). Moreover, Jack Bean mannosidase digestion of wt N-glycans resulted in a major peak (peak 7), attributed to  $\text{Galf/Man}_3\text{GlcNAc}_2$  from its retention time, in addition to  $\text{Man}_1\text{GlcNAc}_2$  (peak 6) expected from digestion of high-mannose type N-glycans (Fig. 3A; panels 7 and 8). These experiments do not allow for the determination of the detailed N-glycan structure but suggest that they resemble the N-glycans of *A. niger*  $\alpha$ -glucosidase and  $\alpha$ -galactosidase (44,45). More importantly, these experiments demonstrate the absence of Galf in the  $\Delta glfA$  N-glycans.



**Figure 5** Field emission scanning electron micrographs of cross-fractured mycelial walls of *A. fumigatus* wt and  $\Delta glfA$ . Panel 1 and 3 display the highest measurement of cell wall thickness. Panel 2 and 4 present two measurements illustrating the 50% reduction of  $\Delta glfA$  cell wall thickness.

**Loss of Galf alters morphology and growth of *A. fumigatus*.** The  $\Delta glfA$  strain exhibited a marked growth defect on solid minimal media or complete media when compared to wt. This effect could be observed for a wide range of temperatures (Fig. 4) and was statistically different in all cases ( $P < 0.001$ ,  $t$  test,  $n = 3$ ). The most severe effect was found at 42 °C with a 75 % reduction in radial growth (Fig. 4C). In parallel,  $\Delta glfA$  conidiation was diminished by 90 % at 37 °C and was almost absent at 42 °C. In contrast, the onset and rate of germination of wt,  $\Delta glfA$  and  $glfA^*$  conidia were similar. In minimal media at 37 °C, the conidia of all strains started forming germ tubes at 3.2 h and reached 100 % germination within 8 to 9 h (data not shown).

Scanning electron micrographs of intact mycelium, conidiophores and conidia of  $\Delta glfA$  did not reveal any obvious morphologic differences. However, the observation of fractured mycelium revealed a marked reduction of the  $\Delta glfA$  cell wall thickness (Fig. 5). Measurements indicated that the cell wall of wt *A. fumigatus* varies from 85 to 315 nm which is in good agreement with earlier findings (39). In contrast,  $\Delta glfA$  cell wall thickness ranged from 85 to 150 nm. The mean values ( $\pm$  standard deviation) of cell wall thickness obtained from 25 measurements were 227.5 nm ( $\pm 15.98$  nm) and 109.7 nm ( $\pm 11.3$  nm) for wt and  $\Delta glfA$  hyphae respectively. The cell wall of  $\Delta glfA$  was thus approximately half the thickness of the wild type cell wall.

**Table 2.** MICs of various antifungal agents against *A. fumigatus* mutants obtained from a broth microdilution assay.

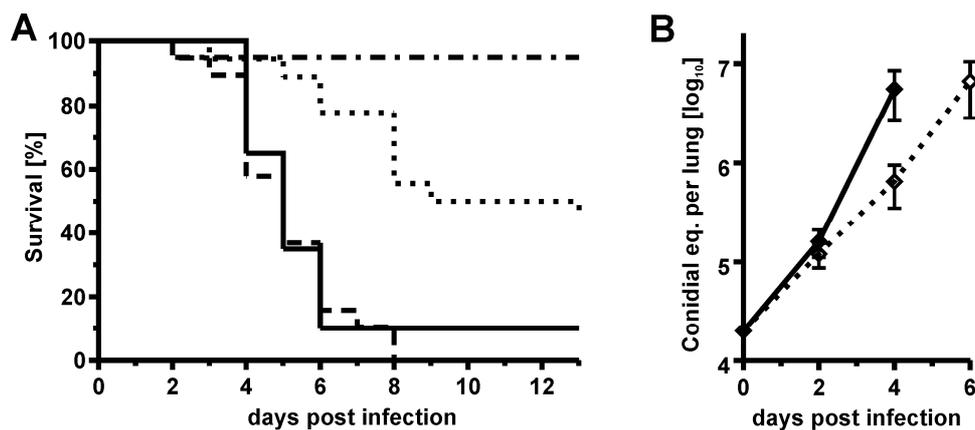
Genotype	AmB <sup>a</sup>	Vor <sup>b</sup>	mg/L			H <sub>2</sub> O <sub>2</sub> <sup>c</sup>
			Cas <sup>c</sup>	NiZ <sup>d</sup>		
Wt	3.9	0.3	62.5	500	218	
$\Delta glfA$	2.0	0.04	31.3	62.5-125	218	
$glfA^*$	3.9	0.3	62.5	500	218	

<sup>a</sup>Amphotericin B (MIC90), <sup>b</sup>Voriconazole (MIC50),  
<sup>c</sup>Caspofungin (MIC90), <sup>d</sup>Nikkomycin Z (MIC50),  
<sup>e</sup>MIC100.

**$\Delta glfA$  is more susceptible to drugs.** The structural cell wall defect caused by Galf deficiency was accompanied by an increased susceptibility to several antifungal agents (Table 2). MICs determined in a broth microdilution test were slightly reduced for amphotericin B and caspofungin in the  $\Delta glfA$  mutant. A more pronounced increase in susceptibility was seen for voriconazole (0.04 mg/L for  $\Delta glfA$  compared to 0.3 mg/L for wt) and nikkomycin Z (63-125 mg/L for  $\Delta glfA$  and 500 mg/mL for wt), suggesting an increased permeability of the cell wall caused by the loss of Galf. In contrast, the sensitivity towards oxidative stress remained unchanged as indicated by equal MICs for H<sub>2</sub>O<sub>2</sub> in both wt and  $\Delta glfA$ .

**$\Delta glfA$  displays attenuated virulence in a murine model of invasive aspergillosis.** The influence of the  $glfA$  deletion on pathogenicity of *A. fumigatus* was assessed in a low-dose infection model of invasive aspergillosis (25). Cyclophosphamide was used to induce neutropenia in female Balb/c mice and a single dose of cortisone acetate was administered before intranasal infection with 20,000 *A. fumigatus* conidia. Neutropenia was maintained throughout the observation period of 13 days and survival was recorded daily (Fig. 6A). 90 % of the animals infected with wt did not survive day seven after infection, whereas half of the mice infected with  $\Delta glfA$  were still alive on day 13. A logrank test on wt and  $\Delta glfA$  survival data confirmed that the observed difference was statistically significant ( $P = 0.0004$ ). The attenuation in virulence could clearly be attributed to the absence of  $glfA$ , since animals infected with the reconstituted wild type strain  $glfA^*$  showed a survival pattern nearly identical to wt (no significant difference in logrank test,  $P = 0.559$ ). Histological examination of lung tissue from mice infected with wt,  $\Delta glfA$  and  $glfA^*$  5 days after inoculation showed evident fungal growth surrounding bronchioles and tissue penetration (Fig. 7). For each strain, inflammatory cells were rarely observed at the sites of infection.

To correlate the delay in the onset and progression of mortality with a growth defect, fungal burden in lungs of infected mice was determined by quantitative PCR (Fig. 6B). Mice were treated and infected as



**Figure 6** A) Survival of immunosuppressed mice infected intranasally with *A. fumigatus* wt (continuous),  $\Delta glfA$  (dotted) or  $glfA^*$  (dashed) and uninfected mice (dot-dashed). Each group consisted of 20 animals. B) Quantitative PCR determination of *A. fumigatus* burden (measured as conidial equivalents, see Material and Methods) in lung tissue from immunosuppressed mice infected with wt (continuous) or  $\Delta glfA$  (dotted). Each datapoint represents mean values obtained from three to five animals. Error bars indicate standard error of the mean.

described above. After two, four and six days, animals were sacrificed and their lungs taken. DNA was isolated from homogenized lung tissue and fungal content determined by amplification of a part of *A. fumigatus* ribosomal DNA. As shown in figure 6B, growth of  $\Delta glfA$  was restricted *in vivo* when compared to wt, which was in agreement with the slower growth observed *in vitro*.

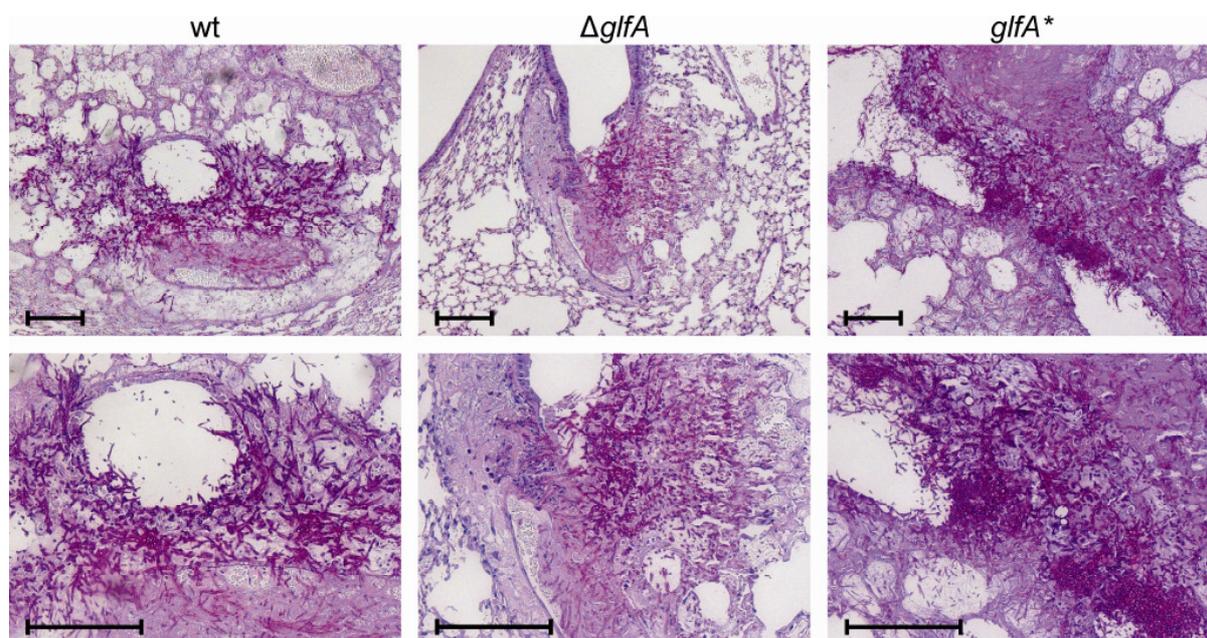
## DISCUSSION

The essential role of the  $\beta$ 1,3-glucan in cell wall organization and growth of several pathogenic fungi has been the basis for the development of the echinocandins (11). Likewise, inhibitors of chitin biosynthesis are currently explored as new antifungal drugs since chitin is an important structural element of the fungal cell wall (6). In contrast, although it is a major component of the cell wall and extracellular matrix, the role of galactomannan had not yet been investigated since the enzymes involved in its biosynthesis are unknown. Recently, we and others characterized the UDP-galactopyranose mutase (UGM) of various pathogenic eukaryotes including *A. fumigatus* (2,5). In prokaryotes, like in the protozoan *Leishmania*, this enzyme is the only route to the formation of UDP-Galf, the donor substrate of galactofuranosyltransferases, and thus controls the biosynthesis of all Galf containing molecules. Likewise, *A. fumigatus* UGM was found to be essential for the biosynthesis of galactomannan as well as some glycosphingolipids and glycoproteins. Like in other organisms (16,32), deletion of the  $glfA$  gene resulted in the complete absence of Galf, as shown for instance by the absence of reactivity to the antibody EB-A2.

Besides demonstrating the lack of Galf in the  $\Delta glfA$  mutant, our analyses provide useful structural

information of *A. fumigatus* N-glycans. Treatment of wild type secreted proteins with PNGase F released galactofuranosylated high-mannose type N-glycans. The size of the oligosaccharides and presence of a single Galf residue is in agreement with previous studies in filamentous fungi (26,29). Moreover, analysis of these oligosaccharides after digestion by Jack bean- or *T. reesei*  $\alpha$ 1,2-mannosidase helps positioning the Galf residue. These data and the comparison with high-mannose standards suggest that the N-glycans from *A. fumigatus* secreted proteins resemble those of *A. niger*  $\alpha$ -D-galactosidase and  $\alpha$ -D-glucosidase (45,44,49). These N-glycans might have simply arisen from trimming of the  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  precursor and substitution by a Galf residue. *Aspergilli* indeed contain several  $\alpha$ 1,2-mannosidase genes and trimming of high mannose glycans has been shown previously (52,13). Interestingly, Galf addition has been suggested to act as a stop signal for mannose addition in analogy to the role proposed for  $\alpha$ 1,3-linked terminal mannose in *Saccharomyces cerevisiae* (29,49). However, preventing the addition of galactofuranose does not result in an increased size of the oligosaccharides. On the contrary,  $\text{Man}_5\text{GlcNAc}_2$  is the main oligosaccharide found in the  $\Delta glfA$  mutant while  $\text{Galf/Man}_6\text{GlcNAc}_2$  is predominant in wild type.

Although  $glfA$  deletion has been shown to be lethal in *Mycobacterium smegmatis* (32), the *in vitro* viability of *A. fumigatus*  $\Delta glfA$  mutant is unsurprising since Galf occupies a non-reducing terminal position in the molecules of this fungus. Hence, the absence of Galf does not perturb the basic organization of the cell wall, as would the absence of the underlying structures. Nevertheless, it resulted in marked alterations of the cell surface and notably a thinner cell wall as revealed by electron microscopy. The basis of this drastic change is unclear and difficult to attribute to a particular cell wall component since



**Figure 7** Periodic Acid Schiff stained lung sections of mice infected with *A. fumigatus* wild type (left),  $\Delta glfA$  (middle) or *glfA\** (right). Fungal colonies appear purple/red. Infected sites are typically surrounded by areas of necrotic tissue but show no or hardly any infiltrating leukocytes. The scale bar represents 100  $\mu$ m.

GPI-/cell wall bound galactomannan, N-glycans, O-glycans and GIPCs are affected by *Galf* deficiency. In other fungi, the loss of terminal sugar residues has sometimes been associated with reduced cell wall strength. For instance, a *Schizosaccharomyces pombe* mutant deficient in cell wall galactosylation displays morphological changes, attenuated growth and a 25–35 % reduction in cell wall thickness (46).

The structural changes originating from the *glfA* deletion are associated with slower growth indicating that *Galf* plays an important role in *A. fumigatus* morphogenesis. The temperature-sensitive growth defect at higher temperature displayed by the  $\Delta glfA$  mutant is reminiscent to that observed in the  $\Delta AfPmt1$  mutant, a mutant characterized by reduced O-glycosylation (53). Interestingly, an influence of *Galf* deficiency on the growth rate was also observed in  $\Delta glfA$  mutants of *Aspergillus nidulans* and *Aspergillus niger* (10). Conversely *glfA* deletion had no effect on the *in vitro* growth of *Leishmania* parasites (16) highlighting that the role of *Galf* cannot be translated to every *Galf* containing organism.

The ability to thrive at 37 °C is a characteristic of human pathogens that has been shown to correlate with virulence potential in the case of *A. fumigatus* (31). Consequently, mutations that affect the growth of fungi at mammalian body temperature are commonly associated with attenuated virulence (40). In this study, we observed slower growth of the *A. fumigatus*  $\Delta glfA$  mutant *in vitro* but also *in vivo* using quantitative PCR. In agreement with this observation, the mutant was clearly attenuated in virulence showing a delay in both the onset and progression of mortality when tested in a low dose mouse infection model of invasive aspergillosis. An altered immune

response caused by the different cell wall structure of the  $\Delta glfA$  mutant may also contribute to the attenuation in virulence. However, no differences in adherence and uptake of wt and  $\Delta glfA$  conidia by murine bone-marrow derived dendritic cells or in the production of TNF- $\alpha$  or IL-10 by infected murine bone-marrow derived macrophages were observed (K. Kotz, F. Ebel and F.H. Routier, unpublished data).

The value of echinocandins in invasive aspergillosis treatment resides in their synergistic effects with azoles and amphotericin B. Similarly, chitin synthesis inhibitors demonstrate synergy with echinocandins and azoles (24). These synergistic effects that offer new options for combination antifungal therapy are most likely due to greater cell wall permeability. We did note an increase in susceptibility of the  $\Delta glfA$  mutant to several antifungal agents, notably to voriconazole. However, in the liquid culture conditions classically used for antifungal susceptibility testing, the fungus is not surrounded by extracellular matrix. This extracellular matrix that delays the penetration of drug is rich in galactomannan (3) and is probably altered in the  $\Delta glfA$  mutant as suggested by the compact appearance of colonies on agar plates. *In vivo* a greater increase in susceptibility of the  $\Delta glfA$  mutant to drugs would therefore be expected. Besides the attenuated virulence, this suggests that inhibitors of UGM might be useful in antifungal therapy. The absence of *Galf* biosynthesis in mammals would represent a considerable advantage for the development of antifungal drugs with selective toxicity.

**ACKNOWLEDGMENTS**

We thank Dr. Florian Länger, Dr. Frank Ebel and Jakob Engel for their help with histopathology, cytokine analysis and glycolipid analysis respectively. Monika Berger, Verena Grosse, Sabine Schild, Olaf Macke and Brigitte Philippons are thanked for excellent technical assistance and Dr. Anita Straus and Dr. Helio Takahashi for the generous gift of MEST-1 monoclonal antibody. We are indebted to Dr. Rita Gerardy-Schahn for her constant support.

