"The Synthesis and Functions of Galactosaminogalactan in *Aspergillus* species"

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Abstract

Invasive pulmonary aspergillosis (IA) is a progressive necrotizing pneumonia that affects immunosuppressed patients. The most frequently recovered isolate in patients with IA is *Aspergillus fumigatus*, a ubiquitous filamentous mold commonly found in decaying organic material and soil. Extensive efforts have been made to identify virulence factors in *A. fumigatus*, especially factors involved in the synthesis of the cell wall, an important site for host-pathogen interactions. Polysaccharides that make up the cell wall play critical roles in biofilm formation, adherence, and virulence of this fungus. Recently, Fontaine *et al.* characterized a novel cell wall polysaccharide of unknown function, galactosaminogalactan. Galactosaminogalactan is composed of varying units of galactose and N-acetylgalactosamine (GalNAc), and is located in the outer, amorphous layer of the cell wall. It is only produced during vegetative/filamentous growth. The overarching goal of this thesis was to investigate the mechanisms underlying the biosynthesis of galactosaminogalactan and to characterize the role of this polysaccharide in the pathogenesis of invasive aspergillosis.

Using comparative transcriptomics, we identified a putative galactosaminogalactan synthesis gene cluster. The genes in the cluster are annotated as having carbohydrate-related biological functions, including a sugar epimerase (Uge3) and a polysaccharide deacetylase (Agd3). Deletion of *uge3* completely blocked galactosaminogalactan synthesis and resulted in the loss of adherence, disruption of biofilm, and attenuated virulence in a murine model of invasive aspergillosis. Importantly, loss of galactosaminogalactan on the hyphal surface resulted in the exposure of β-glucans, and increased dectin-1 mediated host inflammatory responses to *A. fumigatus* hyphae, establishing a role for galactosaminogalactan in immune evasion.

Studies of recombinant Uge3 revealed that this enzyme could convert both galactose from glucose, and GalNAc from GlcNAc (N-acetylglucosamine). Two other sugar epimerases were identified within the *A. fumigatus* genome, *uge4* and *uge5*. Expression of *uge4* was not detected, and deletion of this gene had no effect on galactose metabolism. Uge 5 was found to

mediate conversion of galactose from glucose and was required for the synthesis of the cell wall polysaccharide galactomannan, but only contributed minor amounts of galactose to the production of galactosaminogalactan.

Biochemical studies of galactosaminogalactan purified from *A. fumigatus* suggested that GalNAc residues within galactosaminogalactan are partially deacetylated, resulting in a positively charged polysaccharide. Deletion of the putative deacetylase *agd3*, prevented the deacetylation of galactosaminogalactan, and resulted in a strain that was impaired in biofilm formation, adherence to negatively charged surfaces and virulence in a murine model of invasive aspergillosis. Thus deactylation and the resulting cationic nature of galactosaminogalactan are required for the function of this exopolysaccharide.

The predominance of *A. fumigatus* as a cause of invasive aspergillosis suggests that it expresses unique virulence traits that are absent in other *Aspergillus* species. To determine if differences in galactosaminogalactan production or composition could contribute to these differences in virulence between species, we examined galactosaminogalactan production among *Aspergillus* species. The reported virulence of *Aspergillus* species was found to be correlated with the amount of GalNAc present in galactosaminogalactan in each of these species. Of the species studied, *A. fumigatus* had the highest level of GalNAc, while the non-virulent species, *A. nidulans* had the lowest. Increasing the GalNAc content in galactosaminogalactan in *A. nidulans* by overexpression of *uge3* or *ugeB* (the *A. nidulans* ortholog of *uge3*) not only increased the adherence of this strain but also enhanced virulence of *A. nidulans* to the level of *A. fumigatus*. Further studies probing the mechanisms underlying this increase in virulence found that galactosaminogalactan plays a crucial role in mediating resistance to antimicrobial peptides released by neutrophils.

Collectively, these results shed light on the mechanisms underlying the biosynthesis of galactosaminogalactan, and have identified several mechanisms by which galactosaminogalactan modulates virulence and will provide the framework to guide the development of therapeutic strategies targeting this important polysaccharide.

Résumé

L'aspergillose invasive (AI) de type pulmonaire est une infection touchant des patients immuno-compromis, notamment des patients ayant reçu une greffe d'organe ou subissant une chimiothérapie. L'aspergillose invasive se traduit par une pneumonie nécrotique évolutive. Le germe le plus couramment isolé à partir d'échantillons de patients souffrant d'aspergillose invasive est *Aspergillus fumigatus*, un champignon filamenteux de type moisissure présent dans la plupart des sols et des matières organiques en décomposition. L'identification des facteurs de virulence d'*A. fumigatus* est donc un axe de recherche très actif, en particulier l'identification de facteurs de virulence liés à la production de la paroi cellulaire et à la régulation de cette production. Les principaux composants de la paroi cellulaire sont des polysaccharides dont le rôle dans la formation de biofilms, dans l'adhérence, et dans la virulence du champignon a été démontré. Récemment, la structure et la composition du galactosaminogalactane (GAG), un polysaccharide appartenant à la paroi cellulaire mais jusqu'ici peu étudié, ont été décrites par l'équipe de Dr Latgé. Le GAG est composé d'un nombre variable de résidus galactose et N-acétyle-galactosamine (GalNAc), et est situé dans la couche externe et inerte de la paroi cellulaire - les autres polysaccharides composant la couche externe étant le galactomannane et l'α-glucane. Le GAG est produit seulement pendant la croissance végétative du filament fongique. L'objectif principal de cette thèse était d'étudier les mécanismes responsables de la biosynthèse du GAG et d'identifier le rôle de ce polysaccharide dans la pathogénèse de l'aspergillose invasive.

L'étude comparative du transcriptome de différentes souches nous a permis d'identifier un groupe de gènes possiblement impliqués dans la biosynthèse du GAG. Les protéines des gènes de ce groupe ont été identifiées comme ayant une fonction liée au métabolisme des glucides, en particulier Uge3, qui fonctionnerait comme épimérase, et Agd3, qui fonctionnerait comme déacétylase. La délétion du gène *uge3* a résulté en une interruption de la biosynthèse de GAG, une perte d'adhérence des hyphes fongiques, une incapacité à former des biofilms normaux, et une virulence atténuée dans notre modèle murin d'aspergillose invasive. De plus, il est à noter que la perte de GAG à la surface des

hyphes a résulté en une exposition des β-glucanes pariétaux sous-jacents, d'où un accroissement de la réponse inflammatoire médiée par la dectine-1, démontrant ainsi le rôle du GAG dans le processus d'évasion d'*A. fumigatus* par rapport au système immunitaire de l'hôte.

L'étude d'une protéine recombinante d'Uge3 a révélé que cette enzyme pouvait convertir à la fois le glucose en galactose et le GlcNAc (N-acétyl-glucosamine) en GalNAc. Deux autres gènes codant pour des épimérases, *uge4* et *uge5*, ont été identifiés dans le génome d'*A. fumigatus*. L'expression du gène *uge4* n'a pas pu être mesurée (car en dessous du seuil de détection), et la délétion de ce gène est restée sans effet sur le métabolisme du galactose. Par contre, l'étude de Uge5 a montré que cette enzyme peut convertir le glucose en galactose, est essentielle pour la synthèse de galactomannane, un autre polysaccharide pariétal, mais n'a qu'une contribution modeste à la production du galactose utilisé dans la synthèse de GAG.

L'analyse biochimique du GAG purifié à partir de cultures d'*A. fumigatus* a démontré que ce polysaccharide est positivement chargé, à cause de la présence dans le GAG de résidus partiellement déacétylés. Par ailleurs, la délétion du gène *agd3*, codant pour une potentielle déacétylase, a entrainé une absence de déacétylation du GAG, et s'est traduite par une souche fongique incapable de former un biofilm normal, d'adhérer à des surfaces chargées négativement, ou de présenter une virulence normale dans notre modèle murin d'aspergillose invasive. La déacétylation du GAG, et la charge cationique qui en résulte, est donc essentielle à la fonction de ce polysaccharide.

La prédominance de l'espèce *A. fumigatus* au sein des isolats issus d'aspergillose invasive suggère que cette espèce possède un ou des facteur(s) de virulence absent(s) des autres espèces d'*Aspergillus*. Pour déterminer si les différences dans la production ou dans la composition du GAG pourraient être un de ces facteurs, nous avons examiné la production de GAG dans différentes espèces d'*Aspergillus*. Le niveau de virulence de ces espèces, tel que décrit, correspond bien avec la proportion de GalNAc au sein du GAG de chaque espèce. En effet, l'espèce la plus virulente, *A. fumigatus*, a démontré le pourcentage de GalNAc le plus élevé, et l'espèce la moins virulente, *A. nidulans*, le pourcentage le plus

faible. En augmentant le taux de GalNAc dans le GAG d'*A. nidulans*, soit par introduction et surexpression du gène *uge3*, soit par surexpression du gène *ugeB* (orthologue à *uge3* chez *A. nidulans*), nous avons pu non seulement augmenter le niveau d'adhérence de cette espèce, mais aussi augmenter sa virulence jusqu'à la rendre équivalente à celle d'*A. fumigatus*. Des études additionnelles, cherchant à élucider le mécanisme d'action du GAG dans la virulence, ont montré le rôle central de celui-ci dans la résistance aux neutrophiles, lors de l'attaque médiée par la NAPDH oxydase.

Pris ensemble, ces résultats améliorent la compréhension de la biosynthèse du GAG et des fonctions que le GAG joue dans la virulence d'*A. fumigatus*. De plus, ces résultats permettent de mieux comprendre les raisons pour lesquels *A. nidulans* est le pathogène dominant dans une catégorie particulière d'infection, l'infection des patients atteints de granulomatose chronique, une maladie génétique dans laquelle la NAPDH oxydase est non-fonctionnelle.

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List of Abbreviations

ABPA: allergic bronchopulmonary aspergillosis

AF: Aspergillus fumigatus

AfWT: *Aspergillus fumigatus* A293 strain

AMP: antimicrobial peptide

ANOVA: analysis of variance

Anuge3OE: *A. nidulans uge3* overexpression strain

AnWT: *Aspergillus nidulans* A26 parental strain

BALF: bronchoalveolar lavage fluid

BMDC: Bone marrow-derived dendritic cell

CF: culture filtrate

DC: dendritic cell

DPI: Diphenyleneiodonium

FBS: fetal bovine serum

fMLP: Formyl-Methionyl-Leucyl-Phenylalanine

GAG: Galactosaminogalactan

GalNAc: N-acetyl-galactosamine

GlcNAc: N-acetyl-glucosamine

GC: gas chromatography

GM: galactomannan

GPI: glycosylphosphatidylinositol

GM-CSF: granulocyte macrophage colony stimulating factor

H&E: hematoxylin and eosin

IA: Invasive aspergillosis

IgG: immunoglobulin G

IL: interleukin

KC: cytokine-induced neutrophil chemoattractant

LPS: lipopolysaccharide

NETs: neutrophil extracellular traps

NK cell: natural killer cell

NMR: nuclear magnetic resonance

PAMP: Pathogen-associated molecular pattern

PAS: Periodic acid-Schiff

PBS: phosphate buffered saline

PDA: potato dextrose agar

PRR: pattern recognition receptors

ROS: reactive oxygen species

SE: standard error

SEM: scanning electron microscopy

TEM: transmission electron microscopy

Th cell: T-helper cell

TNF-α: tumor necrosis factor-alpha

YPD: yeast extract/peptone/dextrose

Contributions to Original Scientific Knowledge

The following key results are presented in this thesis:

1) Galactosaminogalactan is a multi-functional cell wall polysaccharide that can mediate:
    a. Adherence to various surfaces, most notably negatively charged surfaces like human cells
    b. Mask PAMPs like β-1,3-glucan from immune recognition
    c. Resistance neutrophil killing through increased resistance to NADPH oxidase dependent release of antimicrobial peptides l

2) Deficiency in galactosaminogalactan attenuates virulence in a murine model of invasive aspergillosis

3) Uge3 and Uge5 are the only active UDP-glucose 4-epimerases in *A. fumigatus*

4) The UDP-glucose 4-epimerase Uge3, bifunctional epimerase with substrate specificity to both galactose and N-acetyl-galactosamine (GalNAc), is required for the synthesis of galactosaminogalactan

5) The UDP-glucose 4-epimerase Uge5 is required for the synthesis of galactomannan but not galactosaminogalactan

6) Deacetylation of galactosaminogalactan by the polysaccharide deacetylase Agd3 converts the GalNAc residue to galactosamine (GalN), which confers a positive charge on the polysaccharide

7) Agd3-mediated deacetylation of galactosaminogalactan is an extracellular process

8) Deacetylation by Agd3 is required to mediate virulence properties associated with galactosaminogalactan

9) The GalNAc/GalN content of galactosaminogalactan correlates to the amount of cell wall bound galactosaminogalactan and the reported virulence of *Aspergillus* species

10) Heterologous expression of *A. fumigatus uge3* or overexpression of *A. nidulans ugeB* increased the virulence of relatively non-pathogenic *A. nidulans*

Contributions of Authors

This doctoral thesis was prepared in accordance with the guidelines stated in the McGill University "Guidelines for Thesis Preparations". The work of this thesis is presented in the "Manuscript-based thesis" format. All the studies have been performed under the sole supervision of Dr. Donald C. Sheppard. The detailed contributions of each author are listed below. Authors are designated by their initials.

Chapter 1: General Introduction

Parts of the introduction were adapted from: **Lee MJ, Sheppard DC. (2014). The Molecular Biology of Cell Wall Polysaccharides.** *The Mycota Vol. III: Biochemistry and Molecular Biology, 3rd Ed. By D. Hoffmeister* (*in press*).

L.M.J. wrote the literature review. D.C.S. edited the manuscript.

Chapter 2: Gravelat FN*, Beauvais A*, Liu H, Lee MJ, Snarr BD, Chen D, Xiu W, Kravstov I, Hoareau CMQ, Vanier G, Urb M, Campoli PV, Al Abdallah Q, Lehoux M, Chabot JC, Ouimet M-C, Baptista SD, Fritz JH, Nierman WC, Latgé JP, Mitchell AP, Filler SG, Fontaine T, Sheppard DC**.** ***Aspergillus*** **Galactosaminogalactan Mediates Adherence to Host Constituents and Conceals Hyphal β-Glucan from the Immune System.**

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* These authors contributed equally.

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M.J.L. performed 25% of experiments: all scanning electron microscopy imaging, the chemical complementation experiments with exogenous GAG, SBA lectin binding assays, dectin-1 neutralization studies, and assisted with Fc-dectin-1 and β-1,3-glucan exposure imaging.

F.N.G. performed 30% of experiments: generated the fungal mutant strains, and performed adherence assays on biotic and abiotic surfaces, complementation of adherence, mRNA expression profiling, and fungal burden analysis.

H.L. performed 20% of experiments: conducted the mouse survival experiments.

The remaining 25% of experiments were performed by the following co-authors: B.D.S. performed cytokine profiling. D.C. performed the microarray studies. W.X. performed the nanostring studies. I.K. assisted with extraction of galactosaminogalactan and mRNA expression profiling. C.M.Q.C. performed Fc-dectin-1 and β-1,3-glucan exposure imaging. G.V. assisted with the microarray studies. M.U. assisted with transmission electron microscopy imaging. P.V.C. assisted with antifungal susceptibility studies. Q.A.A. assisted with cloning. M.L. performed FACS analysis. J.C.C. assisted with cell culture. M-C.O. assisted with cloning of the mutant and mRNA expression profiling. S.D.B. assisted with growth kinetic assays. F.T. performed the cell wall and galactosaminogalactan compositional analysis.

J.H.F., W.C.N., and A.P.M., contributed reagents/materials/analysis tools.

F.N.G., and D.C.S. wrote the manuscript. A.B., J.P.L., A.P.M., S.G.F., F.T., M.J.L edited the manuscript.

<u>Chapter 3:</u> Lee MJ, Gravelat FN, Cerone RP, Baptista SD, Campoli PV, Choe SI, Kravtsov I, Vinogradov E, Creuzenet C, Liu H, Berghuis AM, Latgé JP, Filler SG, Fontaine T, Sheppard DC. Overlapping and distinct roles of *Aspergillus fumigatus* UDP-glucose 4-epimerases in galactose metabolism and the synthesis of galactose-containing cell wall polysaccharides.

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M.J.L. performed 80% of the experiments. R.P.C., I.K., and F.N.G. performed the mRNA expression experiments. RPC and IK assisted with extraction of galactosaminogalactan. RPC assisted with biofilm adherence and Gal*f* detection assays. S.D.B. assisted with growth kinetic assays. H.L. performed the host cell damage assay. T.F. performed galactosaminogalactan compositional analysis.

F.N.G., R.P.C., S.D.B., S.I.C., and P.V.C. generated fungal mutant strains used in this study.

E.V., C.C., A.M.B., J.P.L., and S.G.F. provided technical/analysis support.

M.J.L. and D.C.S. wrote the manuscript and S.G.F. provided edits.

Chapter 4. **Lee MJ, Geller AM, Liu H, Gravelat FN, Snarr BD, Cerone RP, Baptista SD, Bamford NC, Vinogradov E, Fontaine T, Latgé JP, Stajich J, Howell PL, Filler SG, Sheppard DC. Deacetylation of galactosaminogalactan in *Aspergillus fumigatus* is a required post-synthesis modification for adherence and virulence.**

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M.J.L. performed 80% of the experiments. A.M.G. assisted with the adherence assay, extraction of galactosaminogalactan, *Galleria mellonella* survival experiments, and bioinformatics analysis. H.L. conducted the mouse survival experiments. F.N.G. assisted with the adherence complementation assay. F.N.G. and B.D.S. assisted with extracellular detection assays. R.P.C. assisted with antifungal susceptibility assays. S.D.B. assisted with growth kinetic assays. N.C.B. assisted with bioinformatics analysis.

A.M.G. and F.N.G. generated the fungal mutant strains used in this study.

E.V., J.P.L., J.S., P.L.H. provided technical/analysis support.

M.J.L. and D.C.S. wrote the manuscript and S.G.F provided edits.

Chapter 5: **Lee MJ, Liu H, Barker BM, Snarr BD, Gravelat FN, Al Abdallah Q, Xiao T, Solis NV, Lehoux M, Baptista SD, Cerone RP, Ralph B, Gavino C, Kaminskyj SGW, Vinh DC, Guiot M-C, Latgé JP, Fontaine T, Filler SG, Sheppard DC. Galactosaminogalactan Mediates Virulence in *Aspergillus* Species By Enhancing Resistance to NADPH Oxidase-Dependent Neutrophil Killing.**

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M.J.L. performed 80% of the experiments. H.L., B.M.B., M.L., B.R., assisted with the mouse survival experiments. B.D.S. performed the cytokine profiling. F.N.G. performed the fungal burden. Q.A.A. assisted with cloning. T.X. assisted with morphometry analysis. N.V.S. and M.L. ran the FACS analysis. S.D.B. assisted with growth kinetic assays. R.P.C. assisted with antifungal susceptibility assay. C.G. isolated human neutrophils. T.F. performed galactosaminogalactan compositional analysis.

S.G.W.K., D.C.V., M-C.G., J.P.L., S.G.F., provided technical/analysis support.

M.J.L. and D.C.S. wrote the manuscript, S.G.F provided edits.

Chapter 6. Conclusion and General Discussion

L.M.J. wrote the discussion. D.C.S. edited the manuscript.

CHAPTER 1: General Introduction

1. *Aspergillus fumigatus* biology and pathogenesis

Aspergillus is a ubiquitous, saprophytic mold commonly found in the soil and decaying organic materials such as compost and leaf litter. There are over 250 known members of the genus, many of which play an important role in industry, agriculture, and livestock (1). *A. niger* and *A. oryzae*, are used for citric acid production and fermentation for sake and soy sauce, respectively (1). *A. nidulans*, is a commonly used model organism to study genetics and development. Still others, like *A. fumigatus* and *A. flavus*, are known pathogens of plants and animals and can produce a wide variety of diseases. Importantly, these pathogenic *Aspergillus* species can also cause disease in human as opportunists.

A. fumigatus is the species most commonly isolated from human patients. It is responsible for almost 90% of all *Aspergillus* infections, although it represents only a small proportion of airborne *Aspergillus* spores (2). One major exception to the predominance of *A. fumigatus* in human infection is seen in patients suffering from chronic granulomatous disease (CGD), a hereditary disease caused by a genetic defect in NADPH oxidase that results in impaired granulocyte function and increased susceptibility to bacterial and fungal infections (3). In CGD patients, *Aspergillus nidulans* is recovered with the same frequency as *A. fumigatus* (4). While great efforts have gone into elucidating the reason for the uniquely susceptible of CGD patients to *A. nidulans* infections, the explanation for this observation remains elusive.

The spores of *A. fumigatus*, also known as conidia, are ubiquitous in the environment, and the average person can inhale several hundred conidia per day (5). Once inside the lungs, the fate of the conidia depends on the several host factors. In healthy individuals, the inhaled conidia are readily cleared by pulmonary innate defense mechanisms and do not cause disease (2). However, in individuals with chronic lung disease or immunosuppressed patients, inhaled conidia can germinate and cause a spectrum of diseases, collectively called Aspergillosis. Aspergillosis can range from non-invasive colonization to life threatening invasive infection.

1.1. Life cycle of *A. fumigatus*

Although *A. fumigatus* is most commonly thought of as an opportunistic pathogen, its natural habitat is decaying organic material and soil, and not the human host (5). As a

saprophytic fungus, *A. fumigatus* contributes to carbon cycling and decomposition of dead materials in the environment. The life cycle of *A. fumigatus* begins when an airborne spore is deposited in an appropriate environment. These conidia then break dormancy in association with differential regulation of 734 genes in the first 30 minutes of germination, representing roughly 8% of the total genome (6). Conidia then undergo extensive morphological changes associated with germination, which under laboratory conditions lasts about 4-6 hours. One of the first changes seen during germination is loss of the hydrophobin layer that provided both protection and enhanced dispersion properties during dormancy (7). Hydrophobins are highly polar proteins that arrange in the shape of rodlets and which cover the surface of dormant conidia, rendering it highly hydrophobic (8).

The loss of the hydrophobin layers is associated with changes in the cell wall composition and surface morphology (7). Over a two to three hour period, the germinated conidia become larger, and are commonly referred to as swollen conidia. As the swollen conidia mature, polarized growth is initiated by the polar extension of a tube-like structure, commonly referred to as a germ tube. Germination is the beginning of the vegetative, filamentous growth. Germ tubes continue to grow by apical extension, eventually becoming multicellular hyphae. At this stage, colony formation is apparent, all within the first 12 hours of landing on a favorable surface.

As the colony matures, it forms a hyphal mat, also known as a mycelium. Within 24 hours of germination, the organism becomes developmentally competent and can undergo asexual sporulation in response to appropriate stimuli such as nutrient deprivation. During asexual sporulation, multicellular reproductive structures called conidiophores are formed. These are aerial stalk-like structures that protrude perpendicular to the hyphal mat and give rise to conidia. To aid in dissemination of spores, conidiophores are most commonly produced at the air and water/solid interface. Thus, conidiation usually does not occur when the fungus is grown in submerged liquid culture. The structure and morphology of conidiophore from each species are fairly unique, and commonly used as a morphological marker for taxonomic identification. Understanding the life cycle of *A. fumigatus* is important in considering the pathogenesis of this fungus.

1.2. Diseases caused by *A. fumigatus*

A. fumigatus can cause a wide spectrum of pulmonary diseases including: invasive aspergillosis, aspergilloma, chronic pulmonary colonization and allergic pulmonary aspergillosis. Susceptibility to *A. fumigatus,* and the type of disease depend largely on host factors as outlined below.

1.2.1. Invasive Aspergillosis

In highly immunosuppressed patients, such as those undergoing solid organ and stem cell transplantation or those receiving cytotoxic chemotherapy, inhalation of *A. fumigatus* conidia can lead to an acute, invasive, progressive pulmonary infection, which can hematogenously disseminate to other parts of the body. Importantly, after the initial germination of conidia, only hyphae are observed in tissues of infected patients and conidiation does not occur (9). Although not a common infection, the mortality rate associated with patients that develop invasive aspergillosis is extremely high, between 40% and 90% (10). Despite advances in antifungal therapeutics, including the recent introduction of the mold active azoles and echinocandins, the mortality rate associated with invasive aspergillosis remains over 50% (10). The poor response rate to antifungals is due in part to the failure to diagnose infection early. Recovery of tissue invasive hyphae by sputum culture and bronchoalveolar lavage is poor, and molecular diagnostics are positive relatively late in the course of disease.

1.2.2. Aspergilloma

In patients with pre-existing cavitary pulmonary disease such as tuberculosis or sarcoidosis, *Aspergillus* colonization of the cavity can develop, forming non-invasive fungal balls clinically known as aspergilloma (11). While most aspergilloma patients are asymptomatic, some develop severe hemoptysis requiring surgery (12). Since most patients with aspergilloma are asymptomatic, diagnosis is often difficult, and requires the radiographic detection of fungal balls within the lungs. Aspergilloma is the only form of *A. fumigatus* infection in which conidiophores are produced and conidiation occurs, likely due to the presence of the air-tissue interface found within the pulmonary cavity.

1.2.3. Chronic airway colonization and Allergic Bronchopulmonary Aspergillosis (ABPA)

Individuals with underlying chronic pulmonary conditions such as cystic fibrosis, asthma, or chronic obstructive pulmonary disease, have reduced airway clearance of *Aspergillus* spores due to damage of the mucociliary elevator and the production of excess amounts of pulmonary mucus (10,13). As a result, these patients can develop a chronic form of *Aspergillus* infection in which the damaged airways are chronically colonized with hyphae of *A. fumigatus* (11). Chronic colonization is generally associated with worsening of lung function, and can lead to invasive disease if patients are treated with immunosuppressive agents such as corticosteroids. A subset of these patients (10%) can develop allergic bronchopulmonary aspergillosis (ABPA), a severe hypersensitivity reaction to fungal antigens, which leads to progressive obstructive airway disease and is associated with significant morbidity and mortality (14).

1.2.4. Burden of *Aspergillus*-related diseases

While the exact disease burden associated with *A. fumigatus* invasive infection is not known, it is estimated at an annual rate of 200,000 cases of life-threatening infections world-wide, with an associated antifungal market of around $10 billion USD (15). Assessing disease burden associated with colonization and hypersensitivity is challenging since diagnosis is more difficult and individuals normally do not seek medical assistance unless symptoms are severe. From the limited available data, it is estimated that between 15-25 million people worldwide suffer from some form of chronic aspergillosis (15). However, this is a very conservative estimation, and in reality, the burden of colonization is likely many folds higher. Therefore, effective and efficient treatment of all forms of aspergillosis is paramount in reducing both the disease burden, as well as health care costs.

1.3. Virulence factors of *A. fumigatus*

Of the 250 species of *Aspergillus* found in the environment, the fact that 90% of *Aspergillus* isolates recovered from patients is identified as *A. fumigatus* suggests that factors unique to this species are associated with virulence in humans (2). An extensive effort in the past two decades to identify these virulence factors has broadened our understanding of *A.*

fumigatus pathogenesis. Commonly, virulence factors are categorized into those that are specific to the conidial stage and those that are specific to hyphal growth. For the purposes of this review, and the work in this thesis, we will focus our attention on the pathogenesis of invasive aspergillosis.

1.3.1. Virulence factors produced by conidia

Conidia are the first fungal form encountered by the host. The physical attributes of conidia are important in the establishment of the infection. The small size of *A. fumigatus* conidia enables them to travel deep inside the lung, where mucociliary clearance is less efficient, and their marked hydrophobicity enhances their dispersion in the air and adhesion to host tissues (2,9).

Once the conidia are inhaled they must evade immune recognition by microbial pattern recognition receptors (PRR) such as the c-type lectin, dectin-1 (16). Hydrophobic proteins known as hydrophobins, are layered on the outer most part of conidia (7). These hydrophobins are non-immunogenic, and mask pathogen-associated molecular patterns (PAMPs) like β-1,3-glucan, the cognate ligand for dectin-1 (16). Mutants lacking in RodA, the primary hydrophobin in *A. fumigatus*, produce conidia that are hydrophilic and more readily phagocytosed by macrophages in vitro. The loss of *rodA* attenuates virulence in immunosuppressed mice (17). Importantly however, although hydrophobin production attenuates the inflammatory response to conidia, they do not completely block phagocytosis, and the majority of conidia are phagocytosed by pulmonary macrophages and epithelial cells soon after inhalation (18).

Following phagocytosis, conidia are subject to the unfavorable environment of the phagolysosome where the majority of organisms are killed. Some conidia can escape macrophage killing, particularly in the face of impaired macrophage function due to immunosuppressive therapy. Further, *A. fumigatus* conidia produce factors that enhance resistance to phagolysosomal killing such as synthesis of the cytoprotective dissacharide trehalose, and the pigment melanin, both of which enhance resistance to oxidative stress (19,20).

1.3.2. Hyphal virulence factors

Hyphae are the only morphology of *A. fumigatus* found during established infection. The germination of conidia is associated with the activation of a complex cascade of developmental regulatory pathways that leads to apical extension and elongation of the hyphae, and the production of toxic secondary metabolite proteases. Apical growth and elongation is not only an important process of fungal growth (21), but also material to pathogenesis. While yeast or bacteria can invade host tissue by enzymatic and biochemical degradation (22), hyphal growth allows fungi to also mechanically invade host tissue by penetrating through the tissue layer and invading blood vessels (23,24). An in vitro model of hyphal invasion showed that conidia inoculated on the abluminal surface of endothelial cell layer germinate, elongate, and penetrate through the layer onto the luminal side in a polarized manner (24). Hyphal invasion of endothelial cells also induces tissue factor expression and enhances thrombosis of pulmonary blood vessels. This angioinvasion, thrombosis, and the resulting pulmonary infarction are prominent parts of the clinical presentation of invasive aspergillosis, which can even mimic pulmonary embolism.

Germination and hyphal growth is also associated with secretion of secondary metabolites. There are dozens of metabolites that are produced by the growing hyphae, and many more to be identified. The best studied of the *A. fumigatus* secondary metabolites is gliotoxin (25). Gliotoxin was the first epidithiodioxopiperazines that was isolated and characterized (26). Gliotoxin induces apoptosis of leukocytes through several mechanisms. It disrupts normal respiration and oxidative state of the leukocytes, leading to depolarization of the mitochondrial membrane and release of cytochrome c and caspase-3 activation (27,28). Gliotoxin can also induce mitochondrial-independent apoptosis (28). Gliotoxin has been detected in the lungs of mice infected with *A. fumigatus* and serum of patients with invasive aspergillosis (29,30). Gliotoxin deficient mutants fail to induce apoptosis of leukocytes in vivo, and are attenuated in virulence in non-neutropenic models of invasive aspergillosis (31).

Nutrient uptake is an indispensible biological process in hyphae. In the environment, growing hyphae secrete extracellular enzymes that break down organic material into smaller molecules and building blocks that can be absorbed by the growing organism (32). This form of digestion is mediated largely by an arsenal of fungal proteases.

In *A. fumigatus*, close to 100 predicted proteases are found within the genome, and at least a dozen have been studied (2,33). These proteases can also degrade and damage host tissue by degrading host proteins found in the lungs like collagen and fibrinogen, and thereby enhancing invasion (2). In particular, a serine alkaline protease, Alp1, has been recently found to cleave host complement protein and thus potentially enhance host evasion by *A. fumigatus* (34). While in vitro studies of deletion mutants have significantly broadened our understanding of fungal proteases, establishing a role in virulence of Alp1, or any particular protease by gene deletion approach has been challenging due to redundancy and complementary function among the 100 or so proteases (2,32).

Other hyphal-associated virulence factors include iron acquisition by siderophore (35,36), resistance to oxygen radicals by catalases and superoxide dismutase (37,38), efflux transporters, and thermotolerance that allows growth at the host body temperature. These enzymes and proteins, like the proteases, have evolved to support the growth and development of the organism in different environments. In the human host, the fungus grows and develops differently during different forms of disease. Further studies on the life cycle and development within the host are needed to better understand these different environmental conditions and their impact on fungal virulence.

1.3.3. The role of the hyphal cell wall in virulence

The cell wall is an essential component of the fungus. In *A. fumigatus*, without the protection of a dynamic cell wall, the spheroplast cannot last more than several hours in liquid culture, much less in the harsh the environments where it is usually found. Protection from desiccation and providing structure are not the only functions of the cell wall. Being at the interface of the fungus and the environment, the cell wall mediates important functions such as environment sensing, fungi-to-fungi communication and interaction with the host immune system. In addition, the cell wall and associated polysaccharide biofilm play a critical role in mediating adherence to surfaces. The composition of the cell wall, and the role of individual cell wall polysaccharides in virulence are reviewed in the remaining sections of the first chapter of this thesis.

Preface to Chapter 1, Sections 2 – 5

The synthesis of cell wall polysaccharides is a complicated process that requires careful regulation of many metabolic and biosynthetic pathways. Continuous synthesis and remodeling of the polysaccharides of the cell wall is essential for the survival of the fungus during development, reproduction, colonization and invasion. As a reflection of the importance and interest in the fungal cell wall, as of August 8, 2014, a search on PubMed (NCBI) with the search words "fungal cell wall" returns close to 18,000 publications. Additionally, the multidisciplinary nature of cell wall research, which encompasses structural chemistry, biochemistry, immunology, molecular biology, and evolutionary biology, makes cell wall biology a complex field. Therefore to provide a solid background for the remaining chapters of this thesis, we provided a concise review of the cell wall of *Aspergillus fumigatus*.

The Cell Wall Polysaccharides of *Aspergillus fumigatus*

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2. Introduction

Cell wall polysaccharides are integral components of the fungal cell wall. Cell wall polysaccharides interact to form structural and amorphous matrices through covalent bonding and non-covalent associations. The resulting cell wall encases the fungus providing structure and protection, mediates adherence to surfaces and acts as a molecular sieve allowing the selective passage of molecules into and out of the cell. Although all fungi have a cell wall, the polysaccharide structure and composition of the cell wall varies between fungal species. Further, the cell wall can also vary between morphotypes during the life cycle of the fungus, reflecting the need for stage-specific changes to the cell wall. The importance of the cell wall in fungal biology, and its absence from human cells have made cell wall polysaccharides a promising target for the development of antifungal therapeutics directed against pathogenic fungi such as the ubiquitous mold, *Aspergillus fumigatus*.

In immunocompromised patients, *A. fumigatus* can cause invasive pulmonary aspergillosis (IA), a progressive necrotizing pneumonia that often disseminates to other organs such as the brain. In the absence of a normal immune response, inhaled airborne conidia of *A. fumigatus* can swell and germinate to grow as hyphae that invade deeper pulmonary tissues (39). From the time of germination to hyphal invasion of host tissues, the cell wall provides structure and protection of the organism, as well as mediates a variety of host interactions. Cell wall polysaccharides can mediate adherence to host surfaces, modulate inflammation, induce host leukocyte apoptosis, and mask pathogen-associated molecular patterns (PAMPs) produced by *A. fumigatus*. In essence, the cell wall and associated polysaccharides are indispensible in the establishment and progression of fungal infections.

Disrupting the cell wall by interfering with the synthesis of cell wall polysaccharides is therefore an attractive strategy in managing and treating IA. However, the effects of inhibiting synthesis of an individual polysaccharide by molecular approaches or chemical agents can be compensated by changes in the production or metabolism of other glycans (40). These changes can modulate, and in some cases, even enhance virulence (41) or host inflammation (42). These studies highlight that despite decades of research, we are only now beginning to understand the mechanisms underlying the synthesis of the fungal cell

wall and the role of cell wall components in the pathogenesis of fungal infection. In this chapter, we review our current understanding of the biosynthesis and function of the polysaccharide components of the *A. fumigatus* hyphal cell wall, as well as the role of the network of cell wall integrity pathways in governing cell wall composition.

2.1. The Cell Wall of *Aspergillus fumigatus* Hyphae

The cell wall accounts for 20 – 40% of the dry weight of *A. fumigatus* mycelia, (43,44). Electron microscopy has provided valuable insights into the organization of the cell wall. Scanning electron micrographs reveal that the hyphal surface is highly decorated with extracellular matrix material (Figure 1A). Using a high-resolution transmission electron microscopy technique, two distinct cell wall layers with contrasting electron density are visible beneath this looser matrix component (Figure 1B-C). Although, the exact chemical composition of the ECM or cell wall layers remain unclear, cell wall fractionation studies using alkali extraction methods have suggested that the inner cell wall corresponds to alkali-insoluble fractions of cell wall preparations while the outer cell wall components are found within the alkali-soluble fraction (45,46).

The alkali-insoluble, inner cell wall fraction contains predominantly β-1,3-glucans (51%) and chitin (22%), and minor components of chitosan (7%), β-1,3;1,4-glucans (6%), galactosaminogalactan (7%), and galactomannan (8%) (47). Together, these polysaccharides form the rigid, structural scaffold of the cell wall. The current model of the inner cell wall suggest that it is composed of a large network of β-1,3-glucan that is highly branched, giving it a 3-dimensional shape. While the majority of the branching points are β-1,3 linked, about 4% are β-1,6 linked (45). β-1,3-glucans are covalently linked to other inner cell wall polysaccharides, including chitin, which is responsible for the alkali insolubility of the inner cell wall (45,48).

In contrast to the inner cell wall, the alkali-soluble, outer cell wall fraction contains predominantly α-glucans (92%), with minor components of galactosaminogalactan (5%) and galactomannan (3%) (47). Consistent with the alkali solubility of α-1,3-glucan, immunolabeling with anti-α-1,3-glucan antibody confirmed that α-1,3-glucan is localized in

the outer cell wall (49). Although α-1,3-glucans found in *A. fumigatus* are almost all α-1,3 linked, about 1% has been reported to have α-1,4 linkages (44,46).