**REFERENCES**

1. **Aquino, V. R., L. Z. Goldani, and A. C. Pasqualotto.** 2007. Update on the contribution of galactomannan for the diagnosis of invasive aspergillosis. *Mycopathologia*. **163**:191-202.
2. **Bakker, H., B. Kleczka, R. Gerardy-Schahn, and F. H. Routier.** 2005. Identification and partial characterization of two eukaryotic UDP-galactopyranose mutases. *Biol. Chem.* **386**:657-661.
3. **Beauvais, A., C. Schmidt, S. Guadagnini, P. Roux, E. Perret, C. Henry, S. Paris, A. Mallet, M. C. Prévost, and J. P. Latgé.** 2007. An extracellular matrix glues together the aerial-grown hyphae of *Aspergillus fumigatus*. *Cell Microbiol.* **9**:1588-1600.
4. **Bernard, M. and J. P. Latgé.** 2001. *Aspergillus fumigatus* cell wall: composition and biosynthesis. *Med. Mycol.* **39 Suppl 1**:9-17.
5. **Beverley, S. M., K. L. Owens, M. Showalter, C. L. Griffith, T. L. Doering, V. C. Jones, and M. R. McNeil.** 2005. Eukaryotic UDP-galactopyranose mutase (GLF gene) in microbial and metazoal pathogens. *Eukaryot. Cell.* **4**:1147-1154.
6. **Borgia, P. T. and C. L. Dodge.** 1992. Characterization of *Aspergillus nidulans* mutants deficient in cell wall chitin or glucan. *J. Bacteriol.* **174**:377-383.
7. **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.
8. **Callewaert, N., S. Geysens, F. Molemans, and R. Contreras.** 2001. Ultrasensitive profiling and sequencing of N-linked oligosaccharides using standard DNA-sequencing equipment. *Glycobiology.* **11**:275-281.
9. **Costachel, C., B. Coddeville, J. P. Latgé, and T. Fontaine.** 2-12-2005. Glycosylphosphatidylinositol-anchored fungal polysaccharide in *Aspergillus fumigatus*. *J. Biol. Chem.* **280**:39835-39842.
10. **Damveld, R. A., A. Franken, M. Arentshorst, P. J. Punt, F. M. Klis, C. A. van den Hondel, and A. F. Ram.** 2008. A Novel Screening Method for Cell Wall Mutants in *Aspergillus niger* Identifies UDP-Galactopyranose Mutase as an Important Protein in Fungal Cell Wall Biosynthesis. *Genetics.* **178**:873-881.
11. **Denning, D. W.** 2002. Echinocandins: a new class of antifungal. *J. Antimicrob. Chemother.* **49**:889-891.
12. **Denning, D. W.** 4-10-2003. Echinocandin antifungal drugs. *Lancet.* **362**:1142-1151.
13. **Eades, C. J. and W. E. Hintz.** 5-9-2000. Characterization of the class I alpha-mannosidase gene family in the filamentous fungus *Aspergillus nidulans*. *Gene.* **255**:25-34.
14. **Fontaine, T., C. Simenel, G. Dubreucq, O. Adam, M. Delepierre, J. Lemoine, C. E. Vorgias, M. Diaquin, and J. P. Latgé.** 8-9-2000. Molecular organization of the alkali-insoluble fraction of *Aspergillus fumigatus* cell wall. *J. Biol. Chem.* **275**:27594-27607.
15. **Guan, S., A. J. Clarke, and C. Whitfield.** 2001. Functional analysis of the galactosyltransferases required for biosynthesis of D-galactan I, a component of the lipopolysaccharide O1 antigen of *Klebsiella pneumoniae*. *J. Bacteriol.* **183**:3318-3327.
16. **Kleczka, B., A. C. Lamerz, G. van Zandbergen, A. Wenzel, R. Gerardy-Schahn, M. Wiese, and F. H. Routier.** 2007. Targeted gene deletion of *Leishmania major* UDP-galactopyranose mutase leads to attenuated virulence. *J. Biol. Chem.* **282**:10498-10505.
17. **Köplin, R., J. R. Brisson, and C. Whitfield.** 14-2-1997. UDP-galactofuranose precursor required for formation of the lipopolysaccharide O antigen of *Klebsiella pneumoniae* serotype O1 is synthesized by the product of the *rfbD<sub>KPO1</sub>* gene. *J. Biol. Chem.* **272**:4121-4128.
18. **Krappmann, S., Ö. 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.
19. **Kremer, L., L. G. Dover, C. Morehouse, P. Hitchin, M. Everett, H. R. Morris, A. Dell, P. J. Brennan, M. R. McNeil, C. Flaherty, K. Duncan, and G. S. Besra.** 13-7-2001. Galactan biosynthesis in *Mycobacterium tuberculosis*. Identification of a bifunctional UDP-galactofuranosyltransferase. *J. Biol. Chem.* **276**:26430-26440.
20. **Laroy, W., R. Contreras, and N. Callewaert.** 2006. Glycome mapping on DNA sequencing equipment. *Nat. Protoc.* **1**:397-405.
21. **Lass-Flörl, C., M. Cuenca-Estrella, D. W. Denning, and J. L. Rodriguez-Tudela.** 2006. Antifungal susceptibility testing in *Aspergillus* spp. according to EUCAST methodology. *Medical Mycology.* **44**:319-325.
22. **Latgé, J. P., H. Kobayashi, J. P. Debeauvais, M. Diaquin, J. Sarfati, J. M. Wieruszski, E. Parra, J. P. Bouchara, and B. Fournet.** 1994. Chemical and immunological characterization of the extracellular galactomannan of *Aspergillus fumigatus*. *Infect. Immun.* **62**:5424-5433.
23. **Leitão, E. A., V. C. Bittencourt, R. M. Haido, A. P. Valente, J. Peter-Katalinic, M. Letzel, L. M. de Souza, and E. Barreto-Bergter.** 2003. Beta-galactofuranose-containing O-linked oligosaccharides present in the cell wall peptidogalactomannan of *Aspergillus fumigatus* contain immunodominant epitopes. *Glycobiology.* **13**:681-692.
24. **Li, R. K. and M. G. Rinaldi.** 1999. In vitro antifungal activity of nikkomycin Z in combination with fluconazole or itraconazole. *Antimicrob. Agents Chemother.* **43**:1401-1405.
25. **Liebmann, B., T. W. Mühleisen, M. Müller, 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.
26. **Maras, M., D. van, I. R. Contreras, and C. A. van den Hondel.** 1999. Filamentous fungi as production organisms for glycoproteins of bio-medical interest. *Glycoconj. J.* **16**:99-107.

27. Mikusova, K., M. Belanova, J. Kordulakova, K. Honda, M. R. McNeil, S. Mahapatra, D. C. Crick, and P. J. Brennan. 2006. Identification of a novel galactosyl transferase involved in biosynthesis of the mycobacterial cell wall. *J. Bacteriol.* **188**:6592-6598.
28. Mohr, J., M. Johnson, T. Cooper, J. S. Lewis, and L. Ostrosky-Zeichner. 2008. Current options in antifungal pharmacotherapy. *Pharmacotherapy.* **28**:614-645.
29. Morelle, W., M. Bernard, J. P. Debeauvais, M. Buitrago, M. Tabouret, and J. P. Latgé. 2005. Galactomannoproteins of *Aspergillus fumigatus*. *Eukaryot. Cell.* **4**:1308-1316.
30. Nassau, P. M., S. L. Martin, R. E. Brown, A. Weston, D. Monsey, M. R. McNeil, and K. Duncan. 1996. Galactofuranose biosynthesis in *Escherichia coli* K-12: identification and cloning of UDP-galactopyranose mutase. *J. Bacteriol.* **178**:1047-1052.
31. Paisley, D., G. D. Robson, and D. W. Denning. 2005. Correlation between *in vitro* growth rate and *in vivo* virulence in *Aspergillus fumigatus*. *Medical Mycology.* **43**:397-401.
32. Pan, F., M. Jackson, Y. Ma, and M. McNeil. 2001. Cell wall core galactofuran synthesis is essential for growth of mycobacteria. *J. Bacteriol.* **183**:3991-3998.
33. Patterson, T. F. 2006. Treatment of invasive aspergillosis: Polyenes, echinocandins, or azoles? *Med Mycol.* **44 Suppl 1**:357-362.
34. Pedersen, L. L. and S. J. Turco. 2003. Galactofuranose metabolism: a potential target for antimicrobial chemotherapy. *Cell Mol. Life Sci.* **60**:259-266.
35. Perea, S., G. Gonzalez, A. W. Fothergill, W. R. Kirkpatrick, M. G. Rinaldi, and T. F. Patterson. 2002. In vitro interaction of caspofungin acetate with voriconazole against clinical isolates of *Aspergillus* spp. *Antimicrob. Agents Chemother.* **46**:3039-3041.
36. Pontecorvo, G., J. A. Roper, L. M. Hemmons, K. D. MacDonald, and A. W. Bufton. 1953. The genetics of *Aspergillus nidulans*. *Adv. Genet.* **5**:141-238.
37. 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.
38. Reichard, U., S. Büttner, H. Eiffert, F. Staib, and R. Rüchel. 1990. Purification and characterisation of an extracellular serine proteinase from *Aspergillus fumigatus* and its detection in tissue. *J. Med. Microbiol.* **33**:243-251.
39. Reijula, K. E. 1991. Two common fungi associated with farmer's lung: fine structure of *Aspergillus fumigatus* and *Aspergillus umbrosus*. *Mycopathologia.* **113**:143-149.
40. Rementeria, A., N. López-Molina, A. Ludwig, A. B. Vivanco, J. Bikandi, J. Pontón, and J. Garaizar. 2005. Genes and molecules involved in *Aspergillus fumigatus* virulence. *Rev. Iberoam. Micol.* **22**:1-23.
41. Simenel, C., B. Coddeville, M. Delepierre, J. P. Latgé, and T. Fontaine. 2008. Glycosylinositolphosphoceramides in *Aspergillus fumigatus*. *Glycobiology.* **18**:84-96.
42. Stynen, D., J. Sarfati, A. Goris, M. C. Prévost, M. Lesourd, H. Kamphuis, V. Darras, and J. P. Latgé. 1992. Rat monoclonal antibodies against *Aspergillus* galactomannan. *Infect. Immun.* **60**:2237-2245.
43. Suzuki, E., M. S. Toledo, H. K. Takahashi, and A. H. Straus. 1997. A monoclonal antibody directed to terminal residue of beta-galactofuranose of a glycolipid antigen isolated from *Paracoccidioides brasiliensis*: cross-reactivity with *Leishmania major* and *Trypanosoma cruzi*. *Glycobiology.* **7**:463-468.
44. Takayanagi, T., A. Kimura, S. Chiba, and K. Ajisaka. 18-3-1994. Novel structures of N-linked high-mannose type oligosaccharides containing alpha-D-galactofuranosyl linkages in *Aspergillus niger* alpha-D-glucosidase. *Carbohydr. Res.* **256**:149-158.
45. Takayanagi, T., K. Kushida, K. Idonuma, and K. Ajisaka. 1992. Novel N-linked oligo-mannose type oligosaccharides containing an alpha-D-galactofuranosyl linkage found in alpha-D-galactosidase from *Aspergillus niger*. *Glycoconj. J.* **9**:229-234.
46. Tanaka, N., M. Konomi, M. Osumi, and K. Takegawa. 2001. Characterization of a *Schizosaccharomyces pombe* mutant deficient in UDP-galactose transport activity. *Yeast.* **18**:903-914.
47. Toledo, M. S., S. B. Levery, B. Bennion, L. L. Guimaraes, S. A. Castle, R. Lindsey, M. Momany, C. Park, A. H. Straus, and H. K. Takahashi. 2007. Analysis of glycosylinositol phosphorylceramides expressed by the opportunistic mycopathogen *Aspergillus fumigatus*. *J. Lipid Res.* **48**:1801-1824.
48. Trejo, A. G., J. W. Haddock, G. J. Chittenden, and J. Baddiley. 1971. The biosynthesis of galactofuranosyl residues in galactocarlose. *Biochem. J.* **122**:49-57.
49. Wallis, G. L., R. L. Easton, K. Jolly, F. W. Hemming, and J. F. Peberdy. 2001. Galactofuranoic-oligomannose N-linked glycans of alpha-galactosidase A from *Aspergillus niger*. *Eur. J. Biochem.* **268**:4134-4143.
50. Weston, A., R. J. Stern, R. E. Lee, P. M. Nassau, D. Monsey, S. L. Martin, M. S. Scherman, G. S. Besra, K. Duncan, and M. R. McNeil. 1997. Biosynthetic origin of mycobacterial cell wall galactofuranosyl residues. *Tuber. Lung Dis.* **78**:123-131.
51. Wing, C., J. C. Errey, B. Mukhopadhyay, J. S. Blanchard, and R. A. Field. 7-11-2006. Expression and initial characterization of WbbI, a putative D-Gal:alpha-D-Glc beta-1,6-galactofuranosyltransferase from *Escherichia coli* K-12. *Org. Biomol. Chem.* **4**:3945-3950.
52. Yoshida, T., Y. Kato, Y. Asada, and T. Nakajima. 2000. Filamentous fungus *Aspergillus oryzae* has two types of alpha-1,2-mannosidases, one of which is a microsomal enzyme that removes a single mannose residue from Man9GlcNAc2. *Glycoconj. J.* **17**:745-748.
53. Zhuo, H., H. Hu, L. Zhang, R. Li, H. Ouyang, J. Ming, and C. Jin. 28-9-2007. Protein O-mannosyltransferase 1 (AfPmt1p) in *Aspergillus fumigatus* is crucial for cell wall integrity and conidia morphology especially at an elevated temperature. *Eukaryot. Cell.*

## **Chapter 3 – A single UDP-Galactofuranose transporter is required for galactofuranosylation in *Aspergillus fumigatus***

- Manuscript submitted to the Journal of Biological Chemistry -

Jakob Engel\*<sup>1</sup>, Philipp S. Schmalhorst\*<sup>1</sup>, Thilo Dörk-Bousset<sup>2</sup>, Vincent Ferrières<sup>3,4</sup> and Françoise H. Routier<sup>1</sup>

Institute for Cellular Chemistry<sup>1</sup>, Centre for Biochemistry, Hannover Medical School;  
Gynaecology Research Unit<sup>2</sup>, Clinics of Obstetrics and Gynaecology, Hannover Medical School;  
École Nationale Supérieure de Chimie de Rennes<sup>3</sup>, CNRS, Rennes, France;  
Université Européenne de Bretagne<sup>4</sup>, 12, Rennes, France

\*J Engel And PS Schmalhorst contributed equally to this work.

## **Preface – About this manuscript**

This part of my work aimed at the identification of the assumed UDP-Galf transporter in *A. fumigatus*. For this purpose the *A. fumigatus* genome was searched using the BLAST algorithm with known nucleotide sugar transporter genes. Sixteen candidate genes were identified and one of them, *glfB*, was found closely located to the previously described *glfA* gene. The *glfB* cDNA was cloned and the GlfB protein overexpressed in *S. cerevisiae*. The golgi fraction of this yeast strain was subjected to a modified nucleotide sugar transport assay to demonstrate binding specificity of GlfB for UDP-Galf. An *A. fumigatus glfB* deletion mutant was generated ( $\Delta glfB$ ) and analyzed with the methods described in detail in the previous chapter.

My contributions to this manuscript comprised the identification of the candidate gene *glfB* and the *in vitro* transport/binding assays. Moreover, I assisted Jakob Engel, the shared first author, in the design of the experiments dedicated to characterize the molecular and macroscopic phenotype of the  $\Delta glfB$  mutant. Prof. Routier and I wrote the paper.

## SUMMARY

Galactofuranose (Galf) containing molecules have been described at the cell surface of several eukaryotes and shown to contribute to the virulence of the parasite *Leishmania major* and the fungus *Aspergillus fumigatus*. It is anticipated that a number of the surface glycoconjugates such as N-glycans or glycolipids are galactofuranosylated in the Golgi apparatus. This raises the question how the substrate for galactofuranosylation reactions, UDP-Galf, which is synthesized in the cytosol, translocates into the organelles of the secretory pathway. Here we report the first identification of a nucleotide sugar transporter with specificity for UDP-Galf named GlfB. *In vitro* transport assays established binding of UDP-Galf to GlfB and excluded transport of several other nucleotide sugars. Furthermore, the implication of *glfB* in the galactofuranosylation of *A. fumigatus* glycoconjugates and galactomannan was demonstrated by a targeted gene deletion approach. Our data reveal a direct connection between galactomannan and the organelles of the secretory pathway which strongly suggests that the cell wall bound polysaccharide originates from its GPI-anchored form.

---

## INTRODUCTION

The monosaccharide galactofuranose (Gal<sup>f</sup>) is an important constituent of the microbial cell surface (1). It occurs in structures essential for bacterial virulence or growth such as the O-antigen of the outer membrane lipopolysaccharide or the mycobacterial arabinogalactan (2). In eukaryotes, Galf has principally been reported in glycoconjugates and polysaccharide of fungi and protozoan parasites (3) although the distribution of the *glf* gene encoding the UDP-Galf biosynthetic enzyme, UDP-galactopyranose mutase (UGM), suggests its presence in many lower eukaryotes (4,5). Amongst fungi, the cell wall of the opportunistic pathogen *Aspergillus fumigatus* is one of the best studied. In this organism, Galf has been found in the polysaccharide galactomannan (6), on glycoinositolphosphoceramides (7,8) and in N- and O-linked glycans of glycoproteins (9,10). In *Aspergillus fumigatus* like in the parasite *Leishmania major*, the generation of a mutant devoid of Galf resulted in attenuated virulence

highlighting an important role of Galf for eukaryotic pathogens (11,12).

Little is known about the Galf biosynthetic pathways in eukaryotes. Genetic and biochemical studies have shown that UDP-Galf arising from the action of UGM is essential for galactofuranosylation (12). This nucleotide sugar is most likely the substrate of specific galactofuranosyltransferases although genes encoding putative galactofuranosyltransferases have only been identified in trypanosomatid parasites (13,14). One of these putative transferases known as LPG1 is involved in the biosynthesis of the lipophosphoglycan (LPG) of *Leishmania* parasites and is localized in the Golgi apparatus (15). Similarly, enzymes involved in addition of terminal Galf to N-glycans, O-glycans or glycolipids are assumed to be localized in this organelle. UDP-Galf biosynthesis however occurs in the cytosol (11) which makes translocation of UDP-Galf across the Golgi membrane necessary (fig. 1).

Nucleotide sugar transporters (NSTs) are multi-transmembrane proteins present in all kinds of eukaryotic organisms. They consist of typically 8-10 transmembrane  $\alpha$ -helices linked by short loops. Further structural information is very limited because efforts to obtain crystals for X-ray structure determination have been hindered by the high hydrophobicity of NSTs. Many NSTs have been functionally characterized in biochemical assays measuring incorporation of radioactive nucleotide sugars into membrane vesicles. These experiments led to the development of a mechanistic model, in which NSTs work as antiporters that export a nucleotide sugar molecule in exchange for an equally charged nucleoside monophosphate molecule (16).

Because of their structural conservation, putative NSTs can be readily found by database mining. In humans, they belong to the SLC35 (SoLute Carrier 35) family (17) which comprises ten characterized members and 13 proteins whose function is currently unknown. Phylogenetic classification identified subfamilies which contain all of the characterized SLC35 proteins, but do not allow classification of most of the SLC35 proteins with unknown function (18). Furthermore, substrate specificity is hardly conserved within NST subfamilies, thus it is generally not possible to infer substrate specificity from the level of sequence identity or phylogeny.

NSTs are closely related to plastidic phosphate translocators (pPT) that include translocators for triose phosphate (TPT), phosphoenolpyruvate (PPT), glucose-6-phosphate (GPT) and xylulose phosphate (XPT). Additionally, a variety of uncharacterized phosphate translocator-homologous proteins (PTh) are found in plant and other organisms including human (19). In this study, we describe the characterization of an *Aspergillus fumigatus* PTh protein with NST function and demonstrate its specificity for UDP-Galf. The importance for *in vivo* galactofuranosylation is shown by a targeted gene deletion approach.

## EXPERIMENTAL PROCEDURES

**Materials.** Radiolabeled nucleotide sugars were purchased from PerkinElmer (UDP-[<sup>3</sup>H]-Gal, UDP-[<sup>3</sup>H]-GlcNAc, UDP-[<sup>14</sup>C]-GlcA), American Radiolabeled chemicals (UDP-[<sup>3</sup>H]-GalNAc, [<sup>3</sup>H]-UMP) and GE Healthcare (UDP-[<sup>3</sup>H]-Glc). UDP-Galf was chemically synthesized (20).

**Strains, media, growth conditions.** For protein expression, *S. cerevisiae* strain BY4741 in which the gene for the ER UDP-GlcNAc transporter, YEA4, had been deleted (*MATa*; *his3D1*; *leu2D0*; *met15D0*; *ura3D0*; *YEL004w::kanMX4*; EUROSCARF, Frankfurt, Germany) was cultivated in SC minimal media [2 % glucose, 1.7 g/L Difco™ Yeast Nitrogen Base w/o amino acids and ammonium sulfate (BD Biosciences), 5 g/L ammonium sulfate] supplemented with L-histidine (50 mg/L), L-methionine (50 mg/L) and L-leucine (100 mg/L).

*Aspergillus fumigatus* clinical isolate D141 was used in this study. For gene deletion purposes, a D141 strain deficient in non-homologous end-joining (*Afs35*) was used (21). Strains were grown at 37 °C on *Aspergillus* minimal medium (AMM) containing 1 % D-glucose as carbon source and 70 mM NaNO<sub>3</sub> as nitrogen source. Phleomycin was added for selection purposes at 30 mg/L.

**Bioinformatic analyses.** Transmembrane helix prediction was carried out using the ConPred II program (<http://bioinfo.si.hirosaki-u.ac.jp/~ConPred2>) (22). For plant proteins, predicted transmembrane domains were obtained from the ARAMEMNON database (<http://aramemnon.botanik.uni-koeln.de/index.ep>) (23). BLAST searches were performed using default parameter values with the low complexity filter switched off.

**Cloning of the *glfB* gene** Total RNA was isolated from *A. fumigatus* mycelium and *glfB* mRNA was reversely transcribed into single-stranded cDNA using primer JE28. All primer sequences are provided in Table 1. The *glfB* coding sequence was then amplified by PCR (JE26/JE28) and cloned via BamHI/XbaI into plasmid vector pYEScupFLAGK (24).

**Protein expression in yeast, subcellular fractionation and *in vitro* transport assay.** The *glfB* cDNA as well as the human UDP-Gal transporter cDNA (SLC35A2, isoform a) (25) were cloned into the plasmid vector pYEScupFLAGK (complementing uracil auxotrophy) for copper-inducible expression of N-terminally FLAG tagged proteins in yeast. *S. cerevisiae* spheroplasts were transformed using the lithium-acetate method described by Invitrogen. Transformants were selected on SC minimal media without uracil. For protein expression, transformants were grown in 1L-cultures until A<sub>600</sub> reached 0.8-0.9, expression was then induced by addition of 0.5 mM CuSO<sub>4</sub> (final concentration) and culture was continued for 2 h at 30 °C.

Subcellular fractionation of yeast cells and *in vitro* transport assay were performed as previously described (24). Briefly, 50 µl Golgi vesicle preparations (containing typically 80 µg total protein) and 50 µl of 2 µM [<sup>3</sup>H]-labeled nucleotide sugar or [<sup>3</sup>H]-UMP (0.37 kBq/µl) in assay buffer (10 mM Tris-HCl, pH 7.0; 0.8 M sorbitol; 2 mM MgCl<sub>2</sub>) were incubated for 30 s at 30 °C. For competition assays, the assay buffer contained in addition 100 µM unlabeled nucleotide sugar (26). Reactions were stopped by dilution with 1 ml of ice-cold assay buffer. The vesicle suspension was then filtered through a mixed cellulose ester membrane (MF™ membrane filters, 0.45 µm, Millipore, Bedford, MA). Vesicles adhering to the filters were washed three times with 2 ml of ice-cold assay buffer and the radioactivity retained on the membrane was measured by liquid scintillation.

**Generation of *A. fumigatus* Δ*glfB* mutant strain.** The 5' and 3' flanking regions (1.5 kb each) of *A. fumigatus glfB* coding sequence were amplified from genomic DNA by PCR with primers JE15/JE14 and JE20/JE17 respectively and linked by fusion PCR to a *ble/tk* cassette conferring resistance to phleomycin (27) amplified by JE06/JE07. From this construct, a second PCR (JE18/JE19) yielded the final deletion construct. Sequence integrity was verified after cloning into pCR2.1-TOPO

(Invitrogen). Polyethylene glycol mediated fusion of protoplasts was carried out as described in (34). Transformants were grown on AMM plates containing 1.2 M sorbitol as osmotic stabilizer under appropriate selection conditions and singled out twice before further analysis. Accurate gene deletion and reconstitution were confirmed by southern hybridisation. Southern probes were amplified from genomic DNA using primer pairs JE21/JE22, JE25/JE24 and PS20/PS21. All primer sequences are provided in Table 1.

**Western Blots.** Cell wall glycoproteins and soluble polysaccharides were extracted from 30 mg ground *A. fumigatus* mycelium by incubation in 1 mL sample buffer (15 % glycerol, 100 mM Tris/HCl pH 6.8, 1.5 % SDS, 0.25 %  $\beta$ -mercaptoethanol, 0.025 % bromophenol blue) for 12 min at 95 °C. 20  $\mu$ l of the supernatant were separated on a 10 % SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The monoclonal antibody EB-A2 (39) conjugated to horseradish peroxidase (HRP) from the Platelia *Aspergillus* Test (Bio-Rad, Hercules, CA, USA) or HRP-coupled lectin Concanavalin A (ConA, Sigma-Aldrich) were used in a 1:50 dilution or at 0.2  $\mu$ g/mL respectively. HRP activity was visualized by an enhanced chemiluminescence system (Pierce).

**Purification and analysis of glycosylinositolphosphoceramides (GIPCs).** GIPCs were extracted from 0.5 g mycelium and purified as previously described (12). Purified GIPCs were redissolved in 20  $\mu$ l MeOH. High performance thin layer chromatography and immunostaining with the monoclonal antibody MEST-1 were carried out as described (7) using 2  $\mu$ l for immunostainings and 18  $\mu$ l for orcinol/H<sub>2</sub>SO<sub>4</sub> stainings.

**N-glycan analysis.** N-glycan preparation and separation was carried out as described previously (28). Glycoproteins from 12 mL (40x 300  $\mu$ l) *A. fumigatus* culture supernatant were transferred to Immobilon P Multiwell plates (Millipore). After peptide:N-glycanase (PNGase F) mediated N-glycan release and 8-amino-1,3,6-pyrene-trisulfonic acid (APTS) labelling N-glycans were separated on a capillary electrophoresis DNA Sequencer (ABI PRISM® 3100-Avant Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). Reference glycans were purchased from Dextra Laboratories (Reading, UK).

**Growth assay.** For radial growth measurement, a 5  $\mu$ l drop containing 10,000 *A. fumigatus* conidia in PBS was placed in the centre of an AMM agar plate. Plates were incubated at various temperatures and colony diameters were measured twice daily.

## RESULTS

**Selection of a UDP-Galf transporter candidate gene** – BLAST searches of the *A. fumigatus* genome (29) with various characterized NST protein sequences identified 16 putative NST genes (Suppl Table 1) One of them (AFUA\_3G12700) is adjacent to the recently identified *glaA* gene (AFUA\_3G12690) encoding the UDP-Galf biosynthetic enzyme UGM, and was thus considered as a reasonable candidate since clustering of functionally related genes is sometimes observed in *A. fumigatus* (e.g. the siderophore genes *sidF*, *sidD* and *mirB*) (30). This gene will be further referred to as *glaB* because of its implication in galactofuranosylation as demonstrated below. Interestingly, all fungi from the subphylum Pezizomycotina whose genome has been fully sequenced display a clear homolog of *glaB* clustered with *glaA*. When present in basidiomycota (e.g. *Cryptococcus neoformans*), these two genes are more distant and may be found on different chromosomes. In *A. fumigatus*, the predicted GlaB protein comprises 400 amino acids and shares up to 40 % amino acid identity with uncharacterized *Arabidopsis* phosphate translocator homologs. The most similar protein with known function is the plant UDP-Gal transporter AtUDP-GalT1 (At1g77610) (31) that displays 21% identity with GlaB. The UDP-Gal transporters AtUDP-GalT2 (At1g76670) and AtNST-KT1 (At4g39390) (31,32), and the uncharacterized SLC35C2 and SLC35E3 are more distantly related and show 14 to 19 % identity with GlaB. Finally, the human GDP-Fuc transporter is the closest characterized transporter of the SLC35 family (12 % identity). An alignment of GlaB with these sequences is presented in figure 2. This multiple sequence alignment underlines the conservation of two lysine residues (GlaB K59 and K294) that have been proposed to be involved in substrate binding (19,33). GlaB was predicted to contain eleven transmembrane helices of which the first ten aligned well with the predicted transmembrane helices of its homologs and other NSTs (fig. 2). We thus hypothesized that *glaB* encoded a NST and in

line with its location in the genome speculated about a specificity for UDP-Galf.

*In vitro Transport and binding assays*—Uptake of radioactive nucleotide sugars by Golgi-enriched vesicles isolated from yeast expressing a putative NST is a method of choice to determine substrate specificity. Unfortunately, since UDP-Galf was not available in a radiolabeled form, it could not be directly tested in this *in vitro* assay system. Nevertheless, the transport of UDP-Gal, UDP-GlcNAc, UDP-GalNAc or UDP-GlcA could be excluded. Indeed, the uptake of these nucleotide sugars by Golgi vesicles isolated from yeast cells expressing GlfB or mock transformed was virtually absent (background levels of 1.0 to 1.7 pmol mg<sup>-1</sup> min<sup>-1</sup>) while an endogenous UDP-Glc transport of approximately 7 pmol mg<sup>-1</sup> min<sup>-1</sup> demonstrated the quality of the Golgi vesicles preparation. Additionally, transfection of CHO-Lec8 cells with GlfB resulted in Golgi expression of the protein but did not restore galactosylation of the surface glycoconjugates confirming absence of UDP-Gal transport (data not shown).

To assess UDP-Galf binding, we then evaluated the ability of unlabeled UDP-Galf (34) to inhibit the uptake of radioactive UMP, the postulated counter substrate of GlfB (fig 3A). For this purpose, Golgi vesicles obtained from cells expressing either GlfB or the human UDP-Gal transporter were incubated with 1 μM [<sup>3</sup>H]-UMP and 100 μM unlabeled UMP, UDP-Galf, UDP-Gal or UDP-GlcNAc. In GlfB containing vesicles, the addition of UDP-Galf resulted in an 80 % inhibition of the [<sup>3</sup>H]-UMP transport and was thus comparable to addition of unlabelled UMP (88 % inhibition). In contrast, UDP-GlcNAc and UDP-Gal slightly affected the UMP uptake indicating a limited binding of these nucleotide sugars to GlfB. Unfortunately, since UDP-Galf is rather unstable, contamination of a UDP-Galf solution by UMP is difficult to avoid (35) and was in this case estimated to 6-7 % by HPLC (36) (Suppl fig 1). This contaminating UMP explains the 50 % decrease of [<sup>3</sup>H]-UMP uptake observed with vesicles expressing the UDP-Gal transporter since a comparable inhibition is observed with 8 μM unlabelled UMP (Fig. 3B). In contrast, inhibition of GlfB mediated [<sup>3</sup>H]-UMP uptake by the contaminated UDP-Galf solution was significantly higher than the one observed with 8 μM UMP (P = 0.011, t test) indicating that part of the inhibition observed is actually due to UDP-Galf binding.

*Deletion of glfB in A. fumigatus* – To prove UDP-Galf transport activity *in vivo*, evaluation of the effect of *glfB* loss on galactofuranosylation of glycoconjugates in *A. fumigatus* was undertaken. For this purpose a *glfB* deletion cassette was constructed by double-joint PCR (37) containing the selectable phleomycin resistance gene *ble* (27) flanked by up- and downstream regions of the *glfB* coding sequence. *A. fumigatus* wild type protoplasts were transformed with the linearized cassette for exchange of the genomic *glfB* coding sequence for the phleomycin resistance gene by homologous recombination (fig. 4A). Transformants were selected for phleomycin resistance and gene replacement was confirmed by Southern Blot analysis (fig. 4B). A single strain was chosen for further analysis and named  $\Delta glfB$ .

*Analysis of galactofuranosylation in  $\Delta glfB$*  – Since N-glycosylation of proteins is known to take place along the secretory pathway and requires various NSTs, we first concentrated on this modification. Proteins from *A. fumigatus* culture supernatants were immobilized on PVDF membranes and N-glycans were released by peptide:N-glycanase treatment. After labeling with the negatively charged fluorescent dye APTS, N-glycans were separated by capillary electrophoresis on a DNA sequencer (fig. 5A). In the wild type electropherogram (top panel), the peaks labelled 1a to 5a can be assigned to high-mannose type N-glycans bearing a single Galf residue (Gal/Man<sub>5,9</sub>GlcNAc<sub>2</sub>) according to previous findings (10,12). These Galf-containing glycans were completely absent from the  $\Delta glfB$  N-glycan electropherogram (middle panel) that exclusively displays non galactofuranosylated N-glycans (peaks 1-5) co-migrating with Man<sub>5,9</sub>GlcNAc<sub>2</sub> standards (lower panel, fig. 5B). This finding demonstrates the requirement of GlfB for galactofuranosylation of N-glycans.

The contribution of GlfB to glycolipid biosynthesis was also analysed by testing their reactivity to the monoclonal antibody MEST-1 (38). This antibody reacts specifically with  $\beta$ 1-6-Galf found on several glycosphingolipids of *A. fumigatus* (fig 5C, left) (7). However, glycosphingolipids extracted from *A. fumigatus*  $\Delta glfB$  mycelium and separated by HPTLC were not stained with MEST-1 indicating absence of  $\beta$ 1-6 linked galactofuranose in these molecules. As loading control, carbohydrates were stained with orcinol/sulfuric acid (fig. 5C, right).

Finally we tested for reactivity of cell wall components towards the Galf-specific monoclonal antibody EB-A2 (39). A tetrasaccharide of  $\beta$ 1-5-linked Galf has been described as main epitope of EB-A2 (39). This structure is part of the cell wall polysaccharide galactomannan which can be either linked to the cell wall  $\beta$ 1-3/6-glucan (6) or to a GPI-anchor (40). Also N-glycans with a single terminal Galf have been reported to be recognized by EB-A2 (10). Aqueous extracts of wild type *A. fumigatus* mycelium separated on a polyacrylamide gel and transferred to a nitrocellulose membrane strongly bound EB-A2, while in case of the  $\Delta$ *glfB* mutant, binding was totally absent (fig. 5D, left). In contrast, staining with the mannose-specific lectin Concanavalin A appeared slightly stronger for  $\Delta$ *glfB* than for wild type, suggesting a higher exposure of cell surface mannan structures as previously observed with the  $\Delta$ *glfA* mutant indicating a higher exposure of cell surface mannan structures (fig. 5D, right)(12). Thus, the surface glycoconjugates of  $\Delta$ *glfB* resemble closely those of the Galf-deficient  $\Delta$ *glfA* mutant (12) and suggests a complete loss of galactofuranosylation capacity in the  $\Delta$ *glfB* mutant.

*Growth and thermotolerance of the  $\Delta$ glfB mutant* – Galf deficiency has been shown to induce an altered culture morphology accompanied by a substantial growth defect in *A. fumigatus* (12). This effect was more pronounced at higher temperatures than the standard growth temperature of 37 °C indicating a decreased resistance to temperature stress. Indeed, radial colony growth of the  $\Delta$ *glfB* mutant was found to be 45 % slower than wild type at 37 °C and 66-67 % slower at 42 or 47 °C (fig. 6) in agreement with the observations for the  $\Delta$ *glfA* mutant (12).

## DISCUSSION

This report describes the first identification of a nucleotide sugar transporter with specificity for UDP-Galf. The protein called GlfB because of its implication in galactofuranose metabolism was selected from its homology to other member of the NST family, its phylogenetic classification as well as the location on chromosome 3 directly downstream of the *glfA* gene which encodes the UDP-Galf biosynthesis enzyme UGM (12). It is a 400 amino acid protein with 11 predicted transmembrane helices. This represents a particularity of the GlfB protein since NSTs classically exhibit 8 to

10 predicted hydrophobic domains and follow an experimentally determined model in which both the N- and C-terminus are situated on the cytoplasmic side of the organelle (41).

Remarkably, filamentous fungi of the subphylum Pezizomycotina, including many human or plant pathogens, all seem to exhibit adjacent *glfA* and *glfB* genes and the presence of Galf has been reported in many species of this subphylum. These genes are also found in a few basidiomycota such as the human pathogen *Cryptococcus neoformans* but are absent from other fungi, notably from yeasts. Thus, Galf seems particularly important for filamentous fungi. Indeed several studies have already shown the role of this monosaccharide for growth, hyphal morphology and sporulation of *Aspergillus* species (12,42,43). We thus speculate that Galf plays major roles in the hyphal development and/or reproduction of all filamentous fungi of Pezizomycotina and thus has been maintained during evolution.

To address the function of a nucleotide sugar transporter, two approaches are commonly used. Complementation of a mutant strain or cell line not only provides a way to determine NST function *in vivo*, but also allows identification of the underlying gene(s) by expression of a cDNA library combined with sibling selection (31). Alternatively, NST substrates can be identified by measuring transport of radiolabeled nucleotide sugars into vesicles, either prepared from cell lysates or artificially reconstituted proteoliposomes (44,45). Because of the unavailability of a UDP-Galf transporter deficient mutant or cell line and radioactive UDP-Galf, we opted for targeted gene deletion of candidate genes in the opportunistic fungus *Aspergillus fumigatus*. This approach was enabled by the restricted number of candidate genes, the existence of a haploid stage that facilitates the isolation of clones by molecular techniques and a comprehensive knowledge of the galactofuranosylated structures in this organism. Targeted replacement of the most promising candidate gene, *glfB*, led to the absence of Galf bringing a direct evidence of its involvement in Galf metabolism.