Interestingly, galactomannan and galactosaminogalactan are present in both the inner and outer layers of the cell wall. While the presence of galactomannan in the inner cell wall can be explained by its conjugation to β-1,3-glucan, the presence of galactosaminogalactan within the inner cell wall is puzzling since it is not known to be conjugated to other cell wall proteins. Thus, one would expect that the loosely associated galactosaminogalactan in the inner cell wall would be extracted with those found in the outer cell wall in the alkali soluble fraction. Extending outward, polysaccharides found in the outer cell wall are also found in the extracellular matrix (ECM) (49,50), although ratios of the polysaccharides may differ between these two structures (50). Thus, the ECM could be considered an extension of the outer cell wall. The composition of the ECM during infection is not known, however galactosaminogalactan has been detected surrounding hyphae found in lung tissue samples from both patients with aspergilloma and mice with experimental invasive aspergillosis (51). Further investigation is needed to better understand the role of this unusual polysaccharide in the cell wall and extracellular matrix.

3. Synthesis and Function of Cell Wall Polysaccharides

3.1. β-1,3-glucan

β-1,3-glucan is a homopolymer of glucose residues linked in a beta 1 -> 3 fashion (Figure 2). β-1,3-glucan is present in the cell wall of many fungal species. Most importantly, it is a major component of the cell walls of many pathogenic fungi, including *Aspergillus fumigatus*, *Candida albicans*, and *Cryptococcus neoformans* (52). In *A. fumigatus*, β-1,3-glucan is the most abundant cell wall polysaccharide found in the alkali-insoluble fraction of the cell wall, where it can extending up to 1,500 residues long (53). While β-1,3-glucan is essential for maintaining proper cell wall integrity and morphology, it is also a pathogen associated molecular pattern (PAMP) recognized by the pathogen recognition receptor (PRR) dectin-1. Dectin-1 is a member of the c-type lectin family that is commonly expressed on dendritic cells and macrophages, and can activate various immune responses, including secretion of pro-inflammatory cytokines like IL-1β and TNF-α (42,54). Thus,

minimizing exposure of β-1,3-glucan on its surface is a critical strategy for the fungus to evade immune detection.

β-1,3-glucan is synthesized by a cell membrane-bound synthase complex (52). Two subunits of the β-1,3-glucan synthase complex have been identified to date; the catalytic subunit FKS and the regulatory subunit RHO (53). A single Fks1 (afu6g12400) ortholog encodes the catalytic subunit of the beta-1,3-glucan synthase in *A. fumigatus* (55). Inhibition of Fks1 expression by RNAi (56) or by a repressible promoter (57), results in defective growth and swelling of the hyphae due to osmotic pressure.

RHO is a small GTP-binding protein that regulates the activity of the β-1,3-glucan synthase complex by switching between GDP-bound active and GTP-bound inactive states, inducing conformational changes of the catalytic subunit (58). Unlike *FKS*, the genome of *A. fumigatus* contains 6 putative *RHO* genes. Four of these putative Rho-GTPases have been characterized. Localization studies using GFP-tagged Rho1 or Rho3 revealed that both localize to the hyphal tip, where β-1,3-glucan synthesis is most active (59). Conditional downregulation of *rho1* (afu6g06900), or deletion of *rho2* (afu3g10340) or *rho4* (afu5g14060) resulted in mutant susceptible to various cell wall perturbing agents, implicating Rho-GTPases in the cell wall synthesis (60). Deletion of *rho1* has been reported to have the strongest phenotype, suggesting that it is the primary Rho-GTPase involved in cell wall synthesis (60). Rho1 also complexes with Fks1, suggesting that Rho1 is the regulatory subunit of the β-1,3-glucan synthase complex (53).

Rho-GTPases require a guanine exchange factor (GEF) for activation. In *Saccharomyces cerevisiae*, two GEF's, ScRom1 and ScRom2, activate ScRho1 during cell wall stress (61). In *A. fumigatus*, co-localization and immunoprecipitation studies have demonstrated that a single GEF ortholog, Rom2 (afu5g08550), interacts with Rho1 (62). Conditional suppression of *rom2* resulted in a severe growth defect and enhanced susceptibility to cell wall perturbing agents, suggesting that it is required for cell wall integrity (62). Specifically, under suppressive conditions, the *rom2* mutant was highly susceptible to the β-1,3-glucan synthase inhibitor caspofungin.

Caspofungin is a member of the echinocandins class of lipopeptides that non-competitively bind to FKS, thereby inhibiting the synthesis of β-1,3-glucan (63). Although echinocandins are highly effective against *Candida albicans*, they show variable activity

against other fungi (64-66). Caspofungin treatment is fungistatic against *A. fumigatus*, and induces hyphal swelling, especially at the tip, as well as aberrant hyphal branching (63).

Although β-1,3-glucan is a major component of the cell wall, and the only cell wall polysaccharide currently targeted by licensed antifungals, our knowledge of the mechanisms and regulation of β-1,3 glucan synthesis in *A. fumigatus* are only now emerging. A greater understanding of these pathways will be critical for the development of improved next generation inhibitors targeting this important cell wall polysaccharide.

3.2. Chitin

Chitin is a homopolymer of β 1 -> 4 linked N-acetyl-glucosamine (GlcNAc) residues (Figure 2). Chitin is a major component of the fungal cell wall, and it is synthesized through the action of a number of chitin synthases (CHS). Although chitin is present in many fungi, the mechanisms of chitin biosynthesis vary among fungal species (67). In particular, the number of CHS genes varies significantly between species. In *A. fumigatus*, there are eight *CHS* genes categorized into seven different classes based on their amino acid sequence (43). CHS that belong to class I-III, grouped as Family I CHS, require trypsin for activation *in vitro*. Structurally, the catalytic domain of Family I CHS is flanked on either side by transmembrane regions while Family II CHS (class IV-VII) contain only a single C-terminal transmembrane domain (68-70).

Among the Family I CHS genes, single deletions of *chsA* (Class I), *chasB* (Class II), or *chsC* (class III) did not result in noticeable defects in chitin synthesis or growth (69,71). Only deletion of *chsG* (class III) resulted in decreased chitin synthase activity and reduced radial growth (69). Among the Family II CHS genes, single deletions of *chsD* (class VI), *chsF* (class IV), or *csmB* (class VII) was not associated with any modification in the chitin levels of the hyphal cell wall (68,69,71). Only deletion of *csmA* (class V), previously known as *chsE*, resulted in reduced chitin levels in the hyphal cell wall and the production of swollen hyphae (72). A *chsG* and *csmA* double deletion mutant was still viable, although displayed severe growth defect and produced only half the amount of chitin in the cell wall compared to the parental strain (73). Interestingly, this decrease in chitin was associated with a compensatory increase in cell wall α-1,3-glucan content. Recently, using an innovative

molecular approach (74), Muszkieta et al. generated quadruple deletion mutants of the Family I (chsACBG) or Family II (csmABchsFG) CHS genes (75). The resulting quadruple mutants displayed significant growth and cell wall defects. Although, the Δ*chsACBG* mutant was observed to have reduced chitin synthase activity, it was as virulent as the parental strain.

Chitin synthesis can be inhibited by nikkomycin, a nucleoside-peptide secondary naturally produced by *Streptomyces* (76,77). Nikkomycin contains an UDP-N-acetyl-glucosamine moiety that acts as a competitive inhibitor of chitin synthase, disrupting chitin production, and resulting in osmotic lysis and fungal death (78). Nikkomycin is currently not licensed for clinical use, although animal data suggest it is effective against *Histoplasma*, *Blastomyces*, and *Coccidioides* infections, in vivo (77). While nikkomycin is fungicidal against *A. fumigatus* in vitro these effects are seen at much higher concentrations than those required for other fungi such as *Coccidioides posadasii* (77,79).

Collectively, these data suggest that consistent with a critical role for chitin in maintaining structure of the organism, *A. fumigatus* has developed multiple redundant chitin synthesis mechanisms. This complexity offers a substantial challenge in dissecting the regulation and mechanisms governing chitin biosynthesis. Conversely, the importance of this polysaccharide in cell wall structural integrity suggests that it may be an attractive antifungal target if these challenges can be overcome.

3.3. Galactomannan

Mannose-containing polysaccharides, termed mannans, are a common component of the fungal cell wall. However, the side chains, branching, and linkages can vary greatly between mannans of different fungal species. In *A. fumigatus*, the main mannose-containing polysaccharide is galactomannan. Unlike the homopolymers chitin and β-1,3-glucan, galactomannan is composed of two glycosyl chains: mannan and galactofuran chains. The backbone of galactomannan is a homopolymer of mannose residues linked in an α 1 -> 2 or 1 -> 6 fashion (Figure 2)(80). Along the mannan backbone, side chains of galactofuranose (Gal*f*) oligomers are branched in a β-1,3 and β-1,6 linkage (Figure 2)(81). Galactofuranose is a five-member cyclic hexose found in many microbial pathogens, which

is absent in humans (46). Galactofuranosyl side chains are short oligomers of 4 to 5 residues long linked in a β 1 -> 5 fashion (Figure 2)(80).

The synthesis of the galactofuranosyl chain begins in the cytosol, where the precursor of Gal*f*, UDP-galactopyranose (Gal*p*) is produced; UDP-glucose is converted to UDP-Gal*p* through the activity of a UDP-glucose 4-epimerase, Uge5 (82). UDP-Gal*p* is in turn converted to UDP-Gal*f* by the UDP-galactose mutase, UGM (encoded by *ugm1*, also known as *glfA*), (41,83). Cytosolic UDP-Gal*f* is then transported into the *cis*-Golgi by the activity of the UDP-Gal*f* transporter GlfB (84). Deletion of any of these genes results in mutant strains devoid of galactomannan. Interestingly, while Schmalhorst *et al.*, reported attenuated virulence and increased susceptibility to various antifungals in an UGM deficient mutant, Lamarre *et al.*, reported no significant differences in virulence or antifungal susceptibility in an independently constructed UGM deficient mutant (41,83). Differences in strain background or in the design of the phenotypic characterization experiments may contribute to these conflicting observations.

The synthesis of the mannosyl chain of galactomannan is less well defined. In *S. cerevisae*, cell wall mannans originate from mannoproteins with extensive N- and O-glycosylations (85). Initial protein glycosylation of mannoproteins occurs in the endoplasmic reticulum (85). Subsequent extension for cell wall-bound mannoproteins occurs in the *cis*-Golgi, through the activities of multiple mannosyltransferases (86,87). In *A. fumigatus*, while these mannosyltransferases have not yet been identified, the synthesis of the mannosyl chain also occurs inside the Golgi (88), and is dependent on the import of mannose to the Golgi through the action of the GDP-mannose transporter, *gmtA* (89).

Although the exact role of galactomannan in *A. fumigatus* is not clearly defined, it is a unique cell wall polysaccharide in several ways. It is the only polysaccharide known to be synthesized in the Golgi and not through the action of membrane-bound synthases. Also, it is the only cell wall polysaccharide that shares transferases involved in protein glycosylation (90). Galactomannan detection is also in routine clinical use as a diagnostic marker for *Aspergillus* infection. As a result, there is a great interest in elucidating the complete galactomannan biosynthesis pathway. These include the identification and characterization of the glycosyltransferases involved in the synthesis of galactomannan,

and the characterization of the mechanisms underlying the extracellular secretion and cell wall trafficking of this important glycan.

3.4. α-1,3-glucan

α-1,3-glucan is a homopolymer of glucose residues linked in an α 1 -> 3 fashion (Figure 2). Thus, α-1,3-glucan differs from β-1,3-glucan only in the orientation of its linkage. Like β-1,3-glucan, α-1,3-glucan is present in the cell wall of many fungal species, including those of many pathogenic fungi. α-1,3-glucan is essential for correct cell wall morphology and contributes to virulence of several fungal species. In *Schizosaccharomyces pombe*, α-1,3-glucan is an essential cell wall polysaccharide that is required for normal cell wall morphology and cell polarity. In *Histoplasma capsulatum*, α-1,3-glucan masks β-1,3-glucans from immune recognition by dectin-1 (91). In *Cryptococcus neoformans*, α-1,3-glucan is required to anchor the capsule, an important virulence factor of this fungus (92).

In *A. fumigatus*, α-1,3-glucan is synthesized through the action of three synthases: Ags 1, 2 and 3 (93). Localization studies have demonstrated that Ags1 and Ags2 are located within the cell membrane of hyphae (94), and Ags3 is also predicted to be cell membrane-bound. Deletion of either *ags1* or *ags2* resulted in mutants with altered hyphal morphology and impaired conidiation, however, deletion of *ags1* resulted in only partial reduction of α-1,3-glucan levels, while deletion of *ags2* and *ags3* had no effect on levels of this glycan (94). Increased expression of *ags1* and *ags2* in the *ags3* deletion mutant suggested that compensatory upregulation of *AGS* could contribute to these results (94). Deletion of all three *AGS* genes resulted in a mutant that is completely devoid of α-1,3-glucan (95)(Beauvais, Bozza, 2013). While the triple mutant displayed normal growth and germination, it was found to have significant alterations in the cell wall organization, with increased exposure of β-1,3-glucans and the presence of extracellular proteins on its conidial cell surface. Importantly, conidia of the triple mutant were more efficiently phagocytosed and showed attenuated virulence in neutropenic mice. Pulmonary histopathology studies from mice infected with this triple mutant revealed an absence of filamentous growth in mice infected, suggesting that the increased survival of these mice

was due to enhanced immune recognition of the mutant conidia and/or impaired germination of the mutant strain *in vivo*.

3.5. Galactosaminogalactan

Galactosaminogalactan is a heteropolymer composed of galactose and N-acetyl-galactosamine (GalNAc) linked in an α 1 -> 4 fashion (Figure 2). Unlike other cell wall polysaccharide, the component residues do not seem regularly arranged (44). Galactosaminogalactan has been described in other *Aspergillus* species, including *A. nidulans*, *A. parasiticus*, *A. niger*, and *A. fumigatus* (96-99), and in a non-*Aspergillus* species, *Bipolaris sarkholderia* (100). The synthesis of galactosaminogalactan begins with the conversion of UDP-galactose from UDP-glucose and UDP-GalNAc from UDP-GlcNAc. Two epimerases, Uge3 and Uge5, are required for the normal synthesis of these sugars. Uge5 is a group 1 epimerase required for UDP-galactose synthesis while Uge3 is a group 2 bifunctional epimerase that can mediate synthesis of both UDP-galactose and UDP-GalNAc (82). While Uge5 is the main epimerase responsible for mediating the interconversion of UDP-glucose/galactose, in the absence of Uge5, the bifunctional activity of Uge3 is sufficient to produce UDP-galactose for the synthesis of galactosaminogalactan, but not galactomannan. Further studies delineating downstream components would be required to understand this difference in the two synthetic pathways.

Recent studies have found galactosaminogalactan is required for a number of virulence-associated functions. Blocking galactosaminogalactan synthesis by deletion of *uge3* resulted in a mutant associated with defects in biofilm adherence, increased exposure of β-1,3-glucans, and attenuated virulence in leukopenic mice (Gravelat 2013). In addition, studies using purified fractions of galactosaminogalactan have found that galactosaminogalactan induces natural killer (NK) cell-dependent apoptosis of neutrophils (101), and intranasal administration of these preparations induced anti-inflammatory responses in mice (99,102). Thus, purified galactosaminogalactan seems to have immune suppressive properties and could be potentially developed as a therapeutic agent. The composition and structure of galactosaminogalactan produced *in vivo* remains unknown.

Further structural and functional characterization of this heteropolymer is required if it is to be exploited as a therapeutic target.

4. Modifications of cell wall polysaccharides

4.1. Modification of β-1,3-glucans

β-1,3-glucan undergoes multiple modifications following production of the linear β-1,3-glucan chain by the β-1,3-glucan complex. The most common modification involves a cut/paste action in order to add a new chain of β-1,3-glucan onto an existing chain, form a branching point, or conjugate β-1,3-glucan to other cell wall polysaccharides or glycoproteins. A survey of putative β-1,3-glucan modifying enzymes in the genome of *A. fumigatus* was recently performed, and reported that 29 out of 45 candidate genes are expressed during vegetative growth (103). These genes were annotated as having glucanase or transferase activity, belonging to 8 different GH (glycosyl hydrolase) families as classified on the Carbohydrate-Active enZYmes database [CAZy database (http://www.cazy.org)]. While functional evidence is lacking to validate the function of most of these 45 putative β-1,3-glucan modifiers, several of these have been studied as detailed below.

4.1.1. Endo-β-1,3-glucanase (ENG proteins)

The endo-β-1,3-glucanase proteins are glycosylphosphatidylinositol (GPI)-anchored proteins that randomly cleave internal residues of soluble β-1,3-glucans (104). To date, two of the genes coding for ENG enzymes, *eng1* and *eng2*, have been deleted and characterized (103,104). No significant phenotypic differences between the single mutants and the parental strains were observed. Given that there are six other putative ENG in the genome, it is likely that compensatory activity of other ENG proteins can compensate for the loss of a single family member as is the case for chitin and α-1,3-glucan synthase genes. A triple mutant of *eng1*, *eng2*, and *eng4*, the only three ENG genes expressed in vegetative growth, would likely provide important insights into the role of endo-β-1,3-glucanase in cell wall biosynthesis.

4.1.2. β-glucanosyltransferase (GEL proteins)

Another group of GPI-anchored proteins, the GEL family of β-glucanosyltransferases, has been studied extensively. This class of enzyme promotes the elongation of β-1,3-glucan chains by cleaving an internal β-1,3 linkage of a β-1,3-glucan chain, and transferring the newly created reducing end to the non-reducing end of another β-1,3-glucan chain (105). Of the seven putative genes coding for GEL's, *gel1*, *gel2*, *gel4*, and *gel7* have been studied. While the deletion of *gel1* did not result in phenotypic differences from the parental strain, deletion of *gel2* or the double deletion of *gel1* and *gel2* was associated with growth defects, abnormal conidiogenesis, and hypovirulence (106). Deletion of *gel4* was lethal, suggesting that Gel4 is essential for viability (107). Deletion of *gel7* did not significantly alter germination or susceptibility to cell wall perturbing agents, but was associated with a conidiation defect (108).

Unlike the ENG enzymes, individual GEL enzymes seem to play important roles in cell wall biogenesis. This observation suggests that while ENG's have redundant functions, each of the GEL proteins may have a distinct role in β-1,3-glucan modification. It is also possible that *A. fumigatus* favours restructuring the cell wall by "recycling" pre-made β-1,3-glucan by removing these glycans from locations where they are no longer needed and re-attaching them as required. This cell wall remodeling strategy using GEL enzymes would offer an energy-saving approach as compared with de novo biosynthesis of β-1,3-glucan chains.

4.1.3. Branching enzymes (BGT proteins)

Branching enzymes are also involved in modification of β-1,3-glucan to generate a complex polysaccharide structure. Of the five putative branching enzymes, all of which are expressed during vegetative growth, Bgt1 and Bgt2 have been studied. Bgt1 transfers the reducing end of one β-1,3-glucan to C6 of the non-reducing end of another β-1,3-glucan, generating a linear β-1,3;1,6-glucan chain (109,110). Bgt2, on the other hand, transfers the reducing end of a β-1,3-glucan to an internal C6, forming a linear β-1,3-glucan with branched β-1,6 linked β-1,3-glucan (110). Single or double deletions of *bgt1* and *bgt2* had

no effect on *A. fumigatus* growth and resistance to cell wall stressors (110). Like the ENG enzymes, it is possible that the other three branching enzymes have redundant function and are able to compensate for the loss of both Bgt1 and Bgt2. A quintuple mutant would ultimately be required to study the role of the branching enzymes, especially since all five are expressed during hyphal growth.

4.1.4. Other β-1,3-glucan modifying enzymes (EXO and SUN proteins)

Other β-1,3-glucan modification enzymes have also been studied. Exo-β-1,3-glucanases, ExoGI and ExoGII, have been purified and found to have specific affinity against β-1,3-glucan and other β-glucans, respectively (111). To date, no deletion mutants of the corresponding genes have been generated, so their role in cell wall metabolism is unknown. Another group of β-1,3-glucan modification enzymes belonging to the SUN protein family has been recently studied. Initially characterized in *S. cerevisae* for their involvement in cell wall biogenesis, this group of enzymes consists of SIM1, UTH1, NCA3, and SUN4 (112-114). In *A. fumigatus*, two SUN protein homologs have been identified. Recombinant Sun1 binds to and hydrolyzes β-1,3-glucan, and deletion of the gene encoding this protein, led to defects in growth and cell wall integrity, including swelling and leakage of the hyphal tip (115). In contrast, deletion of *sun2* did not result in any major phenotypic differences as compared with the parental strain. Thus, Sun1 seems to be directly involved in cell wall biogenesis and required for β-1,3-glucan modification. Further characterization of Sun1, including its role during growth in vivo, will be needed to ascertain its role in pathogenesis.

4.2. Modification of Chitin

4.2.1. Chitinase (CHI proteins)

Modification of chitin is an important process for remodeling of the hyphal cell wall. Similar to hydrolysis of β-1,3-glucan by β-1,3-glucanases, chitin is hydrolyzed by chitinases. The function of chitinases has been well described in yeast, where chitinase activity is important in cell separation during budding (116-118). In *A. fumigatus*, 14 putative chitinases (CHI) were initially identified and classified into two families based on their

sequences and similarities to either plant or bacterial chitinases (119). These CHI proteins have more recently been re-classified into 3 groups, group A, group B, and group C (116). Single deletion mutants of all *CHI* genes have been constructed and have not displayed unique phenotypes as compared to the parental strain (116,120). In fact, deletion of all five genes in the group B *CHI's* also was not associated with any changes in cell wall associated phenotypes (116). Given that there are 14 putative *CHI* genes in the genome of *A. fumigatus*, it is likely that there are redundancies within this family, and the construction of more multiple deletion mutants will likely be required to elucidate the role of these proteins in cell wall biogenesis.

4.2.2. Chitin deacetylase (CDA proteins)

Chitin can also be modified to produce chitosan through the deacetylation of N-acetyl-glucosamine (GlcNAc) to glucosamine (GlcN). Chitosan is produced on an industrial scale through chemical deacetylation of chitin found in seashells and other biomass sources (121). In microorganisms, chitosan is produced through the activity of a chitin deacetylase, CDA. Although chitosan is applied to deacetylated chitin, the degree of deacetylation can vary between 50-90% (121). In recent years, chitosan has gained increasing popularity due to its expanding industrial, pharmaceutical, and medical uses (122). Among its many uses, chitosan is becoming a promising option as an antimicrobial coating agent on catheters to fight biofilm-related infections (123).

Chitosan is synthesized by many fungi, and is estimated to account for 4% of the cell wall of *A. fumigatus* (44,67). In *Cryptococcus neoformans*, chitosan is necessary for virulence in mice (124). In the pathogenic plant fungi, *Puccinia graminis* and *Uromyces fabae*, chitosan is present on the surfaces of invading hyphae, but absent on surfaces of non-invading hyphae (125). In *A. fumigatus*, two putative chitin deacetylase genes, *CDA* (afu4g09940, and afu6g10430), have been identified (43). However, these genes remain unstudied, and therefore, the contribution of chitin deacetylase to *A. fumigatus* virulence is unknown.

4.2.3. Chitosanase (CSN proteins)

Chitosan remodeling requires the action of chitosan hydrolases, or chitosanases. In *A. fumigatus*, there are four predicted chitosanase genes, (afu3g14980, afu4g01290, afu8g00930, and afu6g00500), in the genome. Secreted chitosanases have been identified in the culture supernatants of *A. fumigatus*, and their expression increases upon exposure to the antifungal voriconazole (126). Recently, CsnB was studied in detail (127). Deletion of *csnB* did not alter growth or hyphal cell wall morphology. However, the *csnB* deletion mutant was unable to use chitosan as a carbon source, suggesting that the other three putative chitosanases cannot compensate for the extracellular activity of CsnB. Further studies of CsnB and the other three chitosanases will be needed to fully assess the contribution of these enzymes to virulence.

4.3. Modification of other cell wall polysaccharides

In contrast to β-1,3-glucan, α-1,3-glucan is not known to be branched or conjugated. Therefore, its post-synthesis modification is predicted to be more limited. One known modification of α-1,3-glucan is hydrolysis of its glycosidic bonds by α-1,3-glucanase, AGN. In *A. fumigatus*, there are eight putative *Agn* genes in the genome, all belonging to the GH family 71 which is classified in the CAZy database to have predicted α-1,3-glucanase activity (Lee et al, unpublished). Interestingly, one of the AGN encoding genes, afu8g0630, is down-regulated in *A. fumigatus* in response to voriconazole exposure (126). Another AGN gene, afu2g03980, is induced by calcium signaling and has been detected in a secretome analysis from patient sera (128). Given the fact that α-1,3-glucan is the most abundant polysaccharide in the hyphal cell wall, future studies examining the function of the AGN enzymes may provide further insights into the biosynthesis of α-1,3-glucan and its role in the virulence of *A. fumigatus*.

Unlike other cell wall polysaccharides, galactosaminogalactan is a heteropolysaccharide with no distinct repeating units or internal segments. Preliminary studies from our laboratory have identified an N-acetyl galactosamine deacetylase, Agd3, which mediates partial deacetylation of galactosaminogalactan. Deletion of *agd3* is associated with a loss of deacetylation, and impairs the ability of galactosaminogalactan to

mediate adherence and biofilm formation (129). The role of galactosaminogalactan deacetylation in virulence is currently under study.

5. Towards an understanding of the regulation of cell wall biosynthesis

Cell wall biosynthesis in *A. fumigatus* is a tightly controlled process that is required for the survival of the fungus. Active cell wall biogenesis begins when resting conidia sense an appropriate environmental cue and initiate swelling and germination. Upon germination, hyphae must constantly change and remodel the cell wall to accommodate for growth, development, and adaptation to the changing environment. These environmental cues include, temperature, moisture, pH, quorum molecules, and nutrients, just to name a few. The need for a constantly adapting cell wall suggests the existence of a sensitive, tightly controlled, and highly rapid set of regulatory pathways that can ensure that enzymes involved in the cell wall biosynthesis are readily available when and where they are needed and efficiently repressed when they are not.

Over the past decade, efforts in deciphering the regulatory pathways and stress-related elements involved in cell wall biosynthesis in *A. fumigatus* have provided us with great insights into this seemingly complicated process. At its core, pathways involved in maintaining the cell wall integrity (CWI) play a prominent role in the regulation of cell wall biosynthesis. These pathways integrate numerous environmental cues and intracellular sensing to activate several signaling cascades that influence the expression of cell wall biosynthetic proteins. Elements and pathways involved in maintaining the CWI include the the mitogen-activated protein kinases (MAPK), including MpkA and Hog1 (60,130,131); calcium signaling through the calcineurin pathway (132,133); heat shock protein (Hsp90)(134), and a number of developmentally regulated transcription factors such as MedA (135) and StuA (136). Understanding these pathways and elements is critical in understanding the complex regulatory web of cell wall biosynthesis and compensatory mechanism, which is emerging as a major challenge in developing cell wall-targeting antifungal agents.

5.1. Chemo-genetic approaches to understanding the regulation of cell wall biosynthesis

Perhaps the best example of the role of the effects of regulatory pathway on cell wall composition comes from studies examining the effect of echinocandins on cell wall composition. As noted previously, echinocandins inhibit the synthesis of β-1,3-glucan by non-competitive inhibition of the β-1,3-glucan synthase (63). Interestingly, echinocandin mediated inhibition of β-1,3-glucan synthesis is associated with a compensatory increase in cell wall chitin content. A number of studies have therefore examined the effects of mutations in various signaling pathways on the susceptibility of *A. fumigatus* to echinocandins in order to better understand the regulatory pathways governing cell wall biosynthesis.

The best characterized pathway involved in cell wall biosynthesis is the calcineurin pathway. Deletion of key components of this pathway, the transcription factor gene *crz1* or the calcineurin catalytic subunit A gene *cnaA*, increased susceptibility to caspofungin (132,137). Importantly, not only did Δ*crz1* or Δ*cnaA* mutants display decreased β-1,3-glucan content, but their chitin levels also remained unchanged compared to the parental wild-type (137). Further, exposure to echinocandins did not result in an increase in chitin in the cell wall of either mutant strain as was seen in the wild-type parent. In fact, both mutants exhibited severe growth defects compared to the parental strain in the presence of either caspofungin or nikkomycin. Thus, not only does the calcineurin pathway govern β-1,3-glucan synthesis, but it is also a critical element in mediating the compensatory upregulation of chitin in response to β-1,3-glucan deficiency.

Similarly, deletions within the MkpA-MAP kinase pathway are associated with altered susceptibility to echinocandins. Deletion of *wsc1*, a cell surface sensor protein, or the downstream GTPase *rho1* or MAP kinase protein *mkpA* also increased susceptibility to echinocandins as compared to the parental strain (59,60). Whether the MkpA-MAP kinase pathway is required for increasing synthesis of β-1,3-glucan in the presence of caspofungin, or mediates a compensatory increase in the synthesis of chitin or another cell wall polysaccharide is not known.

Signal pathways are not the only integral components of the cell wall integrity network. Repression of an essential heat shock protein, *hsp90*, resulted in a mutant strain

with severe cell wall defects, including swollen hyphae with blunted tips, similar to those produced by fungi exposed to echinocandins (134). Localization studies using fluorescence protein-tagged Hsp90 revealed that while Hsp90 is normally cytoplasmic, exposure to echinocandins results in localization of Hsp90 to the cell wall and septum. Chitin levels under repressed conditions were not different in the mutant strain as compared to the parental strain, suggesting that Hsp90 directly effects β-1,3-glucan synthesis rather than compensatory upregulation of chitin synthesis. Further studies will be required to fully understand the role of Hsp90 in the cell wall integrity network.

5.2. Deciphering the regulation of cell wall composition through direct molecular approaches

5.2.1. Global regulators of cell wall biosynthesis

Morphogenesis and development are associated with major changes in the physical structure of fungal cells, and by extension, their cell walls. Studies of development in the model organism *A. nidulans* have identified regulatory pathways governing development whose function seems to be largely conserved in *A. fumigatus*. In both fungi, sequential activation and feedback of transcription factors BrlA, AbaA, and WetA are required for normal development of the fungus, especially in regulation of conidiation (138). (139,140). Although detailed cell wall studies of deletion mutants of these transcription factors are lacking, transcriptomic studies revealed that BrlA is required for the expression of a number of cell wall modifying enzymes (141). These include a putative endoglucanse (afu2g14540), endo-chitinase *csn* (afu4g01290) (142-144), and putative chitosanase (afu8g00930). Given that remodeling of the cell wall is a critical step in later stages of development, the fact that BrlA is required for the regulation of carbohydrate hydrolases fits within this framework.

The core conidiation pathway is also modulated through the action of the temporal and spatial developmental transcription factors MedA and StuA. These development transcription factors have also been studied in the context of cell wall biosynthesis in *A. fumigatus* (135,136,145). Deletion of *medA* or *stuA* resulted in mutants with changes in the cell wall composition, most notably a marked reduction in the production of

galactosaminogalactan (Fig4), (42,135,136). It is likely that future studies of transcriptional regulators will identify other pathways controlling the synthesis and modification of cell wall polysaccharides in *A. fumigatus.*

5.2.2. Other compensatory relationships between cell wall polysaccharides.

Deletion analyses of polysaccharide biosynthetic genes have revealed other examples which altering synthesis of one cell wall polysaccharide leads to increase in the synthesis of another cell wall component. For example, blocking the synthesis of α-1,3-glucan by deletion of the *AGS* genes was associated with an increase in chitin and β-1,3-glucan (93). These compensatory relationships between polysaccharides are complex, as inhibition of synthesis of galactomannan by deletion of *ugm1* was associated with an increase in galactosaminogalactan and β-1,3-glucan, while abrogating the synthesis of galactosaminogalactan by deletion of *uge3* had no effect on galactomannan or β-1,3-glucan production (41,42).

The molecular mechanism underlying these compensatory changes in cell wall content are unknown. It is possible that, as with chitin and β-1,3-glucan, elements of the cell wall integrity regulatory network play a key role in mediating these compensatory changes, although direct evidence is still lacking. Alternatively, alterations in cell wall composition in the face of altered polysaccharide synthesis can reflect substrate shunting whereby the accumulations of sugar substrates from a blocked biosynthetic pathway are utilized for the production of a second cell wall polysaccharide. Substrate shunting is not independent of regulatory effects as it is also likely that the organism detects shifts in intracellular pools of sugars and alters enzyme expression or activity in response to this perturbation in metabolism. One example of this phenomenon can be found in studies examining the biosynthetic pathway of trehalose, a disaccharide that enhances resistance to a variety of stressors and serves as an energy source during germination. Production of trehalose requires dephosphorylation of the intermediate substrate trehalose-6-phosphate (T6P) by a phosphatase OrlA. Deletion of *orlA* leads to the accumulation of T6P, which in turn inhibits hexokinase activity. This inhibition of hexokinase activity in the Δ*orlA* mutant also impairs the synthesis of UDP-GlcNAc, resulting in a mutant deficient in chitin (146). Since UDP-GlcNAc is also a substrate in the galactosaminogalactan biosynthetic pathway, it

is also possible that T6P accumulation could also inhibit the production of this polysaccharide, although this hypothesis has not been tested.

Studying these changes in metabolic activity or intracellular sugar substrate concentration is challenging. Future efforts in developing advanced tools and techniques specific to filamentous fungi will be required to elucidate the role of substrate shunting in *A. fumigatus* cell wall biogenesis.

5.3. Real world applications: targeting compensatory changes in cell wall composition to enhance antifungal efficacy

To date, only one class of antifungals targeting the cell wall, the echinocandins, is approved for clinical use. However, echinocandin mediated inhibition of β-1,3-glucan synthesis results in an increase cell wall chitin content (147), which may reduce the efficacy of these agents. Combining echinocandins with inhibitors of chitin synthesis such as nikkomycin may prove to be an effective strategy to increase the activity of this class of antifungals. (147,148). Further, the development of new agents targeting the synthesis of other cell wall polysaccharides may offer exciting new therapeutic options for the treatment of invasive aspergillosis, alone or in combination with existing antifungals. Indeed, it is possible that agents may be identified that have minimal intrinsic antifungal activity as monotherapy, but which will have important activity when combined with other antifungals that target the cell wall. Thus the study of cell wall mutants, and the corresponding regulatory pathways is likely to be of critical importance in guiding the rational development of combinatorial antifungal strategies that target the cell wall.

6. Rationale and objective of the research

During infection, growing hyphae must adhere to host substrates as anchor for colonization and host cell invasion. Microbial adhesins are usually cell wall associated, as this is the outermost surface of the microorganism which interacts directly with host cells and macromolecular substrates. Recently, our group used a comparative genomics approach to demonstrate that galactosaminogalactan, a cell wall polysaccharide composed of galactose and N-acetyl-galactosamine (GalNAc), is required for hyphal adherence to a variety of substrates, including host cells and macromolecules. Deletion of a UDP-glucose 4-epimerase, *uge3*, completely blocked galactosaminogalactan synthesis and resulted in the loss of adherence, disruption of biofilm, and attenuated virulence in a murine model of invasive aspergillosis. The main goal of this thesis was to investigate the molecular mechanisms underlying the synthesis of galactosaminogalactan and elucidate the molecular mechanisms whereby galactosaminogalactan mediates virulence.

The first objective of this thesis was to examine the role of galactosaminogalactan synthesis in the architecture of the fungal cell wall. Through characterization of a galactosaminogalactan deficient mutant *of A. fumigatus* we demonstrated that galactosaminogalactan within the outer cell wall and extracellular matrix, attenuates inflammatory responses through masking of the pathogen associated molecular pattern β-1,3-glucan and preventing its recognition by the pattern recognition receptor dectin-1.

The second objective of the thesis was to define the early steps of galactosaminogalactan biosynthesis by identifying the mechanism by which galactose and N-acetyl galactosamine are produced in *A. fumigatus*. We demonstrated that a single bifunctional UDP-glucose 4-epimerase, Uge3, is necessary and sufficient for the synthesis of galactosaminogalactan, while another epimerase, Uge5, has a supporting, but dispensable role in this synthetic pathway.

The third objective of the thesis was to investigate the role of galactosaminogalactan deacetylation in mediating the various functions of this exopolysaccharide. We found that deacetylation of galactosaminogalactan by the polysaccharide deacetylase Agd3 is required to convert GalNAc to galactosamine (GalN), rendering the nascent galactosaminogalactan cationic. The cationic nature of galactosaminogalactan mediates adherence and other properties associated with this cell wall polysaccharide.

Finally, the fourth objective of the thesis was to determine if differences in galactosaminogalactan production or composition could contribute to differences in virulence between *Aspergillus* species. These studies found that the GalNAc/GalN content of galactosaminogalactan directly correlates with virulence in these species, and that increasing the GalNAc/GalN content by heterologous expression of *uge3* in the relatively non-pathogenic *A. nidulans*, increased its virulence and resistance to NADPH oxidase-mediated neutrophil killing by antimicrobial peptides.

We hypothesized that the composition of galactosaminogalactan in the most virulence species, *A. fumigatus*, will be different than that of less pathogenic ones like *A. nidulans*. We further hypothesized that this difference could be material in pathogenesis, and that changing the composition of *A. nidulans* galactosaminogalactan will increase its virulence.

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Figure Legends

Figure 1. The cell wall of *Aspergillus fumigatus*. Scanning electron micrograph (A), and transmission electron micrograph (B), of 24 hour grown hyphae. Higher magnification of panel B showing the layers of the cell wall (C). Scale bar indicates (A) 4 μm, (B) 100 nm, and (C) 20 nm. Abbreviations are as follows: ECM, extracellular matrix; CW, cell wall; OCW, outer cell wall; ICW, inner cell wall; PM, plasma membrane.

Figure 2. Cell wall polysaccharides. Depiction of cell wall polysaccharides in *A. fumigatus* showing linkages and monosaccharide components.

Figure 3. Schematic of cell wall polysaccharide synthesis in *A. fumigatus*. Abbreviations are as follows: Ags, alpha-glucan synthase; Cda, chitin deacetylase; Chs, chitin synthase; GT, glycosyltransferase;

Figure 4. Cell wall morphology of galactosaminogalactan-deficient mutants. Scanning electron micrograph of hyphae of (A) wild-type *A. fumigatus* strain Af293 (B) Δ*uge3*, (C) Δ*stuA*, and (D) Δ*medA*

Figure 1

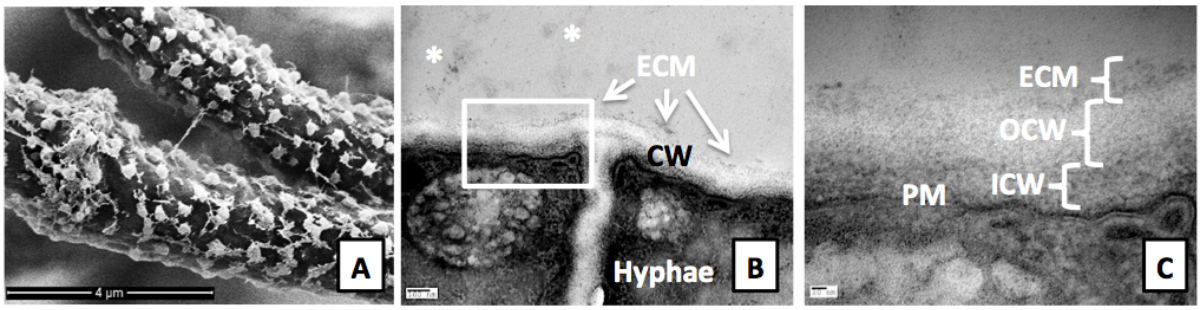
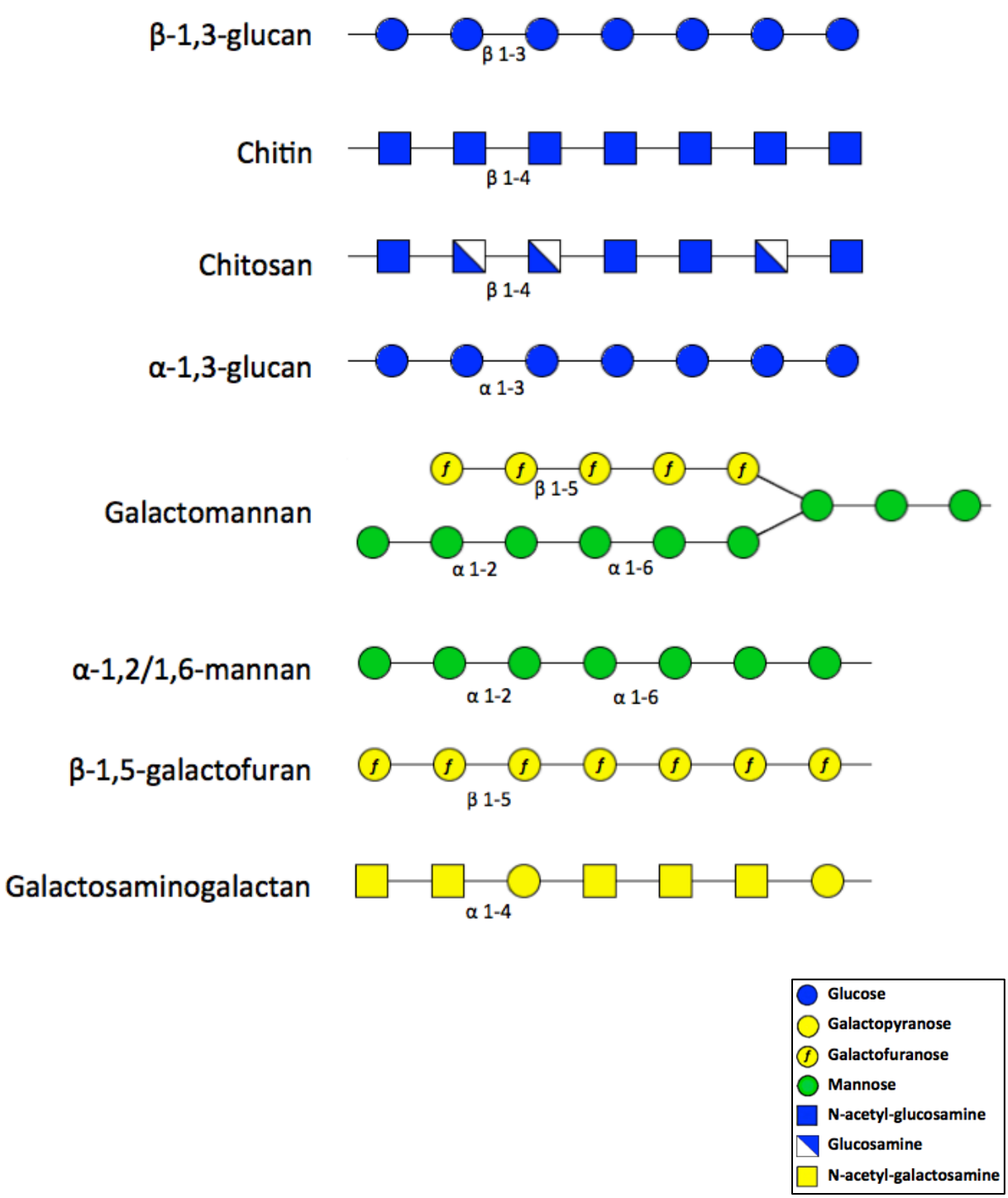


Figure 2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Chitosan
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α 1-2
α 1-6
α-1,2/1,6-mannan
α 1-2
α 1-6
β-1,5-galactofuran
β 1-5
Galactosaminogalactan
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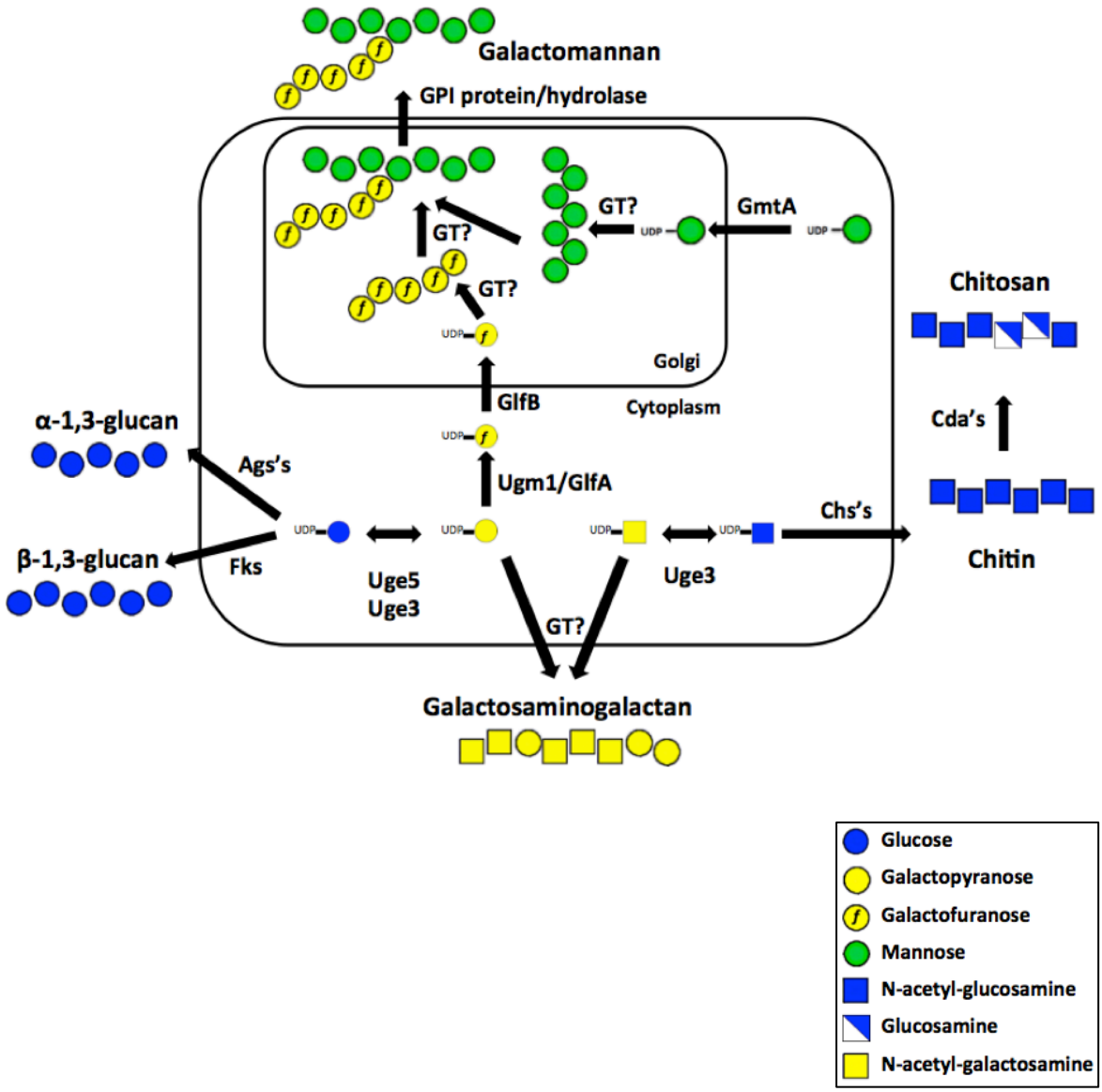
N-acetyl-galactosamine

Figure 3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GmtA
UDP
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GT?
Golgi
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Ags's
β-1,3-glucan
Fks
Uge5
Uge3
Uge3
Chs's
GT?
Chitosan
Cda's
Chitin
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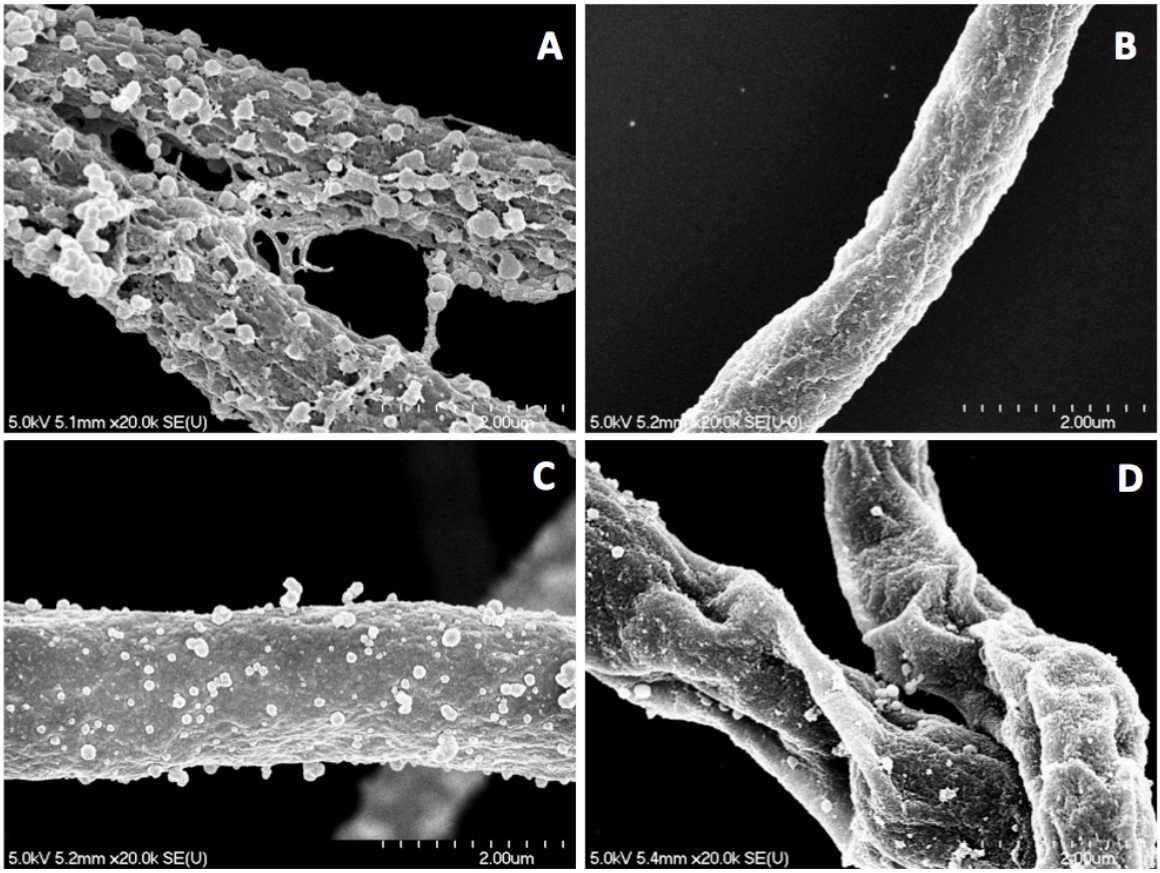
Glucosamine
N-acetyl-galactosamine

Figure 4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Preface to Chapter 2

Galactosaminogalactan is a secreted polysaccharide that has been described in several fungal species decades ago, and has been known to exist in *Aspergillus fumigatus* since the late 1990's. Only recently was the core structure and composition of galactosaminogalactan characterized in *A. fumigatus*. In this chapter, we used a molecular genetics approach to elucidate the role of galactosaminogalactan in host-pathogen interactions. Using comparative transcriptomics, we identified a key enzyme required for the synthesis of galactosaminogalactan, Uge3. We constructed and characterized a mutant strain deficient in the gene encoding for Uge3. These experiments demonstrated a role for galactosaminogalactan in mediating adherence to a variety of host substrates, biofilm formation, and masking of fungal cell wall PAMPs during infection. Consistent with these observations, galactosaminogalactan deficient mutants exhibited attenuated virulence in a murine model of invasive aspergillosis.

CHAPTER 2: *Aspergillus* Galactosaminogalactan Mediates Adherence to Host Constituents and Conceals Hyphal β -Glucan from the Immune System

***Aspergillus* Galactosaminogalactan Mediates Adherence to Host Constituents and Conceals Hyphal β-Glucan from the Immune System.**

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ABSTRACT

Aspergillus fumigatus is the most common cause of invasive mold disease in humans. The mechanisms underlying the adherence of this mold to host cells and macromolecules have remained elusive. Using mutants with different adhesive properties and comparative transcriptomics, we discovered that the gene *uge3*, encoding a fungal epimerase, is required for adherence through mediating the synthesis of galactosaminogalactan. Galactosaminogalactan functions as the dominant adhesin of *A. fumigatus* and mediates adherence to plastic, fibronectin, and epithelial cells. In addition, galactosaminogalactan suppresses host inflammatory responses in vitro and in vivo, in part through masking cell wall β-glucans from recognition by dectin-1. Finally, galactosaminogalactan is essential for full virulence in two murine models of invasive aspergillosis. Collectively these data establish a role for galactosaminogalactan as a pivotal bifunctional virulence factor in the pathogenesis of invasive aspergillosis.

INTRODUCTION

The incidence of invasive mold infections due to the fungus *Aspergillus fumigatus* has increased dramatically in hematology patients receiving intensive cytotoxic chemotherapy or undergoing hematopoietic stem cell transplantation [1]. Despite the advent of new antifungal therapies, the mortality of invasive aspergillosis (IA) remains 60–80% [2]. There is therefore a pressing need for novel therapeutic strategies to treat or prevent IA. A better understanding of the pathogenesis of IA is one approach that may inform the development of new therapeutic targets.

Adherence of *A. fumigatus* to host constituents is thought to be an early and critical step in the initiation of colonization and infection [3]. Upon inhalation, *A. fumigatus* conidia rapidly adhere to pulmonary epithelial cells and resident macrophages before being internalized and germinating within host cells [4], [5], [6]. Following germination, filamentous hyphae remain in intimate contact with host epithelial, endothelial and immune cells and can induce tissue injury and inflammatory responses. Inhibition of these

adherence events may provide a useful therapeutic strategy to reduce morbidity and mortality of *A. fumigatus* mediated disease.

Despite the fact that hyphae play such a critical role in the pathogenesis of invasive aspergillosis, the fungal ligands governing adherence of *A. fumigatus* hyphae to host constituents are largely unknown. A bioinformatic analysis of potential adhesins of *A. fumigatus* has identified several candidate proteins involved in mediating adhesion to host constituents [7], but the adherence of mutant strains that lack these proteins has not been reported. Carbohydrate constituents of the cell wall have been recently implicated in adherence events [8] although their role in mediating adherence to host constituents has not been studied.

We recently identified a fungal regulatory protein, MedA, which governs fungal adhesion to host cells and basement membrane constituents and biofilm formation [9]. In addition, we found that a strain deficient in StuA, a previously described developmental transcription factor [10], was similarly deficient in the formation of adherent biofilms. In contrast deletion of *ugm1*, which encodes a UDP-galactose mutase required for the production of galactofuranose, has been reported to result in a strain of *A. fumigatus* with increased adherence to epithelial cells [11]. Here, we report that carbohydrate analysis of these mutants revealed that the Δ*medA* and Δ*stuA* mutants were defective in galactosaminogalactan production whereas the Δ*ugm1* mutant hyperproduced galactosaminogalactan. A comparative transcriptome analysis of the Δ*medA* and Δ*stuA* regulatory mutants identified a gene encoding a putative UDP-glucose-epimerase, designated *uge3*, which was dysregulated in both the Δ*stuA* and Δ*medA* mutants. Disruption of *uge3* resulted in a complete block in galactosaminogalactan synthesis, and markedly decreased adhesion to host cells and biofilm formation. The Uge3 deficient strain was also attenuated in virulence and induced a hyperinflammatory response in a corticosteroid treated mouse model. The absence of galactosaminogalactan in hyphae resulted in an increased exposure of cell wall β-glucan and in higher levels of dectin-1 binding, in association with the release of higher levels of pro-inflammatory cytokine by dendritic cells. Blocking dectin-1 with an anti-dectin-1 antibody, or pre-incubating hyphae with Fc-dectin-1 blocked this increased cytokine production. Suppression of inflammation in mice treated with cyclophosphamide and cortisone acetate resulted in further attenuation of

virulence of the Δ*uge3* mutant, although the degree of reduction in fungal burden was similar in neutropenic and corticosteroid treated mice. Collectively these data identify galactosaminogalactan as a multifunctional virulence factor that mediates adherence of *A. fumigatus*, cloaks β-glucan and suppresses host inflammatory responses in vivo.

MATERIALS AND METHODS

Fungal strains and growth conditions

A. fumigatus strain Af293 (a generous gift from P. Magee, University of Minnesota, St. Paul, MN) was used as the wild-type strain for all molecular manipulations. The Δ*medA*, Δ*ugm1* and Δ*stuA* mutants and their corresponding parent and complemented strains were described previously [10]. Except where indicated, strains were propagated on YPD agar and at 37°C while exposed to light as previously described [9]. Liquid growth media were synthetic Brian medium [31], Aspergillus Minimum Medium (AspMM) [32], and RPMI 1640 medium (Sigma-Aldrich) buffered with 34.53 g of MOPS (3-(N-morpholino)propanesulfonic acid, Sigma-Aldrich) per liter, pH 7.0 as indicated. When noted, pH and/or iron concentration were modified in AspMM, in order to generate a pH from 4.5 to 8.5, and a [Fe2+] from 0 to 30 μM. Microaerophilic growth was performed using YPD or AspMM, incubated in a candle jar.

Tissue culture

The type II pneumocyte cell line CCL-185 (lung epithelial cells A549) was obtained from the American Type Culture Collection, and was grown in DF12K medium containing 10% foetal bovine serum, streptomycin (100 mg/litre) and penicillin (16 mg/litre) (Wisent).

Bone marrow derived dendritic cells (BMDDCs) were prepared by flushing femurs and tibias of 6–8 week old C57BL/6 mice. Bone marrow cells were then cultured with culture media supplemented with either J558L culture supernatants or rGM-CSF, as previously described [33], [34]. Marrow cells were plated at a density of 4×105 cells/ml in petri dishes containing 10 ml of culture media. For J558L supernatant-supplemented

cultures, on days 3 and 6, cells were fed an additional 1 ml J558L culture supernatant per dish, and on day 8 with 4 ml of culture media with 30% J558L culture supernatant per dish. BMDDCs were used in fungal interaction experiments after 11 days of culture. For rmGM-CSF-supplemented cultures, on day 3 marrow cells were fed an additional 4 ml of culture media, and used in experiments on day 9. BMDDC differentiation was confirmed by flow cytometry via CD11c expression (data not shown).

Mutant transcriptome analysis.

RNA expression analysis of the Δ*medA* mutant was compared to the wild-type and *medA* complemented strains using an *A. fumigatus* Af293 amplicon microarray as described previously [10], [14]. To obtain RNA for microarray analysis, RPMI 1640 medium buffered with 4-morpholinepropanesulfonicacid (MOPS) to pH 7.0 (Sigma-Aldrich) was inoculated with conidia of the various strains at a final concentration of 106 conidia per ml. RNA was harvested after 8, 18, and 24 h, as described above. An *A. fumigatus* Af293 DNA amplicon microarray containing triplicate probes for 9516 genes was used in this study [35]. The labeling reactions with RNA and hybridizations were performed as described in the J. Craig Venter Institute standard operating procedure (http://pfgrc.jcvi.org/index.php/microarray/protocols.html). cDNA from the Δ*medA* mutant was hybridized against cDNA from the wild-type strain in three biological replicates and against cDNA from the Δ*medA*::*medA* complemented strain in two biological replicates. Dye swaps were performed. The gene expression ratios were log2-transformed and imported into JCVI MultiExperiment Viewer (MeV) software (http://www.tm4.org/mev.html) [36]. The Significance Analysis for Microarrays (SAM) method [37] was used (false discovery rate of 0.1%) to determine genes subject to differential transcriptional regulation between the control strains and the Δ*medA* mutant after 18 h and/or 24 h of growth. The results were then compared to the previously published results obtained in the same conditions with the set of strains Af293, Δ*stuA*, and Δ*stuA*::*stuA* [10].

Disruption of uge3

Our standard disruption protocol [10], [32] was adapted to the Gateway® (Invitrogen) system as follows: first plasmid pAN7.1 was modified for Gateway® use by digestion with

BmgBI or NaeI followed by fusion of an attR::ccdB target sequence at the site of each digestion using the Gateway® Vector Conversion system, to generate plasmids pHY, and pYG. To generate the disruption constructs, ~1 kb of the flanking sequences of *uge3* was amplified by PCR from Af293 genomic DNA using primers U1,U2 and U3,U4 to generate fragments FS1 and FS4 respectively (Table S1). The resulting PCR products were then cloned into pENTR-D-TOPO® entry plasmid. A LR recombination allowed recombination of pENTR::FS1 with pHY, and of pENTR::FS4 with pYG, resulting in the fusion of each flanking sequence with the hph cassette in plasmids pHY and pYG plasmids. Finally, the DNA fragments for transformation were generated by PCR, using the primers U1,HY with pHY::FS1 and U4,YG with pYG::FS4. Protoplasts of *A. fumigatus* Af293 were then transformed with 5 µg of each DNA fragment, as previously described [32]. Transformants were selected on 0.025% hygromycin enriched plates. Complete deletion of the *uge3* open reading frame was confirmed by PCR using primers U-ext1, U-ext4, U-RT sense, U-RT antisense, HY and YG (Table S1), by real-time RT-PCR to ensure a complete absence of *uge3* mRNA using primers U-RT sense and U-RT antisense (Table S1).

Construction of the *uge3*-complemented strain.

To verify the specificity of the mutant phenotype, we constructed a complemented strain in which a wild-type copy of *uge3* was reintroduced in the Δ*uge3* strain, using a split marker approach [32]. Briefly, the phleomycin resistance (ble cassette) plasmid p402 was converted to the Gateway system as describe above using XbaI or SacI digestion and ligation of an attR::ccdB target sequence at the site of each digestion to generate plasmids pBL and pLE. A 2.4 kb DNA fragment containing the *uge3* ORF and 1 kb of upstream sequence was amplified by PCR from Af293 genomic DNA using primers U1,U5, and cloned into the Gateway pENTR-D-TOPO plasmid to generate pENTR::FS1-*uge3*. Next, the *uge3* cassette from pENTR::FS1-*uge3* was cloned upstream of the ble cassette in pBL by Gateway mediated LR recombination. Using the same approach, the *uge3* downtream flanking sequences from the previously constructed pENTR::FS4 were cloned downstream of the ble cassette in pLE. Finally, the DNA fragments for transformation were generated by PCR, using primers U1,BL for pBL::FS1-*uge3* and U4,LE for pLE::FS4. Protoplasts of the Δ*uge3*

mutant were then transformed with 5 µg of each PCR product, as previously described [32]. Transformants were selected on 0.015% phleomycin enriched plates. Transformants were tested by PCR and by Southern blotting to ensure re-integration of *uge3*, and *uge3* expression was verified by real-time RT-PCR using primers U-RT sense and U-RT antisense (Table S1) to ensure that *uge3* mRNA production was restored.

Real-Time RT-PCR

In vitro, expression of the genes of interest was quantified by relative real-time RT-PCR analysis as previously described [38]. The primers used for each gene are shown in Table S1. First strand synthesis was performed from total RNA with Quantitec Reverse Transcription kit (Qiagen) using random primers. Real-time PCR was then performed using an ABI 7000 thermocycler (Applied Biosystems) Amplification products were detected with Maxima® SYBR Green qPCR system (Fermentas). Fungal gene expression was normalized to *A. fumigatus* TEF1 expression, and relative expression was estimated using the formula $2 - \Delta\Delta Ct$, where $\Delta\Delta Ct$ = [(Cttarget gene) sample − (CtTEF1) sample] / [(Cttarget gene) reference − (CtTEF1) reference]. To verify the absence of genomic DNA contamination, negative controls were used for each gene set in which reverse transcriptase was omitted from the mix.

Fungal cell wall analysis

Cell wall extraction was performed as previously described [39]. Alkali soluble (AS) and alkali insoluble (AI) fractions were extracted as previously described [40]. Monosaccharides were determined by gas chromatography after hydrolysis, reduction and peracetylation of the AI and AS fractions [41] with meso-inositol as internal standard. Both hexose and hexosamine concentrations were expressed as percentages of the total cell wall.

β-glucan analysis.

Total cell wall β-glucans were assayed by aniline blue staining [42]. Briefly, conidia were grown for 4, 6 or 12 hours at 105 conidia/mL in YPD at 37°C and stained with 0.05% aniline blue (Sigma-Aldrich) in PBS buffered at pH 9.5. Stained cells were imaged with confocal microscopy (IX81, Olympus), excitation 400 nm and emission 455 nm.

Surface exposed β-glucans were assayed by immunostaining with an Fc-dectin-1 fusion construct (a generous gift from Dr G.D. Brown, University of Aberdeen) as described previously [18]. Briefly, fungal cells were fixed in 4% paraformaldehyde in PBS, and blocked in 3% bovine serum albumin supplemented with 0.2% sodium azide in PBS. Cells were then labelled with 10 μg/ml of Fc-dectin-1 [18] followed by FITC-labeled AffiniPure F(ab') fragment donkey anti-human IgG, FCy fragment specific (Jackson ImmunoResearch). Stained cells were imaged with confocal microscopy (IX81, Olympus), excitation 495 nm and emission 519 nm.

Soluble β-glucan release was measured in culture supernatants using the Glucatell assay (Associates of Cape-Cod Inc.), following the manufacturer's instructions. Results were normalized to fungal biomass dry weight.

Scanning electron microscopy

Hyphae were grown for 24 hours in phenol red-free RPMI 1640 medium on glass coverslips, fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4°C overnight, sequentially dehydrated in ethanol, and critical-point dried (Leica, Inc.). Samples were then sputter coated with Au-Pd and imaged with a field-emission scanning electron microscope (S-4700 FE-SEM, Hitachi).

Extracellular galactosaminogalactan and galactomannan production

For both polysaccharide production assays, 50 mL of Brian medium was inoculated with 5×107 conidia, and incubated for 24 h at 37°C. Culture supernatant aliquots were harvested by filtration of the culture on nylon membrane. Extracellular galactosaminogalactan was precipitated by 2.5 volume of ethanol overnight night at 4°C, washed with 60% ethanol, lyophilized and weighed as described previously [16]. The precipitate was homogenized in 40 mM HCl and sonicated. Next, the galactosamine content was analyzed by total acid hydrolysis (HCl 6.6 N, 100°C, 4 h) and quantified with HPAEC on a CarboPAC-PA1 column (4.6×250 mm, Dionex) using NaOH (18.8 mM) and sodium acetate (0.3 M) in 0.1 M NaOH as eluent A and B, respectively, as described [43]. In addition, absence of protein in galactosaminogalactan fractions was verified by Bradford assay. Extracellular galactomannan culture supernatant content was assayed by EIA using the

Platelia® Aspergillus kit (Bio-Rad), following the manufacturer instructions. Both assays were performed on 3 independent occasions.

Adherence assays

The ability of strains to form biofilms was tested by inoculating 6- well culture plates with 10^5 conidia in 1 mL of Brian broth. After incubation at 37°C for 24 h, the plates were washed, fixed and stained as previously described [9].

The capacity of the various strains of *A. fumigatus* to adhere to plastic, fibronectin and epithelial cells was analyzed using our previously described method [9]. Six-well culture plates were prepared with confluent monolayers of A549 epithelial cells, adsorbed with 0.01 mg/ml of fibronectin overnight, or left untreated and then infected with 200 germlings of the strain of interest in each well and incubated for 30 minutes. Following incubation, wells were washed 3 times with 4 mL of PBS in a standardized manner, and overlaid with YPD agar for quantitative culture. The adherence assays were performed in triplicate on at least three separate occasions.

For carbohydrate supplementation experiments, either the plastic plate or the germlings were coated with the carbohydrate of interest. Briefly, the appropriate amount of extracellular galactosaminogalactan isolated as above, or of zymosan (Sigma-Aldrich), was resuspended in 2 mL of PBS by sonication, added to the wells of a 6-well non-tissue culture plate and incubated overnight before being washed and used in the adherence assay described above. For adherence assay with glycan treated fungus, germlings were incubated for 1 hour, at room temperature, in a dilution of galactosaminogalactan or zymozan in PBS; then rinsed three times to remove non-adherent carbohydrate before being tested for adherence as described above.

To measure the adherence of purified galactosaminogalactan to A549 epithelial cells, monolayers of A549 cells were grown to confluence in 96-well plate (Nunclone, Inc) then fixed in 4% paraformaldehyde. Cells were incubated with varying concentrations of galactosaminogalactan suspended in PBS, washed and then stained with fluorescein conjugated Soybean Agglutinin (Vector Labs). Binding was quantified by measuring fluorescence at 495 nm using Spectramax (Molecular Devices). To confirm the specificity of Soybean Agglutinin (SBA) for GalNAc residues of GAG, hyphae of Af293 wild-type, Δ*uge3*,

and Δ*uge3*::*uge3* were grown on poly-D-lysine coated glass coverslip for 12 hours, fixed with 4% paraformaldehyde, co-incubated with fluorescein conjugated SBA, and imaged by confocal microscope at 488 nm (Olympus).

Antifungal drug susceptibility assays

Caspofungin (Merck) and nikkomycin X (Sigma-Aldrich) were diluted in sterile deionized H2O. Calcofluor white (Sigma-Aldrich) was diluted in a solution of 0.8% KOH and 83% glycerol. Antifungal susceptibility testing was performed in accordance with the CLSI M38-A document for broth dilution antifungal susceptibility testing of filamentous fungi [44] as previously described [45]. Final dilutions of antifungals were prepared in RPMI 1640 buffered with MOPS. 100 μL of drug stock was added to 100 μL of 105 conidia/mL solution per well. Plates were examined after 24 and 48 hours of incubation and the minimal inhibitory concentration (MIC) was determined by visual and microscopic inspection resulting in 100% growth inhibition while the minimal effective concentration (MEC) was determined by visual and microscopic inspection resulting in abnormal growth.

Epithelial cell damage assay

The extent of damage to epithelial cells caused by the various strains of *A. fumigatus* was determined using a minor modification of our previously described method [46]. Briefly, A549 cells were loaded with chromium by incubating monolayers grown in 24-well tissue culture plates with 3 μCi of 51Cr at 37°C in 5% CO2 for 24 hours. Excess chromium was removed by washing with HBSS. The labeled A549 cells were then infected with 5×105 conidia in 1 ml serum free DF12K medium. After a 16 h incubation, the medium above the cells was retrieved. The cells were then lysed with 6 N NaOH and the lysate collected. The 51Cr content of the medium and lysates was then measured in a gamma counter and the extent of epithelial cell damage was calculated. Each strain was tested in triplicate on three separate occasions, and all results were corrected for spontaneous chromium release by uninfected epithelial cells.

Dendritic cell stimulation with *A. fumigatus* strains

A. fumigatus conidia were germinated for 9 h in non-tissue culture treated six-well plates in 2 ml phenol red-free RPMI 1640 medium at a concentration of 1.5×106 conidia/well and allowed to germinate. Next, 1.5×106 BMDDCs were then added to each well in 1 ml of RPMI 1640 medium. As a positive control BMDDCs were incubated with 3 μg/ml lipopolysaccharide (purified from S. minnesota, Invitrogen). Following 6 hrs co-incubation, culture supernatants were collected. Total cytokine analysis in culture supernatants was performed using the Mouse Cytokine 20-Plex Panel (Invitrogen), as per manufacturer's instructions, and analyzed using xPONENT analysis software. To investigate neutralization of either dectin-1 or β-glucan, BMDDCs or fungi were co-incubated for 1 h with 10 μg/ml mouse anti-dectin-1 (Invivogen) or 10 μg/ml Fc-dectin-1 recombinant protein (a generous gift from G. D. Brown), respectively. BMDDCs were added to fungi at a MOI of 1:2. As controls, BMDDC or 5 μg/ml zymosan (Sigma-Aldrich) were co-incubated with fungi or BMDDC, respectively. TNF-α analysis in culture supernatant was performed using the Mouse TNF alpha ELISA Ready-SET-Go kit (eBiosciences).

Virulence studies

The virulence of the indicated *A. fumigatus* strains was tested in two different murine models of invasive pulmonary aspergillosis. In the first model, male BALB/C mice were immunosuppressed by administering 10 mg of cortisone acetate (Sigma-Aldrich) subcutaneously every other day, starting on day −4 relative to infection and finishing on day +4, for a total of 5 doses [47]. In the second model, the mice were immunosuppressed with cortisone acetate, 250 mg/kg subcutaneously on days −2 and +3, and cyclophosphamide (Western Medical Supply), 250 mg/kg intraperitoneally on day −2 and 200 mg/kg on day +3 [48], [49]. For each fungal strain tested, groups of 11–13 mice were infected using an aerosol chamber as previously described [48]. An additional 8 mice were immunosuppressed but not infected. To prevent bacterial infections, enrofloxacin was added to the drinking water while the mice were immunosuppressed. Mice were monitored for signs of illness and moribund animals were euthanized. All procedures involving mice were approved by the Los Angeles Biomedical Research Institute Animal Use and Care Committee, and followed the National Institutes of Health guidelines for

animal housing and care. In both models, differences in survival between experimental groups were compared using the log-rank test.

Determination of fungal burden

11 mice per strain were immunosuppressed and infected with the strains of interest in a separate experiment. After 4 days of infection, the mice were sacrificed and their lungs were harvested. They were immediately homogenized in ice cold PBS containing 10 µL of protease inhibitor mix/mL (Sigma Aldrich), and then aliquoted and stored at −80°C until use. The fungal burden was estimated by the total concentration of fungal genomic DNA [50]. Briefly, total DNA was extracted from the lungs using High Pure PCR Template Preparation Kit (Roche diagnostics) following the manufacturer's instructions. Real-Time PCR quantification of fungal DNA was performed on 150 ng of total lung DNA, using TaqMan Universal PCR Master Mix (Roche diagnostics) with a pair of oligonucleotides and a FAM-labelled probe for the amplification and detection of the 18S rRNA gene (Table S1). qPCR run was as follow: 2 min at 50°C; 10 min at 95°C; 50 cycles of (15 sec at 95°C+1 min at 60°C). The concentration of fungal DNA in the total lung extracted DNA was determined from a standard curve of serially diluted genomic DNA from strain AF293 (from 20 pg to 2.5 ng of fungal DNA per qPCR well). Fungal DNA concentration was normalized to lung weight and total DNA yield. To avoid the healthy survivor bias, initial fungal burden studies were performed on the fourth day after infection at which point 90% of infected animals remained alive. Subsequent experiments were performed on the third day of infection to minimize differences in fungal burden between mice infected with the wild-type and Δ*uge3* mutant strains. In experiments in which inflammation was measured, pulmonary galactomannan content of lung homogenates was used as a surrogate measure of fungal burden using the Platelia Aspergillus EIA kit (Bio-Rad) as we have done previously [9], [12], [13], [51], in order to preserve the lung tissue for the assays below. All strains produced similar levels of galactomannan during growth in vitro. Lung homogenates were clarified by centrifugation, diluted 1:20 and assayed as per manufacturer's recommendations. Samples were compared to a standard curve composed of serial dilutions of lung homogenate from a mouse heavily infected with strain Af293.