The specificity of GlfB for UDP-Galf was established by an indirect assay showing competitive inhibition of [<sup>3</sup>H]-UMP transport by GlfB with unlabeled UDP-Galf. In addition, the transport of several other nucleotide sugars was excluded using a direct *in vitro* transport assay suggesting that GlfB is highly specific. In

particular the absence of UDP-Gal transport which was confirmed by the inability of *glfB* to complement the CHO cell line Lec8 indicates that the transporter is able to discern the ring conformation of the monosaccharide. *Vice versa*, our data show that the human UDP-Gal transporter used as control in this study does not recognize UDP-Galf. Similarly *Leishmania major* UDP-Gal transporters LPG5A and LPG5B appear to be specific for the pyranic form of galactose since their concomitant deletion results in the synthesis of glycoconjugates containing Galf but devoid of galactopyranose (46). The obvious difference in the three-dimensional structure of the furanic and pyranic rings certainly plays a role in the ability of these NSTs to differentiate the two cyclic forms. Initially NSTs were thought to be monospecific. However, several NSTs have now been shown to be multifunctional *in vitro* and usually recognize sugars activated with the same nucleotide which led to the assumption that the nucleotide part is a major player in recognition. With the exception of Fringe Connection (47), which is thought to be a general UDP-sugar transporter, the specificity of NSTs is generally restricted to a few related nucleotides sugars demonstrating that the sugar part also plays a significant role in the recognition.

Detailed analyses of *A. fumigatus*  $\Delta glfB$  total extract and purified glycoconjugates revealed the complete absence of galactofuranose in N-glycans, glycolipids as well as galactomannan. It can be inferred from this result that GlfB is the only NST in *A. fumigatus* capable of UDP-Galf transport. In this organism Galf is capping the N-glycans and glycolipids. Galactofuranosylation of these molecules was therefore expected to occur in the Golgi apparatus and thus depend on import of UDP-Galf in this organelle as observed. The lack of Galf in galactomannan was however less predictable since its biosynthesis is currently unknown. Recently, we demonstrated that the terminal sugar of this polysaccharide arises from UDP-Galf synthesized in the cytoplasm by UGM (12). Galactomannan is either linked to the membrane by a GPI anchor, covalently bound to the cell wall  $\beta$ 1,3/1,6-glucan or secreted in the environment (6,40). Since they present the same carbohydrate structure, it has been postulated that these three forms of the polysaccharide share a common biosynthetic pathway. By analogy to the biosynthesis of the *Leishmania* GPI-anchored polysaccharide LPG (15), we

assumed that the biosynthesis of the GPI-linked galactomannan takes place in the Golgi apparatus. The total absence of EB-A2 staining in the  $\Delta glfB$  mutant indicative of the absence of all form of galactomannan supports this location. Galactomannan would then be transferred to the  $\beta$ 1,3/1,6-glucan from the GPI-anchored polymer as it has been proposed for some GPI-anchored proteins in ascomycetous yeasts (48). The secreted form would arise from enzymatic cleavage of surface galactomannan. Our data demonstrating absence of galactofuran in the  $\Delta glfB$  mutant establish a clear link between galactomannan including the cell wall bound form and the secretory pathway and thus strongly support this model. In contrast, the synthesis of  $\alpha$ 1-3-glucan,  $\beta$ 1-3-glucan and chitin seems to occur at the plasma membrane (49).

In agreement with the complete absence of Galf in glycoconjugates and galactomannan, the growth phenotype of the *glfB* gene deletion mutant resembles closely the one of the previously described Galf-deficient  $\Delta glfA$  (12). In the case of the  $\Delta glfA$ , the growth defect was correlated with a reduction of virulence. It can thus be extrapolated that *glfB* is most probably required for full virulence of the fungus. Another example of nucleotide sugar transporter implicated in pathogenicity and restricted to certain organisms is the Golgi GDP-Man transporter. Deletion of this NST led to avirulence of the parasite *Leishmania major* (50) and is lethal in *Saccharomyces cerevisiae*, *Candida albicans*, and *Candida glabrata* (51-53). In the latter organisms, Golgi mannosylation comprises N-glycan outer chain elongation, O-mannosylation of proteins and GIPC biosynthesis. The *Aspergillus fumigatus* genome contains a clear GDP-Man transporter homolog (AFUA\_5G05740) whose importance is difficult to predict. It has recently been shown that protein O-mannosylation is dispensable in this fungus but required for cell wall stability and full virulence (54,55). Yet the importance of N-glycan branching and elongation, and mannosylation of GIPCs, is currently undetermined. The absence of Galf is however not sufficient to abolish growth of *A. fumigatus* as shown previously and confirmed in this study. Even chitin, a polysaccharide which is believed to contribute to the rigidity of the fungal cell wall is not strictly essential for *S. cerevisiae* (56,57). Therefore, therapeutic strategies directed against cell wall biosynthesis will likely have to address several targets to be successful.

### **Acknowledgments**

We thank Dr. Sven Krappmann for support and *A. fumigatus* strain AfS35, Dr. Anita Straus and Dr. Helio Takahashi for the monoclonal antibody MEST-1, Dr. C. Nugier-Chauvin, Dr. R. Daniellou and Dr. P. Peltier for their involvement in the synthesis of UDP-Galf and Dr. Rita Gerardy-Schahn and Dr. Hans Bakker for helpful discussion and critical reading of the manuscript.

This work was funded by the Graduate School 745 of the German Research Foundation (DFG), the French Research Agency (ANR JCJC06\_140075) and the Région Bretagne.

**REFERENCES**

1. Peltier, P., Euzen, R., Daniellou, R., Nugier-Chauvin, C., and Ferrières, V. (2008) *Carbohydr.Res.* **343**, 1897-1923
2. Pan, F., Jackson, M., Ma, Y., and McNeil, M. (2001) *J.Bacteriol.* **183**, 3991-3998
3. de Lederkremer, R. M. and Colli, W. (1995) *Glycobiology* **5**, 547-552
4. Beverley, S. M., Owens, K. L., Showalter, M., Griffith, C. L., Doering, T. L., Jones, V. C., and McNeil, M. R. (2005) *Eukaryot.Cell* **4**, 1147-1154
5. Bakker, H., Kleczka, B., Gerardy-Schahn, R., and Routier, F. H. (2005) *Biol.Chem.* **386**, 657-661
6. Latgé, J. P., Kobayashi, H., Debeaupuis, J. P., Diaquin, M., Sarfati, J., Wieruszkeski, J. M., Parra, E., Bouchara, J. P., and Fournet, B. (1994) *Infect.Immun.* **62**, 5424-5433
7. Toledo, M. S., Levery, S. B., Bennion, B., Guimaraes, L. L., Castle, S. A., Lindsey, R., Momany, M., Park, C., Straus, A. H., and Takahashi, H. K. (2007) *J.Lipid Res.* **48**, 1801-1824
8. Simenel, C., Coddeville, B., Delepierre, M., Latgé, J. P., and Fontaine, T. (2008) *Glycobiology* **18**, 84-96
9. Leitão, E. A., Bittencourt, V. C., Haido, R. M., Valente, A. P., Peter-Katalinic, J., Letzel, M., de Souza, L. M., and Barreto-Bergter, E. (2003) *Glycobiology* **13**, 681-692
10. Morelle, W., Bernard, M., Debeaupuis, J. P., Buitrago, M., Tabouret, M., and Latgé, J. P. (2005) *Eukaryot.Cell* **4**, 1308-1316
11. Kleczka, B., Lamerz, A. C., van Zandbergen, G., Wenzel, A., Gerardy-Schahn, R., Wiese, M., and Routier, F. H. (2007) *J.Biol.Chem.* **282**, 10498-10505
12. Schmalhorst, P. S., Krappmann, S., Vervecken, W., Rohde, M., Müller, M., Braus, G. H., Contreras, R., Braun, A., Bakker, H., and Routier, F. H. (2008) *Eukaryot.Cell* **7**, 1268-1277
13. Zhang, K., Barron, T., Turco, S. J., and Beverley, S. M. (2004) *Mol.Biochem.Parasitol.* **136**, 11-23
14. Späth, G. F., Epstein, L., Leader, B., Singer, S. M., Avila, H. A., Turco, S. J., and Beverley, S. M. (2000) *Proc.Natl.Acad.Sci.U.S.A* **97**, 9258-9263
15. Ha, D. S., Schwarz, J. K., Turco, S. J., and Beverley, S. M. (1996) *Mol.Biochem.Parasitol.* **77**, 57-64
16. Gerardy-Schahn, R., Oelmann, S., and Bakker, H. (2001) *Biochimie* **83**, 775-782
17. Ishida, N. and Kawakita, M. (2004) *Pflugers Archiv-European Journal of Physiology* **447**, 768-775
18. Jack, D. L., Yang, N. M., and Saier, M. H., Jr. (2001) *Eur.J.Biochem.* **268**, 3620-3639
19. Knappe, S., Flügge, U. I., and Fischer, K. (2003) *Plant Physiol* **131**, 1178-1190
20. Peltier, P., Guégan, J. P., Daniellou, R., Nugier-Chauvin, C., and Ferrières, V. (2008) *European Journal of Organic Chemistry* 5988-5994
21. Krappmann, S., Sasse, C., and Braus, G. H. (2006) *Eukaryot.Cell* **5**, 212-215
22. Arai, M., Mitsuke, H., Ikeda, M., Xia, J. X., Kikuchi, T., Satake, M., and Shimizu, T. (2004) *Nucleic Acids Res.* **32**, W390-W393
23. Schwacke, R., Schneider, A., van der Graaff, E., Fischer, K., Catoni, E., Desimone, M., Frommer, W. B., Flügge, U. I., and Kunze, R. (2003) *Plant Physiol* **131**, 16-26
24. Ashikov, A., Routier, F., Fuhlrott, J., Helmus, Y., Wild, M., Gerardy-Schahn, R., and Bakker, H. (2005) *J.Biol.Chem.* **280**, 27230-27235
25. Kabuss, R., Ashikov, A., Oelmann, S., Gerardy-Schahn, R., and Bakker, H. (2005) *Glycobiology* **15**, 905-911
26. Muraoka, M., Miki, T., Ishida, N., Hara, T., and Kawakita, M. (2007) *J.Biol.Chem.* **282**, 24615-24622
27. Krappmann, S., Bayram, Ö., and Braus, G. H. (2005) *Eukaryot.Cell* **4**, 1298-1307
28. Laroy, W., Contreras, R., and Callewaert, N. (2006) *Nat.Protoc.* **1**, 397-405
29. Nierman, W. C., Pain, A., Anderson, M. J., Wortman, J. R., Kim, H. S., Arroyo, J., Berriman, M., Abe, K., Archer, D. B., Bermejo, C., Bennett, J., Bowyer, P., Chen, D., Collins, M., Coulsen, R., Davies, R., Dyer, P. S., Farman, M., Fedorova, N., Fedorova, N., Feldblyum, T. V., Fischer, R., Fosker, N., Fraser, A., Garcia, J. L., Garcia, M. J., Goble, A., Goldman, G. H., Gomi, K., Griffith-Jones, S., Gwilliam, R., Haas, B., Haas, H., Harris, D., Horiuchi, H., Huang, J., Humphray, S., Jimenez, J., Keller, N., Khouri, H., Kitamoto, K., Kobayashi, T., Konzack, S., Kulkarni, R., Kumagai, T., Lafon, A., Latge, J. P., Li, W., Lord, A., Lu, C., Majoros, W. H., May, G. S., Miller, B. L., Mohamoud, Y., Molina, M., Monod, M., Mouyna, I., Mulligan, S., Murphy, L., O'Neil, S., Paulsen, I., Penalva, M. A., Pertea, M., Price, C., Pritchard, B. L., Quail, M. A., Rabinowitsch, E., Rawlins, N., Rajandream, M. A., Reichard, U., Renauld, H., Robson, G. D., Rodriguez, d. C., Rodriguez-Pena, J. M., Ronning, C. M., Rutter, S., Salzberg, S. L., Sanchez, M., Sanchez-Ferrero, J. C., Saunders, D., Seeger, K., Squares, R., Squares, S., Takeuchi, M., Tekaiia, F., Turner, G., Vazquez de Aldana, C. R., Weidman, J., White, O., Woodward, J., Yu, J. H., Fraser, C., Galagan, J. E., Asai, K., Machida, M., Hall, N., Barrell, B., and Denning, D. W. (2005) *Nature* **438**, 1151-1156

30. Schrettl, M., Bignell, E., Kragl, C., Sabiha, Y., Loss, O., Eisendle, M., Wallner, A., Arst, H. N., Jr., Haynes, K., and Haas, H. (2007) *PLoS.Pathog.* **3**, 1195-1207
31. Bakker, H., Routier, F., Oelmann, S., Jordi, W., Lommen, A., Gerardy-Schahn, R., and Bosch, D. (2005) *Glycobiology* **15**, 193-201
32. Rollwitz, I., Santaella, M., Hille, D., Flügge, U. I., and Fischer, K. (2006) *FEBS Lett.* **580**, 4246-4251
33. Gao, X. D., Nishikawa, A., and Dean, N. (2001) *J.Biol.Chem.* **276**, 4424-4432
34. Peltier, P., Daniellou, R., Nugier-Chauvin, C., and Ferrières, V. (2007) *Organic Letters* **9**, 5227-5230
35. Marlow, A. L. and Kiessling, L. L. (2001) *Organic Letters* **3**, 2517-2519
36. Lee, R., Monsey, D., Weston, A., Duncan, K., Rithner, C., and McNeil, M. (1996) *Anal.Biochem.* **242**, 1-7
37. Yu, J. H., Hamari, Z., Han, K. H., Seo, J. A., Reyes-Dominguez, Y., and Scazzocchio, C. (2004) *Fungal.Genet.Biol.* **41**, 973-981
38. Suzuki, E., Toledo, M. S., Takahashi, H. K., and Straus, A. H. (1997) *Glycobiology* **7**, 463-468
39. Stynen, D., Sarfati, J., Goris, A., Prévost, M. C., Lesourd, M., Kamphuis, H., Darras, V., and Latgé, J. P. (1992) *Infect.Immun.* **60**, 2237-2245
40. Costachel, C., Coddeville, B., Latgé, J. P., and Fontaine, T. (2005) *J.Biol.Chem.* **280**, 39835-39842
41. Eckhardt, M., Gotza, B., and Gerardy-Schahn, R. (1999) *J.Biol.Chem.* **274**, 8779-8787
42. Damveld, R. A., Franken, A., Arentshorst, M., Punt, P. J., Klis, F. M., van den Hondel, C. A., and Ram, A. F. (2008) *Genetics* **178**, 873-881
43. El Ganiny, A. M., Sanders, D. A., and Kaminskyj, S. G. (2008) *Fungal.Genet.Biol.* **45**, 1533-1542
44. Puglielli, L. and Hirschberg, C. B. (1999) *J.Biol.Chem.* **274**, 35596-35600
45. Segawa, H., Soares, R. P., Kawakita, M., Beverley, S. M., and Turco, S. J. (2005) *J.Biol.Chem.* **280**, 2028-2035
46. Capul, A. A., Barron, T., Dobson, D. E., Turco, S. J., and Beverley, S. M. (2007) *J.Biol.Chem.* **282**, 14006-14017
47. Goto, S., Taniguchi, M., Muraoka, M., Toyoda, H., Sado, Y., Kawakita, M., and Hayashi, S. (2001) *Nature Cell Biology* **3**, 816-822
48. Kollár, R., Reinhold, B. B., Petràková, E., Yeh, H. J., Ashwell, G., Drgonová, J., Kapteyn, J. C., Klis, F. M., and Cabib, E. (1997) *J.Biol.Chem.* **272**, 17762-17775
49. Klis, F. M., Ram, A. F. J., and De Groot, P. W. J. (2007) A Molecular and Genomic View of the Fungal Cell Wall. In Howard, R. J. and Gow, N. A. R., editors. *Biology of the Fungal Cell*, 2nd Ed., Springer, Berlin Heidelberg New York
50. Späth, G. F., Lye, L. F., Segawa, H., Sacks, D. L., Turco, S. J., and Beverley, S. M. (2003) *Science* **301**, 1241-1243
51. Dean, N., Zhang, Y. B., and Poster, J. B. (1997) *J.Biol.Chem.* **272**, 31908-31914
52. Nishikawa, A., Mendez, B., Jigami, Y., and Dean, N. (2002) *Yeast* **19**, 691-698
53. Nishikawa, A., Poster, J. B., Jigami, Y., and Dean, N. (2002) *J.Bacteriol.* **184**, 29-42
54. Zhou, H., Hu, H., Zhang, L., Li, R., Ouyang, H., Ming, J., and Jin, C. (2007) *Eukaryot.Cell* **6**, 2260-2268
55. Wagener, J., Echtenacher, B., Rohde, M., Kotz, A., Krappmann, S., Heesemann, J., and Ebel, F. (2008) *Eukaryot.Cell* **7**, 1661-1673
56. Roy, S. K., Chiba, Y., Takeuchi, M., and Jigami, Y. (2000) *J.Biol.Chem.* **275**, 13580-13587
57. Schmidt, M. (2004) *Microbiology* **150**, 3253-3260

## FOOTNOTES

- <sup>1</sup> Abbreviations used in this paper: *Galf*, Galactofuranose, Gal, Galactopyranose; UDP, Uridine-5'-diphosphate; UGM, UDP-galactopyranose mutase; NST, nucleotide sugar transporter; GlcNAc, N-Acetylglucosamine; GlcA, Glucuronic acid; GalNAc, N-Acetylgalactosamine; Glc, Glucose; UMP, Uridine-5'-monophosphate; ER, Endoplasmic Reticulum; AMM, Aspergillus Minimal Medium.
- <sup>2</sup> The *glfB* mRNA sequence of *Aspergillus fumigatus* D141 has been deposited in the GenBank database under GenBank Accession Number FJ746723.