Characterization of pulmonary inflammation

Mice were sacrificed and the airway contents were recovered by instillation and retrieval of 1 ml of sterile PBS through a needle inserted in the trachea. A total of three lavages were performed and pooled. LDH determination in BAL fluid was performed using a commercial assay (Abcam) following the manufacturer's instructions. 50 µl of each sample was assayed without dilution. Pulmonary TNF-α content was assayed using the Mouse TNF alpha ELISA Ready-SET-Go kit (eBiosciences) and the MPO content of the lungs was quantified by enzyme immunoassay (Cell Sciences). Lung homogenate samples were clarified by centrifugation, and assayed undiluted as per manufacturer's recommendations. Results of MPO, LDH and TNF-α determination were normalized for differences in fungal burden by multiplying values by the ratio of the galactomannan content of lungs infected with the experimental strain divided by the galactomannan content of lungs infected with wild-type Af293.

Ethics statement

The mouse studies were carried out in accordance with the National Institutes of Health guidelines for the ethical treatment of animals. This protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center (Animal Welfare Assurance Number A3330-01).

RESULTS

Identification of galactosaminogalactan as a major adhesin of *A. fumigatus*

Previously, a mutant deficient in UDP-galactofuranose mutase (*ugm1*) was reported to have increased adherence to host cells and abiotic substrates, while an *A. fumigatus* mutant deficient in the regulatory factor MedA had markedly impaired adherence to multiple substrates and was defective in biofilm formation [9]. To identify other mutants with alterations in adhesion, we screened a collection of regulatory mutants including mutants

deficient in StuA, BrlA, AcuM and DvrA [10], [12], [13], [14] for their ability to form biofilms on plastic surfaces. Using this approach, we found that the Δ*stuA* mutant previously described by our group [10] was also markedly impaired in the formation of adherent biofilms on plastic (Fig. 1A, 1B). We hypothesized that these differences in the adherence properties of these three strains might stem from alterations in expression of a single adhesion factor. To test this hypothesis, we performed an analysis of the cell wall carbohydrate composition of these mutant strains in comparison to their respective complemented strains and wild-type *A. fumigatus*. The cell walls of the hypoadherent Δ*medA* and Δ*stuA* strains, but not the hyperadherent Δ*ugm1* strain, were found to contain a significant reduction in N-acetyl galactosamine (GalNAc) (Fig. 1C). Since N-acetyl galactosamine is a key component of galactosaminogalactan, a glycan found within the amorphous cell wall and extracellular matrix of *A. fumigatus* during infection [15], [16], these results suggested that galactosaminogalactan is involved in the differential adhesive properties of these mutants. Consistent with this hypothesis, culture supernatants from the Δ*medA* mutant contained no detectable galactosaminogalactan, while only trace amounts of galactosaminogalactan were found in supernatants from the Δ*stuA* mutants (Fig. 1D). In contrast, culture supernatants from the Δ*ugm1* mutant contained markedly increased galactosaminogalactan as compared to the wild-type and ugm1 complemented strains.

To test the hypothesis that galactosaminogalactan mediates *A. fumigatus* adherence, we examined the ability of a suspension of extracellular GAG harvested from wild-type hyphae to rescue the adherence defects of the Δ*stuA* and Δ*medA* hyphae. The addition of supplemental galactosaminogalactan resulted in a dose dependent increase in adherence to tissue culture treated plates (Fig. 1E). The addition of exogenous galactosaminogalactan also increased the adherence of wild-type *A. fumigatus*, although to a lesser extent than was seen with the adhesion deficient mutants treated with galactosaminogalactan. Collectively these results suggested that galactosaminogalactan was responsible for the adherence of *A. fumigatus* to plastic and other substrates.

Comparative transcriptome analysis of Δ*medA* and Δ*stuA* strains identifies a co-regulated gene *uge3*, encoding a candidate galactosaminogalactan biosynthetic enzyme

Since MedA and StuA control the expression of hundreds of genes, to remove any pleiotrophic effect and to test the specific role of GAG production in adherence, we sought to identify the specific genes required for GAG synthesis. Whole genome microarray analysis of the ΔmedA strain was performed during hyphal growth and development, and compared with the wild-type and *medA* complemented strain. Genes that were significantly dysregulated in the Δ*medA* strain were then compared with the list of previously identified Δ*stuA* dependent genes [10]. Ten genes were identified as being significantly dysregulated in both mutant strains (Fig. 2A). Among these genes was afu3g07910, predicted to encode a UDP-glucose epimerase. Given the role of glucose epimerases in the biosynthesis of galactose and galactosamine, this gene, designated *uge3*, was selected for further study. Real-time RT-PCR confirmed that *uge3* expression was reduced in both the Δ*stuA* and Δ*medA* strains (Fig. 2B).

Disruption of *uge3* specifically abrogates galactosaminogalactan production

To test the role of Uge3 in the synthesis of GAG, a Δ*uge3* mutant strain was constructed. Deletion of *uge3* had no observable effects on growth or morphology including conidiation, conidia size, germination, and radial hyphal growth on solid media in a wide variety of conditions including: minimal media, nutrient rich media (YPD), pH range 4.5 to 8.5, varying iron concentrations (from 0 to 30 μM), and microaerophilic or normoxic conditions (Fig. S1). Scanning electron microscopy of Δ*uge3* mutant hyphae revealed a complete loss of surface decoration and of intercellular matrix (Fig. 3A). Cell wall analysis of the Δ*uge3* mutant strain demonstrated an undetectable level of N-acetyl galactosamine (Fig. 3B), and no galactosaminogalactan was detected in culture filtrates from this strain (Fig. 3C). The production of soluble galactofuranose was unaffected (Fig. 3D). When compared with wild-type *A. fumigatus*, a slight increase in cell wall GlcNAc content was observed in the Δ*uge3* mutant (Fig. 3B), as well as a minimally increased resistance to the anti-chitin agent nikkomycin but not to the chitin binding agent calcofluor white (Table 1). However, complementation of the Δ*uge3* mutant with an intact allele of *uge3* had no effect on these

observations, despite completely restoring N-acetyl galactosamine and galactosaminogalactan synthesis. Collectively these results suggest that Uge3 is necessary for the production of galactosaminogalactan.

The *uge3* deficient mutant is markedly deficient in adherence to and damage of pulmonary epithelial cells

To test the hypothesis that galactosaminogalactan was required to mediate *A. fumigatus* adherence to substrates, we compared the adherence of the Δ*uge3* mutant with the wild-type and *uge3* complemented strains to a variety of substrates. The Δ*uge3* mutant strain exhibited a near complete absence of adherence to all substrates tested, including plastic, pulmonary epithelial cells and fibronectin (Fig. 4A, 4B). As it was observed with the Δ*stuA* and Δ*medA* mutant strains, preincubation of either plastic plates or hyphae of the Δ*uge3* mutant with a suspension of wild-type galactosaminogalactan produced a dose dependent increase in adherence to plastic (Fig. 4C, 4D). This increased adherence was not observed when the wells or hyphae were supplemented with a suspension of zymosan, a β-glucan-rich fungal cell wall preparation, suggesting that the increased adherence is specific to galactosaminogalactan. Scanning electron microscopy of Δ*uge3* hyphae incubated with a suspension of galactosaminogalactan demonstrated a partial restoration of the cell wall decoration seen in wild-type hyphae, suggesting that the Δ*uge3* mutant was able to bind extracellular galactosaminogalactan (Fig. 4E). To confirm that galactosaminogalactan directly binds to epithelial cells, we tested the ability of a suspension of galactosaminogalactan isolated from wild-type *A. fumigatus* to bind directly to A549 cells. FITC conjugated Soy Bean Agglutinin (SBA) was used to quantify galactosaminogalactan binding. This lectin is specific for terminal GalNAc residues, and does not bind to galactosaminogalactan deficient *uge3* mutant hyphae (Fig. 5A). Using this approach, purified galactosaminogalactan was observed to bind to A549 epithelial cells in a dose dependent manner (Fig. 5B). Collectively these results demonstrate that galactosaminogalactan is required for adherence to, and injury of epithelial cells, and suggest that galactosaminogalactan is an important adhesin of *A. fumigatus*.

Deletion of *uge3* also completely blocked the ability of *A. fumigatus* to induce pulmonary epithelial cell injury as measured by a chromium release assay (Fig. 6).

Restoration of *uge3* expression in the Δ*uge3* mutant completely restored the ability of *A. fumigatus* to adhere to host constituents and damage epithelial cells, confirming the specificity of these observations and suggesting that GAG is necessary for adherence to host constituents and subsequent induction of epithelial cell injury.

The *uge3* deficient mutant is attenuated in virulence and induces an increased inflammatory response in vivo

To determine if blocking galactosaminogalactan synthesis and fungal adherence alters virulence, we compared the virulence of the Δ*uge3*, wild-type *A. fumigatus* and the *uge3* complemented strain in a corticosteroid treated mouse model of invasive aspergillosis. Mice infected with the Δ*uge3* mutant strain survived significantly longer than mice infected with either the wild type of *uge3*-complemented strain (Fig. 7A), although this effect was modest. Consistent with the increased survival of mice infected with the Δ*uge3* mutant, these mice were found to have a significantly reduced pulmonary fungal burden after four days of infection as compared with mice infected with wild-type *A. fumigatus* (Fig. 7B). Histopathologic examination confirmed that infection with the Δ*uge3* mutant strain produced fewer and much smaller fungal lesions than did the wild-type *A. fumigatus* (Fig. 7C). Surprisingly, despite the lower abundance of hyphae in pulmonary lesions of mice infected with the Δ*uge3* mutant, these lesions contained more neutrophils than did those of mice infected with wild-type *A. fumigatus*. These results suggest that infection with the Δ*uge3* mutant strain induced an increased host inflammatory response.

Galactosaminogalactan masks β-glucan exposure on the surface of *A. fumigatus* during hyphal growth

Galactosaminogalactan has been localized to the amorphous outer layer of the fungal cell wall [15]. We therefore hypothesized that extracellular galactosaminogalactan might mask the surface exposure of other fungal pathogen-associated molecular pattern (PAMP) molecules such as β-1,3 glucan, and as a result the increased inflammatory response seen during infection with the Δ*uge3* mutant might be a consequence of unmasking of these PAMPs. To test this hypothesis, we performed immunofluorescent microscopy to compare the binding of recombinant Fc-dectin-1 [17] to the Δ*uge3* mutant and wild-type *A.*

fumigatus. Consistent with previous reports [18], we found that binding of Fc-dectin-1 to swollen conidia could be detected in both strains (Fig. 8A). However, during germination and hyphal growth, there was much more intense staining of Δ*uge3* mutant hyphae as compared with the wild-type parent strain, in which Fc-dectin-1 binding decreased over time. In contrast, total β-1,3 glucan content, as assessed by aniline blue staining and release of soluble β-1,3 glucan in the culture supernatant, was not different between the wild-type and the Δ*uge3* mutant strains (Fig. 8B, 8C). Similarly, sensitivity of the Δ*uge3* mutant to the β-1,3 glucan synthase inhibitor casofungin was unchanged from the wild-type parent strain (Table 1). Therefore, the increased binding of Fc-dectin-1 to the Δ*uge3* cells was due to greater surface exposure of β-1,3 glucan rather than increased synthesis of this glycan.

Galactosaminogalactan deficient strains induce a dectin-1 dependent increase production of pro-inflammatory cytokines by dendritic cells in vitro

To test if the increased exposure of β-glucan, or other fungal cell wall PAMPs, on the surface of Δ*uge3* hyphae might induce an increased inflammatory response by immune cells, we determined the cytokine response of bone marrow derived dendritic cells (BMDDCs) upon co-culture with wild-type or Δ*uge3* hyphae. After 6 hours of co-incubation, BMDDCs infected with the Δ*uge3* mutant strain produced significantly higher levels of pro-inflammatory cytokines, including TNF-α, KC, MIP-1α, IL-6, and a trend to higher IL-12 levels, as compared to BMDDCs infected with the wild-type strain (Fig. 9). In addition, a trend to lower levels of the anti-inflammatory cytokine IL-10 produced by BMDDCs infected with the Δ*uge3* mutant as compared with hyphae of wild-type *A. fumigatus* was observed, although this difference was not statistically significant.

To confirm these results and determine if this increased pro-inflammatory response induced by the Δ*uge3* mutant was mediated by increased binding to dectin-1, we examined the ability of an anti-dectin-1 neutralizing antibody and Fc-dectin-1 to block the increase in TNF-α production by BMDDCs in response to hyphae of the Δ*uge3* mutant strain. Pre-incubation of BMDDCs with a monoclonal anti-dectin-1 antibody completely blocked the increased TNF-α production by BMDDCs in response to hyphae of the Δ*uge3* mutant strain (Fig. 10). Similarly, pre-incubating hyphae of the Δ*uge3* mutant strain with Fc-dectin-1

completely blocked the increased TNF-α production by BMDDCs. Collectively these results support the hypothesis that galactosaminogalactan inhibits host inflammatory responses in part by masking of PAMPs such as β-glucan.

Galactosaminogalactan is essential for virulence in highly immunocompromised mice

The results of these in vitro and in vivo studies suggest that the unmasking of fungal PAMPs in the absence of galactosaminogalactan induces an increased inflammatory response to hyphae that is detrimental to the host. To test this hypothesis, the virulence of the Δ*uge3* mutant and wild-type strain was compared for their virulence in highly immunosuppressed mice treated with both corticosteroids and cyclophosphamide. In this model, *A. fumigatus* infection does not induce a detectable cellular or cytokine inflammatory response during the neutropenic period [19]. In these highly immunosuppressed mice, the Δ*uge3* mutant strain exhibited markedly attenuated virulence as compared with the wild-type parent strain (Fig. 11A). This difference in virulence was unlikely related to differences in the initial infectious inoculum, since the fungal burden was similar between mice infected with the wild-type and Δ*uge3* mutant and sacrificed one hour after infection (a median of 1900 vs. 1850 colony forming units per animal, respectively). Mice infected with the Δ*uge3* mutant strain had a reduction in pulmonary fungal burden that was similar in magnitude to that seen in the non-neutropenic mouse model (Fig. 11B). Histopathologic examination of lungs after 5 days of infection confirmed an absence of infiltrating leukocytes surrounding the sites of wild-type *A. fumigatus* infection (Fig. 11C). These data suggest that the increased inflammatory response induced by the Δ*uge3* strain in non-neutropenic mice is non-protective and increases mortality, because inhibiting inflammation in the highly immunosuppressed mouse model was associated with improved survival.

To confirm this hypothesis, we compared the inflammatory response during infection with the wild-type and the Δ*uge3* mutant in both the non-neutropenic model and the highly immunosuppressed models. To minimize the effects of differences in fungal burden between strains, mice were studied earlier in the course of disease, after three days of infection. In non-neutropenic immunosuppressed mice, a significantly lower fungal

burden was again observed in mice infected with the Δ*uge3* mutant strain as compared with those infected with wild-type *A. fumigatus* (Fig. 12A). Relative to this lower fungal burden, Δ*uge3* mutant strain was found to induce significantly higher pulmonary myeloperoxidase levels (MPO) suggesting that this mutant has a higher capacity to mediate pulmonary leukocyte recruitment as compared with wild-type *A. fumigatus* (Fig. 12A). Similarly, the relative induction of pulmonary TNF-α, as well as the ability to induce pulmonary injury, as measured by LDH levels in BAL fluid, was significantly greater with the Δ*uge3* mutant than with wild-type *A. fumigatus* (Fig. 12A). In contrast, in the highly immunosuppressed mouse model there was no significant difference in pulmonary fungal burden, myeloperoxidase content, TNF-α levels or LDH release between mice infected with the wild-type and with the Δ*uge3* mutant strain at this earlier time point (Fig. 12B). Further, these measures of inflammation were significantly lower in these highly immunosuppressed mice as compared with non-neutropenic animals. Collectively these data suggest that in non-neutropenic mice, infection with the Δ*uge3* mutant stimulates a non-protective hyper-inflammatory response.

DISCUSSION

In *A. fumigatus*, galactosaminogalactan is a heterogeneous linear polymer consisting of α1–4 linked galactose and N-acetylgalactosamine residues in variable combination [16]. Galactosaminogalactan is secreted and also a component of both the amorphous cell wall and extracellular matrix. In addition, galactosaminogalactan has been detected in lung lesions of experimentally infected animals [15]. The present study adds significantly to our understanding of the biosynthesis and function of this fungal polysaccharide.

First, the results of our in vitro studies strongly suggest that galactosaminogalactan is the principal mediator of *A. fumigatus* adherence and plays a key role in biofilm formation. The mechanism by which this carbohydrate mediates adherence to substrates and binds to hyphae remains undefined. Although specific host or fungal lectins may mediate binding of *Aspergillus* galactosaminogalactan, binding to plastic is clearly

independent of host receptors and must be mediated by physicochemical interactions such as charge or hydrophobicity. Further, the lack of competition observed when galactosaminogalactan in suspension was added to wild-type hyphae would argue against a receptor-ligand interaction. Overall, these data are most consistent with a model in which galactosaminogalactan functions as a glue that mediates attachment between hyphae and substrates in a highly promiscuous fashion. The findings of this study add to the growing body of evidence implicating polyhexosamine glycans as key adhesion factors for microorganisms. Work from the 1970's identified polygalactosamine compounds from *Neurospora crassa* and *Bipolaris sorokiniana* and suggested that they could potentially play a role in the adherence of fungal spores to glass surfaces [20,21]. Similarly, a large number of gram positive and gram negative bacterial biofilms contain polysaccharide intercellular adhesin (PIA), a homopolymer of N-acetylglucosamine, which mediates adherence between bacteria and the surfaces they colonize [22]. Although composed of a different amine sugar, the similarities between these mechanisms of adherence are striking. The adhesive characteristics of PIA are in large part governed by de-acetylation of N-acetyl glucosamine residues. PIA differs from *A. fumigatus* galactosaminogalactan in which the galactosamine residues have been reported to be uniformly acetylated [16]. Nevertheless, these data suggest that the use of polyhexosamine glycans is a widespread microbial adherence strategy, and could potentially serve as a useful target for the development of antimicrobial strategies with broad applicability.

The present results suggest that Uge3 is a key enzyme in the galactosaminogalactan biosynthetic pathway. The absence of N-acetyl galactosamine in the cell wall of the *uge3* mutant strain and the absence of effects on galactofuranose synthesis suggest that this enzyme functions in the production of N-acetyl galactosamine, although experimental validation of this hypothesis is required. A minimal increase in the GlcNAc content of the cell wall of the Δ*uge3* mutant was also noted. Although this finding could suggest accumulation of substrate in the absence of conversion to GalNAc, it is unclear if this is a significant finding. A similar increase in GlcNAc content was also seen in the *uge3* complemented strain despite a restoration of GalNAc and GAG synthesis. Similarly, we observed no difference in susceptibility to classic cell wall perturbing agents between the Δ*uge3* mutant and complemented strains, suggesting that the increased GlcNAC seen in

both strains does not contribute to the marked reduction of adherence and virulence that was seen only in the Δ*uge3* mutant. Although these data suggests Uge3 therefore mediates synthesis of the N-acetyl galactosamine component of galactosaminogalactan, the pathways responsible for the production of galactose for the synthesis of galactosaminogalactan remain unknown. It is possible that Uge3 also mediates the interconversion of UDP-glucose to UDP-galactose, as epimerases with dual substrate affinity have been described [23], however the normal levels of galactose in the Δ*uge3* mutant strain argue against this hypothesis. Alternately, galactose synthesis may be dependent on one of the other two putative epimerases identified within the *A. fumigatus* genome.

The results of this and previous studies strongly suggest that galactosaminogalactan modulates immune responses in vivo. Previous work has suggested that galactosaminogalactan may be recognized by the host as a PAMP and mediate immunosuppression. Fontaine *et. al.* observed that a urea-soluble fraction of galactosaminogalactan induced neutrophil apoptosis in vitro, and that vaccination of mice with a soluble fraction of galactosaminogalactan enhanced the progression of invasive aspergillosis in immunocompetent and immunosuppressed mice in association with increasing Th2 and Th17 responses [16]. The experiments described here add substantially to these findings by testing the effects of live organisms deficient in galactosaminogalactan production in both non-neutropenic and highly immunosuppressed leukopenic mice. Our findings of increased local inflammation surrounding the Δ*uge3* strain support the role of galactosaminogalactan as an immunosuppressive molecule. The increased inflammatory response to galactosaminogalactan-deficient hyphae was not protective, but rather attenuated the survival advantage in mice infected with this strain when compared with highly immunosuppressed animals. These findings add support to the growing body of literature suggesting that non-protective inflammatory responses can increase mortality during infection with *A. fumigatus* [24], [25], [26].

In addition to its direct effects on the immune system, galactosaminogalactan likely also modulates host immune responses through cloaking β-glucan and possibly other PAMPs on the surface of hyphae. Masking of β-glucan and other cell wall PAMPs by the hydrophobin RodA has been previously demonstrated in conidia, and is thought to play an important role in immune evasion [27]. These cell wall β-glucans and other PAMPs are

then exposed when the hydrophobin layer is shed during germination. However, studies examining β-glucan exposure during the growth and development of *A. fumigatus* hyphae have found that, as hyphae mature, the recognition of β-glucan exposure by dectin-1 decreases when compared with swollen conidia and early germinated hyphae [18]. Our results suggest that the production of galactosaminogalactan by maturing hyphae may account for this reduced exposure of β-glucan, and as in the case of conidia, results in an attenuation of inflammatory responses. A similar immune evasion strategy has been reported in the dimorphic fungus *Histoplasma capsulatum*, in which surface expression of α-(1,3)-glucan has been shown to mask exposure of β-glucan and reduce inflammatory responses [28]. This modulation of β-glucan exposure is the natural converse to the effects of the echinocandin antifungals, in which increasing the exposure of β-glucan is postulated to increase host inflammatory responses and improve fungal killing [29], [30].

The findings of this study also suggest that galactosaminogalactan-mediated adherence may play a role in virulence. Suppression of inflammation in mice infected with the Δ*uge3* mutant resulted in a reduced pulmonary fungal burden and increased survival of the mice infected with the Δ*uge3* mutant as compared to mice infected with the wild-type *A. fumigatus*. It is therefore possible that this attenuated virulence and reduced fungal burden reflects the impaired ability of this mutant to adhere to, and form colonies in the lung, rather than alterations in immune mediated fungal killing. Alternately, loss of galactosaminogalactan may render hyphae more susceptible to host killing by microbicidal peptide or other neutrophil-independent host defences, or result in a unique growth defect seen under in vivo conditions.

These studies suggest that anti-galactosaminogalactan strategies could be useful in the therapy of invasive aspergillosis. Importantly, however, our data would suggest that blocking galactosaminogalactan function would likely be a superior approach to inhibiting the synthesis of galactosaminogalactan, in order to avoid potentially increasing the inflammatory response, and potentially mortality, attributable to unmasking β-glucan or other PAMPs.

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FIGURE LEGENDS

Table 1. Sensitivity of fungal strains to cell wall perturbing agents.

Table S1. PCR primers used in this study.

Figure 1. MedA and StuA are required for biofilm formation, adherence to plastic, and galactosaminogalactan production.

(A) Formation of biofilms by hyphae of the indicated strains after 24 h growth on polystyrene plates. After washing, hyphae were stained with crystal violet for visualization.

(B) Adherence of germlings of the indicated strains to polystyrene plates.

(C) GalNAc content of the hyphal cell wall of the indicated A. fumigatus strains. Results are expressed as a percentage of the total carbohydrate content of the alkali insoluble fraction of the cell wall.

(D) Amount of galactosaminogalactan released in the culture supernatant of the indicated strains after 48 h growth, normalized to the culture dry weight.

(E) Effects of galactosaminogalactan supplementation on germling adherence of the indicated strains. 6-well polystyrene culture plates were coated with galactosaminogalactan extracted from Af293 culture at the indicated concentration overnight at 4°C before testing the adherence of germlings. All graphs indicate mean ± standard error and represent data obtained from at least three independent experiments performed on separate days.

For graphs (B–D) *indicates a significant reduction as compared with the wild-type Af293 strain, with p value<0.05 by factor ANOVA.

For graph (E): * and § indicate increased adherence of strains with the addition of galactosaminogalactan with p values of <0.05 and = 0.08, respectively, by factor ANOVA.

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Figure 2. The gene *uge3* (Afu3g07910) encodes a UDP-glucose-4-epimerase and is regulated by both MedA and StuA.

(A) Genes found to be differentially regulated in both Δ*medA* and Δ*stuA* strains by microarray analysis.
(B) Expression of *uge3*, measured by real-time RT-PCR in the indicated strains after 18 h growth. Results indicate the mean expression level from at least three independent experiments ± standard error.
* indicates a significantly reduced *uge3* mRNA levels as compared with Af293 strain, $p<0.05$ by factor ANOVA.

Figure 3. Uge3 is necessary for the production of galactosaminogalactan but dispensable for galactofuranose synthesis.
(A) Scanning electron micrographs of hyphae of the indicated strains after 24 h of growth. Magnification was 20,000X.
(B) Hexose and hexosamine content of the hyphal cell wall of the indicated *A. fumigatus* strains. Results are expressed as a percentage of the total carbohydrate content of the cell wall.
(C) Quantity of GalNAc and (D) of galacto-furanose (Gal*f*) released in the culture supernatant of the indicated strains after 48 h growth, normalized to the culture dry weight.
Graphs B, C and D indicate the mean ± standard error of at least three independent experiments.
* indicates a significant reduction as compared with the wild-type Af293 strain, $p<0.05$ by factor ANOVA.

Figure 4. Uge3 is necessary for normal adherence of *A. fumigatus* hyphae.
(A) Formation of biofilms by hyphae of the indicated strains after 24 h growth on polystyrene plates. After washing, hyphae were stained with crystal violet for visualization.
(B) Adherence of germlings of the indicated strains to cell culture treated polystyrene, fibronectin and A549 epithelial cells.
(C) Adherence of the Δ*uge3* strain to cell culture treated polystyrene supplemented with a suspension of galactosaminogalactan or zymosan at the indicated concentrations.

(D) Adherence to cell culture treated polystyrene of the Δ*uge3* strain pre-incubated for 30 min with a suspension of GAG or zymosan at the indicated concentrations.
(E) Scanning electron micrographs of hyphae of the indicated strains after 24 h of growth and co-incubation with a suspension of galactosaminogalactan or zymozan for 30 min, when indicated. Magnification was 20,000X.
All assays were performed on at least three independent occasions. Graphs indicate mean ± standard error.
* indicates a significantly reduced adherence of the Δ*uge3* mutant as compared with Af293 and *uge3*-complemented strains (B) or a significantly increased adherence of the *uge3* mutant after carbohydrate addition as compared with *uge3* mutant alone (C), $p<0.05$ by factor ANOVA.

Figure 5. Galactosaminogalactan binds to epithelial cells.
(A) Soy Bean Agglutinin (SBA), a GalNAc binding lectin, detects galactosaminogalactan on hyphae. Hyphae were grown for 12 h, fixed, stained with FITC-conjugated SBA and imaged using confocal microscopy. Magnification was 40X.
(B) Dose dependent binding of purified galactosaminogalactan particles to A549 cells. Cells were co-incubated with the indicated concentration of galactosaminogalactan, and then washed and bound galactosaminogalactan was quantified by detection with FITC-conjugated SBA.
The assay was performed on three independent occasions. Graphs indicate mean ± standard error.

Figure 6. Uge3 is required for the induction of epithelial cell injury by *A. fumigatus*.
A549 pulmonary epithelial cells were incubated with conidia of the indicated strains for 16, 20 or 24 h, after which the extent of epithelial cell injury was measured with a chromium release assay.
Graphs indicate mean ± standard error of 3 independent experiments, each performed in triplicate.
* indicates a significantly reduced injury to cells induced by the Δ*uge3* mutant, as compared with injury induced by Af293 and *uge3*-complemented strains, $p<0.05$ by factor ANOVA.

Figure 7. Uge3 is required for full virulence in mice model of IA.

(A) Survival of cortisone acetate-treated Balb/C mice infected with the indicated A. fumigatus strains. Data are the combined results of 2 independent experiments for a total of 16 mice per strain.

* indicates a significant increase in survival between mice infected with the Δ*uge3* mutant and mice infected with the wild-type or with the *uge3* complemented strain, p = 0.017 and <0.001 respectively by the log rank test.

(B) Quantification of fungal DNA in lung homogenates of mice after 4 days of infection. Results are median ± interquartile of 8 mice per strain.

* indicates a significantly reduced fungal DNA content in lungs of mice infected with the Δ*uge3* mutant compared with those infected with the wild-type strain, p = 0.015 by the Wilcoxon rank sum test.

(C) Photomicrographs of PAS stained sections of mouse lungs obtained 4 days after infection with the indicated strains. White arrows indicate hyphae, black arrows indicate infiltrating leukocytes.

Figure 8. β-glucans are exposed on hyphae in the absence of galactosaminogalactan.

Detection of Fc-dectin-1 binding using indirect immunofluorescence. Conidia of A. fumigatus strains Af293 and Δ*uge3* were grown in Brian media for the indicated times, to produce swollen conidia (6 h), early germinating conidia (8 h) or hyphae (12 h). Samples were then fixed and stained with Fc-dectin-1 (A) or with aniline blue (B) and imaged with epifluorescent microscopy at 40X magnification.

(C) β -glucan content of supernatants harvested from the indicated strains, as determined by the (1→3)-β -D-Glucan Detection Reagent Kit.

Results are the mean ± standard error of two independent experiments.

Figure 9. Dendritic cells produce an increased pro-inflammatory cytokine profile in response to the galactosaminogalactan deficient mutant.

Graphs show the cytokine content of culture supernatant after 6 h of infection of BMDDCs with hyphae of the indicated strains. LPS was used as a positive control, and medium as a negative control. Cytokine concentrations in culture supernatants were determined by multiplex EIA.
Results are mean ± standard error of duplicate determination of cytokine concentrations, indicated in pg/mL.
* indicates a significantly increased cytokine concentration induced by the Δ*uge3* mutant, as compared with the one induced by Af293, $p<0.05$ by factor ANOVA.
§ indicates that actual value is above 5,000 pg TNFα/mL (measures exceeded upper limit of the test).

Figure 10. Dectin-1 blockade abrogates the increased TNFα secretion induced by the galactosaminogalactan deficient Δ*uge3* mutant.
BMDDCs were infected with hyphae of the indicated strains for 6 h, after which the TNFα content of culture supernatants was determined by EIA. Dectin-1 recognition of β-glucan was inhibited by preincubating BMDDCs with a monoclonal anti-dectin 1 antibody or by preincubating hyphae with Fc-dectin-1.
Results are mean ± SEM of duplicate experiments, each performed in triplicate.
* indicates a significant reduction of TNFα production compared to BMDDCs exposed to the Δ*uge3* mutant without dectin-1 blocking, $p<0.05$ by factor ANOVA.

Figure 11. Uge3 is required for full virulence in a highly immunosuppressed mouse model of IA.
(A) Survival of highly immunosuppressed mice treated with cyclophosphamide and cortisone acetate and infected with the indicated *A. fumigatus* strains.
* indicates significantly increased survival of mice infected with the Δ*uge3* mutant as compared with those infected with the wild-type, $p = 0.002$ by the log rank test ($n = 12$ mice per fungal strain).
(B) Quantification of fungal DNA in lung homogenates of mice after 5 days of infection.
Results are median ± interquartile range of 8 mice per strain.

* indicates a significantly reduced fungal DNA content in lungs of mice infected with the Δ*uge3* mutant as compared with those infected with the wild-type strain, p = 0.11 by the Wilcoxon rank sum test.
(C) Photomicrographs of Gomori methenamine silver stained sections of mouse lungs obtained 4 days after infection with the wild-type strain Af293. No fungal lesions could be identified in the lungs of mice infected with the Δ*uge3* mutant strain. Magnification was ×100 and ×400. White arrows indicate hyphae. Note the lack of infiltrating leukocytes within fungal lesions.

Figure 12. The *A. fumigatus* Δ*uge3* mutant induces a hyperinflammatory response in non-neutropenic mice that is attenuated in highly immunocompromised mice.
(A.) Corticosteroid treated mice were infected by inhalation with the indicated strains of *A. fumigatus* and sacrificed three days after infection. Fungal burden was determined by pulmonary galactomannan content and pulmonary inflammation was measured by determining MPO, and TNF-α content. Pulmonary injury was quantified by measuring LDH release in BAL fluid. MPO, TNF-α, and LDH levels were normalized to the fungal burden of each strain in Panel 1.
Results are median ± interquartile range of 8 mice per strain.
* indicates a significant decrease in fungal burden or a significant increase in MPO, TNFα or LDH content in lungs of mice infected with the Δ*uge3* mutant as compared to the lungs of mice infected with the wild-type strain, $p<0.01$ by the Wilcoxon rank sum test.
(B) Corticosteroid and cyclophosphamide treated mice were infected by inhalation with the indicated strains, sacrificed and the lungs processed as in (A). MPO, TNF-α, and LDH levels were normalized to the fungal burden of each strain in Panel 1.
Results are median ± interquartile range of 9 mice per strain.
Note: y-axis values for all graphs are lower than those in (A).

Figure S1. The Δ*uge3* mutant exhibits normal growth under a variety of conditions.
Radial growth of hyphae of each of the indicated strains was determined under the indicated growth conditions. No significant difference between strains was observed in response to hypoxia, changes in pH, or low iron conditions.

Table 1

Fungal strain	Minimum Inhibitory/Effective Concentration (μg/mL)		
	Caspofungin	Nikkomycin	Calcofluor White
Af293	0,42	1,12	10,42
Δ*uge3*	0,50	1,92	10,42
Δ*uge3*+*uge3*	0,42	1,92	10,42

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Table S1

Primer name	Target gene	Sequence 5'-3'
U1	*uge3*	CACCAAGGTTTACTATTTCTAGTGGATGG
U2	*uge3*	CGAGGACGATATGCTCAGG
U3	*uge3*	CACCTTTTCATTTTGGTATGGCGT
U4	*uge3*	CGTGTCTGCTTTGGACTTGA
U5	*uge3*	CGCTCGATATTACGACCTGAGAATG
U-ext1	*uge3*	GCAGGCACCGTCGATTGCTC
U-ext4	*uge3*	CCGTTGTTGACTTCCCGCGT
U-OE1	*uge3*	GGGCCATGGATATCATGGACAGCTACCAGCAAT
U-OE2	*uge3*	GGGTCCACTAGTCTAAGTAGATAACCCACTGAC
U-RT sense	*uge3*	GCTGTTAGCCTCCCAGTACC
U-RT antisense	*uge3*	GGACTTGGTCGTACCCCAT
HY	*hph*	CAACCACGGCCTCCAGAAGAAGA
YG	*hph*	GCGAGAGCCTGACCTATTGCATCT
tef1-RT sense	*tef1*	CCATGTGTGTCGAGTCCTTC
tef1-RT antisense	*tef1*	GAACGTACAGCAACAGTCTGG
18S forward	18S	GGCCCTTAAATAGCCCGGT
18S reverse	18S	TGAGCCGATAGTCCCCCTAA
18S probe	18S	6-FAM-AGCCAGCGGCCCGCAAATG-MGB

Figure 1

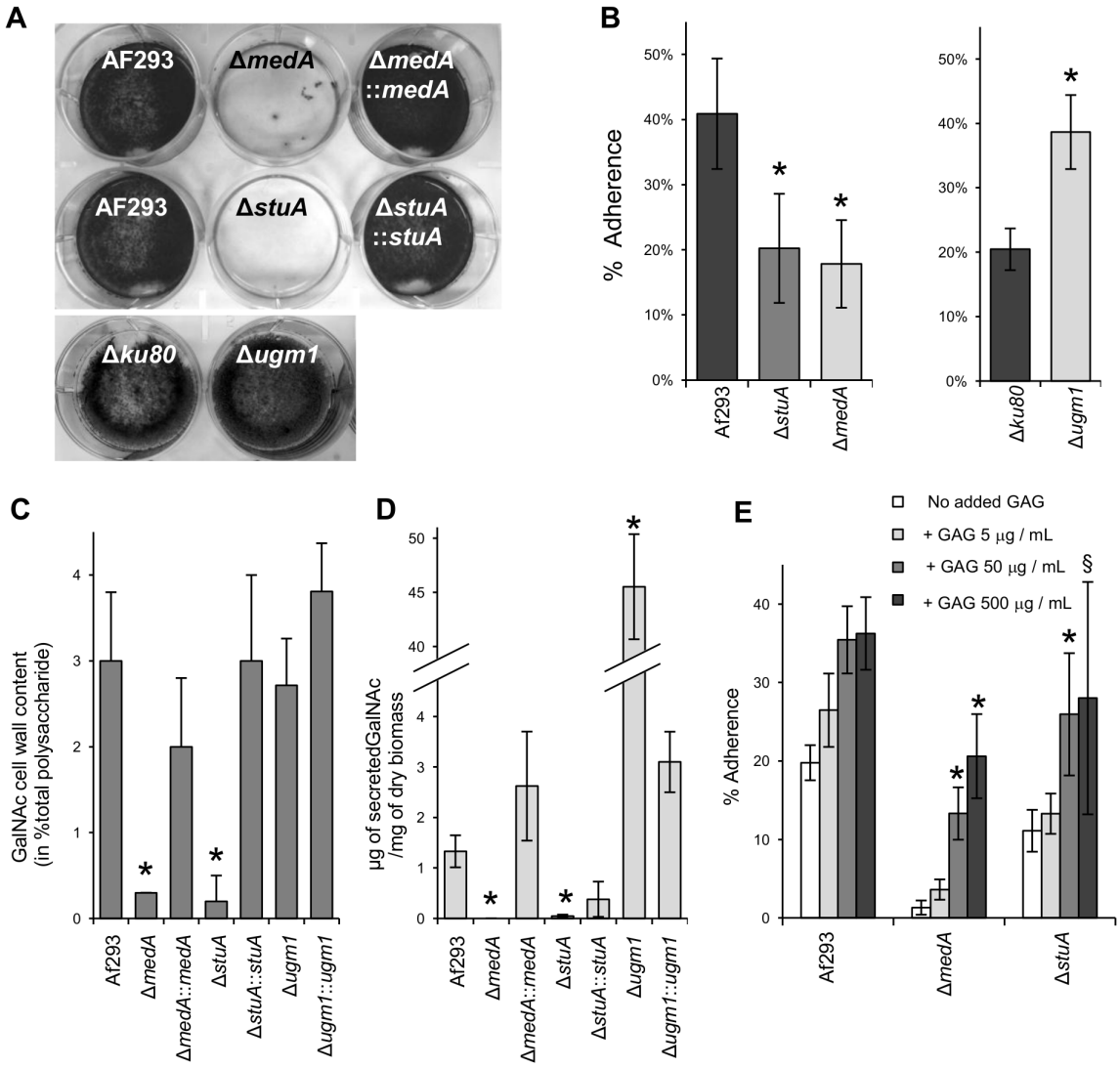
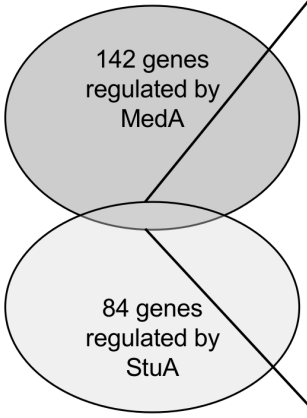

A
AF293
ΔmedA
ΔmedA ::medA
AF293
ΔstuA
ΔstuA ::stuA
Δku80
Δugm1
B
% Adherence
50%
40%
30%
20%
10%
0%
Af293
ΔstuA
ΔmedA
Δku80
Δugm1
C
GalNAc cell wall content (in %total polysaccharide)
Af293
ΔmedA
ΔmedA::medA
ΔstuA
ΔstuA::stuA
Δugm1
Δugm1::ugm1
D
µg of secretedGalNAc /mg of dry biomass
E
No added GAG
+ GAG 5 µg / mL
+ GAG 50 µg / mL
+ GAG 500 µg / mL
§
% Adherence

Figure 2

A



Locus	Encoded protein putative function
Afu1g10390	ABC multidrug transporter
Afu1g14300	Fasciclin domain family
Afu3g07870	extracellular serine-rich protein
Afu3g07910	**UDP-glucose 4-epimerase**
Afu4g12700	hypothetical protein
Afu5g10950	hypothetical protein
Afu6g00680	hypothetical protein
Afu7g06780	hypothetical protein
Afu8g00190	cytochrome P450
Afu8g00200	O-methyltransferase

B

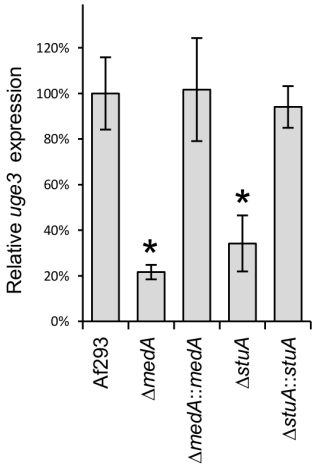
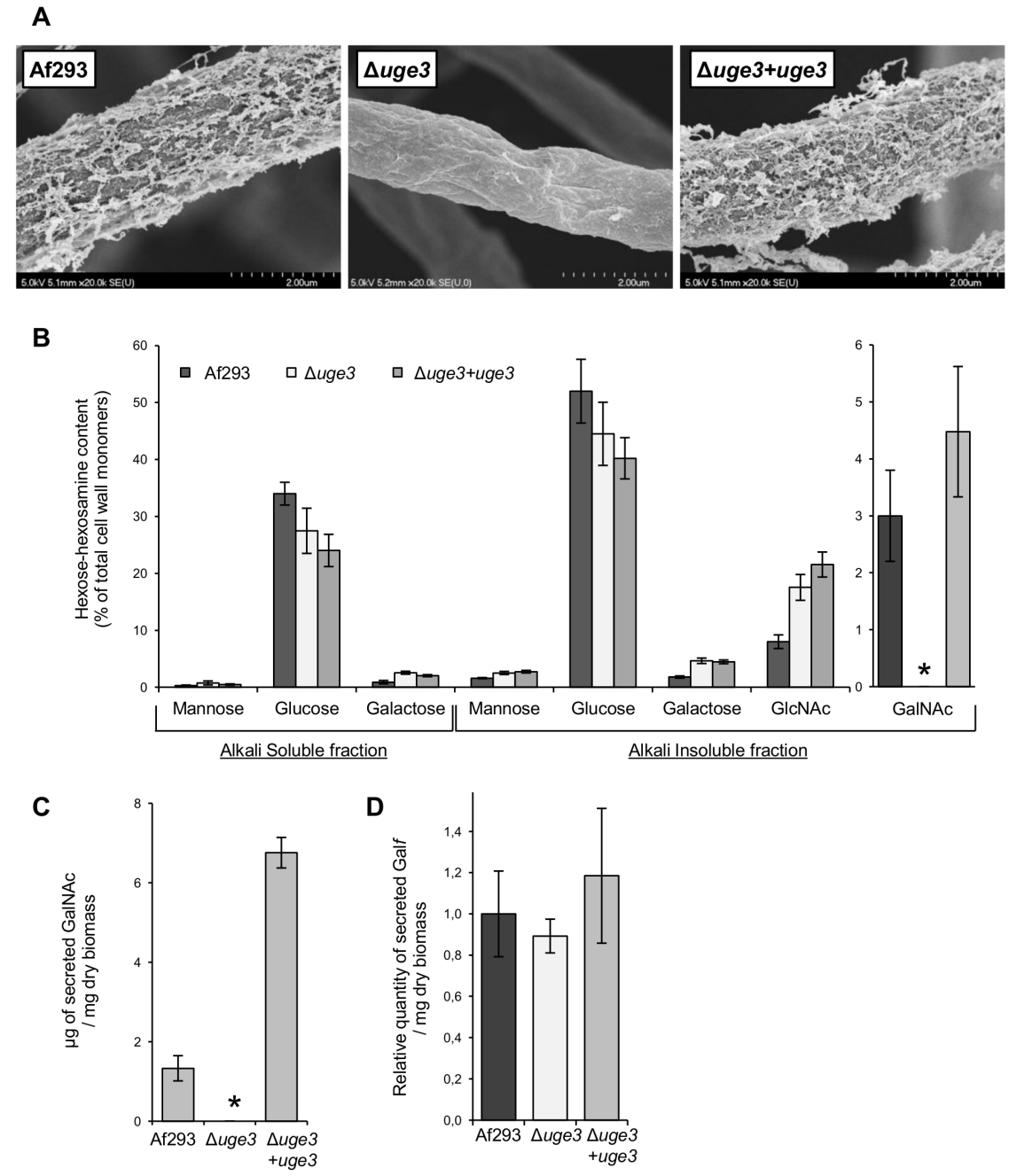


Figure 3


A
Af293
Δuge3
Δuge3+uge3
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2.00um
5.0kV 5.2mm x20.0k SE(U,0)
2.00um
5.0kV 5.1mm x20.0k SE(U)
2.00um
B
Hexose-hexosamine content
(% of total cell wall monomers)
Af293
Δuge3
Δuge3+uge3
Mannose
Glucose
Galactose
Mannose
Glucose
Galactose
GlcNAc
GalNAc
Alkali Soluble fraction
Alkali Insoluble fraction
C
µg of secreted GalNAc
/ mg dry biomass
Af293
Δuge3
Δuge3
+uge3
D
Relative quantity of secreted Galf
/ mg dry biomass
Af293
Δuge3
Δuge3
+uge3

Figure 4

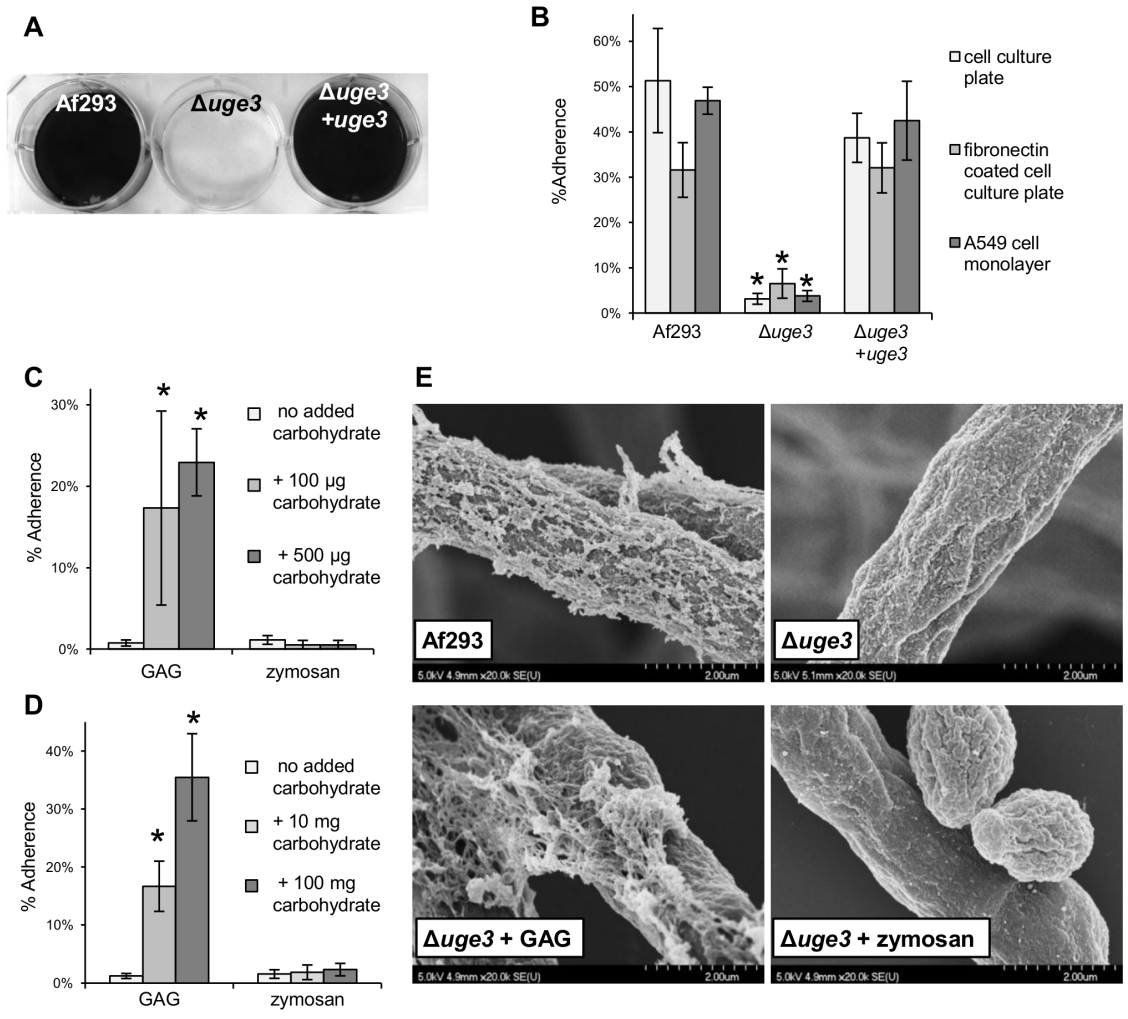

A
Af293
Δuge3
Δuge3 +uge3
B
%Adherence
60%
50%
40%
30%
20%
10%
0%
Af293
Δuge3
Δuge3 +uge3
cell culture plate
fibronectin coated cell culture plate
A549 cell monolayer
C
% Adherence
30%
20%
10%
0%
GAG
zymosan
no added carbohydrate
+ 100 µg carbohydrate
+ 500 µg carbohydrate
D
% Adherence
40%
30%
20%
10%
0%
GAG
zymosan
no added carbohydrate
+ 10 mg carbohydrate
+ 100 mg carbohydrate
E
Af293
Δuge3
Δuge3 + GAG
Δuge3 + zymosan

Figure 5

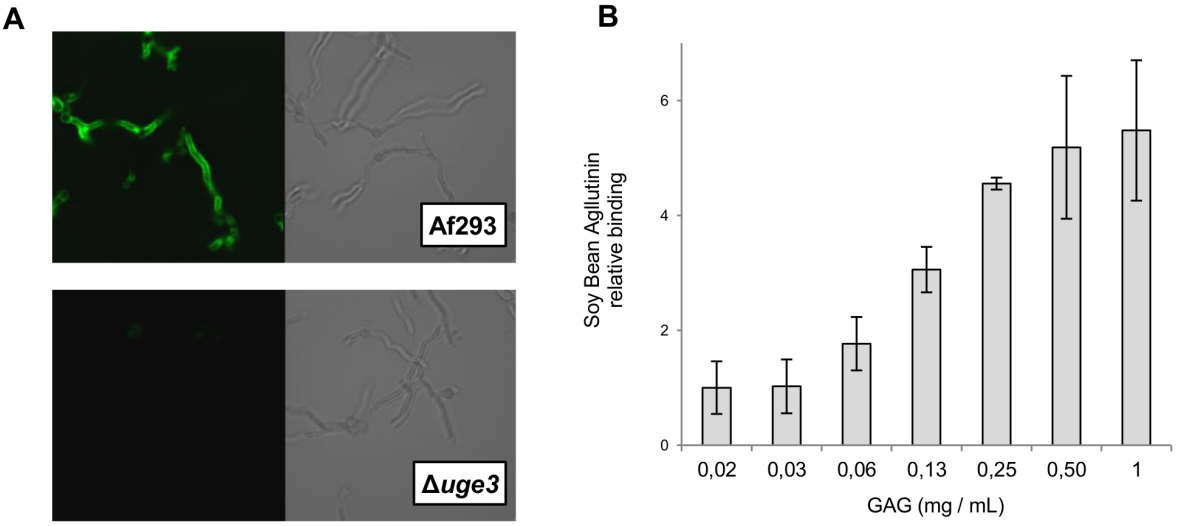

A
Af293
Δuge3
B
Soy Bean Agllutinin
relative binding
0
2
4
6
0,02
0,03
0,06
0,13
0,25
0,50
1
GAG (mg / mL)

Figure 6

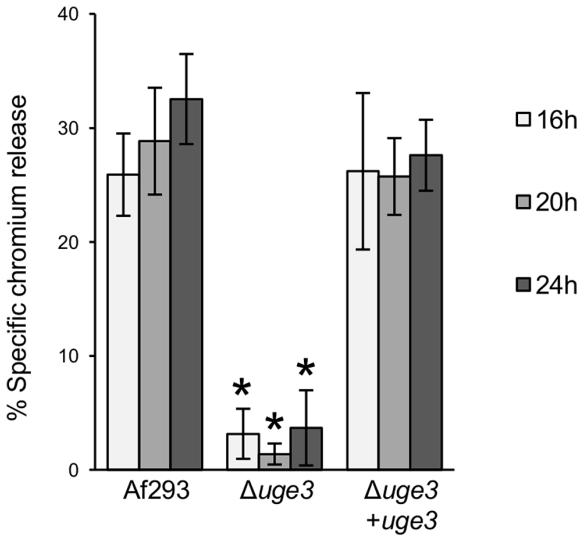

40
30
20
10
0
% Specific chromium release
*
*
*
Af293
Δuge3
Δuge3
+uge3
16h
20h
24h

Figure 7

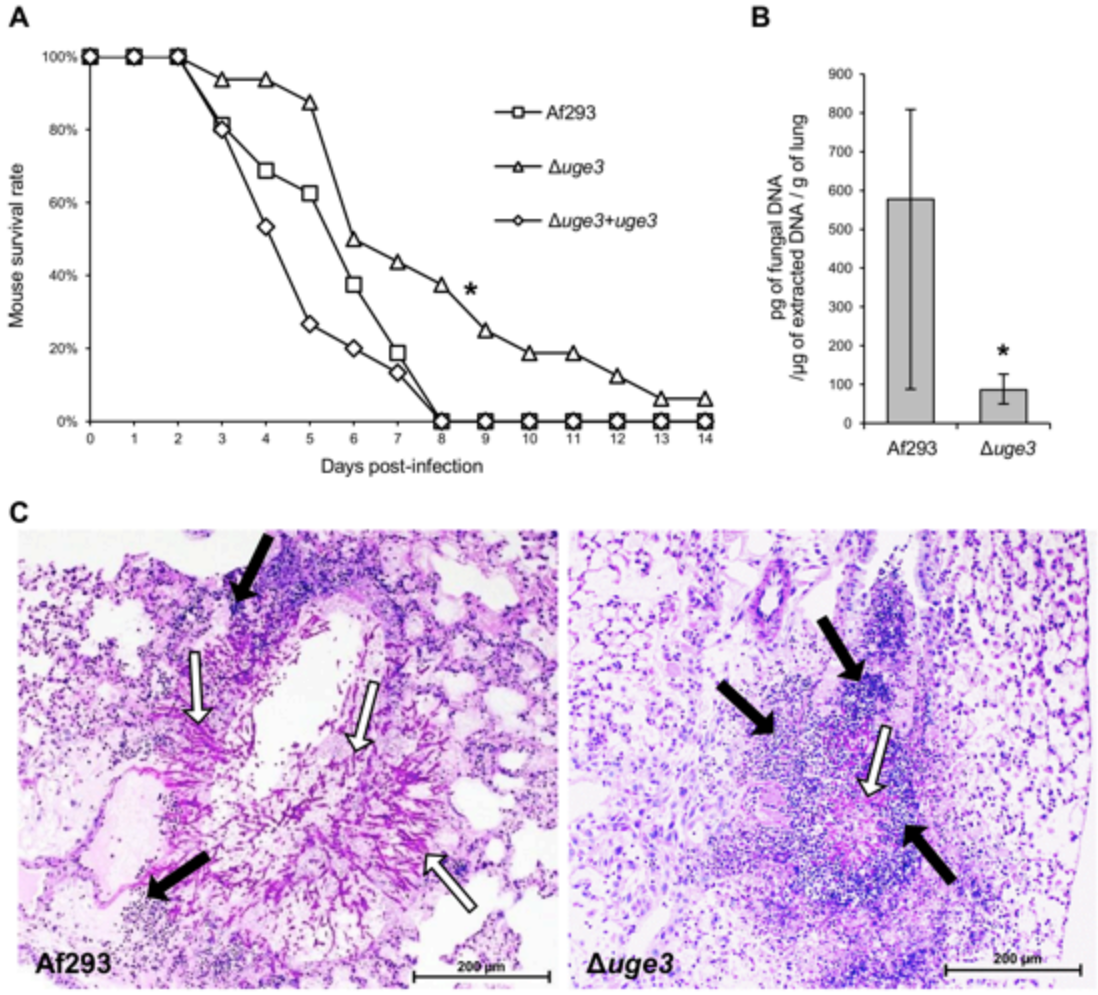

A
100%
80%
60%
40%
20%
0%
Mouse survival rate
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days post-infection
Af293
Δuge3
Δuge3+uge3
*
B
900
800
700
600
500
400
300
200
100
0
pg of fungal DNA
/µg of extracted DNA / g of lung
*
Af293
Δuge3
C
Af293
200 µm
Δuge3
200 µm

Figure 8

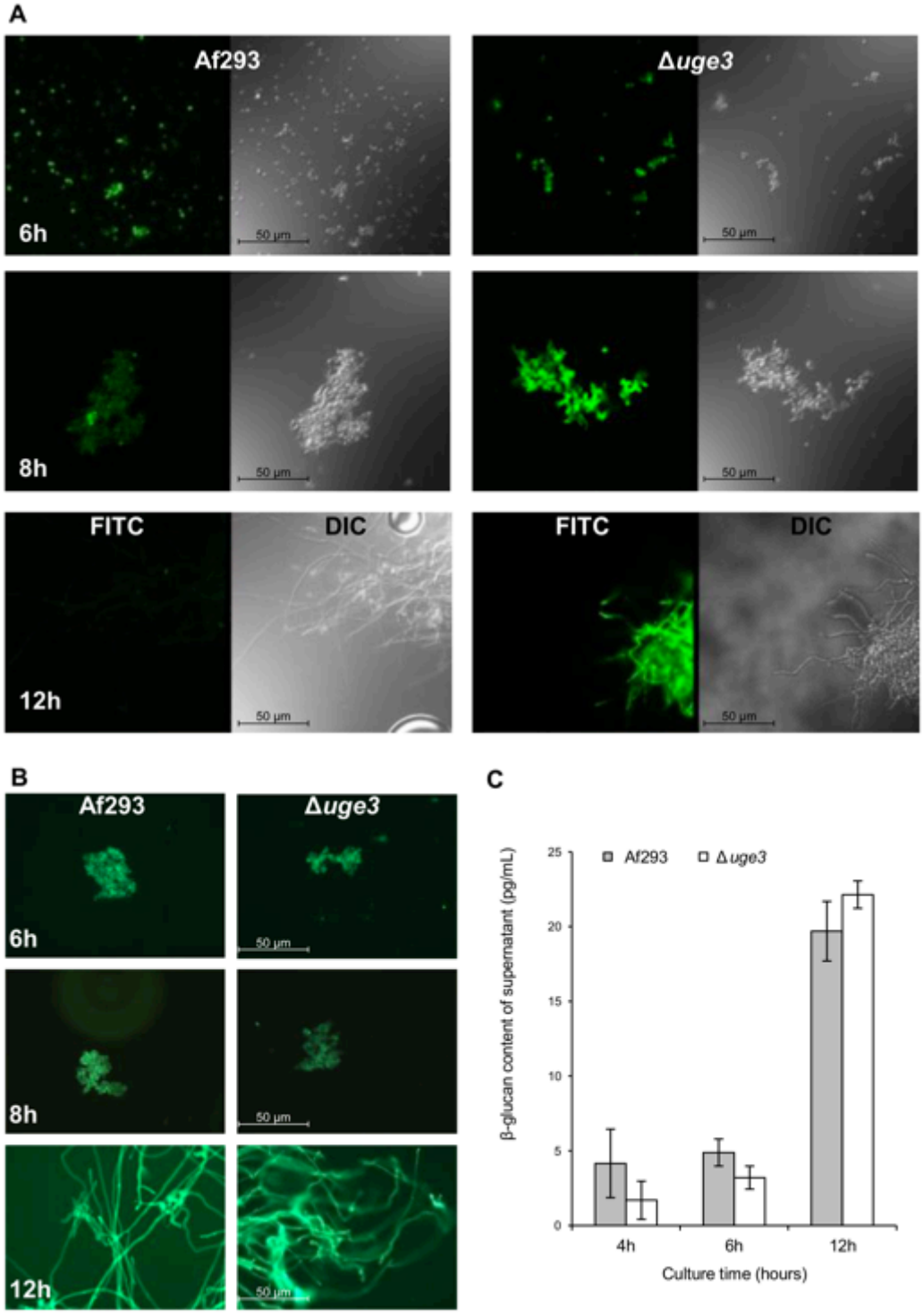

A
Af293
Δuge3
6h
8h
12h
FITC
DIC
50 μm
B
Af293
Δuge3
6h
8h
12h
50 μm
C
Af293
Δuge3
β-glucan content of supernatant (pg/mL)
0
5
10
15
20
25
4h
6h
12h
Culture time (hours)

Figure 9

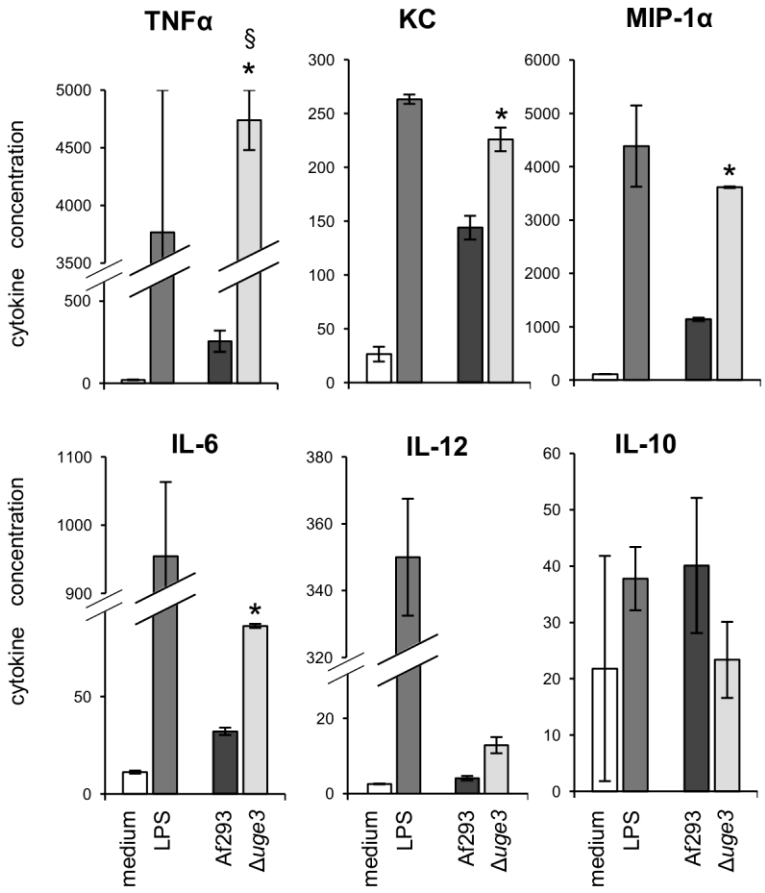

TNFα
KC
MIP-1α
IL-6
IL-12
IL-10
cytokine concentration
cytokine concentration
§
*
*
*
*
*
5000
4500
4000
3500
500
0
300
250
200
150
100
50
0
6000
5000
4000
3000
2000
1000
0
1100
1000
900
50
0
380
360
340
320
20
0
60
50
40
30
20
10
0
medium
LPS
Af293
Δuge3
medium
LPS
Af293
Δuge3
medium
LPS
Af293
Δuge3

Figure 10

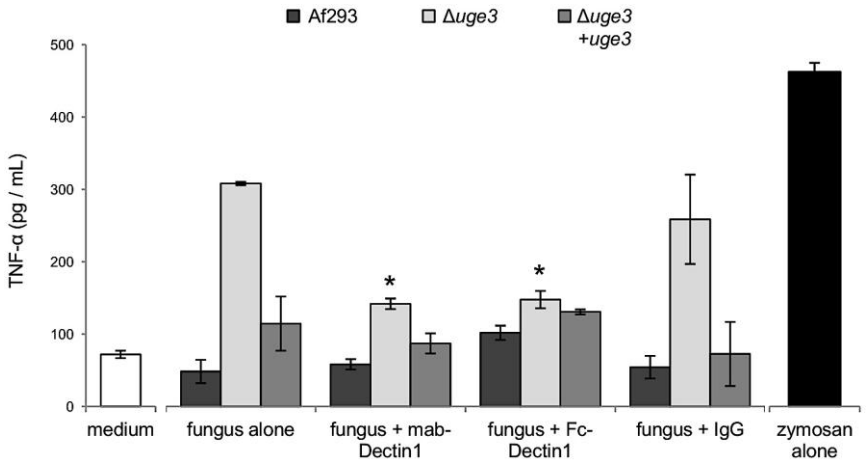

Af293
Δuge3
Δuge3 +uge3
TNF-α (pg / mL)
500
400
300
200
100
0
*
*
medium
fungus alone
fungus + mab-Dectin1
fungus + Fc-Dectin1
fungus + IgG
zymosan alone

Figure 11

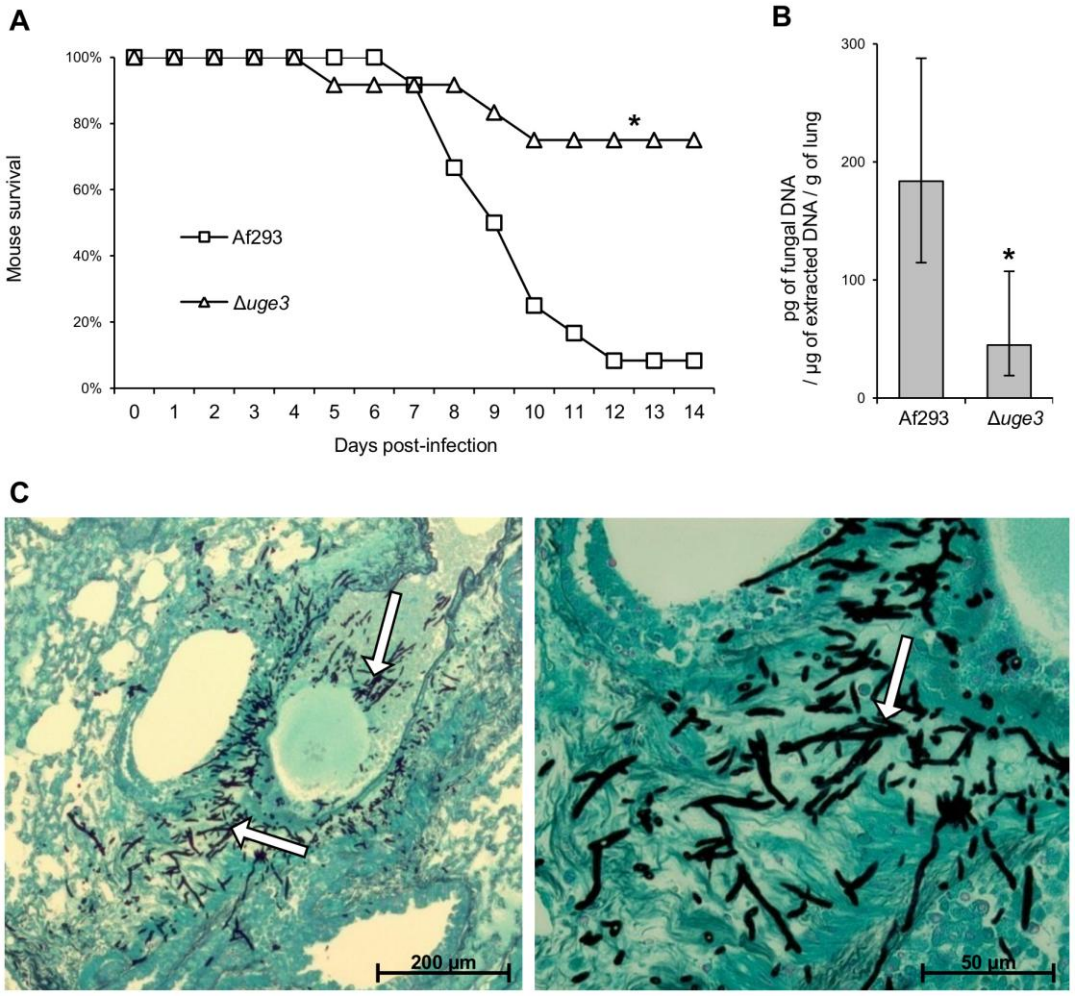

A
100%
80%
60%
40%
20%
0%
Mouse survival
Af293
Δuge3
*
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days post-infection
B
300
200
100
0
pg of fungal DNA
/ µg of extracted DNA / g of lung
*
Af293
Δuge3
C
200 µm
50 µm

Figure 12

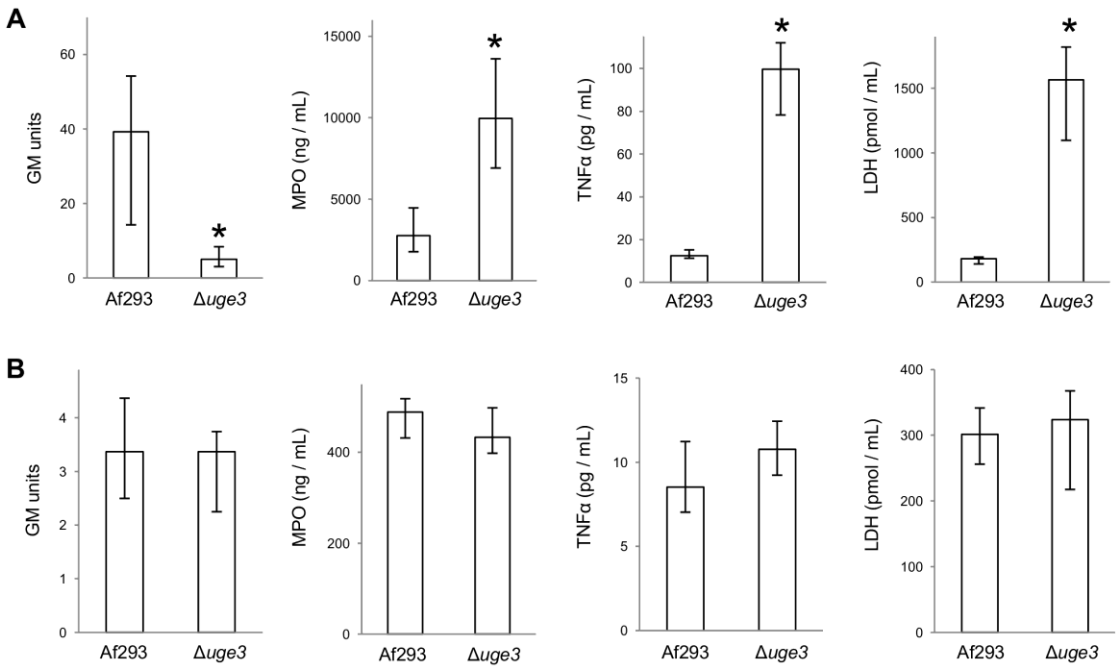
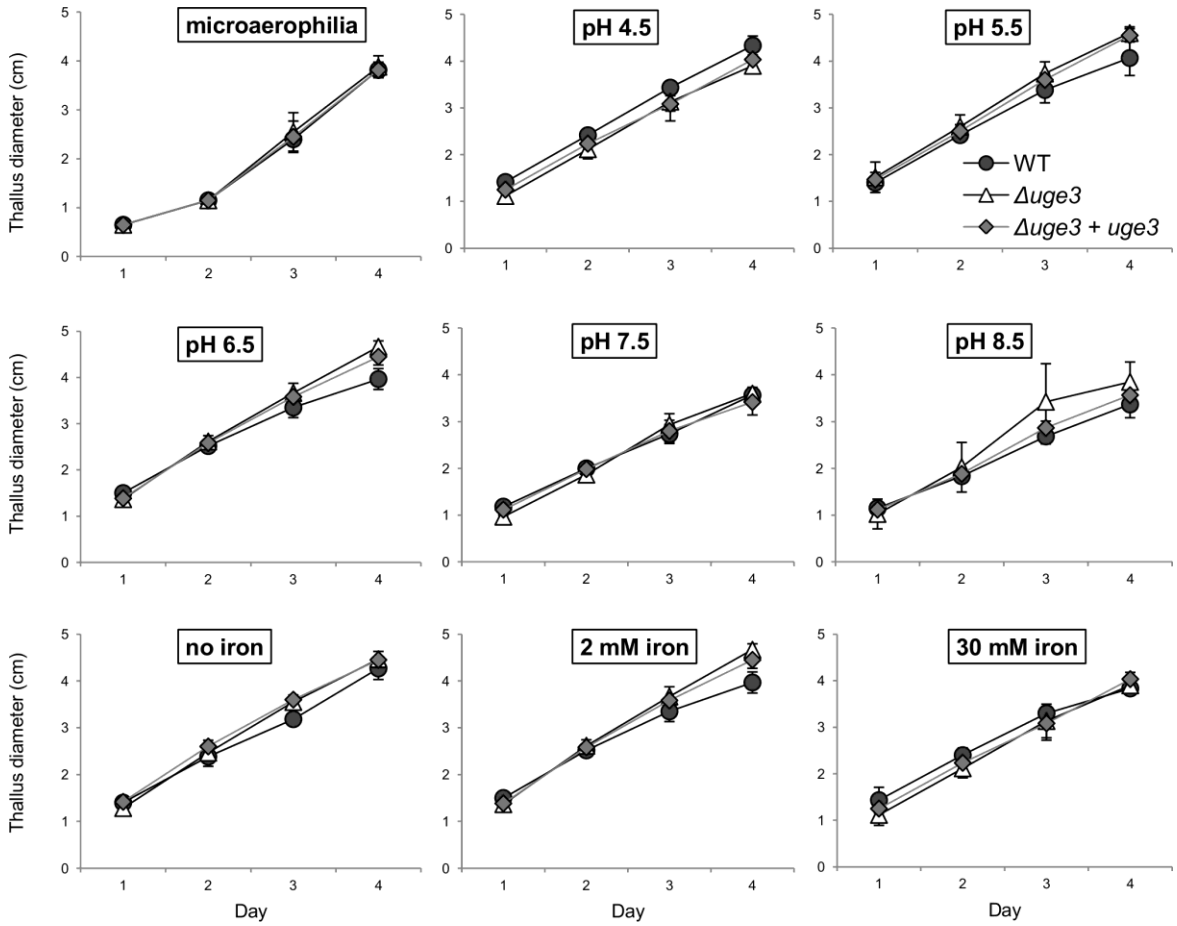

A
GM units
60
40
20
0
*
Af293
Δuge3
MPO (ng / mL)
15000
10000
5000
0
*
Af293
Δuge3
TNFα (pg / mL)
100
80
60
40
20
0
*
Af293
Δuge3
LDH (pmol / mL)
1500
1000
500
0
*
Af293
Δuge3
B
GM units
4
3
2
1
0
Af293
Δuge3
MPO (ng / mL)
400
200
0
Af293
Δuge3
TNFα (pg / mL)
15
10
5
0
Af293
Δuge3
LDH (pmol / mL)
400
300
200
100
0
Af293
Δuge3

Figure S1


microaerophilia
pH 4.5
pH 5.5
pH 6.5
pH 7.5
pH 8.5
no iron
2 mM iron
30 mM iron
Thallus diameter (cm)
Day
WT
Δuge3
Δuge3 + uge3

Preface to Chapter 3

In Chapter 2, we discovered that Uge3 is required for the synthesis of galactosaminogalactan, and specifically, the GalNAc component. However, the origin of the galactose component of galactosaminogalactan remained unknown. In this chapter, we performed a characterization of all the putative glucose epimerases found within the genome of *A. fumigatus* through gene disruption as well as characterization of purified enzymes in vitro. We found that of the three candidate epimerases in the genome of *A. fumigatus,* only two were expressed and required for galactose catabolism – Uge3 and Uge5. Uge3 was found to be a bifunctional epimerase that mediates both galactose/glucose and GlcNAc/GalNAc interconversion. Uge5 is the galactose/glucose epimerase responsible for the majority of galactose synthesis and contributes galactose to the synthesis of galactosaminogalactan and the related galactose containing polysaccharide galactomannan. Uge3 was required for formation of the GalNAc component of galactosaminogalactan. Interestingly, in the absence of Uge5, Uge3 could provide sufficient galactose to support the production galactosaminogalactan but not galactomannan. Finally we demonstrate that reducing the galactose content of this galactosaminogalactan does not impair the function of this polysaccharide.