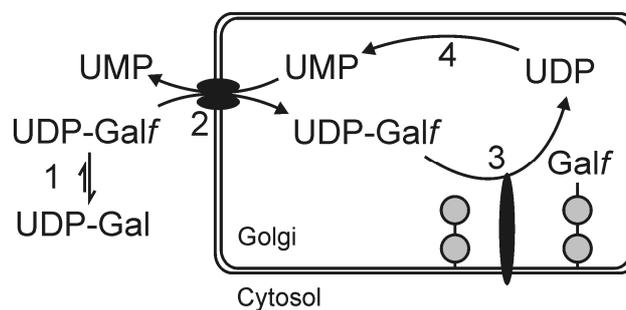
## TABLES

**Table 1** DNA primer sequences for PCR

Primer	Sequence (5' -3'), restriction sites underlined	Description (restriction site)
JE06	TCGAGATCTTTTCGACACTG	forward primer <i>ble/tk</i> cassette
JE07	CTAGAAAGAAGGATTACCTC	reverse primer <i>ble/tk</i> cassette
JE14	TCGACGTATTTTCAGTGTGCGAAAGATCTCGATGTGAAGAGTTGACTCTGGT	reverse primer 5' flanking region +30 nt <i>ble/tk</i> cassette overlap
JE15	GATGGAGAGTACGTATAACG	forward primer 5' flanking region
JE17	CGAAACTGTTGCGTTCTGTCC	reverse primer 3' flanking region
JE18	CAAGCTAGAGTAACCTGTGGAT	forward primer nested PCR
JE19	TATCCTTCGACGCTTCACTG	reverse primer nested PCR
JE20	CACTTGTTTAGAGGTAATCCTTCTTTCTAGGCTGGGAATGCGTAAAGAAAATG	forward primer 3' flanking region +30 nt <i>ble/tk</i> cassette overlap
JE21	CTCCGTCCGGTAGATAGGCACTGG	forward primer Southern Blot probe 1
JE22	GCCCGAAAATCAGACAAGCAGAGA	reverse primer Southern Blot probe 1
JE24	CCGGTGCGAGGATTGATAAGTGTC	reverse primer Southern Blot probe 2
JE25	CGGATTCACCCTCTTAACCACCTTC	forward primer Southern Blot probe 2
JE26	GCAAGGATCCAGTAACGAAGGAGAAAAAGCC	forward primer <i>glfB</i> cDNA (BamHI)
JE28	CGAGTCTAGATTACGCATTCCCAGCAGT	reverse primer <i>glfB</i> cDNA (XbaI)
PS20	AAGGTCGTTGCGTCAGTCCA	forward primer Southern Blot probe 3
PS21	TCGATGTGTCTGCCTCC	reverse primer Southern Blot probe 3

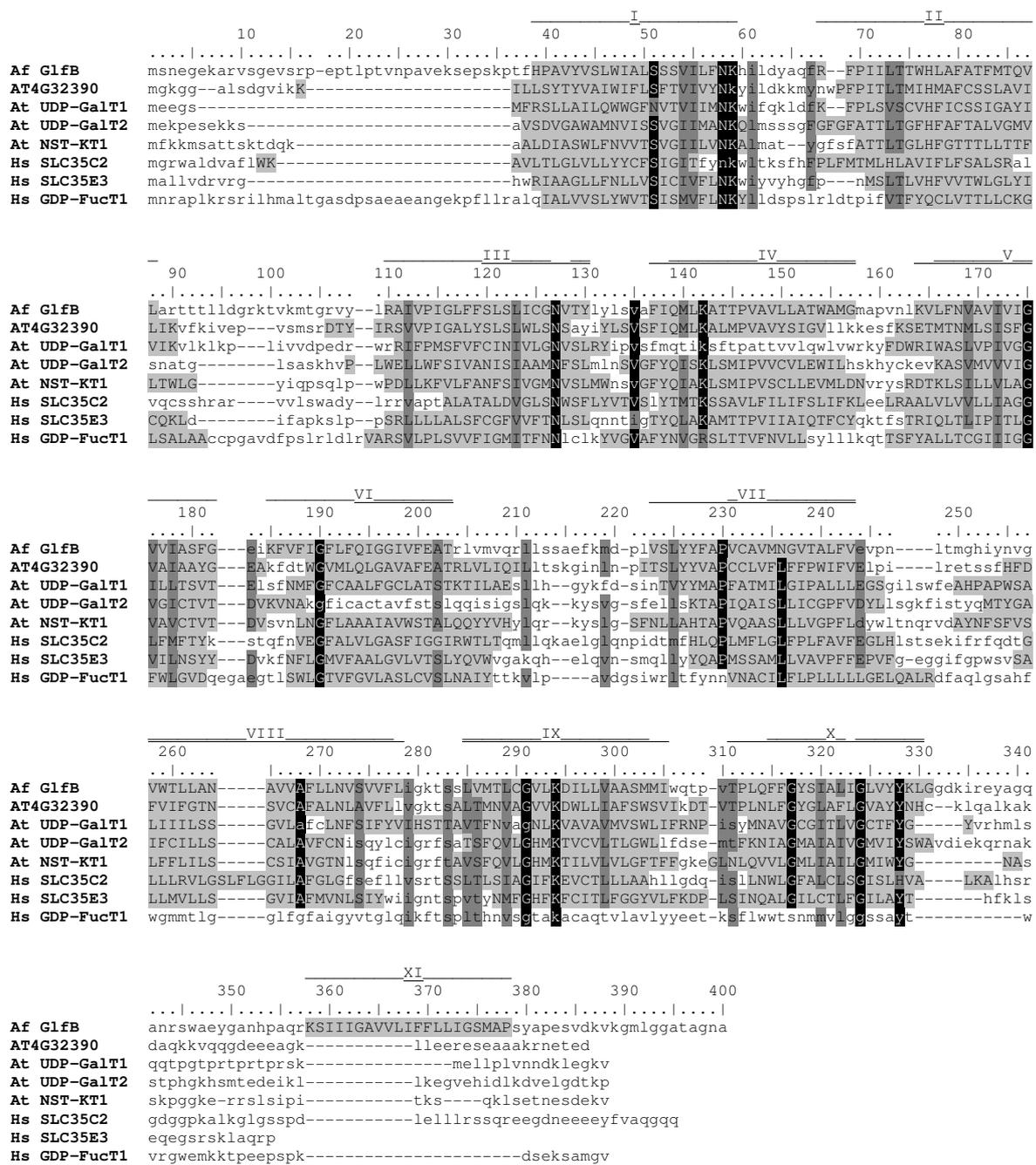
FIGURES

Figure 1



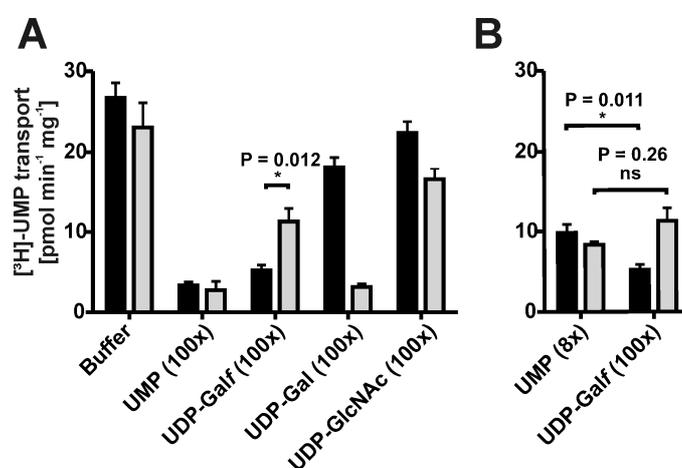
**Figure 1** Schematic model of galactofuranosylation. UDP-Galactofuranose (UDP-Galf) is synthesized from UDP-Galactopyranose (UDP-Gal) (1) and translocated via an antiporter into the Golgi in exchange for UMP (2). Galactofuranosyltransferases transfer Galf moieties from UDP-Galf on various glycoconjugates (3) and UDP is converted to UMP by a nucleoside diphosphatase (4).

Figure 2



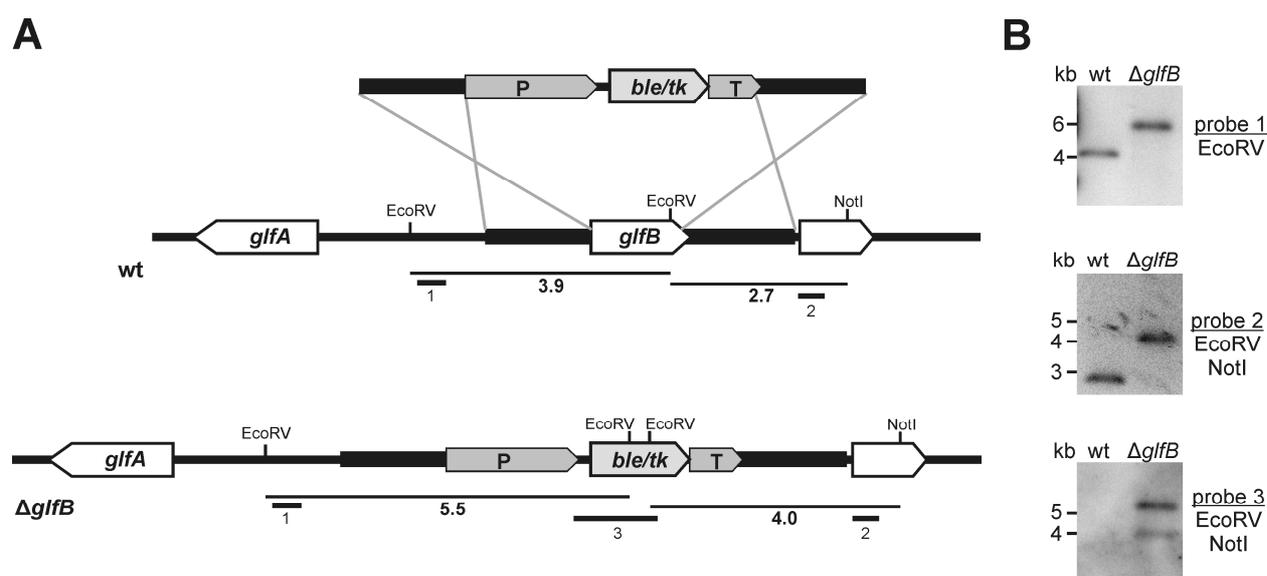
**Figure 2** Multiple sequence alignment of *Aspergillus fumigatus* (Af) GlfB and related proteins from *Arabidopsis thaliana* (At) and humans (Hs) with prediction of transmembrane helices. The *Arabidopsis* uncharacterized protein encoded by the gene AT4G32390 and UDP-Gal transporter AtUDP-GalT1 are the closest GlfB homologs while the plant UDP-Gal transporters At UDP-GalT2 and At NST-KT1, the human proteins SLC35C2 and SLC35E3 and the characterized GDP-Fucose transporter (SLC35C1) are more distantly related. Conserved residues, black shading; similar residues, dark-grey shading; predicted transmembrane domains, capital letters and light-grey shading; Roman numerals, predicted transmembrane domains for GlfB.

Figure 3



**Figure 3** UDP-Galf binds to GlfB. A) [<sup>3</sup>H]-UMP uptake by Golgi vesicles isolated from yeast expressing GlfB (black bars) or the human UDP-Gal transporter (grey bars) in absence or presence of a 100-fold molar excess of unlabelled UMP, UDP-Galf (containing 7 % UMP), UDP-Gal or UDP-GlcNAc. B) Comparison of [<sup>3</sup>H]-UMP uptake in presence of a 100-fold molar excess UDP-Galf containing 7 % UMP or an 8-fold molar excess UMP. Each value represents the average of three independent experiments with duplicate measurements. ns, not significant.

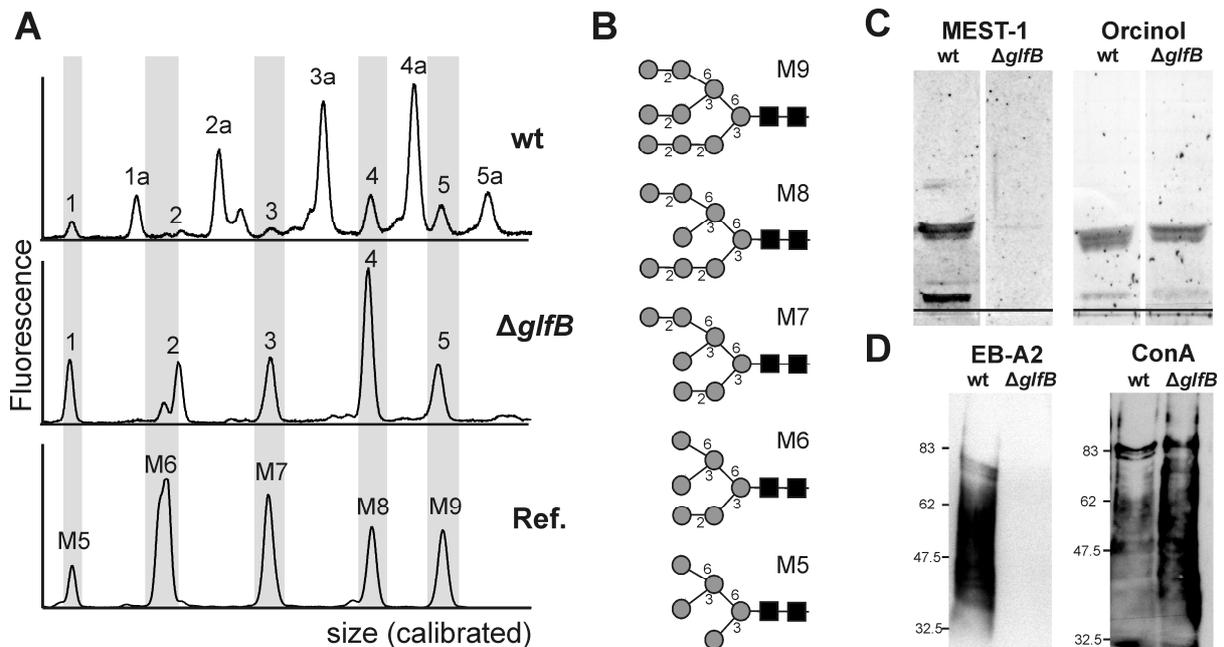
Figure 4



**Figure 4** Gene replacement of *glfB* in *Aspergillus fumigatus*. A) Strategy for the targeted replacement of *glfB* by the *ble/tk* selection marker cassette mediated by homologous recombination (thick black bars). The positions of probes (1-3) used for Southern Blot hybridisation along with respective restriction fragments (size in kb) are indicated. B) Southern Blots of genomic DNA digested with indicated restriction enzymes and hybridized to three different digoxigenin-labelled probes.

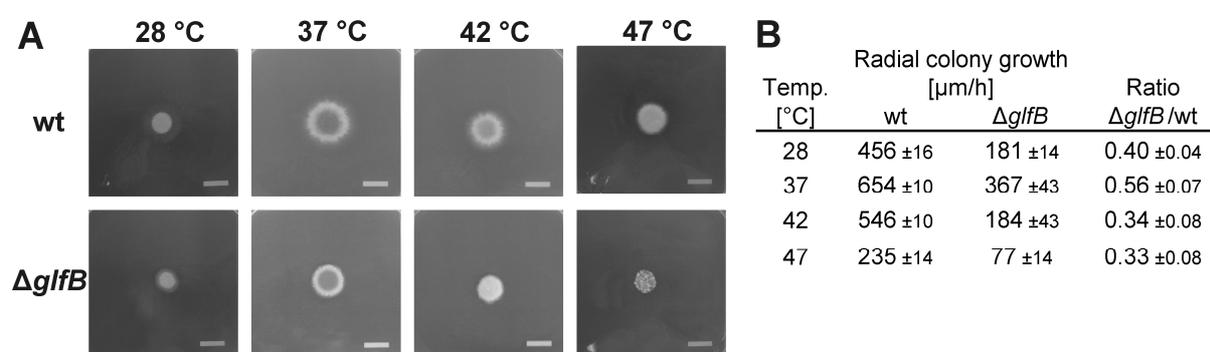
wt, wild type; *ble/tk*, phleomycin resistance/thymidine kinase fusion gene; P, *A. nidulans gpdA* promoter; T, *A. nidulans trpC* terminator; *glfA*, UDP-galactopyranose mutase coding sequence

Figure 5



**Figure 5** The *Aspergillus fumigatus*  $\Delta glfB$  mutant lacks Galf A) Electropherograms of fluorescently labelled N-glycans enzymatically released from secreted glycoproteins of *A. fumigatus* wild type (wt) and the  $\Delta glfB$  mutant. Commercial oligosaccharides (Dextra Laboratories) served as reference (Ref.). The x-axis was calibrated to the fragment sizes of the GeneScan-500 ROX standard (Applied Biosystems). B) Schematic structures of reference oligosaccharides. Black squares, N-acetylglucosamine; grey circles, mannose. C) Glycoinositolphosphoceramides (GIPCs) extracted from *A. fumigatus* mycelium, separated by HPTLC and stained with either the Galf( $\beta$ 1-6/  $\beta$ 1-3)-specific antibody MEST-1 (left) or with orcinol/ $H_2SO_4$  (right). The black line indicates the loading spot. D) Water-soluble extracts of *A. fumigatus* mycelium separated by SDS-PAGE, transferred onto nitrocellulose membrane and stained with either the Galf-specific monoclonal antibody EB-A2 (left) or mannose-specific lectin Concanavalin A (ConA, right).

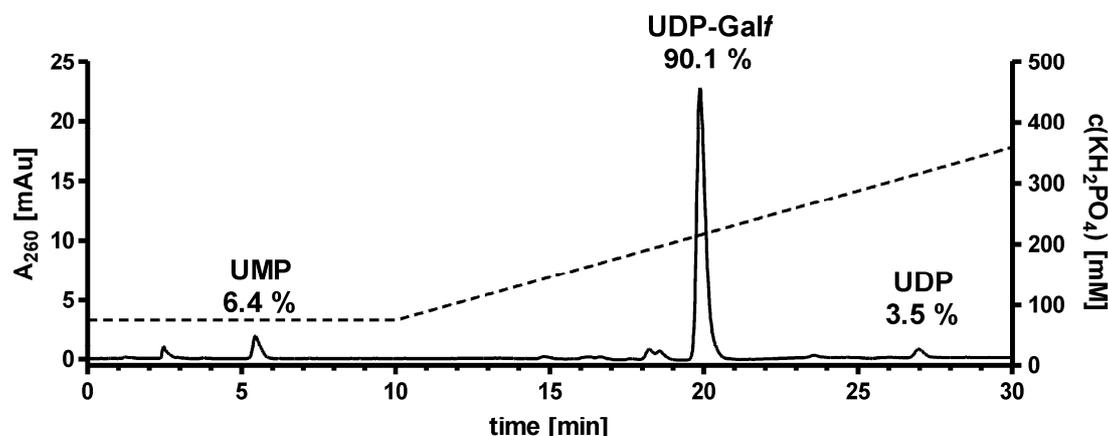
Figure 6



**Figure 6** The morphology and growth of *Aspergillus fumigatus*  $\Delta glfB$  is altered. A) Colony morphology of *A. fumigatus* wild type (wt) and the  $\Delta glfB$  mutant after two days of growth on minimal agar at various temperatures. B) Absolute and relative growth rates obtained from colony diameter measurements (+/- Standard Error of the Mean, n = 3).