CHAPTER 3: Overlapping and distinct roles of *Aspergillus fumigatus* UDP-glucose 4-epimerases in galactose metabolism and the synthesis of galactose-containing cell wall polysaccharides

Overlapping and distinct roles of *Aspergillus fumigatus* UDP-glucose 4-epimerases in galactose metabolism and the synthesis of galactose-containing cell wall polysaccharides

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ABSTRACT

The cell wall of *Aspergillus fumigatus* contains two galactose-containing polysaccharides, galactomannan and galactosaminogalactan, whose biosynthetic pathways are not well understood. The *A. fumigatus* genome contains three genes encoding putative UDP-glucose-4-epimerases, *uge3*, *uge4*, and *uge5*. We undertook the present study to elucidate the function of these epimerases. We found that *uge4* is minimally expressed and is not required for the synthesis of galactose-containing exopolysaccharides or galactose metabolism. Uge5 is the dominant UDP-glucose-4-epimerase in *A. fumigatus* and is essential for normal growth in galactose-based medium. Uge5 is required for synthesis of the galactofuranose (Gal*f*) component of galactomannan, and contributes galactose to the synthesis of galactosaminogalactan. Uge3 can mediate production of both UDP-galactose and UDP-N-acetyl-galactosamine (GalNAc), and is required for the production of galactosaminogalactan but not galactomannan. In the absence of Uge5, Uge3 activity is sufficient for growth on galactose and the synthesis of galactosaminogalactan containing lower levels of galactose, but not the synthesis of Gal*f*. A double deletion of *uge5* and *uge3* blocked growth on galactose and synthesis of both Gal*f* and galactosaminogalactan. This study is the first survey of glucose epimerases in *A. fumigatus*, and contributes to our understanding of the role of these enzymes in metabolism and cell wall synthesis.

INTRODUCTION

In immunosuppressed patients, the mold *Aspergillus fumigatus* causes an invasive pulmonary infection that can disseminate hematogenously to the brain and other deep organs. In recent years, the incidence of invasive aspergillosis due to *A. fumigatus* has risen dramatically in patients undergoing immunosuppressive and cytotoxic chemotherapy (1). Although earlier classes of antifungals such as the polyenes and azoles target the fungal plasma membrane, the carbohydrate cell wall is emerging as an effective target for

antifungals (2). A better understanding of the composition and biosynthesis of the cell wall and its components will be important in developing new cell wall active antifungals.

The cell wall of *A. fumigatus* is composed of an inner fibrillar layer and an outer amorphous layer (3). The inner layer is comprised of a mesh of chitin, β-1,3-glucans, and associated glycoproteins. The outer layer contains α-1,3-glucans, galactomannan, and galactosaminogalactan. Of note, both galactomannan and galacto-saminogalactan contain galactose residues, indicating the importance of fungal galactose metabolism in cell wall biosynthesis.

Galactomannan is a branched glycan consisting of an alpha-linked mannosyl backbone with branches of 4 to 5 beta-linked galactofuranose (Gal*f*) units (4,5). Gal*f* residues are also found in a variety of other fungal glycoproteins and glycolipids (6,7). Although the role of galactomannan, and Gal*f*, in virulence remains unclear (6,8), immunodetection of Gal*f* antigen by EB-A2 antibody is a widely used diagnostic test for invasive aspergillosis (9-11). Galactomannan biosynthesis has been best studied in the non-pathogenic species *Aspergillus nidulans*. In this species, UDP-glucose is converted to UDP-galactose (UDP-galactopyranose) in the cytoplasm by the UDP-glucose 4-epimerase, UgeA (12). UDP-galactopyranose is in turn modified to UDP-galactofuranose through the activity of the UgmA, an UDP-glucose mutase. UDP-galactofuranose is then transported by the glycosyl transporter, UgtA (13), into the Golgi where the glycosylation of carbohydrates, proteins, and lipids occurs. While the glycosyl transferases involved in chain formation remain unknown in *Aspergillus*, galactofuranosyltransferases have been studied in bacteria (14-17) and protozoans (18). Although, the *A. fumigatus* orthologues of *ugmA* and *ugtA*, termed *ugm1* (*glfA*) and *glfB*, respectively, have been studied and found to have a similar function to their *A. nidulans* orthologues, the *A. fumigatus* ortholog of the *ugeA* epimerase has yet to be identified or characterized (5,8,19-21).

Galactosaminogalactan is a recently described galactose-containing cell wall polysaccharide that plays an important role in virulence (22,23). This glycan mediates adhesion to a variety of host substrates and is immunosuppressive both through directly inducing leukocyte apoptosis, as well as by concealing fungal β-1,3-glucan from immune recognition by the pattern recognition receptor Dectin-1 (22,23). Galactosaminogalactan is

a linear heteropolysaccharide composed of varying combinations of alpha-1,4-linked galactose and N-acetyl-galactosamine (GalNAc) (23). The pathways governing synthesis of galactosaminogalactan are largely unknown. Previously, we reported that disruption of *uge3* (afu3g07910), predicted to encode an UDP-glucose 4-epimerase, resulted in a complete absence of cell wall GalNAc and galactosaminogalactan (22). Gal*f* production and the galactose content of the cell wall were not altered in the Δ*uge3* mutant, suggesting that *uge3* encodes a GlcNAc-GalNAc epimerase. The source of the galactose component of galactosaminogalactan remains unknown.

In addition to *uge3*, the genome of *A. fumigatus* contains genes predicted to encode two other UDP-glucose 4-epimerases, *uge5* (afu5g10780) and *uge4* (afu4g14090). Although neither of these epimerases has been studied in *A. fumigatus*, Uge5 is most homologous to the *A. nidulans*, UgeA, which was previously reported to have UDP-glucose 4-epimerase activity (12). Uge4 shares 55% amino acid homology with Uge3, and 60% with a putative epimerase of *A. niger* (an12g10410), but has not been studied in either organism. We undertook the present study to elucidate the role of these three UDP-glucose 4-epimerases in galactose metabolism and the biosynthesis of *A. fumigatus* cell wall galactose containing glycans.

MATERIALS AND METHODS

Fungal strains and growth conditions

A. fumigatus strain Af293 (a generous gift from P. Magee, University of Minnesota, St. Paul, MN) was used as the parent wild-type strain for all molecular manipulations. The Δ*uge3* mutant was described previously (22). Unless otherwise noted, strains were grown and harvested on YPD agar (Fisher Scientific) at 37^{o}C as previously described (24). For growth in liquid medium, Brian medium (23), Aspergillus Minimum Medium (AspMM) (25), and RPMI 1640 (Wissent) were used as indicated. For agar plates and liquid medium with galactose as the sole source of carbon, Brian agar and liquid medium were modified to include galactose as the only carbon source. For adherence and apoptosis assays,

germinated conidia (germlings) were obtained growing either $1x10^5$ or $2x10^5$ conidia in either 24-well plates or 1.5 mL microcentrifuge tubes for 9h at 37°C, 5% CO_2 incubation, in phenol-free RPMI 1640.

Molecular and genetic manipulations

Deletion of *uge4* and *uge5,* was performed as previously described (22). Briefly, pAN7.1 plasmid was modified for Gateway® (Invitrogen) use by digestion with restriction enzymes *BmgBI* or *NaeI* followed by fusion of an *attR*::*ccdB* target sequence at the site of each digestion using the Gateway® Vector Conversion system, to generate plasmids pHY, and pYG (22). To generate the disruption constructs, ~1 kb of the flanking sequences of *uge5* was amplified by PCR from Af293 genomic DNA using primers U5-1, U5-2 and U5-3, U5-4 to generate fragments FS1 and FS4 respectively. The resulting PCR products were then cloned into pENTR-D-TOPO® entry plasmid. A LR recombination resulted in recombination of pENTR::FS1 with pHY, and of pENTR::FS4 with pYG, yielding the fusion of each flanking sequence with the *hph* cassette in plasmids pHY and pYG plasmids. Finally, the DNA fragments for transformation were generated by PCR, using the primers U5-1,HY with pHY::FS1 and U5-4,YG with pYG::FS4. For Δ*uge4*, the same strategy was used as with Δ*uge5*, except that U4-1, U4-2, U4-3, and U4-4 were used as flanking sequence primers to amplify genomic DNA from Af293. *A. fumigatus* wild-type strain Af293 was then transformed with each pair of disruption cassettes using protoplasting (24). All integrations were confirmed by PCR and the expected gene expression profile was confirmed real-time RT-PCR for the gene of interest.

For the generation of the Δ*uge3* Δ*uge5* double mutant, plasmid p402 was modified for Gateway® use by digestion with *BsaBI* or *BamHI,* followed by a Mung Bean Nuclease treatment in the case of *BamHI*, and fusion of an *attR*::*ccdB* target sequence at the site of each digestion to generate plasmids pBL, and pLE. A LR recombination allowed recombination of previously produced pENTR::FS1 with pBL, and of pENTR::FS4 with pLE, resulting in the fusion of each flanking sequence with the *ble* cassette in plasmids pBL and pLE plasmids. Finally, the DNA fragments for transformation were generated by PCR, using the primers U5-1,BL with pBL::FS1 and U5-4,LE with pLE::FS4. Protoplasts of the *A.*

fumigatus Δ*uge3* mutant strain (22) were then transformed with each DNA fragment, as previously described (22). Transformants were selected on 0.015% phleomycin enriched plates. Complete deletion of the *uge5* open reading frame was confirmed as described in the previous paragraph.

For construction of the Δ*uge5::uge5* complemented strain, the plasmid pSK485, bearing a pyrithiamine resistance cassette (26) and obtained from the Fungal Genetics Stock Center (27), was modified by PCR using Sbf-pSK and Asc-pSK primers, in order to add unique *SbfI* and *AscI* restriction sites upstream of the β*rec*::*trpA* cassette. The plasmid was then linearized by *SbfI* and *AscI* digestion. A 2.9kb DNA fragment, corresponding to the restriction site *SbfI*, 1.2 kb of the *uge5* promoter, the entire *uge5* ORF, 0.8 kb of the *uge5* terminator and the restriction sites *AscI*, was amplified by PCR using Af293 DNA as a template and the primers U5-1b and U5-5. The resulting PCR product was cloned into the linearized pSK485plasmid via the Infusion® cloning reaction, following manufacturer instructions. The resulting pSK485::*uge5* plasmid was linearized at the unique Dra1 site, located upstream of the *uge5* partial promoter, and was used to transform protoplasts of the Δ*uge5* strain. Transformants were selected on 0.1 μg/ml pyrithiamine-enriched plates, and *uge5* expression was confirmed by RT-PCR

To generate the *uge3-gfp* construct, the *uge3* gene was amplified by PCR using primers uge3-gfp fw and uge3-gfp rev. This PCR fragment was then cloned into the pGFP plasmid previously digested with NcoI and NotI (28,29). The resulting plasmid, designated p*uge3*-GFP, was used to transform wild-type *Aspergillus fumigatus* Af293 as previously described (30). Transformants were selected by phleomycin resistance and Uge3-GFP expression was verified using confocal microscopy (IX81, Olympus), excitation wavelength 495 nm and emission wavelength 519nm.

To generate the *uge5-rfp* construct, *uge5* was amplified by PCR using primers uge5-rfp fw and uge5-rfp rev. This fragment was then cloned in frame with mRFP1 in the pRFP-HYG plasmid using EcoRV (31). The resulting plasmid pRFP-Uge5-HYG was used to transform wild-type *A. fumigatus* Af293. Transformants were selected using hygromycin and expression was verified using confocal microscopy (IX81, Olympus), excitation 543 nm and emission wavelength 563nm.

For 6his-Uge3, the ORF of *uge3* was produced by PCR with an additional CACC at the 5' end, using the primers U3-start ORF, U3-end ORF, and the Af293 genomic DNA as template. The resulting PCR product was then cloned into pENTR-D-TOPO® entry plasmid. A LR recombination allowed recombination of pENTR::U3-ORF with pDest17®, a plasmid engineered to produce 6his proteins with a high rate in proper *E. coli* strains. The resulting pDest17::*uge3* plasmid was transformed in *E. coli* BL21 DE3 strain.
All primer sequences are listed on Table 1.

Real-Time RT-PCR

Expression of genes of interest was quantified by relative real-time RT-PCR analysis as previously described (32). The primers used for each of the genes are shown in Table 1 or in Gravelat *et al* 2013 (22). First strand synthesis was performed from total RNA with Quantitec Reverse Transcription kit (Qiagen) using random primers. Real-time PCR was then performed using an ABI 7000 thermocycler (Applied Biosystems). Amplification products were detected with Maxima® SYBR Green qPCR system (Fermentas). Fungal gene expression was normalized to *A. fumigatus TEF1* expression. To verify the absence of genomic DNA contamination, negative controls were used for each gene set in which reverse transcriptase was omitted from the mix.

Localization studies

Conidia from *uge3-gfp, uge5-rfp,* or *A. fumigatus* Af293 were grown for 9h at 37°C on coverslips in a 24-well polystyrene plate in RPMI 1640 without phenol. Young hyphae were washed in PBS, stained with Draq® nuclear staining at 1:100 dilution (Cell Signaling, Inc), mounted in Slow Fade® Gold Antifade (Invitrogen), and imaged under confocal microscope (IX81, Olympus) at excitation 495nm, 543nm, and 633 nm, respectively.

Cell culture assays

Type II pneumocyte cell line CCL-185 (lung epithelial cells A549) and murine bone marrow derived dendritic cells (BMDDCs) were cultured as previously described (22). *Aspergillus* adherence to A549 cells was determined by co-incubating germlings of the strain of

interest on a monolayer of A549 cells for 30-45 minutes, as previously described (24). To determine induction of apoptosis via caspase-3 activity, germlings of each strain were co-incubated with BMDDCs at an MOI of 10:1 for 3h. Caspase-3 activity was measured using EnzChek® Caspase-3 Assay Kit following manufacturer's instruction (Invitrogen). Samples containing fungus or BMDDCs alone were included as controls.

Polysaccharide analysis

Galactosaminogalactan and Gal*f* production were assayed as previously described, with minor modifications (22,23). Briefly, $4x10^6$ conidia grown in modified Brian medium for 72h. Culture supernatants were filtered, and extracellular galactosaminogalactan or galactomannan was precipitated by 2.5 or 4.0 volumes of ethanol, respectively. Galactos-aminogalactan composition was determined by gas chromatography after hydrolysis, reduction, and peracetylation with meso-inositol as internal standard. Total neutral hexoses were quantified by the phenol sulfuric assay. Gal*f* quantification was assayed by EIA using the Platelia® *Asgergillus* kit (bio-Rad), following the manufacturer's instructions. For quantification of β-1,3-glucan exposure, $1x10^5$ conidia were grown in Brian medium for 12h in a 96-well opaque bottom plate (Nunclon, Inc.), fixed in 4% paraformaldehyde, and then labeled with 10 μg/mL of Fc-Dectin-1 (a generous gift from Dr. G.D. Brown, University of Aberdeen), followed by FITC-labeled AffiniPure F(ab') fragment donkey anti- human IgG, FCy fragment specific (Jackson ImmunoResearch, Inc.). Fluorescence was measured at 495 nm excitation and 515nm emission using Spectramax® fluorescence microplate reader (Molecular Devices, Inc.). The biofilm adherence assay and scanning electron microscopy were performed as previously described (22,24).

His-tagged Uge3 extraction and purification

Protein expression of 6-his-Uge3 was performed in *E. coli* BL21(DES) in autoinduction medium supplemented with 100ug/mL of ampicillin (33). Cells were grown for 20-24 hours at 28°C, and harvested by centrifugation. Pellets were flash frozen then lysed and filtered through 0.2 μm nylon membrane filter and incubated for 1.5h in Ni^{2+} agarose beads (Qiaqen). After successive washing with increasing imidazole concentrations, bound

6his-Uge3 was eluted with 250mM imidazole. Fractions containing 6his-uge3 determined by SDS-PAGE coomassie blue staining were pooled, concentrated and quantified with either Bradford assay or NanoDrop®. Alternatively, after cell lysis, 6his-Uge3 was purified through metal affinity chromatography on a POROS-MC20 perfusion chromatography column with Ni^{2+} as chelating metal. Elution fractions were pooled and further purified on a preparative scale high-resolution Superdex-200 gel filtration column. Purification of 6his-Uge3 was validated though Western blotting using HRP-tagged anti-6-his (Abcam) and protein mass spectrometry. Enzymes were stored at -20°C in PBS supplemented with 25% glycerol and 0.5mM DTT.

Enzyme activity assays

Product formation: Evolution of product was detected using NMR by incubating 5 µg of 6his-Uge3 with 1mM of either UDP-Glucose or UDP-GlcNAc (Sigma-Aldrich) in a reaction mix of 250 µL for 1h at 37°C. No co-factor was added since preliminary experiments did not show any changes to the reaction rate by adding co-factors such as NAD^+, Mg^{2+}, or Ca^{2+} (data not shown), as similarly reported in other epimerases (34-36). All reagents were in D_2O (Calbiochem) and spectra were recorded at 25°C with acetone internal reference (2.23 ppm) using standard pulse COSY, TOCSY (mixing time 120 ms) and ^1H, ^{31}P HMQC. NMR experiments were performed on Varian INOVA® 500 MHz spectrometer with 3 mm gradient probe. Spectra assignment was performed using Bruker Topspin v 3.1 program for spectra visualization.

Enzyme kinetics: Rate of product formation, linearity, and kinetics studies were performed using Beckman Gold Capillary Electrophoresis with a 57 cm bare silica capillary, and 32 Karat software application, as previously described (37). Concentrations of substrates, ranging from 0.2mM – 5.0mM for UDP-GlcNAc and 0.01mM – 1mM for UDP-galactose, were used to obtain product formation data in the presence of 0.25 pmol or 0.53 pmol of Uge3 for each substrate. For kinetics study, enzyme with varying concentrations of substrates in a 10 µL reaction volume of 0.1 M Tris/HCl buffer at pH 8.0 was incubated at 37°C for the specified amount of time, quenched by boiling at 95°C for 5 minutes, and immediately stored at -80°C. Prior to incubation, all reagents and enzymes were kept at 4°C.

Electrophoregram peaks were integrated using 32 Karat software application to estimate substrate conversion. Vmax of the reactions were calculated by taking the reciprocal of the Y-intercept and Km of the reactions were calculated by taking the slope over the Y-intercept (data not shown).

Bioinformatics analysis

Annotation and homology: UDP-glucose 4-epimerase genes and their respective amino acid sequences were retrieved from the Aspergillus Genome Database (38). The amino acid sequences of each of the candidate epimerases were analyzed using Eukaryotic Linear Motif (39), ConSurf (40), Conserved Domain Database (41), and HHpred (42) including SCOP domains. Clusters and protein families by domain predictions were cross-referenced using InterPro (43,44) and Pfam (45). Further, each of the *Aspergillus* epimerases were matched with the closest protein entry available in the Protein Databank (46) and further annotated.

Homology structural modeling: The amino acid sequence of Uge3 was aligned with human GalE PDB:1HZJ (47), *T. brucei* GalE PDB:1GY8 (48), or *P. aeruginosa* WbpP PDB:1SB8 (49) using ClustalW (50). Aligned Uge3 sequences were then modeled against each of the template structures using Modeller v9.11 (51). Resulting models were verified using Pairwise Structure Alignment (46). MacPyMOL v1.3 (academic license, Schrodinger LLC) was used to align respective structures and identify and analyze key residues in the catalytic site.

Statistical analysis

All charts and graphs were produced and analyzed using Prism 6 (GraphPad Software). All tables were created using MS Excel (Microsoft Inc.).

RESULTS

Uge5 is the most highly expressed epimerase gene in *A. fumigatus*, and is required for normal galactose metabolism

We first performed expression analysis of the three epimerase genes in wild-type *A. fumigatus* grown in Brian medium (Fig. 1). Of the three epimerase encoding genes, *uge5* was the most highly expressed, at a level more than 5 fold higher than that of *uge3*. Expression of *uge4* was minimal, approaching the limits of detection by real time RT-PCR. We therefore hypothesized that Uge5 likely plays an important role in galactose metabolism and cell wall glycan synthesis.

To test the role of each of these three epimerases in galactose metabolism, Δ*uge4* and Δ*uge5 A. fumigatus* deletion mutants were constructed. The ability of these mutants to utilize glucose and galactose was compared with the previously constructed Δ*uge3* mutant and wild-type *A. fumigatus* strains (22). All three mutant strains exhibited wild-type growth on medium with glucose as the sole carbon source (Figures 2A-E). Similarly, both the Δ*uge3* and Δ*uge4* mutants grew normally on medium with galactose as a sole carbon source (Figures 2F-H). Deletion of *uge5* resulted in a marked impairment of growth in medium containing galactose as a sole carbon source (Figures 2I-J), consistent with the findings reported with the deletion of *ugeA,* the *A. nidulans* orthologue of *uge5* (12). However, unlike the *A. nidulans* Δ*ugeA* mutant, the Δ*uge5* mutant was not completely blocked in hyphal growth under these conditions, and was able to grow and form hyphae after 30 hours of growth in galactose (Figures 2O). Collectively these results suggest that *A. fumigatus* differs from *A. nidulans* in that while *uge5* is the major epimerase responsible for the interconversion of UDP-galactose and UDP-glucose, other pathways or enzymes in *A. fumigatus* can mediate galactose metabolism in the absence of Uge5.

Deletion of uge5 blocks Galf synthesis, and results in the production of galactosaminogalactan with reduced galactose content

To test the contribution of each of the three epimerases to the synthesis of galactose-containing glycans, we measured the production of galactosaminogalactan and Gal*f* by each of these mutant strains. We previously found that deletion of *uge3* results in a

complete block in galactosaminogalactan synthesis but had no effect on Gal*f* synthesis (22). Deletion of *uge4* had no effect on Gal*f* detection or galactosaminogalactan production (Figures 3A-B). Consistent with reports of *ugeA* deletion in *A. nidulans* (12), deletion of *uge5* resulted in the absence of detectable Gal*f* antigen and by extension, the absence of galactomannan (Figure 4A). Unexpectedly, however, multiple assays demonstrated that deletion of *uge5* did not block galactosaminogalactan synthesis. Scanning electron microscopy of the Δ*uge5* mutant identified normal production of the cell wall decorations that have been associated with galactosaminogalactan production (Figure 4E) (22), and levels of total galactosaminogalactan produced by the Δ*uge5* mutant were slightly higher than those seen with the wild-type, although this was not statistically significant (Figure 4B). Compositional analysis of galactosaminogalactan from the Δ*uge5* mutant revealed a significant reduction in the galactose content of this heteropolysaccharide (Figure 4C). This decrease in galactose was not associated with an increase in other hexose such as glucose or mannose (data not shown). The Δ*uge5* mutant also exhibited increased staining with the GalNAc-specific soybean agglutinin (SBA) lectin (Figure 4D), consistent with the production of GalNAc-rich, galactose-poor galactosaminogalactan. Collectively, these data suggest that while Uge5 activity is required for UDP-Gal*f* synthesis for the production of galactomannan, other enzymes or pathways can also contribute UDP-galactose to the synthesis of galactosaminogalactan in the absence of Uge5 activity.

Uge3 activity may compensate for the lack of Uge5

The Δ*uge5* mutant was able to utilize galactose as a carbon source and produced galactosaminogalactan that still contained galactose. These data suggest that this strain retained some glucose/galactose epimerase activity, possibly mediated by Uge4 or Uge3. Although disruption of *uge4* had no effect on galactose metabolism, galactosaminogalactan or Gal*f* production, *uge4* was only expressed at very low levels in the wild-type strain of *A. fumigatus*, and therefore, could be upregulated in the absence of Uge5. To test for compensatory upregulation of *uge4* in the absence of Uge5, we performed real-time RT-PCR analysis of *uge4* expression in this mutant. The expression of *uge4* remained minimally detectable in the Δ*uge5* mutant under galactosaminogalactan-inducing

conditions (Figure 5). In contrast, not only was *uge3* expression detectable in wild-type *A. fumigatus*, but a trend towards increased *uge3* expression was observed in the absence of Uge5 (Figure 5). Collectively, these data suggest that Uge3 may have dual substrate specificity and can mediate the interconversion of both UDP-glucose to UDP-galactose and UDP-GlcNAc to UDP-GalNAc. Consistent with this model, expression of *uge3-gfp* or *uge5-rfp* in wild-type *A. fumigatus* demonstrated that both of these epimerases are located in the cytoplasm (Figure 6), and thus could provide galactose to the same downstream glycosyltransferases or mediate conversion of galactose to glucose for metabolic use.

Homology modeling suggests Uge3 is a Group 2 epimerase with dual substrate specificity

To examine the possibility that Uge3 could exhibit dual substrate binding and catalysis, the structure of Uge3 was compared with those of other UDP-glucose 4-epimerases by homology modeling. UDP-glucose 4-epimerases are comprised of 3 groups based on substrate specificity (52): Group 1 enzymes have specificity for hexoses, Group 2 for both hexoses and hexosamines, and group 3 for hexosamines. Uge3 was therefore modeled using *T. brucei* tGalE (PDB: 1GY8) as a Group 1 template, human hGalE (PDB: 1HZJ) as a Group 2 template, and *Pseudomonas aeruginosa* WbpP (PBD: 1SB8) as a Group 3 template. The N-terminus portion of Uge3 encoding a predicted signal peptide was omitted from modeling. Inspection of amino acid residues in the catalytic sites of Uge3 model and templates revealed similarities and differences in the predicted substrate binding pocket region that may provide further insights to Uge3's catalytic activity. Similar to tGalE, hGalE, and WbpP, the SYK triad required for catalytic activity is conserved in the Uge3 model (Ser^{191}, Tyr^{238}, and Lys^{242}) (53,54). Previously, six amino acid residues forming the hexagonal substrate-binding pocket were identified to be important in enzymatic function (52,54). Identification of the corresponding residues on Uge3 revealed that these residues are not only present in Uge3, but that they are identical to hGalE, which belongs to the bifunctional Group 2 (Table 2). In fact, these six residues in Uge3 and hGalE align almost in a complete overlap around the cofactor and substrate (Figure 7). Thus, the *in silico* analysis suggests that Uge3 is structurally more closely related to the group 2 epimerase hGALE,

and may play a role in the interconversion of both UDP-Glc/UDP-Gal, as well as, UDP-GlcNAc/UDP-GalNAc.

Uge3 is a dual substrate epimerase that can utilize both UDP-glucose and UDP-N-acetyl glucosamine as substrates

To test if Uge3 has dual substrate activity, recombinant Uge3 was produced (Figures 8A-B) and its enzymatic activity measured. Using ^{1}H NMR, product formation of both UDP-galactose and UDP-GalNAc was detected in the presence of Uge3 when either UDP-glucose or UDP-GlcNAc was provided as a substrate, respectively (Figures 8C-D, Table 3). To obtain better resolution of the peaks, COSY and TOCSY two-dimensional ^{1}H NMR experiments were performed, and demonstrated that the cross peaks of coupled protons show both substrate and product chemical shifts in the respective reaction mixes (data not shown). To further validate that the products retained their UDP moieties and were not derivatives or different species of galactose or GalNAc, ^{31}P NMR was performed and detected the same phosphorus chemical shifts in both respective substrate and product proton peaks (data not shown). Uge3 activity was further characterized using capillary electrophoresis. At steady-state equilibrium, Uge3 converted 30% of UDP-GlcNAc to UDP-GalNAc and 15% of UDP-glucose to UDP-galactose (Figure 8E). The range of reaction time that resulted in linearity of product formation of less than 10% for Michaelis-Menten analysis was determined to be between 0-30 minutes for both substrates (data not shown). Conversion from UDP-GlcNAc to UDP-GalNAc had Km of 3.2 mM and Vmax of 417 pmol/min (data not shown). Epimerization from UDP-glucose to UDP-galactose could not be quantified due to a strong reverse reaction; however conversion of UDP-galactose to UDP-glucose had a Km of 0.9 mM and Vmax of 146 pmol/min (data not shown). Collectively, these results support the hypothesis that the galactose component of galactosaminogalactan in the Δ*uge5* mutant strain likely originates from activity of Uge3.

A mutant deficient in both Uge3 and Uge5 is completely auxotrophic for galactose and produces no galactomannan or galactosaminogalactan

To verify that Uge3 activity is responsible for the residual galactose metabolism and galactose content of galactosaminogalactan in the Δ*uge5* mutant strain, we constructed a mutant deficient in both Uge3 and Uge5. The Δ*uge3*Δ*uge5* double mutant was unable to grow in medium containing galactose as the sole carbon source (Figures 9A-B). Further, the Δ*uge3*Δ*uge5* double mutant was found to have undetectable levels of both Gal*f* and galactosaminogalactan (Figures 9C-D). Collectively these data confirm that Uge3 and Uge5 are the only functional UDP-glucose/galactose epimerases in *A. fumigatus*.

Reducing the galactose component of galactosaminogalactan does not impair its function

To assess whether the galactose-poor galactosaminogalactan produced by the Δ*uge5* mutant is altered in function, we characterized the effects of *uge5* deletion on the reported functions of galactosaminogalactan (22,23). Deletion of *uge5* did not alter biofilm adherence to polystyrene surface (Figure 10A). Furthermore, Δ*uge5* mutant displayed slightly increased adherence to A549 epithelial cells as compared to wild-type *A. fumigatus* and the *uge5* complemented strain, possibly reflecting the slight increase in total galactosaminogalactan production noted in this strain (Figure 10B). Similarly the reduced galactose content of galactosaminogalactan did not impair β-(1,3) glucan masking, as there was no difference between these three strains in the immunodetection of β-(1,3) glucan by recombinant Fc-dectin-1 (Figure 10C). Finally, since leukocyte apoptosis has been reported as a mechanism of galactosaminogalactan immunosuppression (23), the ability of the Δ*uge5* mutant to induce apoptosis of bone-marrow derived dendritic cells was quantified by measuring cellular caspase-3 activity. No difference in the induction of apoptosis by the Δ*uge5* mutant as compared to wild-type *A. fumigatus* (Figure 10D) was observed. Collectively, lowering the galactose content of galactosaminogalactan did not seem to significantly impair adherence, β-(1,3)-glucan masking, or the induction of apoptosis by this glycan.

DISCUSSION

The results of our studies highlight important differences in galactose metabolism, glycan synthesis and epimerase function between *A. fumigatus* and *A. nidulans*. Although deletion of *ugeA* in *A. nidulans* resulted in a strain that was completely auxotrophic for galactose, the *A. fumigatus* Δ*uge5* mutant exhibited only a partial growth defect in galactose containing medium. In light of our findings that Uge3 has dual substrate specificity and that other putative epimerases are silent in the Δ*uge5* mutant, it is likely that Uge3 mediates the interconversion between UDP-glucose and UDP-galactose in the absence of Uge5 (Figure 11). Although the genome of *A. nidulans* contains *ugeB*, an ortholog of *uge3*, this gene has been reported to be silent (55), and as a result likely does not contribute to UDP-galactose/UDP-glucose interconversion in this species.

Disruption of *uge5* was associated with normal to increased galactosaminogalactan production and adherence to host cells. These data are consistent with those from mutations in other components of the galactomannan biosynthetic pathways. For example, deletion of *ugm1* in *A. fumigatus* resulted not only in an absence of galactomannan but also an increase in galactosaminogalactan synthesis and biofilm adherence (6). While galactosaminogalactan production has not been studied in *A. nidulans*, it was reported that the deletion of *ugmA* and *ugeA* was associated with an increase in adherence (55), suggesting a similar phenomenon may also occur in this organism. One possibility is that impaired galactomannan synthesis results in accumulation of precursors which are then redirected to the galactosaminogalactan pathway. If this is true, then such substrate flux must occur at the level of UDP-glucose or earlier given that the Δ*uge5* mutant retained the ability to produce galactosaminogalactan. Alternately, the increase in galactosaminogalactan could reflect activation of a regulatory response to alterations in cell wall integrity as has been observed to occur with other mutations or perturbing agents that alter cell wall composition (56-59).

It is surprising that, in the absence of Uge5, UDP-galactose production by Uge3 was sufficient to permit production of galactosaminogalactan but not Gal*f*. Although compartmental sequestration of the UDP-galactose produced by these two epimerases could account for this observation, our localization studies suggest that both enzymes are

cytoplasmic. Alternatively, differences in the transport of UDP-galactose or the substrate affinity of downstream enzymes specific for each of the two pathways could mediate this preferential funnelling of UDP-galactose into galactosaminogalactan synthesis. It is not known if either epimerase complexes with other elements in their respective biosynthetic pathways, however this explanation could account for differences in accessibility of UDP-galactose between pathways. Finally, there may be different regulatory controls at various levels of gene expression and protein synthesis of other pathway components. While a deficiency in galactosaminogalactan is not lethal, it renders the strain non-adherent, which is not the case for galactomannan deficiency (5,6,8,12,22). Thus in the absence of a specific requirement for galactomannan, it is possible that the fungus preferentially diverts its resources to preserve galactosaminogalactan synthesis for adherence to surfaces or biofilm homeostasis. Testing of these hypotheses will require identification of other elements of the galactosaminogalactan biosynthetic pathways, an area under active research by our group.

We initially hypothesized that enzymes upstream of Uge3 or Uge5 in the Leloir pathway, such as galactokinase or UDP-galactose-1-phosphate uridylyltranferase, may contribute to the residual galactose found in the Δ*uge5* mutant. Although much of the Leloir pathway is uncharacterized in *A. fumigatus*, it is well characterized in *A. nidulans*. In this species, deletion of galactokinase gene *galE* or UDP-galactose-1-phosphate uridylyltranferase *galD*, results in mutants that are viable, albeit with partial growth defects, in galactose-based carbon source (60). However the complete lack of growth of the Δ*uge3*Δ*uge5* double mutant in galactose-based medium strongly suggests that these epimerases are solely responsible for galactose metabolism under the conditions studied in this report and other salvage enzymes are insufficient to provide adequate glucose from galactose under the conditions of growth that we tested.

Our findings demonstrate that Uge3 is dispensable for galactose metabolism and Gal*f* synthesis. This observation is likely due, at least in part, to the much lower expression levels of *uge3* as compared to *uge5*. Indeed, it is possible that interconversion of UDP-galactose and UDP-glucose by Uge3 does not occur in the presence of physiologic levels of Uge5, and is only unmasked upon Uge5 deletion. Interestingly, we found that, *in vitro*, Uge3

produced twice as much UDP-GalNAc compared to UDP-galactose from respective substrates at steady-state equilibrium, thus suggesting a possible preference towards N-acetylated hexosamines as substrates. Nonetheless, this redundancy in UDP-galactose and UDP-glucose interconversion by two epimerases suggests that interconversion of hexoses may be more critical to *A. fumigatus* than the interconversion of UDP-GlcNAc-UDP-GalNAc, which is mediated by Uge3 alone (22). Indeed, sugar epimerase have important functions outside of cell wall polysaccharide synthesis, such as metabolism. Redundancy in hexose interconversion would ensure that the organism can adapt to different carbon sources and maintain glycolysis and generate derivatives required for other cellular activities (12,13,61-63). In contrast, GalNAc seems to be required by *A. fumigatus* primarily for the synthesis of galactosaminogalactan, and is not required for normal growth (22). The need for redundant hexose epimerase activity is unlikely related to galactosaminogalactan synthesis, since production of galactose-poor galactosaminogalactan by the Δ*uge5* mutant did not impair adherence, biofilm formation or other virulence associated properties. Indeed, our studies failed to identify a functional role for the galactose component of galactosaminogalactan and suggest that the adherence, apoptosis inducing, and PAMP masking phenotypes of galactosaminogalactan are mediated by the GalNAc fraction of this glycan. One possibility is that altering the galactose content of galactosaminogalactan may provide a mechanism for the organism to modulate galactosaminogalactan activity through secondarily changing the relative GalNAc content of the resulting glycan. We are currently investigating the effects of lowering the GalNAc content of galactosaminogalactan to better understand the mechanism of action of galactosaminogalactan in these phenotypes.

Although we found three genes annotated as UDP-glucose 4-epimerases in *A. fumigatus*, only *uge3* and *uge5*, seem to be active. One explanation could be that the *in silico* annotation is not correct, and that in fact, *uge4* is not an UDP-glucose 4-epimerase. However, based on the close homology of *uge4* to the other two epimerases, and the fact that all important domains are are predicted with high certainty to be intact in the *uge4* sequence, it is likely that *uge4* is an UDP-glucose 4-epimerase. It is possible that *uge4* is expressed under conditions that were not tested, however, an alternative explanation is that *uge4* is a product of gene duplication that could have served a purpose

earlier in evolution but has now become silenced. Similar silent gene duplications have been reported in other cell wall-related genes in *A. fumigatus* (64,65). Interestingly, silencing of epimerases in *Aspergillus* species may play a role in virulence since in the non-pathogenic species *A. nidulans, ugeB,* the ortholog of *A. fumigatus uge3*, has been reported to be silent (55).