SUPPLEMENTAL DATA

Suppl Figure 1



**Suppl Fig 1** UDP-Galf HPLC profile. 10  $\mu$ l of a 2 mM UDP-Galf solution were loaded on a CarboPac PA-100 column (Dionex) and eluted with a linear gradient of 75 to 500 mM KH<sub>2</sub>PO<sub>4</sub> (dashed line, flow rate 1 ml/min) (36). UMP and UDP were identified from retention times of genuine species, and UDP-Galf retention time was derived from (36). Peak area comparison (assuming identical extinction coefficients) yielded a UMP content of about 6-7 % in the UDP-Galf preparation used in our study.

Suppl Table 1

Acc. No.	Description in the RefSeq Database	Locus Tag	TMDs	TC #	TC Subfamily	Possible Substrate
XP_750355	nucleotide-sugar transporter	AFUA_1G06050	8	2.A.7	No subfamily yet	
XP_752298	DUF6 domain protein	AFUA_1G09310	9	2.A.7	No subfamily yet	
XP_756111	integral membrane protein	AFUA_2G17760	9	2.A.7	No subfamily yet	
XP_754351	DUF250 domain membrane protein (GlfB)	AFUA_3G12700	11	2.A.7	No subfamily yet	UDP-Galf
XP_750700	nucleotide-sugar transporter	AFUA_6G08100	10	2.A.7	No subfamily yet	
XP_747139	solute transporter	AFUA_8G00660	8	2.A.7	No subfamily yet	
XP_747138	integral membrane protein	AFUA_8G00670	9	2.A.7	No subfamily yet	
XP_753855	DUF914 domain membrane protein	AFUA_5G07810	10	2.A.7	No subfamily yet	
XP_747907	ER to Golgi transport protein (Sly41)	AFUA_5G04360	8	2.A.7.9	Triose-phosphate Transporter (TPT) Family UDP-N-	
XP_746924	UPD-GlcNAc transporter (Mnn2-2)	AFUA_8G02830	9	2.A.7.10	Acetylglucosamine:UMP Antiporter (UAA) Family	UDP-GlcNAc
XP_750293	UDP-Glc/Gal ER nucleotide sugar transporter	AFUA_1G05440	10	2.A.7.11	UDP-Galactose:UMP Antiporter (UGA) Family	UDP-Gal, PAPS
XP_751184	UDP-galactose transporter	AFUA_6G13070	7	2.A.7.12	CMP-Sialate:UMP Antiporter (CSA) Family	UDP-Gal
XP_746997	nucleotide-sugar transporter	AFUA_8G02090	5	2.A.7.12	CMP-Sialate:UMP Antiporter (CSA) Family	UDP-Gal
XP_754058	Golgi GDP-mannose transporter	AFUA_5G05740	10	2.A.7.13	GDP-Mannose:GMP Antiporter (GMA) Family	GDP-Man
XP_752704	DUF6 domain protein	AFUA_1G13340	10	2.A.7.24	Thiamine Pyrophosphate Transporter (TPPT) Family	Thiamine derivatives
XP_753437	integral membrane protein	AFUA_5G12140	9	2.A.7.24	Thiamine Pyrophosphate Transporter (TPPT) Family	Thiamine derivatives

**Suppl Table 1** *Aspergillus fumigatus* (Af293) nucleotide sugar transporter candidates. The number of transmembrane domains (TMDs) predicted by ConPredII (<http://bioinfo.si.hirosaki-u.ac.jp/~ConPred2/>) is given, together with family classification according to the TC system (<http://www.tcdb.org>) and putative substrates.

## Chapter 4 – General Discussion

Standard therapy of invasive fungal infections comprises two classes of antifungals, amphotericin B and triazoles. The former has been the gold standard in the therapy of invasive fungal infections for decades despite limited efficacy, severe side effects and nephrotoxicity. First-generation triazoles (fluconazole, itraconazole) have been suffering from a limited activity spectrum and a multiple drug interactions. Newly-developed triazoles (voriconazole, posaconazole) have considerably improved efficacy and broad-spectrum activity and are now an established part of standard antifungal therapy and prophylaxis (Sable et al. 2008, Zonios and Bennett 2008).

Yet for Invasive Aspergillosis the mortality remains unacceptably high (20-80 %), which is why much of current mycological research is devoted to the identification of new drug targets in its major causative agent, *A. fumigatus*. This has been difficult due to the opportunistic nature of *A. fumigatus* pathogenicity and the resulting absence of specific virulence traits. Moreover, the conservation of most of the vital cellular pathways between fungi and humans impedes their use as drug targets. As a consequence, only few targets have been identified that are both fungus-specific and truly essential for survival or virulence: 1) The biosynthesis of siderophores that scavenge and store iron (Schrettl et al. 2004, Hissen et al. 2005); 2) The biosynthesis of essential amino acids (Liebmann et al. 2004); 3) The biosynthesis of the cofactor folate (Sandhu et al. 1976, Brown et al. 2000); and 4) the biosynthesis of cell wall  $\beta$ 1,3-glucan (Firon et al. 2002, Mouyna et al. 2004).

The latter example demonstrates the suitability of cell wall polysaccharide biosynthesis as a source of new drug targets. The major part of the cell wall consists of  $\beta$ 1,3-glucan and perturbation of its biosynthesis is likely to have consequences for proper cell wall function, such as protection against mechanical and osmotic stress. Notably, inhibitors of fungal  $\beta$ 1,3-glucan synthesis form the third and newest class of antifungals, the echinocandins (caspofungin, micafungin, anidulafungin) which show good efficacy against *Candida* spp. and *Aspergillus* spp. (Denning 2003).

Little is known on the importance of the other major cell wall polysaccharides, chitin and galactomannan, for *A. fumigatus*. Chitin is required for the yeast *S. cerevisiae* to withstand osmotic stress (Schmidt 2004), however the efficacy of chitin inhibitors (nikkomycins, polyoxins) is hampered by their low penetration of fungal cell walls (Munro and Gow 2001). Nevertheless, nikkomycin Z shows synergistic effects with echinocandins against *A. fumigatus in vitro* and in experimental murine infection (Ganesan et al. 2004, Clemons and Stevens 2006).

Studies on the importance of galactomannan had not been possible because no enzyme involved in its biosynthesis was known until the identification of UDP-galactopyranose mutase (UGM) which represents the first step in the galactofuranose biosynthesis pathway (Bakker et al. 2005a). One goal of this study was thus to evaluate the drug target potential of UDP-Galf biosynthesis in *A. fumigatus* by means of a UGM deficient mutant. Moreover, the identification of a UDP-Galf transporter

presented in this study showed that galactomannan biosynthesis and all other galactofuranosylation reactions depend on this UDP-Galf transporter and thus are situated in the secretory pathway, most likely the Golgi apparatus. This is an unexpected finding since the other cell wall polysaccharides are synthesized at the plasma membrane.

#### **4.1 Importance of galactofuranosylation for growth and morphology of *A. fumigatus***

Single deletion of either UDP-galactopyranose mutase (UGM), encoded by or the newly identified UDP-Galf transporter resulted in a complete loss of galactofuranosylated glycans. This comprised not only N- and O-glycans and glycoinositolphosphoceramides (GIPCs) but also cell wall galactomannan whose biosynthesis had not been studied to date. The requirement of UGM for galactofuranosylation was not surprising as this enzyme is known to be the only route to UDP-Galf biosynthesis in bacteria and protozoa (Stevenson et al. 1994, Pan et al. 2001, Kleczka et al. 2007).

The severity of the phenotype induced by Galf deficiency varies between different organisms. In mycobacteria, deletion of the UGM gene is lethal (Pan et al. 2001). This is a peculiarity as these organisms possess a unique cell wall structure composed of outer cell wall mycolic acids that are linked to the inner cell wall peptidoglycan through Galf containing arabinogalactan. Without Galf, the mycolic acids would detach from the peptidoglycan which is likely to cause the lethal effect. However this cell wall structure is not found in other organisms and thus the importance of Galf for mycobacteria cannot be generalized. In *E. coli*, UGM deficiency causes loss of LPS O-antigen, and thus virulence, but results in perfectly viable mutants (Stevenson et al. 1994). This resembles the findings in *L. major*, the only eukaryote in which the UGM gene has been deleted so far. This mutant is characterized by the loss of its most abundant cell surface polysaccharide, LPG, resulting in attenuated virulence but no detectable growth Galf defect (Kleczka et al. 2007).

In all these mutants the Galf deficiency phenotype is caused by molecules that contain *internal* Galf residues (arabinogalactan/mycolic acids, LPS, LPG) and thus become severely truncated in the case of Galf absence. This is totally different in *A. fumigatus* and other filamentous fungi, in which Galf or short chains of Galf are found exclusively in *terminal* positions.

However, the loss of Galf leads still to a considerably thinner cell wall, altered culture morphology, increased drug susceptibility, reduced sporulation and thermotolerance and a growth defect. Similar effects were observed recently in UGM deficient mutants of *A. niger* and *A. nidulans* and might thus be generalized to other filamentous fungi (Damveld et al. 2008, El Ganiny et al. 2008). These findings resemble the phenotype of a galactosylation deficient mutant of *Schizosaccharomyces pombe*. In this yeast the loss of galactose on the cell wall galactomannan, induced by deletion of the Golgi UDP-

galactose transporter, results in a thinner cell wall, a growth defect and increased drug susceptibility similar to the defects seen in the *A. fumigatus*  $\Delta glfA$  and  $\Delta glfB$  mutants (Tanaka et al. 2001).

More than 100 fungal genomes have been sequenced to date and in these species the occurrence of Galf can be predicted from the presence of *glfA* and *glfB* homologs. Remarkably, within the ascomycota all pezizomycotina (i.e. the mycelial ascomycetes) seem to produce Galf. This correlates perfectly with chemical analyses on the cell wall composition of members of this subphylum. In contrast, no ascomycetous yeast (subphyla saccharomycotina and taphrinomycotina) is predicted or has been shown to contain Galf. It might thus be possible that there is a link between the occurrence of Galf and the mycelial lifestyle of the pezizomycotina. Little is known about fungi of other phyla, however some basidiomycetous yeasts, such as *Cryptococcus neoformans*, also contain Galf.

## 4.2 The *Aspergillus fumigatus* UDP-Galf transporter

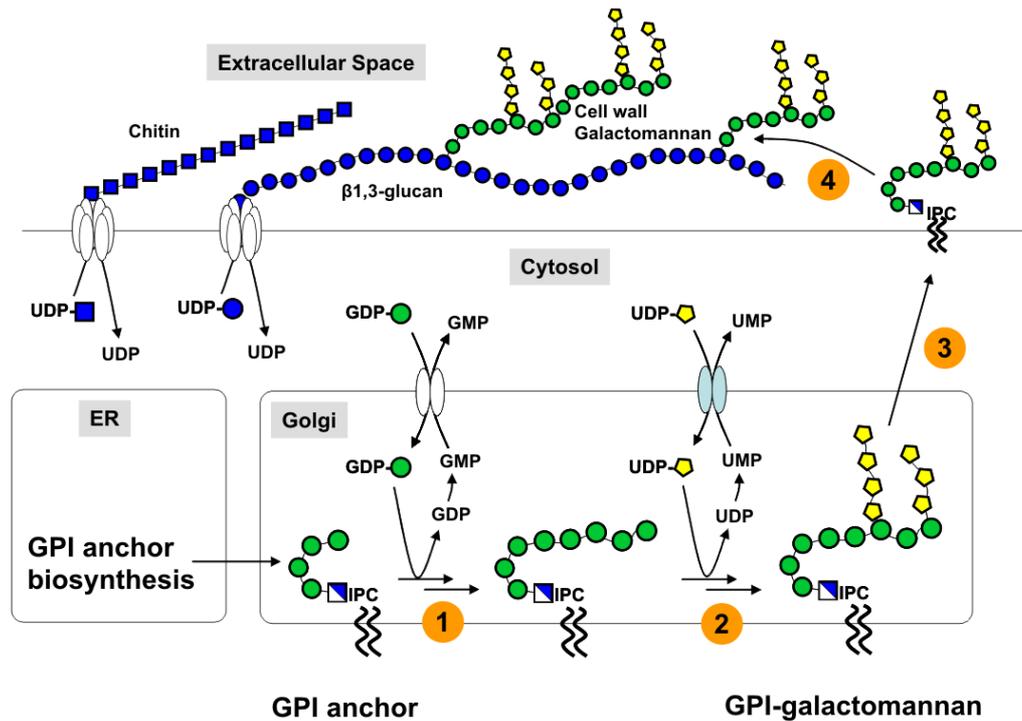
From studies in yeast and other eukaryotes it is known that processing of the Man<sub>8</sub>GlcNAc<sub>2</sub> N-glycan precursor, O-glycosylation and GIPC biosynthesis occur in the Golgi (Dean 1999, Goto 2007, Lisman et al. 2004), thus this is the likely site of galactofuranosylation of N- and O-glycans and GIPCs. From studies in *Leishmania* it is known that the galactofuranosyl donor UDP-Galf is synthesized in the cytosol (Kleczka et al. 2007), therefore it has to be transported into the Golgi lumen to be available for galactofuranosyltransferases. However, no UDP-Galf specific nucleotide sugar transporter (NST) has been described to date nor had any NST been studied in filamentous fungi. As the sequence conservation between NST genes is generally low, candidate genes might be identified by database mining but it is not possible to predict substrate specificity.

Sixteen NST candidate genes were identified in the *A. fumigatus* genome by homology to known NST genes from other species. Intriguingly, one of them was found next to the UGM encoding gene *glfA*. BLAST comparison of this gene, named *glfB*, to characterized NST genes did not allow classification into one of the six established NST families (Saier et al. 2009) but showed relationship to three recently identified plant UDP-Gal transporters (Bakker et al. 2005b, Rollwitz et al. 2006). To determine the substrate specificity of *GlfB*, the uptake of radiolabeled nucleotide sugars into Golgi vesicles of a yeast strain overexpressing *glfB* was measured. It was not possible though to test UDP-Galf directly as it was available only in a non-radioactive form. No transport activity was found for the UDP-sugars UDP-Gal, UDP-GlcNAc, UDP-GalNAc or UDP-GlcA. Many NSTs work preferentially as antiporters, translocating a nucleotide sugar in exchange for the corresponding nucleoside monophosphate, e.g. UDP-Gal for UMP or GDP-Man for GMP (Muraoka et al. 2007, Hong et al. 2000). Thus the NST binding site shows affinity for both, the nucleoside monophosphate and the nucleotide sugar. In a competition experiment it was shown that UDP-Galf, but not UDP-Gal

or UDP-GlcNAc, could inhibit GlfB-mediated UMP transport. This supported the assumption that GlfB was an NST with specificity for UDP-Galf.

To test this hypothesis, a *glfB* gene deletion mutant was created in *A. fumigatus* and analyzed for defects in galactofuranosylation. Remarkably, the  $\Delta glfB$  mutant was completely devoid of Galf as seen for galactomannan and GIPCs by reactivity towards Galf-specific antibodies and electrophoretic N-glycan analysis. These findings were exactly mimicking the molecular phenotype of the  $\Delta glfA$  mutant and as expected, the  $\Delta glfB$  mutant displayed likewise a growth defect, reduced sporulation and decreased thermotolerance. Thus, the  $\Delta glfB$  mutant showed a total loss of galactofuranosylation and combined with the *in vitro* findings on *GlfB* transport activity it could be concluded that *GlfB* encodes a UDP-Galf transporter. Moreover, the total loss of Galf indicates that *GlfB* is the sole UDP-Galf transporter in *A. fumigatus*.

Remarkably, the UDP-Galf transporter deficiency also affects cell wall galactomannan biosynthesis which suggests that galactofuranosylation of galactomannan occurs likewise in the secretory pathway, most likely the Golgi. In contrast, the homopolymeric cell wall polysaccharides  $\beta$ 1,3/1,6-glucan,  $\alpha$ 1,3-glucan, and chitin are synthesized in multi-enzyme complexes at the plasma membrane (Latgé 2007). In this context the existence of a GPI-linked galactomannan is of particular interest as it suggests a galactomannan biosynthesis model (fig. 4-1). In this model, the GPI-galactomannan would be synthesized in the Golgi and subsequently transported to the plasma membrane. There GPI-galactomannan could serve as a donor of ‘galactomannan units’ for a yet unknown extracellular ‘galactomannosyltransglycosidase’ which would transfer galactomannan from its GPI-anchor onto the cell wall  $\beta$ 1,3/1,6-glucan. Two transglycosidases with a comparable activity are known in *A. fumigatus*. The extracellular glucanosyltransferases Gel1 and Gel2 cleave  $\beta$ 1,3-glucan chains and transfer the resulting oligosaccharide on another  $\beta$ 1,3-glucan chain (Hartland et al. 1996, Mouyna et al. 2005). Furthermore, in yeasts some cell wall proteins are covalently linked to the cell wall  $\beta$ 1,3-glucan through a remnant of a GPI-anchor (Klis et al. 2007). This suggests the existence of GPI-specific transglycosidases in the yeast cell wall and it has been speculated that similar mechanisms exist in *Aspergilli* (Damveld et al. 2005). This model would be consistent with an exclusively Golgi-localized UDP-Galf transporter, however this has to be confirmed in additional experiments.



**Figure 4-1** Proposed model of galactomannan biosynthesis. Galactomannan units are synthesized on a GPI anchor by subsequent action of mannosyltransferases (1) and galactofuranosyltransferases (2) in the Golgi. GPI-galactomannan is then transported to the plasma membrane (3) where galactomannan is finally transferred from its GPI anchor to the  $\beta$ 1,3-glucan (4).

The described model of galactomannan biosynthesis allows furthermore to predict that deletion of the Golgi GDP-Man transporter should lead to a stronger glycosylation defect than the one observed in the  $\Delta glfA$  and  $\Delta glfB$  mutants. Not only galactomannan biosynthesis would be completely abrogated, but also O-glycosylation, GIPC biosynthesis, and the putative N-glycan Golgi mannosylation (fig. 3-6). The *A. fumigatus* genome indeed contains a single GDP-mannose transporter candidate gene, and its gene product was characterized *in vitro* as a GDP-Man transporter in the course of this study (data not shown). However, attempts to produce a gene deletion mutant in *A. fumigatus* have failed so far. The repeated difficulties in isolating a GDP-Man transporter deficient mutant might indicate that the GDP-Man transporter is essential for *A. fumigatus*. In several yeast species, the GDP-Man transporter has turned out to be essential as demonstrated by gene deletion mutants in *Saccharomyces cerevisiae*, *Candida albicans* and *Candida glabrata* (Dean et al. 1997, Nishikawa et al. 2002b, Nishikawa et al. 2002a). The importance of the GDP-mannose transporter for yeasts likely results from the combined loss of hypermannosylated N-glycans (fig. 3-4) and elongated O-glycans. However the hypermannosylation of N-glycans is a yeast-specific process that has not been observed in filamentous fungi and protein O-glycosylation seems dispensable in *A. fumigatus* (Zhou et al. 2007, Wagener et al. 2008). GIPC biosynthesis is not essential for *S. cerevisiae* but required for full virulence of *C. albicans* (Beeler et al. 1997, Mille et al. 2004) and current analyses carried out in collaboration with F. Ebel (LMU Munich) indicate that GIPCs are dispensable for *A. fumigatus*, too.