The present study broadens our understanding of *Aspergillus* epimerases and their role in metabolism and carbohydrate synthesis, and also begins to identify some of the critical steps in the biosynthesis of galactosaminogalactan. These results suggest a model of galactose-containing cell wall polysaccharide synthesis in which Uge5 activity alone mediates production of UDP-galactose as a precursor to Gal*f* and subsequent galactomannan synthesis. Conversely, Uge3 activity is required for the synthesis of UDP-GalNAc for the production of galactosaminogalactan and both epimerases can contribute to the pool of UDP-galactose used in the synthesis of galactosaminogalactan. Further, Uge5 is responsible for the majority of the epimerase activity within the Leloir pathway, as deletion of *uge5*, but not *uge3*, was associated with a defect in growth on galactose containing medium. A deeper understanding of the biochemical pathways underlying galactose metabolism and the biosynthesis of cell wall glycans in this pathogenic fungus may provide the basis for the development of future antifungal therapies.

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FIGURE LEGENDS

Table 1. List of primers for plasmid construction and gene expression experiments.

Table 2. Alignment of the six amino acid residues within the catalytic fold of Uge3, hGALE, tGALE, and WbpP shows highest alignment between Uge3 and hGALE.
Six amino acid residues in the catalytic pocket were identified on the Uge3 model. These residues were then compared with those in tGALE and hGALE. Visualization was performed using MacPyMOL v1.3.

Table 3. ^{1}H NMR chemical shift and constant for product and substrate.

Figure 1. The *uge5* gene is the most highly expressed of the three epimerase genes in *A. fumigatus*, while *uge4* mRNA expression is barely detectable.
Strain Af293 was grown in Brian medium for 18h, and the levels of *uge3, uge4* and *uge5* mRNA were measured by real-time RT-PCR. *Indicates significantly different expression as compared with *Tef1* reference gene (22).

Figure 2. Deletion of *uge5* results in a partial galactose auxotrophy.
The indicated strains were grown for the indicated time periods in Aspergillus minimal medium with either glucose (A-E) or galactose (F-P) as the sole carbon source. Brightfield images at a magnification of 200x are shown.

Figure 3. Deletion of *uge4* does not alter galactosaminogalactan or Gal*f* production.
(A) Relative Gal*f* detection by ELISA (EB-A2) in culture supernatant of Af293 or Δ*uge4* after 72h growth of indicated strains in Brian medium.
(B) Galactosaminogalactan production by biomass from culture supernatant of Af293 or Δ*uge4* after 72h growth of the indicated strains in Brian medium.

Figure 4. Deletion of *uge5* blocks Gal*f* synthesis, and results in production of galactosaminogalactan with a reduced galactose content.

(A) Gal*f* content of culture supernatants as determined by ELISA.

(B) Galactosaminogalactan content of culture supernatants, normalized to mycelia biomass.

(C) Galactose content of galactosaminogalactan from the indicated strains as quantified by hexose and hexosamine assays followed by gas chromatography.

(D) FITC-tagged GalNAc-specific soybean agglutenin (SBA) lectin binding on 12h, Brian medium grown hyphae of indicated strains. Total fluorescence was measured with Spectramax® fluorescence microplate reader.

(E) Scanning electron micrograph of hyphae of indicated strains after 24h growth at 37°C, 5% CO_2 in phenol-free RPMI 1640. Hyphae were fixed, sequentially dehydrated in ethanol, dried in CO_2, coated in Pd-Au, and imaged under scanning electron microscope (Hitachi). Arrows indicate surface decorations associated with galactosaminogalactan production.

* Significant reduction compared with Af293 wild-type, ANOVA with pairwise comparison $p<0.05$.

± Not statistically significant compared with Af293, but statistically significant compared to the Δ*uge5* mutant, $p<0.05$

(A-C) The indicated strains were grown for 72 hours in Brian medium.

Figure 5. Deletion of *uge5* is not associated with significant upregulation of expression of *uge3* or *uge4.*

The indicated strains were grown in Brian medium for 18h, and the levels of *uge3* (black bar) and *uge4* (gray bar) mRNA from indicated strains were measured by real-time RT-PCR. * Significant difference between *uge3* and *uge4* expression in the indicated strain as compared with *Tef1* reference gene, FANOVA with pairwise comparison $p<0.05$.

Figure 6. Uge3 and Uge5 are cytoplasmic.

Af293 strains expressing *uge3-gfp* or *uge5-rfp* were grown in Brian medium for 12 hours and imaged by confocal microscopy. For nuclear staining, Draq5® stain was used and pseudocolor blue was added for visualization. Magnification was 1000X, with a 4X digital zoom. GFP was imaged at 488nm, RFP at 543nm, and Draq5® at 633 nm.

Figure 7. The substrate-binding pocket of Uge3 model aligns with hGalE.

The hexagonal substrate-binding pocket of Uge3 model (green) was aligned to hGalE (cyan), with the six residues required for catalytic activity annotated. For Uge3 homology modeling, the N-terminal non-alignment regions were discarded, and resulting aligned sequences were modeled using Modeller v9.11. Bound UDP-GalNAc and NAD+ are indicated in gray at top and bottom, respectively.

Figure 8. Uge3 exhibits bifunctional UDP-Glc/UDP-Gal and UDP-GlcNAc/UDP-GalNAc epimerase activity.

(A) SDS-PAGE of lysate and purified 6his-Uge3 stained with Commassie Blue from 6his-Uge3 expressing BL21(DE) *E. coli* strain. Lanes are as follows: non-induced cells, cells grown in auto-induction medium, crude lysate, wash fraction eluted using 20mM imidazole, and four fractions of 6his-Uge3 eluted with 250mM imidazole.

(B) Western blot of purified lysates for detection of 6his-Uge3 using HRP-tagged anti-6his antibody. Arrow indicates expected band for 6his-Uge3.

(C-D) ^{1}H NMR spectra were measured in a reaction mix containing either UDP-glucose **(C)** or UDP-GlcNAc **(D)** in phosphate buffer, in the presence or absence of Uge3. For all NMR experiments, products were detected after 1h of co-incubation of Uge3 and respective substrates at 37°C using a Varian 500 MHz NMR spectroscopy.

(E) The rate of product formation was measured using capillary electrophoresis over time in a reaction mix containing 20 pmol Uge3 and 0.1mM of either UDP-glucose or UDP-GlcNAc as substrate. Reactions took place at 37°C in a total volume of 10 µL. Products were detected at 254 nm (UV) measuring UDP- moiety endogenous fluorescence. UDP-linked sugars were separated by borate adduct formation.

Figure 9. Deletion of *uge3* and *uge5* renders the resulting *Δuge3Δuge5* double mutant deficient in Gal*f* and galactosaminogalactan.

(A) The indicated strains were grown for the indicated time periods in Aspergillus minimal medium using either glucose or galactose as the sole carbon source. Brightfield images at a magnification of 200x are shown. Glucose is abbreviated as Glc, and galactose as Gal.

(B) The indicated strains were grown for 2 days on either glucose or galactose based Aspergillus minimal medium agar plates.

(C) Relative Gal*f* detection by ELISA in culture supernatant after 72h growth in Brian medium of the indicated strains.

(D) Galactosaminogalactan production after 72h growth in Brian medium of the indicated strains

* Significant reduction compared with Af293 wild-type, ANOVA with pairwise comparison $p<0.05$.

Figure 10. Galactose-poor Δ*uge5* galactosaminogalactan retains normal virulence associated functions.

(A) Biofilm adherence of indicated strains after 24h growth on polystyrene plates, after several washes and visualized by staining with crystal violet.

(B) Adherence of germinated hyphae of the indicated strains to A549 epithelial cells after 30 minutes.

(C) Detection of Fc-dectin-1 binding using immune staining. Conidia from respective strains were grown for 12h in Brian medium, fixed, blocked, stained with Fc-dectin-1 and FITC labeled F(ab) fragment, and total fluorescence was measured with Spectramax® fluorescence microplate reader.

(D) Induction of bone marrow derived dendritic cell apoptosis as determined by caspase-3 activity. Conidia from indicated strains were grown for 9h in RPMI, then co-incubated with mouse bone marrow-derived dendritic cells at MOI of 10:1. Caspase-3 activity was measured by commercial assay following the manufacturer's instructions (Invitrogen, Inc.).

Figure 11. Schematic of galactosaminogalactan and galactomannan pathways. Pathway diagrams showing common and distinct components in the biosynthesis of galactosaminogalactan and galactomannan. For Gal*f* symbol representation, an "*f*" has been inserted to the galactosepyranose symbol. All other symbols follow common nomenclature convention (66).

*Note that the depiction of galactosaminogalactan structure is representative of multiple potential combinations of galactose and GalNAc residues.

FOOTNOTES

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Table 1

Primer name	Target	Sequence
U5-1	uge5 5′ flanking sequence	CACCTTCAAGCGGAACTGGAC
U5-2	uge5 5′ flanking sequence	AAAGGAAGCAGAAGAGCAGAAAGAA
U5-3	uge5 3′ flanking sequence	CACCTGTGAGCGAGAAAGACTGGAAA
U5-4	uge5 3′ flanking sequence	ACATGAAGCAATTAGAGGCAGCA
U5-ext 1	analysis of uge5 locus	CTTAGTGGACAGCAGACCAGGGG
U5-ext 4	analysis of uge5 locus	GCGAGAGCCTGACCTATTGCATCT
uge5 RT-sense	uge5 cDNA	ATGAGGCCGAGAAGTGGAAC
uge5 RT-antisense	uge5 cDNA	CGTGAGAGGCATAGTCGTCA
U5-1b	uge5 transcription unit	TACCTGCAGGGTGACATTGATGACGGA
U5-5	uge5 transcription unit	TGGCGCGCCGACAAATATCCAAACGGTA
U4-1	uge4 5′ flanking sequence	CACCGTCGGCTACATTCGT
U4-2	uge4 5′ flanking sequence	AAGGGTTCGCAGTCATCCTC
U4-3	uge4 3′ flanking sequence	CACCAATCTCACAGCTAACG
U4-4	uge4 3′ flanking sequence	CGCAGACATACCACTTCTTG
U4-ext 1	analysis of uge5 locus	GGCCCCAAAATCAGGAGT
U4-ext 4	analysis of uge5 locus	ATCGCATCTACGCCATGATT
uge4 RT-sense	uge4 cDNA	CATCCACACCCCTCTGAAGT
uge4 RT-antisense	uge4 cDNA	CGGACGAGGAGAAGATGAAG
U3-start ORF	6his-Uge3 production	CACCATGGACAGCTACCAGCAATC
U3-end ORF	6his-Uge3 production	AAGGGACATGCGACAACATC
HY	hph	CAACCACGGCCTCCAGAAGAAGA
YG	hph	GCGAGAGCCTGACCTATTGCATCT
tef1-RT sense	tef1	CCATGTGTGTCGAGTCCTTC
tef1-RT antisense	tef1	GAACGTACAGCAACAGTCTGG
Sbf-pSK	pSK485 modification	AGCTTCCTGCAGGTAAATCAAAAGAATAGACCGAGATA
Asc-pSK	pSK485 modification	TGGCGCGCCTAAGGGATTTTGCCGATTTC
Uge5-RFP FWD	Campoli et al 2013	CATCACCCCATGGATATGTCTGCTGGTTCAGTT
Uge5-RFP-REV	Campoli et al 2013	GGAGGAGGCCATGATCTTCTTGAGCTGTTCCAG
uge3-gfp fw	Choe et al 2011	AGACATCACCCCATGGATGGACAGCTACCAGCA
uge3-gfp rev	Choe et al 2011	CTCACCATCGCGGCCGCAGTAGATAACCCACTGA

Table 2

Species	Enzyme	Residues						Group
A. fumigatus	Uge3	Lys151	Ser191	Tyr238	Asn268	Asn288	Cys391	2
H. sapien	hGalE	Lys92	Ser132	Tyr157	Asn187	Asn206	Cys307	2
T. brucei	tGalE	Leu102	Ser142	Try173	Asn202	His202	Leu342	1
P. aeruginosa	WbpP	Gly102	Ser142	Tyr166	Asn195	Ala209	Ser306	3

Table 3

Unit	H/C 1	H/C 2	H/C 3	H/C 4	H/C 5	H/C 6
UDP-Glc	5.6	3.53	3.78	3.47	3.9	3.78; 3.86
$\mathbf{J_{Hn,Hn+1}}$	3	9	9	9		
UDP-Gal	5.64	3.8	3.92	4.03	4.17	3.74; 3.77
$\mathbf{J_{Hn,Hn+1}}$	3	9	<2	<2		
UDP-GlcNAc	5.51	3.99	3.81	3.55	3.93	3.87; 3.93
$\mathbf{J_{Hn,Hn+1}}$	3	9	9	9		
UDP-GalNAc	5.55	4.26	3.97	4.05	4.19	3.76; 3.78
$\mathbf{J_{Hn,Hn+1}}$	3	9	<2	<2		

Figure 1

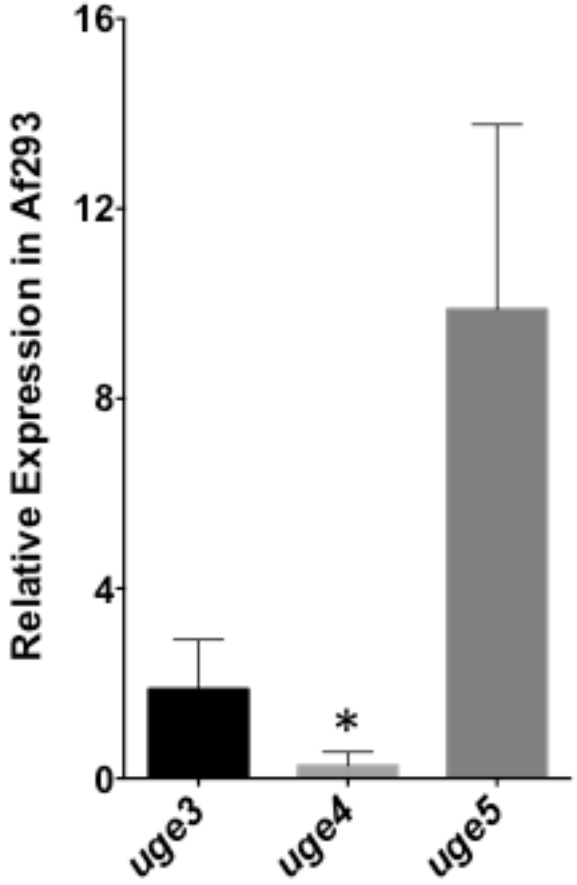

Figure 2

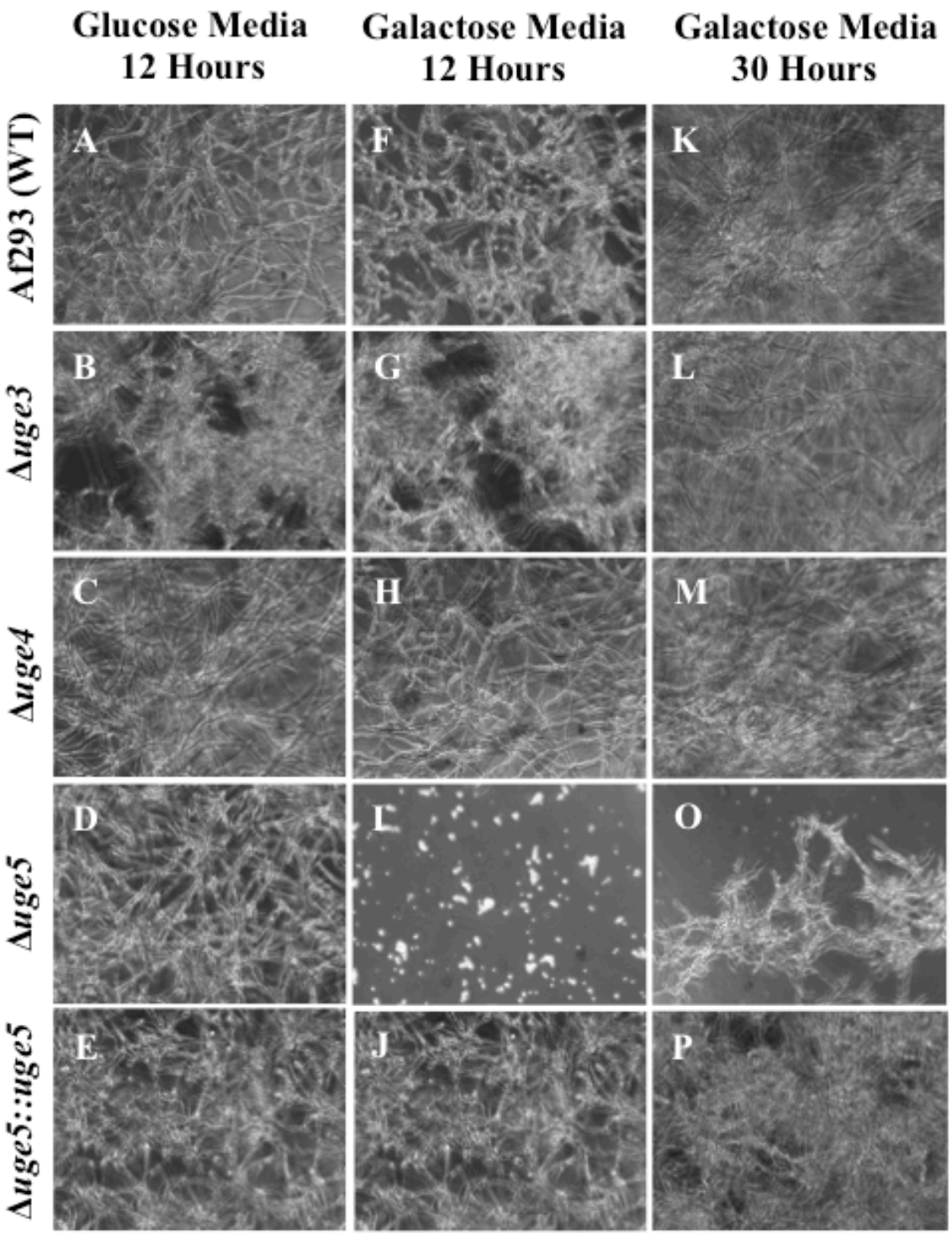
Glucose Media
12 Hours
Galactose Media
12 Hours
Galactose Media
30 Hours
Af293 (WT)
Δuge3
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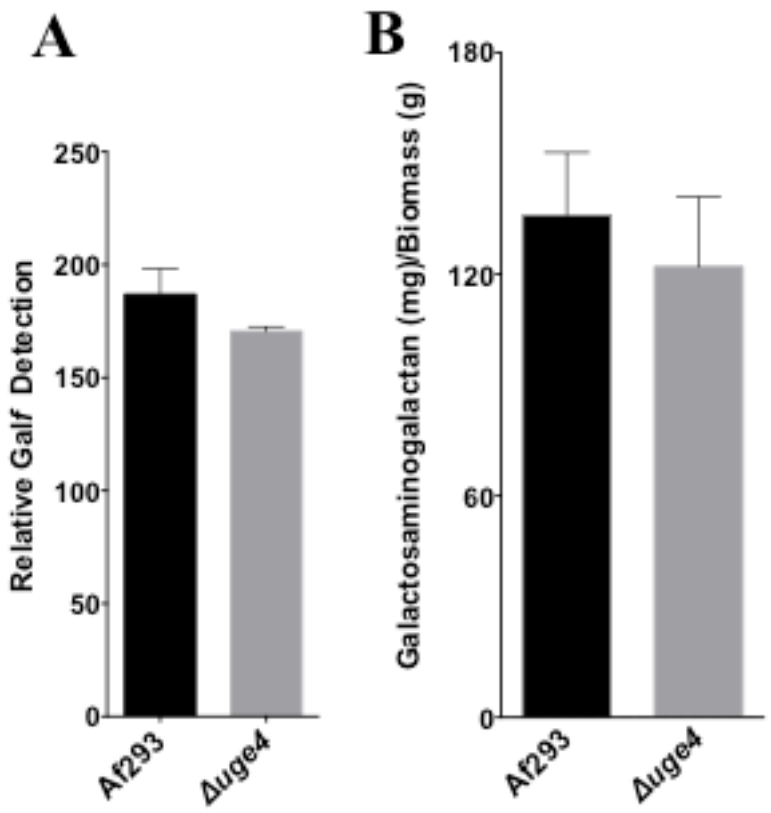
Figure 3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100
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Δuge4
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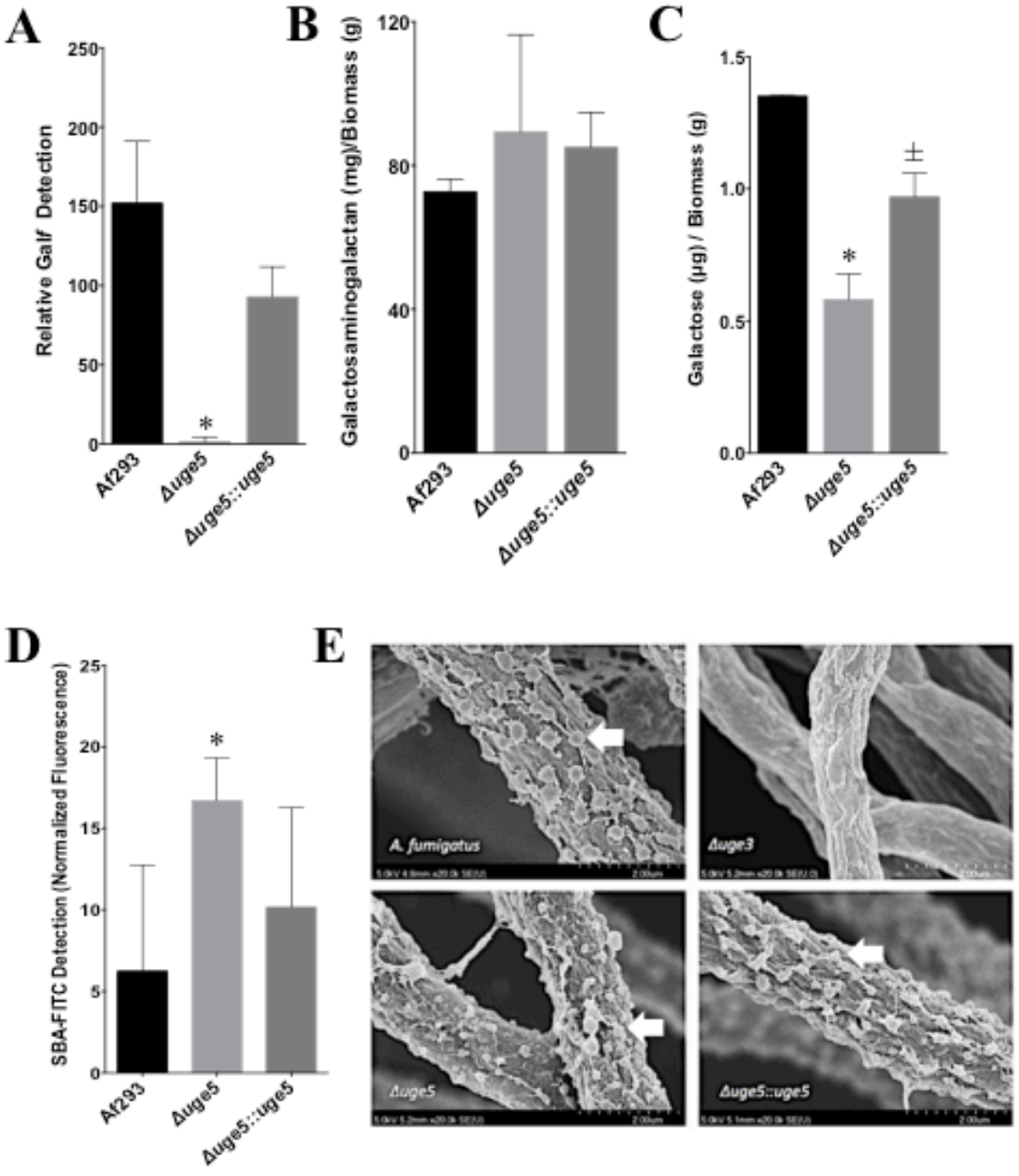
60
0
Af293
Δuge4

Figure 4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B
Galactosaminogalactan (mg)/Biomass (g)
120
80
40
0
Af293
Δuge5
Δuge5::uge5
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Galactose (μg) / Biomass (g)
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Figure 5

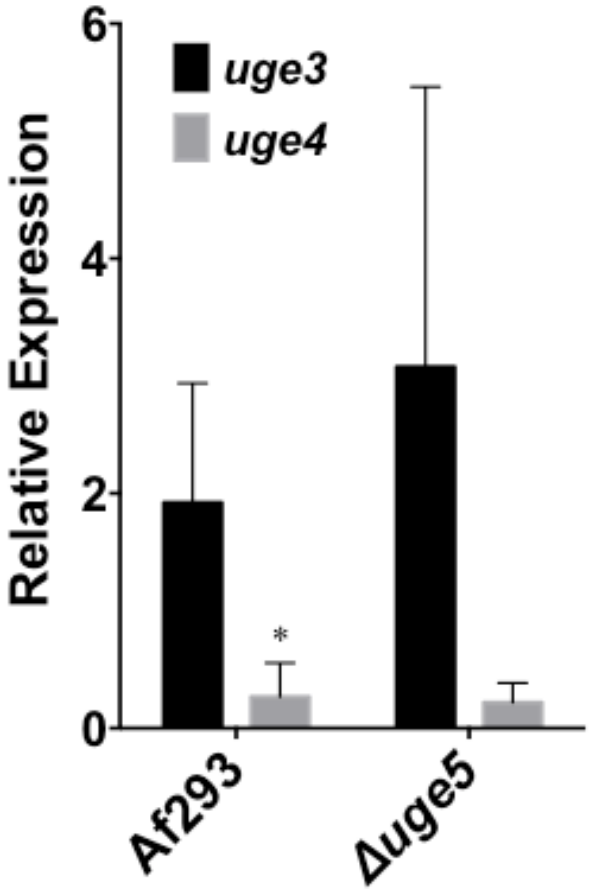


Figure 6

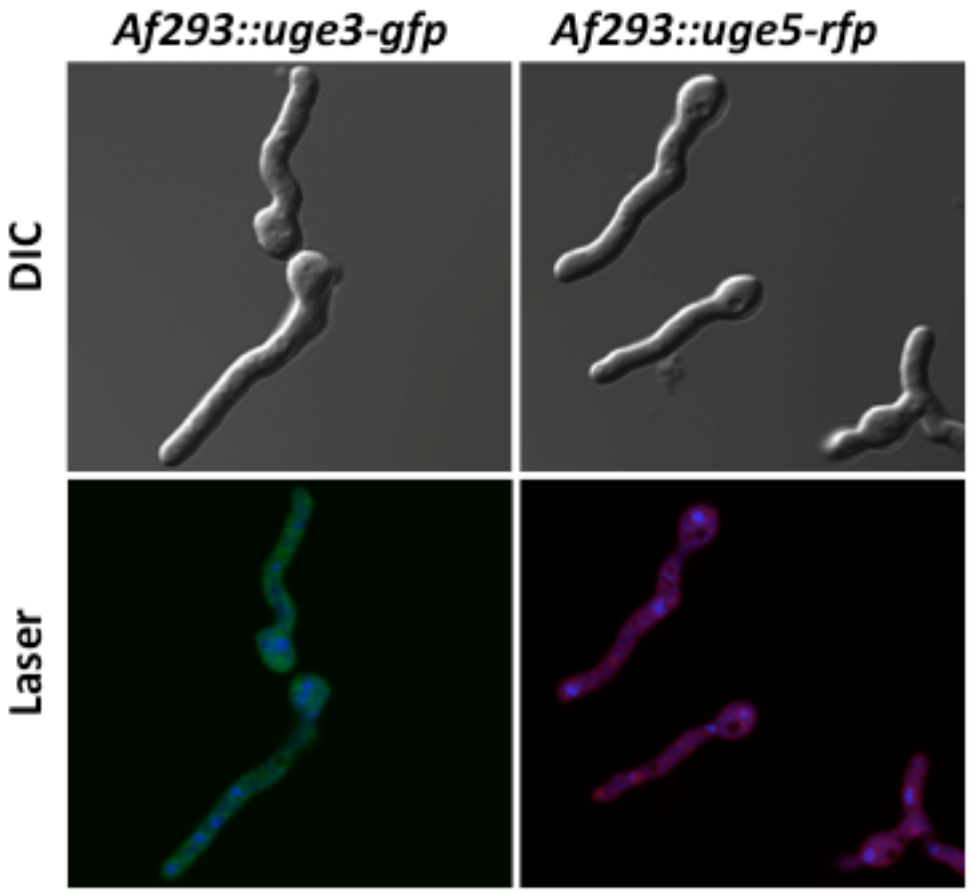


Figure 7

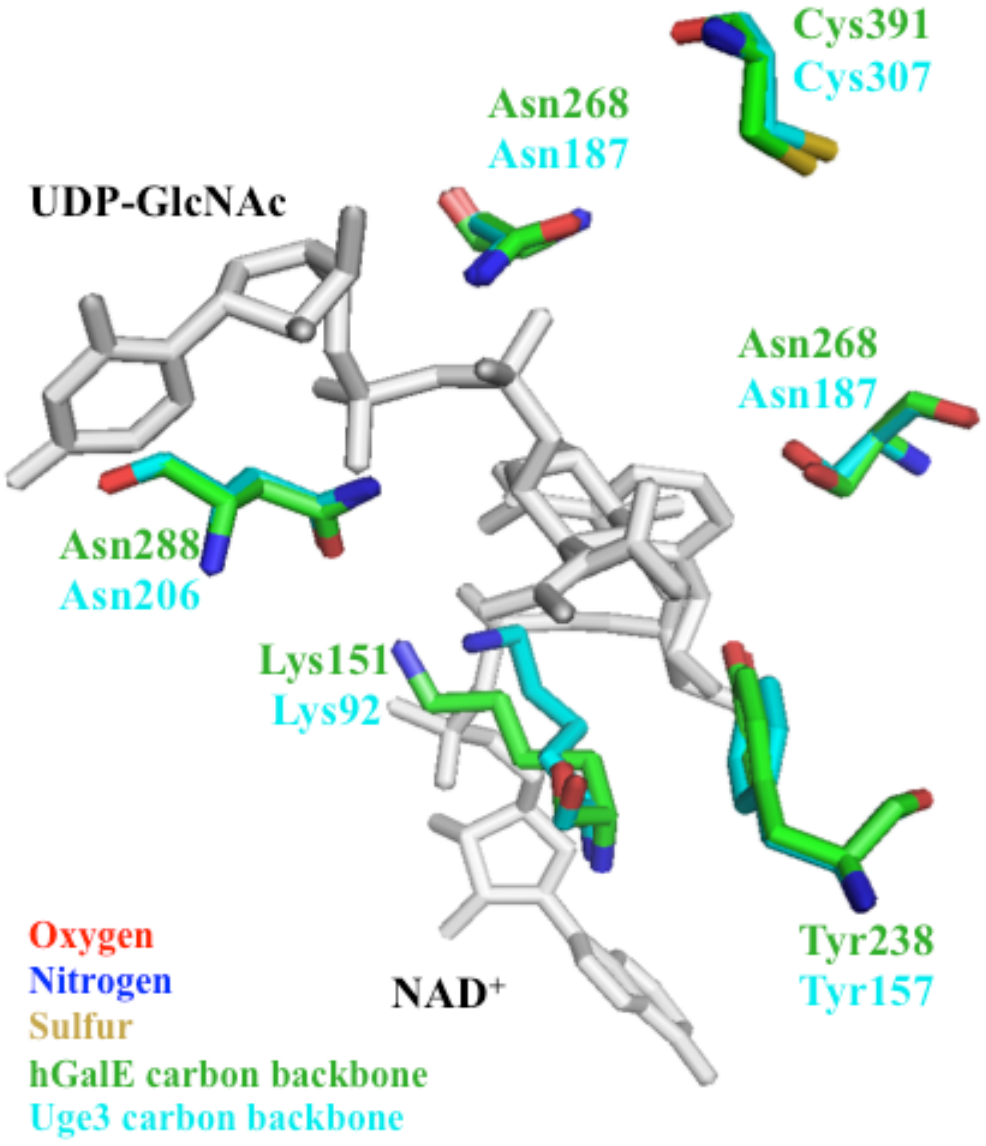
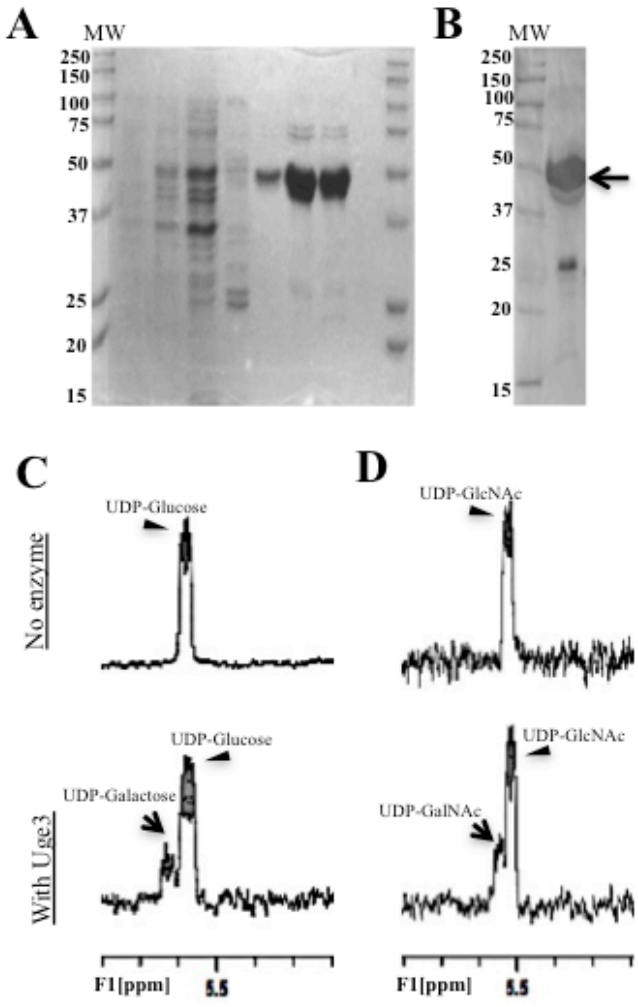

Cys391
Cys307
Asn268
Asn187
UDP-GlcNAc
Asn268
Asn187
Asn288
Asn206
Lys151
Lys92
Tyr238
Tyr157
NAD+
Oxygen
Nitrogen
Sulfur
hGalE carbon backbone
Uge3 carbon backbone

Figure 8


A
MW
250
150
100
75
50
37
25
20
15
B
MW
250
150
100
75
50
37
25
20
15
C
D
No enzyme
UDP-Glucose
UDP-GlcNAc
With Uge3
UDP-Glucose
UDP-Galactose
UDP-GlcNAc
UDP-GalNAc
F1[ppm]
5.5
F1[ppm]
5.5

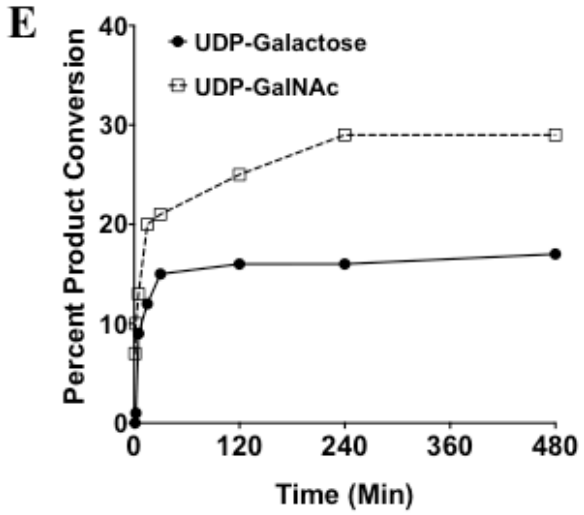

E
UDP-Galactose
UDP-GalNAc
Percent Product Conversion
0
10
20
30
40
0
120
240
360
480
Time (Min)