### 4.3 The galactofuranose biosynthesis pathway as drug target

The absence of *Galf* in the  $\Delta glfA$  mutant resulted in attenuated virulence in a murine model of Invasive Aspergillosis. A growth defect comparable to the one seen under culture conditions was observed in the mouse lung and provides a reasonable explanation for the delayed onset of infection. Additional experiments carried out to elucidate whether an altered immune response was elicited towards the  $\Delta glfA$  mutant did not show any difference compared to wild type (data not shown).

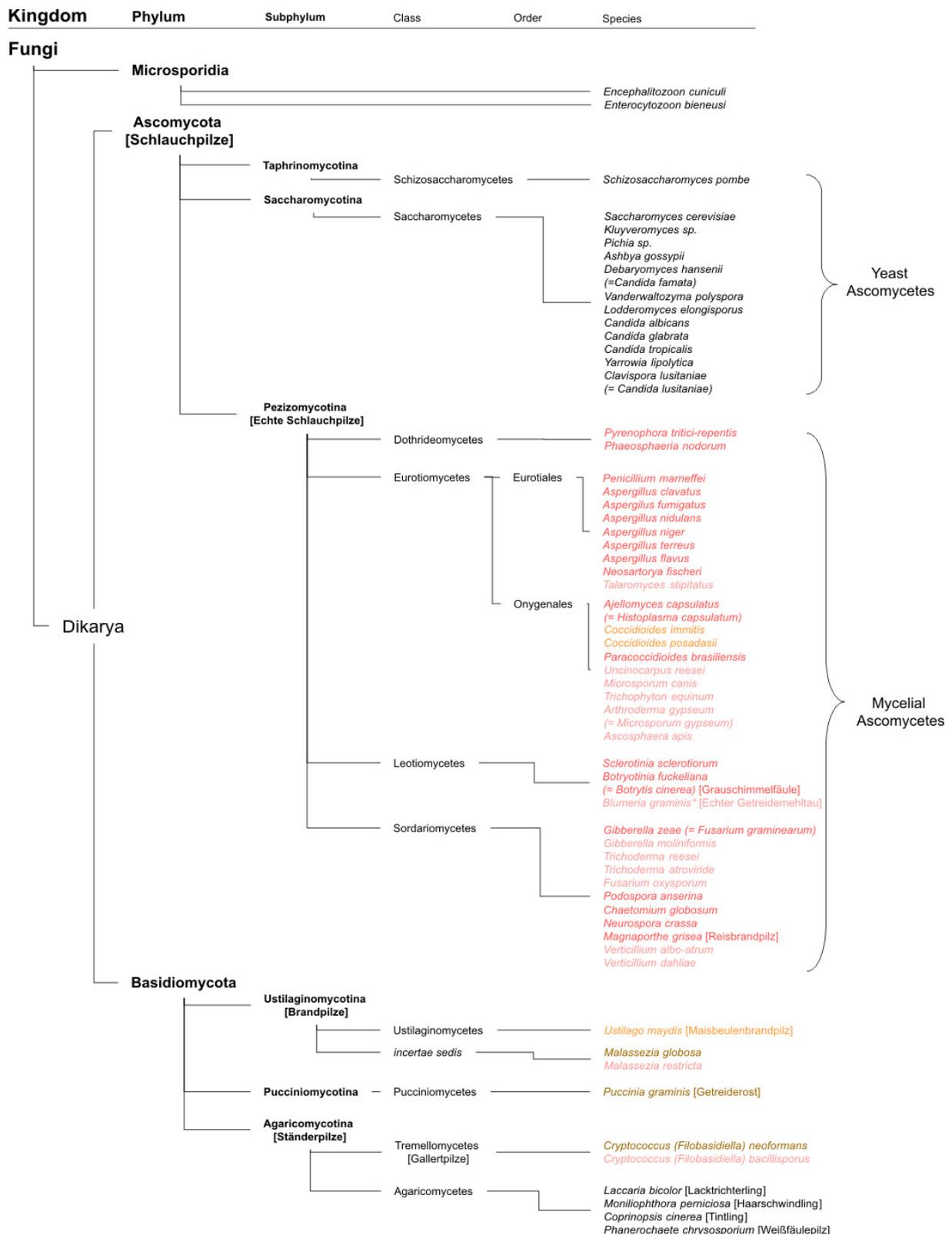
Comparison with other cell wall glycosylation mutants shows that the impact of *Galf* absence can be compared with impaired (not absent)  $\beta$ 1,3-glucan or chitin biosynthesis. The double knockout of two of the seven chitin synthase genes (*ChsG* and *ChsE*) or the deletion of cell wall  $\beta$ 1,3-glucanosyltransferase *Gel2* each lead to a growth defect and reduced virulence (Mellado et al. 1996, Mouyna et al. 2005). However other glycosylation defects, such as loss of cell wall  $\alpha$ 1,3-glucans or partial loss of O-glycans, do not affect virulence or even growth of the mutant strains (Beauvais et al. 2005a, Zhuo et al. 2007). Often the deletion of a gene involved in cell wall biosynthesis of *A. fumigatus* yields unexpectedly viable mutants. In some cases this can be explained by multi-copy genes that functionally compensate for each other, most impressively demonstrated by the seven different chitin synthase genes (Latgé 2007). Besides, fungi possess a ‘cell wall integrity pathway’ that senses various kinds of cell wall stress and responds with the upregulation of numerous cell wall biosynthesis genes, among them synthases for chitin,  $\beta$ 1,3-glucan and  $\alpha$ 1,3-glucan (Lagorce et al. 2003). Thus, cell wall defects caused by the absence of a specific cell wall polysaccharide can often be compensated by an increased production of other cell wall components. Beta-1,3-glucan biosynthesis however, is strictly essential for *A. fumigatus* as demonstrated by RNAi-mediated downregulation of the  $\beta$ 1,3-glucan synthase *Fks1* (Mouyna et al. 2004).

To overcome the compensatory mechanism provided by the cell wall integrity pathway it has been attempted to target the biosynthesis of different cell wall polysaccharides simultaneously. Such synergistic effect is demonstrated by the chitin synthase inhibitor nikkomycin Z. When administered alone it poorly inhibits growth of *A. fumigatus*, both *in vitro* and *in vivo* (Clemons and Stevens 2006, Ganesan et al. 2004). However it shows synergistic effects with the echinocandins caspofungin and micafungin (Ganesan et al. 2004, Stevens 2000). Similarly, the  $\Delta glfA$  mutant was four to eight times more sensitive to nikkomycin Z. Moreover, the susceptibility to caspofungin and amphotericin B was increased twofold and even eightfold to voriconazole. Remarkably, the MIC of voriconazole was decreased to 0.04  $\mu$ g/ml in the  $\Delta glfA$  mutant which is 100 times less than the MIC of the ‘gold standard’ amphotericin B in the wild type. These findings encourage the evaluation of UGM inhibitors as adjunct drugs to second-generation triazole antifungals like voriconazole or posaconazole.

UDP-*Galf* biosynthesis has been recognized as attractive drug target in mycobacteria since arabinogalactan is essential for mycobacterial growth (Pan et al. 2001). A range of 2-aminothiazoles

have been designed as inhibitors of mycobacterial UGM and some of them prevent mycobacterial growth as effectively as currently used antimycobacterial drugs (Dykhuizen et al. 2008). These inhibitors are also effective against *Klebsiella pneumoniae* UGM. However, the sequence divergence between bacterial UGMs and *A. fumigatus* UGM is high and the three-dimensional structure of *A. fumigatus* UGM is not yet known, so inhibitors against bacterial UGMs have a high chance to be inefficient against *A. fumigatus* UGM (Beverley et al. 2005). In collaboration with L. Kiessling (Wisconsin University), a panel of 2-aminothiazoles have been identified that inhibit both bacterial and *A. fumigatus* UGM with good efficacy (T. Gruber, L.L. Kiessling, unpublished results). Preliminary experiments with *A. fumigatus* cultures showed that these inhibitors recapitulated the  $\Delta glfA$  phenotype on minimal medium agar plates at 50  $\mu\text{M}$  (data not shown). These findings demonstrate the cell wall/plasma membrane permeability of these compounds which is an essential property of a drug and suggests that the 2-aminothiazole backbone is a good starting point for the development of a new antifungal. The soon-awaited solution of the three-dimensional structure of *A. fumigatus* UGM will greatly aid the optimization of a 2-aminothiazole drug lead and holds out the prospect of an efficient adjunct drug against *A. fumigatus*.

### 4.4 Supplemental figure



**Suppl Figure** Occurrence of *Galf* in the fungal kingdom. Species containing adjacent *glfA* and *glfB* homologs (red); a *glfA* homolog in unknown relation to *glfB* (faint red); *glfA* and *glfB* in moderate (yellow) or greater (brown) chromosomal distance; or no *glfA* and *glfB* homologs (black).

---

## References

1. Antachopoulos, C., Walsh, T. J., and Roilides, E. (2007) *European Journal of Pediatrics* **166**, 1099-1117
2. Aoki, K., Ishida, N., and Kawakita, M. (2003) *Journal of Biological Chemistry* **278**, 22887-22893
3. Bakker, H., Kleczka, B., Gerardy-Schahn, R., and Routier, F. H. (2005a) *Biol.Chem.* **386**, 657-661
4. Bakker, H., Routier, F., Oelmann, S., Jordi, W., Lommen, A., Gerardy-Schahn, R., and Bosch, D. (2005b) *Glycobiology* **15**, 193-201
5. Barreto-Bergter, E., Gorin, A. J., and Travassos, L. R. (1981) *Carbohydrate Research* **95**, 205-217
6. Beauvais, A., Maubon, D., Park, S., Morelle, W., Tanguy, M., Huerre, M., Perlin, D. S., and Latgé, J. P. (2005a) *Applied and Environmental Microbiology* **71**, 1531-1538
7. Beauvais, A., Maubon, D., Park, S., Morelle, W., Tanguy, M., Huerre, M., Perlin, D. S., and Latgé, J. P. (2005b) *Applied and Environmental Microbiology* **71**, 1531-1538
8. Beauvais, A., Schmidt, C., Guadagnini, S., Roux, P., Perret, E., Henry, C., Paris, S., Mallet, A., Prévost, M. C., and Latgé, J. P. (2007) *Cellular Microbiology* **9**, 1588-1600
9. Beeler, T. J., Fu, D., Rivera, J., Monaghan, E., Gable, K., and Dunn, T. M. (1997) *Molecular and General Genetics* **255**, 570-579
10. Bernard, M. and Latgé, J. P. (2001) *Medical Mycology, Supplement* **39**, 9-17
11. Beverley, S. M., Owens, K. L., Showalter, M., Griffith, C. L., Doering, T. L., Jones, V. C., and McNeil, M. R. (2005) *Eukaryot.Cell* **4**, 1147-1154
12. Beynon, L. M., Richards, J. C., and Perry, M. B. (1997) *European Journal of Biochemistry* **250**, 163-167
13. Bollig, K., Lamshöft, M., Schweimer, K., Marner, F. J., Budzikiewicz, H., and Waffenschmidt, S. (2007) *Carbohydrate Research* **342**, 2557-2566
14. Bonnett, C. R., Cornish, E. J., Harmsen, A. G., and Burritt, J. B. (2006) *Infection and Immunity* **74**, 6528-6539
15. Bourne, E. J., Hartigan, J., and Weigel, H. (1961) *Journal of the Chemical Society (Resumed)* 1088-1092
16. Brakhage, A. A. and Langfelder, K. Menacing mold: The molecular biology of *Aspergillus fumigatus*. 56, 433-455. 2002.
17. Brown, J. S., Aufauvre-Brown, A., Brown, J., Jennings, J. M., Arst, J., and Holden, D. W. (2000) *Molecular Microbiology* **36**, 1371-1380
18. Caillot, D., Thiébaud, A., Herbrecht, R., De Botton, S., Pigneux, A., Bernard, F., Larché, J., Monchecourt, F., Alfundari, S., and Mahi, L. (2007) *Cancer* **110**, 2740-2746
19. Campbell, C. K. (1970) *Journal of General Microbiology* **64**, 373-376
20. Cenci, E., Mencacci, A., Bacci, A., Bistoni, F., Kurup, V. P., and Romani, L. (2000) *Journal of Immunology* **165**, 381-388
21. Clemons, K. V. and Stevens, D. A. (2006) *Medical Mycology* **44**, 69-73
22. Costachel, C., Coddeville, B., Latge, J. P., and Fontaine, T. (2005) *J.Biol.Chem.* **280**, 39835-39842
23. Damveld, R. A., Arentshorst, M., VanKuyk, P. A., Klis, F. M., Van Den Hondel, C. A. M. J., and Ram, A. F. J. (2005) *Fungal Genetics and Biology* **42**, 873-885

24. Damveld, R. A., Franken, A., Arentshorst, M., Punt, P. J., Klis, F. M., Van Den Hondel, C. A. M. J., and Ram, A. F. J. (2008) *Genetics* **178**, 873-881
25. de Lederkremer, R. M., Casal, O. L., Alves, M. J., and Colli, W. (1980) *FEBS Letters* **116**, 25-29
26. Dean, N. (1999) *Biochimica et Biophysica Acta - General Subjects* **1426**, 309-322
27. Dean, N., Zhang, Y. B., and Poster, J. B. (1997) *J.Biol.Chem.* **272**, 31908-31914
28. Denning, D. W. (2003) *Lancet* **362**, 1142-1151
29. Diamond, R. D., Krzesicki, R., Epstein, B., and Jao, W. (1978) *American Journal of Pathology* **91**, 313-328
30. Dubourdeau, M., Athman, R., Balloy, V., Huerre, M., Chignard, M., Philpott, D. J., Latgé, J. P., and Ibrahim-Granet, O. (2006) *Journal of Immunology* **177**, 3994-4001
31. Dykhuizen, E. C., May, J. F., Tongpenyai, A., and Kiessling, L. L. (2008) *Journal of the American Chemical Society* **130**, 6706-6707
32. El Ganiny, A. M., Sanders, D. A. R., and Kaminskyj, S. G. W. (2008) *Fungal Genetics and Biology* **45**, 1533-1542
33. Firon, A., Beauvais, A., Latgé, J. P., Couvé, E., Grosjean-Cournoyer, M. C., and D'Enfert, C. (2002) *Genetics* **161**, 1077-1087
34. Fontaine, T., Magnin, T., Melhert, A., Lamont, D., Latgé, J. P., and Ferguson, M. A. J. (2003) *Glycobiology* **13**, 169-177
35. Fontaine, T., Simenel, C., Dubreucq, G., Adam, O., Delepierre, M., Lemoine, J., Vorgias, C. E., Diaquin, M., and Latgé, J. P. (2000) *J.Biol.Chem.* **275**, 27594-27607
36. Fukuda, T., Boeckh, M., Carter, R. A., Sandmaier, B. M., Maris, M. B., Maloney, D. G., Martin, P. J., Storb, R. F., and Marr, K. A. (2003) *Blood* **102**, 827-833
37. Ganesan, L. T., Manavathu, E. K., Cutright, J. L., Alangaden, G. J., and Chandrasekar, P. H. (2004) *Clinical Microbiology and Infection* **10**, 961-966
38. Gersuk, G. M., Underhill, D. M., Zhu, L., and Marr, K. A. (2006) *Journal of Immunology* **176**, 3717-3724
39. Goto, M. (2007) *Bioscience, Biotechnology and Biochemistry* **71**, 1415-1427
40. Guan, S., Clarke, A. J., and Whitfield, C. (2001) *The Journal of Bacteriology* **183**, 3318-3327
41. Handford, M., Rodriguez-Furlán, C., and Orellana, A. (2006) *Brazilian Journal of Medical and Biological Research* **39**, 1149-1158
42. Hartland, R. P., Fontaine, T., Debeauvais, J. P., Simenel, C., Delepierre, M., and Latgé, J. P. (1996) *Journal of Biological Chemistry* **271**, 26843-26849
43. Haworth, W. N., Raistrick, H., and Stacey, M. (1937) *Biochem.J.* **31**, 640-644
44. Herbrecht, R., Denning, D. W., Patterson, T. F., Bennett, J. E., Greene, R. E., Oestmann, J. W., Kern, W. V., Marr, K. A., Ribaud, P., Lortholary, O., Sylvester, R., Rubin, R. H., Wingard, J. R., Stark, P., Durand, C., Caillot, D., Thiel, E., Chandrasekar, P. H., Hodges, M. R., Schlamm, H. T., Troke, P. F., and De Pauw, B. (2002) *N.Engl.J.Med.* **347**, 408-415
45. Hibbett, D. S., Binder, M., Bischoff, J. F., Blackwell, M., Cannon, P. F., Eriksson, O. E., Huhndorf, S., James, T., Kirk, P. M., Lücking, R., Thorsten Lumbsch, H., Lutzoni, F., Matheny, P. B., McLaughlin, D. J., Powell, M. J., Redhead, S., Schoch, C. L., Spatafora, J. W., Stalpers, J. A., Vilgalys, R., Aime, M. C., Aptroot, A., Bauer, R., Begerow, D., Benny, G. L., Castlebury, L. A., Crous, P. W., Dai, Y. C., Gams, W., Geiser, D. M., Griffith, G. W., Gueidan, C., Hawksworth, D. L., Hestmark, G., Hosaka, K., Humber, R. A., Hyde, K. D., Ironside, J. E., Köljal, U., Kurtzman, C. P., Larsson, K. H., Lichtwardt, R., Longcore, J., Miadlikowska, J., Miller, A., Moncalvo, J. M., Mozley-Standridge, S., Oberwinkler, F., Parmasto, E., Reeb, V., Rogers, J. D., Roux, C., Ryvarden, L., Sampaio, J. P., Schüssler, A., Sugiyama, J., Thorn, R.

- G., Tibell, L., Untereiner, W. A., Walker, C., Wang, Z., Weir, A., Weiss, M., White, M. M., Winka, K., Yao, Y. J., and Zhang, N. (2007) *Mycological Research* **111**, 509-547
46. Hissen, A. H. T., Wan, A. N. C., Warwas, M. L., Pinto, L. J., and Moore, M. M. (2005) *Infection and Immunity* **73**, 5493-5503
47. Hohl, T. M., Van Epps, H. L., Rivera, A., Morgan, L. A., Chen, P. L., Feldmesser, M., and Pamer, E. G. (2005) *PLoS pathogens*. **1**,
48. Hong, K., Ma, D., Beverley, S. M., and Turco, S. J. (2000) *Biochemistry* **39**, 2013-2022
49. Igarashi, T., Satake, M., and Yasumoto, T. (1999) *Journal of the American Chemical Society* **121**, 8499-8511
50. Jack, D. L., Yang, N. M., and Saier, M. H., Jr. (2001) *Eur.J.Biochem.* **268**, 3620-3639
51. Kleczka, B., Lamerz, A. C., van Zandbergen, G., Wenzel, A., Gerardy-Schahn, R., Wiese, M., and Routier, F. H. (2007) *J.Biol.Chem.* **282**, 10498-10505
52. Klis, F. M., Ram, A. F. J., and de Groot, P. W. J. (2007) A Molecular and Genomic View of the Fungal cell Wall. In Howard, R. J. and Gow, N. A. R. (eds.) *Biology of the Fungal Cell*, 2nd ed., Springer (Berlin Heidelberg NewYork).
53. Kremer, L., Dover, L. G., Morehouse, C., Hitchin, P., Everett, M., Morris, H. R., Dell, A., Brennan, P. J., McNeil, M. R., Flaherty, C., Duncan, K., and Besra, G. S. (2001) *Journal of Biological Chemistry* **276**, 26430-26440
54. Lagorce, A., Hauser, N. C., Labourdette, D., Rodriguez, C., Martin-Yken, H., Arroyo, J., Hoheisel, J. D., and François, J. (2003) *Journal of Biological Chemistry* **278**, 20345-20357
55. Langlois, R. A. and Legge, K. L. (2007) *Immunologic Research* **39**, 128-145
56. Latgé, J. P. (1999) *Clin.Microbiol.Rev.* **12**, 310-350
57. Latgé, J. P. (2007) *Molecular Microbiology* **66**, 279-290
58. Li, H., Zhou, H., Luo, Y., Ouyang, H., Hu, H., and Jin, C. (2007) *Molecular Microbiology* **64**, 1014-1027
59. Liebmann, B., Mühleisen, T. W., Müller, M., Hecht, M., Weidner, G., Braun, A., Brock, M., and Brakhage, A. A. (2004) *Arch.Microbiol.* **181**, 378-383
60. Lionakis, M. S. and Kontoyiannis, D. P. (2003) *Lancet* **362**, 1828-1838
61. Lisman, Q., Pomorski, T., Vogelzangs, C., Urli-Stam, D., Van Delwijnen, W. D. C., and Holthuis, J. C. M. (2004) *Journal of Biological Chemistry* **279**, 1020-1029
62. Lübke, T., Marquardt, T., Etzioni, A., Hartmann, E., von Figura, K., and Körner, C. (2001) *Nat.Genet.* **28**, 73-76
63. Madan, T., Eggleton, P., Kishore, U., Strong, P., Aggrawal, S. S., Sarma, P. U., and Reid, K. B. M. (1997) *Infection and Immunity* **65**, 3171-3179
64. Maksyutina, N. P. (1967) *Chemistry of Natural Compounds* **3**, 188-191
65. Martino, R., Subirá, M., Rovira, M., Solano, C., Vázquez, L., Sanz, G. F., Urbano-Ispizua, A., Brunet, S., and de la Cámara, R. (2002) *British Journal of Haematology* **116**, 475-482
66. Mellado, E., Aufauvre-Brown, A., Gow, N. A. R., and Holden, D. W. (1996) *Molecular Microbiology* **20**, 667-679
67. Mikušová, K., Belánová, M., Korduláková, J., Honda, K., McNeil, M. R., Mahapatra, S., Crick, D. C., and Brennan, P. J. (2006) *Journal of Bacteriology* **188**, 6592-6598
68. Mille, C., Janbon, G., Delplace, F., Ibata-Ombetta, S., Gaillardin, C., Strecker, G., Jouault, T., Trinel, P. A., and Poulain, D. (2004) *J.Biol.Chem.* **279**, 47952-47960

- 
69. Morelle, W., Bernard, M., Debeaupuis, J. P., Buitrago, M., Tabouret, M., and Latgé, J. P. (2005) *Eukaryot. Cell* **4**, 1308-1316
  70. Mouyna, I., Henry, C., Doering, T. L., and Latgé, J. P. (2004) *FEMS Microbiology Letters* **237**, 317-324
  71. Mouyna, I., Morelle, W., Vai, M., Monod, M., Léchenne, B., Fontaine, T., Beauvais, A., Sarfati, J., Prévost, M. C., Henry, C., and Latgé, J. P. (2005) *Molecular Microbiology* **56**, 1675-1688
  72. Munro, C. A. and Gow, N. A. R. (2001) *Medical Mycology, Supplement* **39**, 41-53
  73. Muraoka, M., Miki, T., Ishida, N., Hara, T., and Kawakita, M. (2007) *Journal of Biological Chemistry* **282**, 24615-24622
  74. Nassau, P. M., Martin, S. L., Brown, R. E., Weston, A., Monsey, D., McNeil, M. R., and Duncan, K. (1996) *J. Bacteriol.* **178**, 1047-1052
  75. Neofytos, D., Horn, D., Anaissie, E., Steinbach, W., Olyaei, A., Fishman, J., Pfaller, M., Chang, C., Webster, K., and Marr, K. (2009) *Clinical Infectious Diseases* **48**, 265-273
  76. Nishikawa, A., Mendez, B., Jigami, Y., and Dean, N. (2002a) *Yeast* **19**, 691-698
  77. Nishikawa, A., Poster, J. B., Jigami, Y., and Dean, N. (2002b) *J. Bacteriol.* **184**, 29-42
  78. Nogueira, N. F. S., Gonzalez, M. S., Gomes, J. E., de Souza, W., Garcia, E. S., Azambuja, P., Nohara, L. L., Almeida, I. C., Zingales, B., and Colli, W. (2007) *Experimental Parasitology* **116**, 120-128
  79. O'Gorman, C. M., Fuller, H. T., and Dyer, P. S. (2009) *Nature* **457**, 471-474
  80. Odds, F. C., Brown, A. J. P., and Gow, N. A. R. (2003) *Trends in Microbiology* **11**, 272-279
  81. Pagano, L., Caira, M., Candoni, A., Offidani, M., Fianchi, L., Martino, B., Pastore, D., Picardi, M., Bonini, A., Chierichini, A., Fanci, R., Caramatti, C., Invernizzi, R., Mattei, D., Mitra, M. E., Melillo, L., Aversa, F., Van Lint, M. T., Falcucci, P., Valentini, C. G., Girmenia, C., and Nosari, A. (2006) *Haematologica* **91**, 1068-1075
  82. Pan, F., Jackson, M., Ma, Y., and McNeil, M. (2001) *J. Bacteriol.* **183**, 3991-3998
  83. Philippe, B., Ibrahim-Granet, O., Prévost, M. C., Gougerot-Pocidallo, M. A., Perez, M. S., Van der Meeren, A., and Latgé, J. P. (2003) *Infection and Immunity* **71**, 3034-3042
  84. Reijula, K. E. (1991) *Mycopathologia* **113**, 143-149
  85. Riccio, R., Minale, L., Bano, S., and Ahmad, V. U. (1987) *Tetrahedron Letters* **28**, 2291-2294
  86. Rollwitz, I., Santaella, M., Hille, D., Flügge, U. I., and Fischer, K. (2006) *FEBS Lett.* **580**, 4246-4251
  87. Ryan, K. A., Garraway, L. A., Descoteaux, A., Turco, S. J., and Beverley, S. M. (1993) *Proceedings of the National Academy of Sciences of the United States of America* **90**, 8609-8613
  88. Sable, C. A., Strohmaier, K. M., and Chodakewitz, J. A. *Advances in antifungal therapy.* 59, 361-379. 2008.
  89. Saier, J., Yen, M. R., Noto, K., Tamang, D. G., and Elkan, C. (2009) *Nucleic Acids Research* **37**,
  90. Sanchez, A. and Larsen, R. (2007) *Current Opinion in Organ Transplantation* **12**, 579-584
  91. Sandhu, D. K., Sandhu, R. S., Khan, Z. U., and Damodaran, V. N. (1976) *Infection and Immunity* **13**, 527-532
  92. Schmidt, M. (2004) *Microbiology* **150**, 3253-3260
  93. Schrettl, M., Bignell, E., Kragl, C., Joechl, C., Rogers, T., Arst, J., Haynes, K., and Haas, H. (2004) *Journal of Experimental Medicine* **200**, 1213-1219
-

- 
94. Sean Ha, D., Schwarz, J. K., Turco, S. J., and Beverley, S. M. (1996) *Molecular and Biochemical Parasitology* **77**, 57-64
  95. Segawa, H., Soares, R. P., Kawakita, M., Beverley, S. M., and Turco, S. J. (2005) *Journal of Biological Chemistry* **280**, 2028-2035
  96. Serrano-Gómez, D., Domínguez-Soto, A., Ancochea, J., Jimenez-Heffernan, J. A., Leal, J. A., and Corbí, A. L. (2004) *Journal of Immunology* **173**, 5635-5643
  97. Simenel, C., Coddeville, B., Delepierre, M., Latgé, J. P., and Fontaine, T. (2008) *Glycobiology* **18**, 84-96
  98. Singh, N., Limaye, A. P., Forrest, G., Safdar, N., Muñoz, P., Pursell, K., Houston, S., Rosso, F., Montoya, J. G., Patton, P., Del Busto, R., Aguado, J. M., Fisher, R. A., Klintmalm, G. B., Miller, R., Wagener, M. M., Lewis, R. E., Kontoyiannis, D. P., and Husain, S. (2006) *Transplantation* **81**, 320-326
  99. Singh, N. and Paterson, D. L. (2005) *Clin.Microbiol.Rev.* **18**, 44-69
  100. Soltero-Higgin, M., Carlson, E. E., Gruber, T. D., and Kiessling, L. L. (2004) *Nature Structural and Molecular Biology* **11**, 539-543
  101. Späth, G. F., Epstein, L., Leader, B., Singer, S. M., Avila, H. A., Turco, S. J., and Beverley, S. M. (2000) *Proceedings of the National Academy of Sciences of the United States of America* **97**, 9258-9263
  102. Steele, C., Rapaka, R. R., Metz, A., Pop, S. M., Williams, D. L., Gordon, S., Kolls, J. K., and Brown, G. D. (2005) *PLoS pathogens.* **1**,
  103. Stevens, D. A. (2000) *Antimicrob.Agents Chemother.* **44**, 2547-2548
  104. Stevenson, G., Neal, B., Liu, D., Hobbs, M., Packer, N. H., Batley, M., Redmond, J. W., Lindquist, L., and Reeves, P. (1994) *Journal of Bacteriology* **176**, 4144-4156
  105. Takayanagi, T., Kimura, A., Chiba, S., and Ajisaka, K. (1994) *Carbohydr.Res.* **256**, 149-158
  106. Tanaka, N., Konomi, M., Osumi, M., and Takegawa, K. (2001) *Yeast* **18**, 903-914
  107. Toledo, M. S., Levery, S. B., Bennion, B., Guimaraes, L. L., Castle, S. A., Lindsey, R., Momany, M., Park, C., Straus, A. H., and Takahashi, H. K. (2007) *J.Lipid Res.* **48**, 1801-1824
  108. Toyotome, T., Adachi, Y., Watanabe, A., Ochiai, E., Ohno, N., and Kamei, K. (2008) *Microbial Pathogenesis* **44**, 141-150
  109. Trejo, A. G., Haddock, J. W., Chittenden, G. J., and Baddiley, J. (1971) *Biochemical Journal* **122**, 49-57
  110. Turco, S. J., Orlandi, J., Homans, S. W., Ferguson, M. A. J., Dwek, R. A., and Rademacher, T. W. (1989) *Journal of Biological Chemistry* **264**, 6711-6715
  111. Wagener, J., Echtenacher, B., Rohde, M., Kotz, A., Krappmann, S., Heesemann, J., and Ebel, F. (2008) *Eukaryotic Cell* **7**, 1661-1673
  112. Wing, C., Errey, J. C., Mukhopadhyay, B., Blanchard, J. S., and Field, R. A. (2006) *Organic and Biomolecular Chemistry* **4**, 3945-3950
  113. Zhang, K., Barron, T., Turco, S. J., and Beverley, S. M. (2004) *Molecular and Biochemical Parasitology* **136**, 11-23
  114. Zhou, H., Hu, H., Zhang, L., Li, R., Ouyang, H., Ming, J., and Jin, C. (2007) *Eukaryotic Cell* **6**, 2260-2268
  115. Zhuo, H., Hu, H., Zhang, L., Li, R., Ouyang, H., Ming, J., and Jin, C. (2007) *Eukaryot.Cell*
  116. Zonios, D. I. and Bennett, J. E. (2008) *Seminars in Respiratory and Critical Care Medicine* **29**, 198-210
-

---

## Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
DC	Dendritic Cell
Dol	Dolichol
ER	Endoplasmic Reticulum
ERK	Extracellular signal Regulated Kinase
FAD	Flavin Adenine Dinucleotide
Fuc	Fucose
Gal	Galactopyranose
Gal <sub>f</sub>	Galactofuranose
GalNAc	N-Acetyl-galactosamine
GIPC	Glycosylinositolphosphoceramide
GIPL	Glycoinositolphospholipids
Glc	Glucose
GlcN	Glucosamine
GlcNAc	N-Acetyl-glucosamine
GPI	Glycophosphatidylinositol
HSCT	Hematopoietic Stem Cell Transplantation
IA	Invasive Aspergillosis
IPC	Inositolphosphoceramide
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
Man	Mannose
MAPK	Mitogen-associated Protein Kinase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
Neu5Ac	N-Acetylneuraminic acid
NF-κB	Nuclear Factor κB
NST	Nucleotide Sugar Transporter
P	Phosphate
UDP	Uridine-5'-diphosphate
UGM	UDP-galactopyranose mutase

## Curriculum vitae

### Personal Data

---

Address	Philipp Sebastian Schmalhorst Minister-Stüve-Straße 23 30449 Hannover
Date and Place of Birth	29.06.1979, Essen
Nationality	German
Marital Status	Unmarried

### Scientific Education

---

University	1998 – 2004, Studies in biochemistry at the Gottfried Wilhelm Leibniz University Hannover
Diploma thesis	Apr – Oct 2004, Title 'Deletion of the UDP-galactopyranose mutase gene in <i>Aspergillus fumigatus</i> ' under supervision of Prof. Françoise Routier, Dept. of Cellular Chemistry, Hannover Medical School.
Graduation	October 2004 (Diplom-Biochemiker)
Doctoral thesis	Dec 2004 – 2009, under supervision of Prof. Françoise Routier, Dept. of Cellular Chemistry, Hannover Medical School.
Scholarship	Dec 2004 – Nov 2007, PhD scholarship as part of the DFG Graduate School 745 (Mucosal host-pathogen interactions), project title 'Identification of enzymes in the biosynthetic pathway of galactofuranose glycoconjugates in <i>Aspergillus fumigatus</i> '

### Publication

---

P.S. Schmalhorst, S. Krappmann, W. Vervecken, M. Rohde, M. Müller, G.H. Braus, R. Contreras, A. Braun, H. Bakker, and F.H. Routier (2008) Contribution of Galactofuranose to the Virulence of the Opportunistic Pathogen *Aspergillus fumigatus*. *Eukaryot.Cell* **7**, 1268-1277.

### Submitted Manuscript

---

J. Engel\*, P.S. Schmalhorst\*, T. Dörk-Bousset, V. Ferrières, F.H. Routier. A single UDP-Galactofuranose transporter is required for galactofuranosylation in *Aspergillus fumigatus*, submitted to the Journal of Biological Chemistry

\* contributed equally.

## Danksagung

Vielen Dank an

**Prof. Dr. Françoise Routier** für die Überlassung eines ausgezeichneten Projektes, die exzellente Betreuung, fortwährende Unterstützung und die geduldige Korrektur der Manuskripte.

**PD Dr. Sven Krappmann** für die bereitwillige Weitergabe allerlei nützlicher *Aspergillus*-Tools, die große Hilfsbereitschaft in allen Fragen rund um *Aspergillus fumigatus* und die Übernahme des Korreferats.

**Prof. Dr. Peter Valentin-Weigand** für die Initiierung und umsichtige Leitung des Graduiertenkollegs 745 und die Übernahme des zweiten Korreferats.

**Prof. Dr. Rita Gerardy-Schahn** für die begleitende Unterstützung dieses Projekts und die Schaffung einer Arbeitsgruppe mit außergewöhnlicher Atmosphäre.

**Dr. Hans Bakker** für seine unvergleichliche Betreuung und die vielen unschätzbaren Tipps, nicht nur in den Kaffeepausen.

**Dr. Angel Ashikov** für seine unverzichtbare Hilfe nicht nur zu den Transport-Assays und die Erfindung einer Ritual gewordenen, sehr fruchtbaren Kaffeepause.

**die Doktoranden des Graduiertenkollegs 745** für das große Maß an freundschaftlicher Kollegialität.

**Dr. Meike Müller** und **Prof. Armin Braun** für die Unterstützung bei den Maus-Experimenten.

**Dr. Mike Hasenberg** und **Dr. Priyanka Narang** für ihre freundliche Hilfe und die Durchführung immunologischer *Aspergillus*-Experimente.

**Monika Berger, Verena Große, Sabine Schild** und **Olaf Macke** für ihre wertvolle Hilfe und Erklärungen rund um Pilze und Mäuse.

**Jakob Engel**, für großes experimentelles Talent, ohne ihn wäre das *glfB*-Projekt nicht da, wo es jetzt ist.

**Claudia Otto, Manuela Rollenhagen** und **Julia Sürth** für ihre praktische Hilfe im Rahmen diverser Praktika.

**Jens Mittag** für neun Jahre großartigen Zusammenwohnens.

**alle Mitarbeiterinnen und Mitarbeiter des Instituts für Zelluläre Chemie** für diese außergewöhnliche, produktive und zugleich freundliche Atmosphäre.

**meine Eltern** und **Kriki** für darüberhinausgehende unschätzbare Unterstützung in jeglicher Hinsicht.