Figure 9

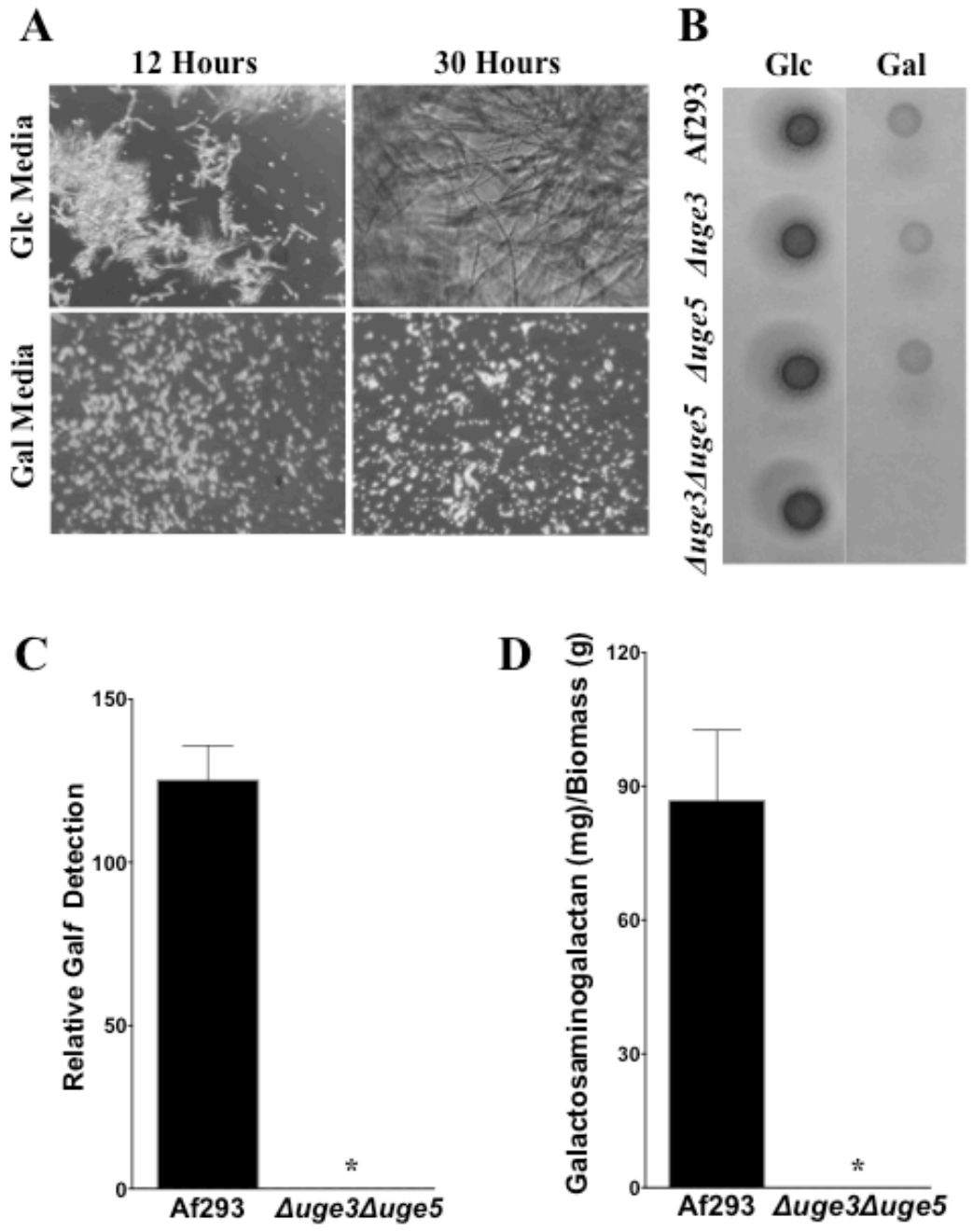

A
12 Hours
30 Hours
Glc Media
Gal Media
B
Glc
Gal
Af293
Δuge3
Δuge5
Δuge3Δuge5
C
Relative Galf Detection
150
100
50
0
*
Af293
Δuge3Δuge5
D
Galactosaminogalactan (mg)/Biomass (g)
120
90
60
30
0
*
Af293
Δuge3Δuge5

Figure 10

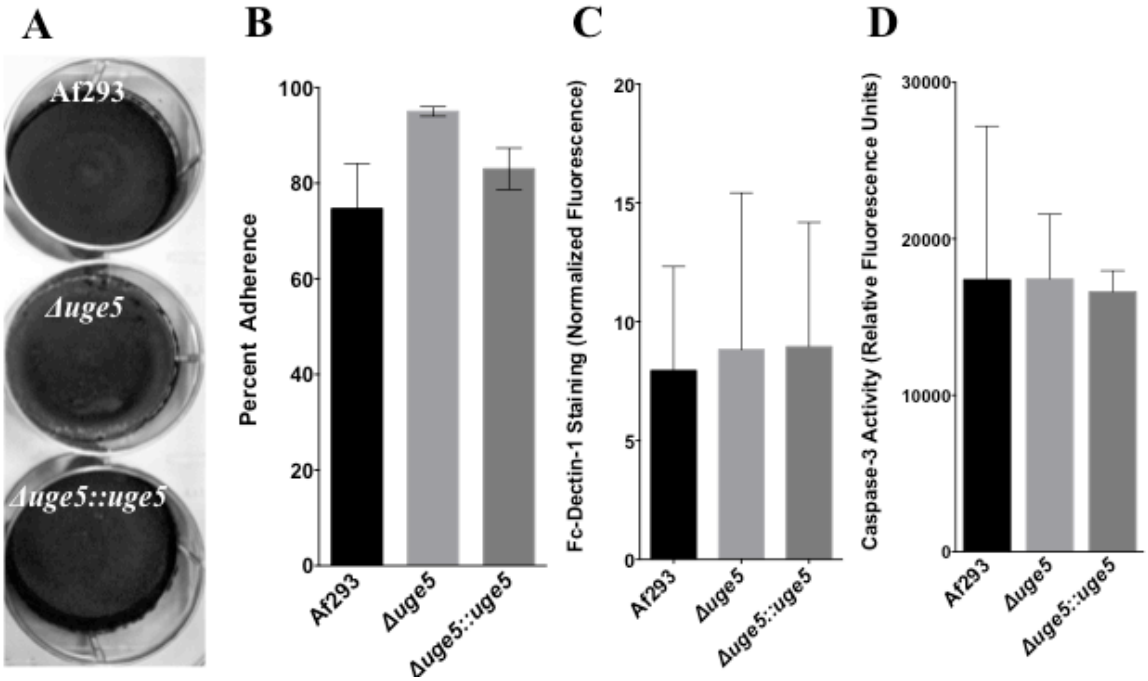
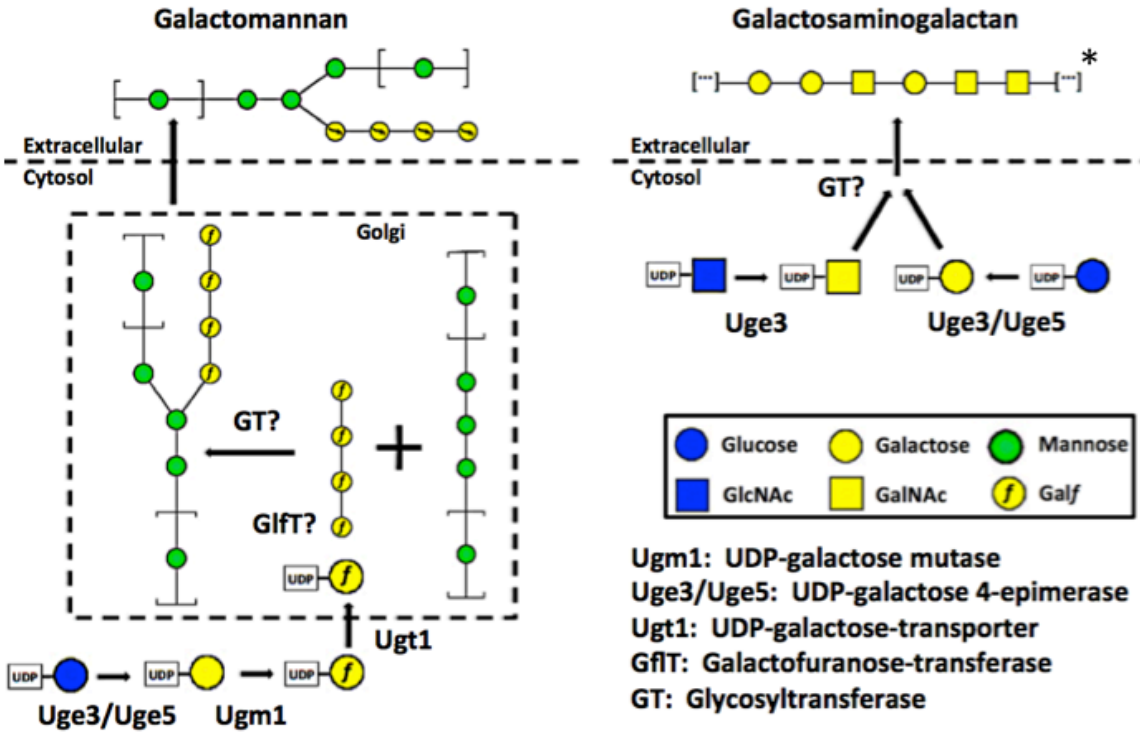

A
Af293
Δuge5
Δuge5::uge5
B
Percent Adherence
100
80
60
40
20
0
Af293
Δuge5
Δuge5::uge5
C
Fc-Dectin-1 Staining (Normalized Fluorescence)
20
15
10
5
0
Af293
Δuge5
Δuge5::uge5
D
Caspase-3 Activity (Relative Fluorescence Units)
30000
20000
10000
0
Af293
Δuge5
Δuge5::uge5

Figure 11


Galactomannan
Extracellular
Cytosol
Golgi
GT?
GlfT?
Ugt1
UDP
Uge3/Uge5
Ugm1
Galactosaminogalactan
*
Extracellular
Cytosol
GT?
Uge3
Uge3/Uge5
Glucose
Galactose
Mannose
GlcNAc
GalNAc
Galf
Ugm1: UDP-galactose mutase
Uge3/Uge5: UDP-galactose 4-epimerase
Ugt1: UDP-galactose-transporter
GflT: Galactofuranose-transferase
GT: Glycosyltransferase

Preface to Chapter 4

In Chapters 2 and 3, we elucidated the essential role of the GalNAc component of galactosaminogalactan in mediating its function. However, the mechanism of how GalNAc can mediate these functions was not known. N-acetyl-hexosamine deacetylation is a strategy employed by some bacteria to produce a cationic polysaccharide that has been reported to mediate similar functions to galactosaminogalactan. We therefore hypothesized that GalNAc in galactosaminogalactan is similarly deacetylated to galactosamine (GalN). In this chapter, we examined the role of a putative polysaccharide deacetylase, Agd3, by constructing and characterizing an *agd3* deletion mutant. Agd3 is required to mediate adherence, masking of cell wall PAMPs, and virulence in a murine model of invasive aspergillosis. We demonstrate that Agd3 mediates deacetylation of galactosaminogalactan extracellularly, highlighting its potential use as an antifungal target. Importantly, these studies demonstrate similarities between the bacterial exopolysaccharide synthesis complex and the galactosaminogalactan synthesis complex, suggesting convergent evolution of bacterial-fungal exopolysaccharide synthesis and function.

CHAPTER 4: Deacetylation of galactosaminogalactan in *Aspergillus fumigatus* is a required post-synthesis modification for adherence and virulence

Deacetylation of galactosaminogalactan in *Aspergillus fumigatus* is a required post-synthesis modification for adherence and virulence

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INTRODUCTION

Aspergillus fumigatus is an opportunistic mold that can cause invasive infection in immunosuppressed patients. Despite treatment with the currently available antifungals, the mortality of invasive aspergillosis remains between 50-95% (1), highlighting the urgent need for new effective therapeutic agents for this disease. Identify and targeting virulence factors unique to this fungus is one approach to the development of novel treatments for invasive aspergillosis.

Galactosaminogalactan is recently identified exopolysaccharide that plays a number of roles in the pathogenesis of invasive aspergillosis. Galactosaminogalactan is a linear, heteropolymer composed of alpha 1 -> 4 linked galactose and N-acetyl-galactosamine (GalNAc) that is bound to the outer cell wall and within the extracellular matrix of biofilms of *Aspergillus* and other ascomycetes (2-5). As has been reported with bacterial exopolysaccharide (6,7), cell wall associated galactosaminogalactan has been found to mediate a number of virulence associated traits including: adherence to host cells and other substrates; biofilm formation; masking of cell wall β-1,3-glucans from immune recognition (8). However, the biosynthetic pathways underlying galactosaminogalactan synthesis and the molecular mechanism by which cell wall galactosaminogalactan mediates these virulence traits remains poorly understood.

Previously, we used comparative genomics to identify *uge3*, a gene encoding a bifunctional epimerase that is required for galactosaminogalactan synthesis (9,10). In this analysis, the *uge3* gene was found to be flanked by 4 other co-regulated genes. These included genes encoding a putative glycosyl transferase, deacetylase, and two hydrolases (Fig 1A). Interestingly, exopolysaccharide synthesis in many bacterial species is governed by an operon containing a similar arrangement of carbohydrate active enzymes. (Figure 1C). In many bacterial species, including many pathogenic species, this exopolysaccharide is composed of beta-1,6 linked N-acetyl-glucosamine (GlcNAc) and has been reported to mediate many of the same virulence mechanisms reported to be associated with galactosaminogalactan including: mediating adherence of the organism to both biotic and abiotic surfaces, resistance to antimicrobial peptide killing, and masking of pathogen associated molecular patterns. (Figure 1B) (6,11-13). This polysaccharide, known as

polysaccharide intracellular adhesion (PIA) in *Staphylococcus aureus* (14) or poly-N-acetyl-glucosamine (PNAG) in *Escherichia coli* (11), is produced gene products of the Ica or Pga operons, respectively. Briefly, poly-GlcNAc is synthesized at the cell membrane by the action of a glycosyl transferase (IcaC/PgaC), and extruded into the periplasm. A critical step for the generation of functional exopolysaccharide is partial deacetylation of GlcNAc residues by IcaB/PgaB, resulting in interspersed glucosamine (GlcN) residues (13,15,16). Under acidic conditions, these GlcN residues are protonated, conferring a positive charge on the mature peptide. The cationic nature of bacterial exopolysaccharide mediates adherence to negatively charged surfaces including host cells and the organism itself, as well as enhances resistance to cationic molecules such as aminoglycoside antibiotics and antimicrobial peptides.

Although the type of hexosamine and linkages differ between bacterial exopolysaccharide and galactosaminogalactan, we hypothesized that the biosynthetic pathways for these two glycans are similar (Figure 1C) and that partial deacetylation of galactosaminogalactan is required for its function. To test this model, we first confirmed that galactosaminogalactan is partially deacetylated, then investigated the function of the putative deacetylase Agd3. Deletion of *agd3* results in a strain of *A. fumigatus* that produces normal amounts of galactosaminogalactan, that was completely acetylated. This loss of deacetylase activity was associated with a strain that lacked cell wall bound galactosaminogalactan, could not form biofilms, displayed higher β-1,3-glucan exposure and was attenuated in virulence in a murine model of invasive aspergillosis. Further, multiple lines of evidence suggested that Agd3 mediated deacetylation of galactosaminogalactan occurred extracellularly.

Collectively, our data suggest a convergent evolution of between bacteria and fungi to develop a similar strategy for biofilm formation and host resistance through the production of hexosamine rich exopolysaccharide. Further, the importance of deacetylation for exopolysaccharide function, and the extracellular location of this process suggest that inhibition of galactosaminogalactan deacetylase activity may be a viable therapeutic strategy to improve outcomes in invasive aspergillosis.

MATERIALS AND METHODS

Fungal Strains and Growth Conditions

A. fumigatus strain Af293 (a generous gift from P. Magee, University of Minnesota, St. Paul, MN, USA) was used as the parental strain for all genetic manipulations and experimental controls. Unless otherwise noted, all strains were maintained on YPD agar (Fisher Scientific) at 37°C. For growth in liquid culture, Brian medium or phenol-free RPMI 1640 (Wisent) was used as indicated.

Molecular and Genetic Manipulations

A split marker, double homologous recombination approach was used to generate the Δ*agd3* mutant (Gravelat et al 2012). An upstream and a downstream portion were amplified from the genomic DNA using PCR methods (Table 1). The resulting PCR fragments were then cloned into the entry vector pENTR-D-TOPO® following manufacturer's instructions (Gateway, Inc.), and transformed into competent Mach T1 *Escherichia coli*. Entry vector plasmid carrying the upstream and downstream fragments was recombined with a previously constructed destination plasmid containing the split marker sites (pHY and pYG)(Gravelat et al 2013) in competent Mach T1 *Escherichia coli*. Resistant colonies were selected, plasmids were extracted, and recombination was verified by restriction enzyme digestion. From the recombined plasmids, target DNA was amplified by PCR then transformed into *A. fumigatus* following a previously described transformation protocol (Gravelat et al 2012). Resulting fungal transformants were verified by one on/one off PCR amplification strategy and expression of *agd3* by qPCR (Table 1).

To generate the Δ*agd3::agd3* complemented strain, the *agd3* locus, including 680 bp downstream and 560 bp upstream of the ORF, was PCR amplified using Sgf-compl-fow and Asc-compl-rev primers, respectively (Table 1). The resulting PCR fragment was inserted into a destination plasmid containing the phleomycin selection marker. Using the split marker approach (Gravelat et al 2012), two fragments were amplified by PCR from the destination plasmid using primers Sgf-compl-fow and LE4, and BL4 and Asc-compl-rev primers, where primers BL4 and LE4 (Table 1) extend the length of the phleomycin resistance gene ORF on opposing directions. The resulting fragments were transformed into the Δ*agd3* mutant following a previously described transformation protocol (Gravelat et al 2012). Phleomycin-resistant transformants of the triple homologous recombination

events were selected and verified by one on/one off PCR amplification strategy and expression of *agd3* by qPCR (Table 1).

In silico analysis

Amino acid sequence of Agd3 (Afu3g07870) was retrieved from the Aspergillus Genome Database. Functional domain was analyzed using Eukaryotic Linear Motif (39), ConSurf (40), Conserved Domain Database (41), and HHpred (42), including SCOP domains. For graphical representation, protein domains were depicted using DOGS software. For homology modeling, amino acid sequence of Agd3 was queried and modeled using Phyre2 (ref). Homology model was also built by aligning Agd3 with *Mycobacterium smegmatis* polysaccharide deacetylase protein as a template (PDB code 3RXZ) (47). Aligned sequences were then modeled using Modeler Version 9.11 (51). Resulting models were verified using Pairwise Structure Alignment (46). MacPyMOL Version 1.3 (academic license, Schrodinger LLC) was used to align respective structures and identify and analyze key residues in the predicted catalytic site.

In vitro characterization assays

To assess adherence, cell surface morphology, and lectin-binding, experiments were performed as previously described (Gravelat et al 2013; Lee et al 2014). Briefly, to test adherence of biofilm, 24 h grown mycelia in Brian medium, on tissue culture-treated 6-well plates were vigorously agitated and shaken. Biofilm that detached were removed and those that remained adherent were stained with crystal violet for visualization, as previously described (Gravelat et al 2010). To examine cell surface morphology, 24 h grown mycelia were fixed with 2.5% gluteraldehyde/cacodylate buffer, ethanol dehydrated, critical point dried in CO_2, and sputter coated with Au-Pd, as previously described (Gravelat et al 2013). Resulting samples were imaged using field emission FEI-50 scanning electron microscopy (FEI, Inc). To determine the degree of soybean-agglutinin or dectin-1 binding on hyphal surface, strains were grown for 7-9 h on poly-D-lysine coated coverslip (BD Biosciences, Inc). Young hyphae were fixed and stained as previously described (Gravelat et al 2013). For all microscopy experimentation, strains were grown in phenol red-free RPMI.

For the complementation assay, culture filtrate of 72 h grown fungi were collected by filtrating through a 0.22 μm membrane (Millipore, Inc.) and stored at -20°C until use. To test complementation of adherence, conidia from indicated strains were grown for 24h on tissue culture-treated 6-well plate in the presence of culture filtrate from either Δ*agd3* mutant or Δ*uge3* mutant. Biofilm adherence was assessed as described above. Before use, culture filtrates were diluted by 50% with fresh Brian medium. For galactosaminogalactan indirect ELISA, culture filtrate from respective strains, and combination of Δ*uge3* and Δ*agd3* mutant culture filtrates, were incubated in a high-binding ELISA plate (Nunclon Inc.). After 1 hour incubation, culture filtrates were removed, wells were washed in washing buffer and incubated for 1 hour with anti-galactosaminogalactan antibody on a rotator. Then, the wells were washed and incubated with anti-IgG HRP-tagged secondary antibody (Jackson Laboratories, Inc.). Subsequently, wells were washed and chromogen was developed by adding the HRP substrate solution (Clontech, Inc.). To test complementation of cell surface morphology, conidia from indicated strains were grown for 24h on poly-D-lysine coated coverslips (BD Biosciences, Inc.) in the presence of culture filtrate from either Δ*agd3* mutant or Δ*uge3* mutant. Scanning electron microscopy was performed as described above. Before use, culture filtrates were diluted by 50% with fresh phenol red-free RPMI. To minimize crystal formation from Brian medium during SEM sample preparation,

For antifungal susceptibility studies, initial experiments were performed using a checkerboard-type approach. $1x10^4$ conidia of indicated strains were inoculated in 100μL of either Brian medium or phenol-red RPMI in a tissue culture-treated 96-well plate and treated with 50 μL of varying concentrations of caspofungin (Sigma, Inc) and 50 μL of varying concentrations of nikkomycin (Sigma, Inc), for a total of 100 μL of antifungal agents. For single drug treatment wells, 50 μL of phenol red-free RPMI was added instead of the second antifungal agent. The fungi were grown at 37°C, 5% CO_2, and examined every 12 hours under the microscope for inhibition and/or morphological changes. A narrower range of antifungal concentrations was subsequently tested in 24-well plates. $1x10^5$ conidia of indicated strains were inoculated in 300μL of either Brian medium or phenol red-free RPMI in a tissue culture-treated 24-well plate and treated with 300 μL of either

single antifungal or combination treatments. All images were taken under brightfield (Zeiss microscope, Inc.) using a mounted camera (Leica, Inc.) at 400X magnification.

In vivo virulence study

Balb/c mice, 5-6 weeks old, were immunosuppressed with 250 mg/kg of cortisone acetate (Sigma-Aldrich) by subcutaneous injection on days -2 and +3, and with 250 mg/kg of cyclophosphamide (Western Medical Supply, Inc.) intraperitoneally on day -2 and 200 mg/kg on day +3 per mouse, relative to conidial challenge, as previously described (Sheppard et al 2004). Mice were infected with the indicated fungal strain (N=26 mice per fungal strain) or sham infected with PBS (N=24 mice) using an aerosol chamber, as previously described (Sheppard et al 2004). To prevent bacterial infection, enrofloxacin was added to the drinking water (Baytril, Inc). Mice were monitored for a period of 2 weeks for signs of illness and moribund animals were euthanized. All procedures involving mice were approved by the Los Angeles Biomedical Research Institute Animal Use and Care Committee.

RESULTS

Bioinformatic analyses suggest that Agd3 functions as a carbohydrate deacetylase.

Agd3 (afu3g07870) contains 806 amino acid residues, and is predicted to have a signal peptide region and two globular domains (Figure 1B). The N-terminus of Agd3 contains a stretch of serine-rich region predicted to be compositionally biased (Figure 2A). The first domain from the N-terminus is predicted to be globular, but of unknown function as it does not correspond to any of the currently known domain family. The second domain is annotated as a partial carbohydrate-4 esterase (CE4) (17). The fact that the CE4 domain in Agd3 only partially aligns to known CE4 domain could reflect specificity for GalNAc rather than the more common GlcNAc. To our knowledge, there is no reported structure of a GalNAc-specific deacetylase and proteins belonging to the CE4 superfamily include enzymes like chitin deacetylases (EC 3.5.1.41) and bacterial peptidoglycan N-

acetylglucosamine deacetylases (EC 3.5.1.104) all of which target GlcNAc (18). Structurally, the CE4 domain of Agd3 is most similar to a polysaccharide deacetylase from *Mycobacterium smegmatis* (PDB code 3RXZ) and a putative peptidoglycan deacetylase from *Heliobacter pylori* (PDB code 3QBU). In fact, Agd3 models against the polysaccharide deacetylase from *Mycobacterium smegmatis* with over 95% confidence (19). Metal coordinating triad shown to be required for catalytic activity in these orthologs are also present in the predicted CE4 domain of Agd3 (20) (Figure 1C).

Deletion of *agd3* blocks deacetylation of galactosaminogalactan.

Using primary amine analysis, secreted galactosaminogalactan was found to be ~ 15-25% deacetylated (Figure 3A). Deletion of *agd3* did not significantly affect the production of total secreted galactosaminogalactan (Figure 3A), however, galactosaminogalactan produced by this mutant was devoid of primary amines (Figure 3B). Complementation of the Δ*agd3* mutant with a wild-type allele of *agd3* completely restored deactylation to wild-type levels. Thus Agd3 is required for partial deactylation of galactosaminogalactan.

Deletion of *agd3* is associated with the loss of adherence and changes in the cell wall.

To examine the role of deacetylation on the function of galactosaminogalactan deacetylation, the Δ*agd3* mutant was compared with the wild-type parent and agd3-complemented strain for a variety of galactosaminogalactan dependent phenotypes. Deletion of *agd3* was associated with a loss of adherence to negatively charged, tissue culture-treated surfaces (Figure 4). However, consistent with the hypothesis that galactosaminogalactan mediated adherence is dependent on charge-charge interactions, the Δ*agd3* mutant retained the ability to form wild-type biofilms on a positively charged poly-D-lysine-coated surface, or on non-polar polystyrene (data not shown). Thus, deacetylation seems to mediate adherence to negatively charged surface, but not on non-polar or positively charged surfaces.

Consistent with defects in biofilm adherence, the loss of Agd3 was associated with alterations in the surface morphology of hyphae as visualized by SEM (Figure 4). The production of fully acetylated galactosaminogalactan resulted in the loss of detectable cell

wall galactosaminogalactan as measured by direct immunofluourescence with the GalNAc-specific lectin, soybean agglutinin (SBA), and confirmed by scanning electron microscopy which demonstrated a loss of the galactosaminogalactan dependent cell wall decorations. Consistent with this loss of hyphal bound galactosaminogalactan, hyphae of the Δ*agd3* mutant were found to have increased exposure of β-1,3-glucan exposure as detected by enhanced binding of recombinant Fc-Dectin-1 (Figure 4). In all of these assays, the Δ*agd3* mutant was indistinguishable from the galactosaminogalactan-deficient Δ*uge3* mutant Collectively, these data suggest that Agd3 mediated deacetylation of galactosaminogalactan is also required for adherence of the polysaccharide to the fungal cell wall and as a result, mediate adherence and masking of β-1,3-glucans.

Deletion of *agd3* attenuates virulence.

To extend these findings in vivo, the virulence of the Δ*agd3* mutant was compared to the wild-type strain Af293 strain, and the Δ*agd3::agd3* complemented strain in a leukopenic murine model of invasive aspergillosis (Figure 7) (8,21). In this model, the galactosaminogalactan-deficient Δ*uge3* exhibits attenuated virulence (8). Consistent with the in vitro findings of impaired galactosaminogalactan function, the Δ*agd3* mutant was hypovirulent compared to wild-type *A. fumigatus* or the *agd3*-complemented strain. Thus deactylation of galactosaminogalactan is also required to mediate virulence.

Galactosaminogalactan deacetylation is an extracellular process.

Since the Δ*agd3* mutant produces fully acetylated galactosaminogalactan but no Agd3, and the Δ*uge3* mutant produce Agd3 but no galactosaminogalactan, we hypothesized that if deacetylation is an extracellular process than co-culture of these either strain with culture supernatants from the other mutant would result in the production of deacetylated galactosaminogalactan and restored biofilm formation. Growth of the Δ*agd3* mutant grown in the presence of culture filtrates from the Δ*uge3* mutant restored adherent biofilm formation (Figure 6A). In addition, hyphae of the Δ*agd3* mutant grown in the presence of culture filtrates from the Δ*uge3* mutant showed wild-type levels of surface GalNAc staining, and were found to have restored cell wall decorations by scanning electron microscopy

(Figure 6B). Growth of the Δ*agd3* or Δ*uge3* mutant in the presence of their own respective culture filtrates had no effects on adherence or hyphal morphology.

To confirm these findings, we also examined the ability of cell free culture filtrates from both Δ*agd3* and Δ*uge3* mutants to generate deacetylated galactosaminogalactan *in vitro*. Culture supernatants of each mutant, or a 1:1 mixture of both culture supernatants were incubated in polystyrene EIA plates to capture adherent, deacetylated galactosaminogalactan. Deacetylated galactosaminogalactan adherent to the microtiter wells was then quantified using an anti-galactosaminogalactan antibody (4). Adherent galactosaminogalactan could be easily detected from culture supernatants of wild-type *A. fumigatus*, while no adherent galactosaminogalactan was detected from the culture filtrate from either Δ*agd3* or Δ*uge3* mutant alone. A 1:1 mixture of cultures supernatants of the Δ*agd3* and Δ*uge3* mutant resulted in detection of adherent galactosaminogalactan at similar levels to that recovered from culture supernatants of wild-type *A. fumigatus* (Figure 6C). Collectively, these results support the proposed model of galactosaminogalactan biosynthesis and highlight the similarities between the biosynthesis and function of this glycan and bacterial exopolysaccharide.

DISCUSSION

Previously, we reported that galactosaminogalactan is required for adherence and biofilm formation, masking of β-1,3-glucan, and virulence (8). In the present study, we report a cluster of co-regulated genes on chromosome 3 with similar composition to bacterial operons encoding proteins required for exopolysaccharide synthesis. We characterized one of the enzymes in this cluster in detail and demonstrated that, as with bacterial exopolysaccharide, a hexosamine deacetylase is required for the synthesis and function of cell wall bound galactosaminogalactan.

These data suggest that galactosaminogalactan synthesis is mediated through a pathway that is similar to that of bacterial exopolysaccharide in that following synthesis of the nascent polymer by a membrane bound glycosyltransferase, the emergent chain

containing N-acetylated hexosamines is then partially deacetylated. Multiple lines of evidence suggest that these biosynthetic pathways developed through convergent evolution rather than having been present in a common ancestor. First, while PIA/PNAG is composed of β-linked GlcNAc residues, galactosaminogalactan is a heteropolymer of α-linked galactose and GalNAc. Further, outside of the conserved enzymatic domains themselves, there is little sequence similarity between the bacterial and fungal enzymes within the biosynthetic clusters. The observation that bacteria and fungi have developed similar strategies for cationic exopolysaccharide production suggests that they have been subject to similar environmental pressures during their evolution. As humans are dead end hosts for *Aspergillus* species, it is likely that the pressures responsible for the development of galactosaminogalactan were found in the soil or other natural environment. Indeed, a shared requirement for adherence to substrates and resist environmental stresses through the production of an adherent layer of exopolysaccharide may underlie the development of exopolysaccharide synthesis by these microorganisms. Future studies examining the role of exopolysaccharide in fungal and bacterial growth in non-human environments may shed light on the factors leading to the evolution of these glycans in microorganisms.

Deacetylation of the GalNAc unit of galactosaminogalactan by Agd3 is required to produce cell wall bound galactosaminogalactan and for mediating the phenotypes that have been associated with cell wall galactosaminogalactan as well as virulence. Further, the studies reported here would suggest that, as with bacterial exopolysaccharide, deacetylation occurs in the extracellular space. This observation suggests that inhibition of galactosaminogalactan deacetylation may represent an attractive antifungal target since intracellular penetration of a candidate deacetylase inhibitor would not be required for activity. Further, deacetylation of GalNAc within human cells has not been reported, suggesting that antifungal specificity may be achievable.

Although a previous report did not identify the presence of deacetylated GalNAc (GalN) within secreted galactosaminogalactan from *A. fumigatus* (4), compositional analysis of galactosaminogalactan from *A. niger* has reported the presence of GalN in preparations from this species (22). Since deacetylated galactosaminogalactan adheres

avidly to glass and plastics, it is possible that during the purification of *A. fumigatus* galactosaminogalactan the preparation was enriched for fully acetylated, non-adherent polysaccharide. Alternately, it is possible that deacetylation varies under different growth conditions. Further experiments are required to test these possibilities.

In conclusion, we report that deacetylation of galactosaminogalactan by Agd3 is essential for the function of this exopolysaccharide, including adherence of galactosaminogalactan to the fungal cell wall and other substrates. These experiments shed light on the molecular mechanisms underlying galactosaminogalactan-mediated adhesion, and draw important parallels between this glycan and bacterial exopolysaccharide. Finally, these data suggest the exciting possibility that targeting deacetylation of galactosaminogalactan may represent a promising antifungal strategy.

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FIGURE LEGEND

Table 1. List of Primers

Figure 1. Galactosaminogalactan biosynthetic gene cluster

(A) Schematic representation of the galactosaminogalactan gene cluster.

(B) Schematic representation of the ica operon.

(C) Comparative diagram of *Staphylococcus aureus* PIA and *Aspergillus fumigatus* galactosaminogalactan (GAG) synthesis systems. 1: synthesis of the polysaccharide chain by the glycosyltransferase; 2: extrusion of the polysaccharide into the extracellular area; 3: deacetylation of the N-acetyl-hexosamine unit of the polysaccharide.

Figure 2. Agd3 is a putative carbohydrate deacetylase.

(A) Putative functional diagram of Agd3 showing 2 predicted domains. S.P.: signal peptide; S.R.: serine-rich region; UNK: unknown domain; CE4: deacetylase domain.

(B) Predicted mechanism of galactosaminogalactan deacetylation converting the GalNAc component of galactosaminogalactan to GalN.

(C) Predicted protonation of primary amine on GalN, conferring a positive charge.

Figure 3. Deletion of *agd3* blocks deacetylation of galactosaminogalactan.

(A) Secreted of total galactosaminogalactan production as measured by ethanol precipitation.

(B) Deacetylation of galactosaminogalactan as measured by presence of primary amine using TNBS reaction.

For all graphs: data are represented as mean +/- SEM. * indicates a significant difference between *A. fumigatus* and other species, $p<0.05$ by ANOVA.

Figure 4. Deletion of *agd3* is associated with loss of adherence and changes in the cell wall.

Panels from left to right: biofilm adherence to tissue culture treated 6-well plates of 24 h grown hyphae; scanning electron micrograph of 24 h hyphae; confocal microscopy images

of 9 h grown hyphae stained with FITC-tagged soybean lectin; confocal microscopy images of 9 h grown hyphae stained with Fc-dectin-1 detected by FITC-tagged Fc-receptor antibody.

Figure 5. Deletion of *agd3* attenuates virulence.
Survival of cortisone and cyclophosphamide treated Balb/c mice infected with the indicated conidial strains. N = 26 for all fungal strains, N=24 for PBS sham infection. * indicates a significant difference in survival of *A. fumigatus* or for Δ*agd3::agd3* compared with for Δ*agd3* strains as determined by the Meir-Kaplan test.

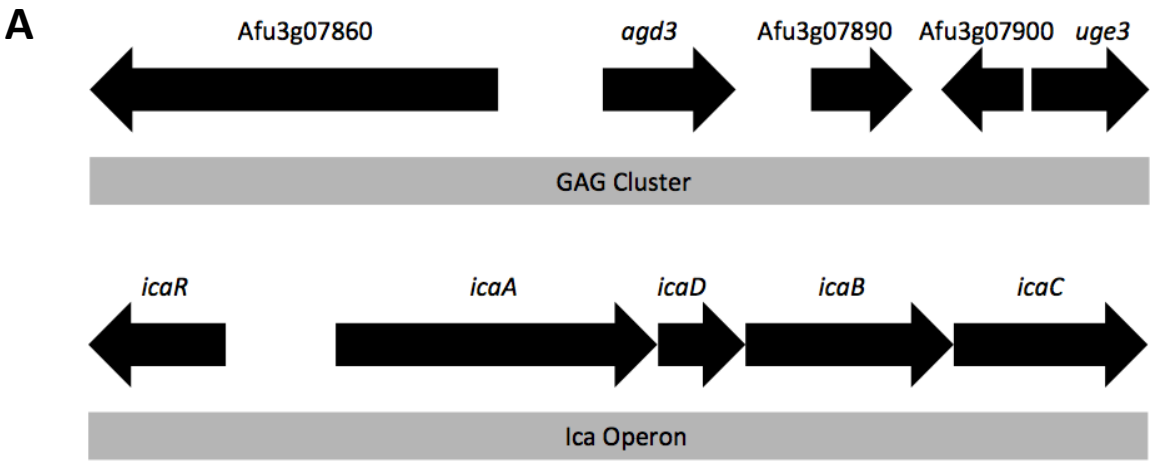
Figure 6. Culture filtrate from Δ*uge3* mutant complements defects in adherence, cell wall morphology, and production of functional galactosaminogalactan in Δ*agd3* mutant.
(A) Biofilm adherence of the indicated strains grown in the presence of 72 hours grown Δ*uge3* culture filtrate.
(B) Cell wall morphology of the indicated strains grown in the presence of 72 hours Δ*uge3* culture filtrate. Standard SEM preparation protocols were followed, and images were taken using field emission FEI-50 scanning electron microscopy.
(C) Absorbance reading of indirect ELISA using anti-galactosaminogalactan antibody to measure galactosaminogalactan presence in culture filtrate after 1 hour incubation in a high-binding plate.

Figure S1. Corresponding DIC images from Figure 4

Table 1

Primer name	Target	Sequence
Gate 1	agd3 flanking sequence	CACCCGAGATAGTTGGTATTTCC
Gate 2	agd3 flanking sequence	GAACGAGAGACTGGGTGGAG
Gate 3	agd3 flanking sequence	CACCATGCCAAGAATCAAGTAA
Gate 4	agd3 flanking sequence	CTCATCACGTAAGCCTGCAA
Sgf-compl-fow	agd3 flanking sequence	ATTAATTAAGCGATCGCGAAGGACCAGGGGA
Asc-compl-rev	agd3 flanking sequence	AGGCGCGCCGTTGAACTCGATATGC
BL4	Phleomycin resistance	CTGATGAACAGGGTCACGTC
LE4	Phleomycin resistance	CAAGTTGACCAGTGCCGTT
agd3-Ext1	agd3	CCCCCTGAAAACCATCGGG
agd3-Ext4	agd3	GCAAACACTCCTTCCCTTCCA
agd3-int-rev	agd3	TACGACTCCCCCAGG
agd3-int-fow	agd3	CGACATTGCTGGAGTTGAGA
agd3-RT-fow	agd3	CACCCACCAAGAGATGTCCG
agd3-RT-rev	agd3	CGTCACGGCGGTGATTTTCT
HY	Hygromycin resistance	CAACCACGGCCTCCAGAAGAAGA
YG	Hygromycin resistance	GCGAGAGCCTGACCTATTGCATCT
tef1-RT-F	tef1	CCATGTGTGTCGAGTCCTTC
tef1-RT-R	tef1	GAACGTACAGCAACAGTCTGG

Figure 1


A
Afu3g07860
agd3
Afu3g07890
Afu3g07900
uge3
GAG Cluster
icaR
icaA
icaD
icaB
icaC
Ica Operon

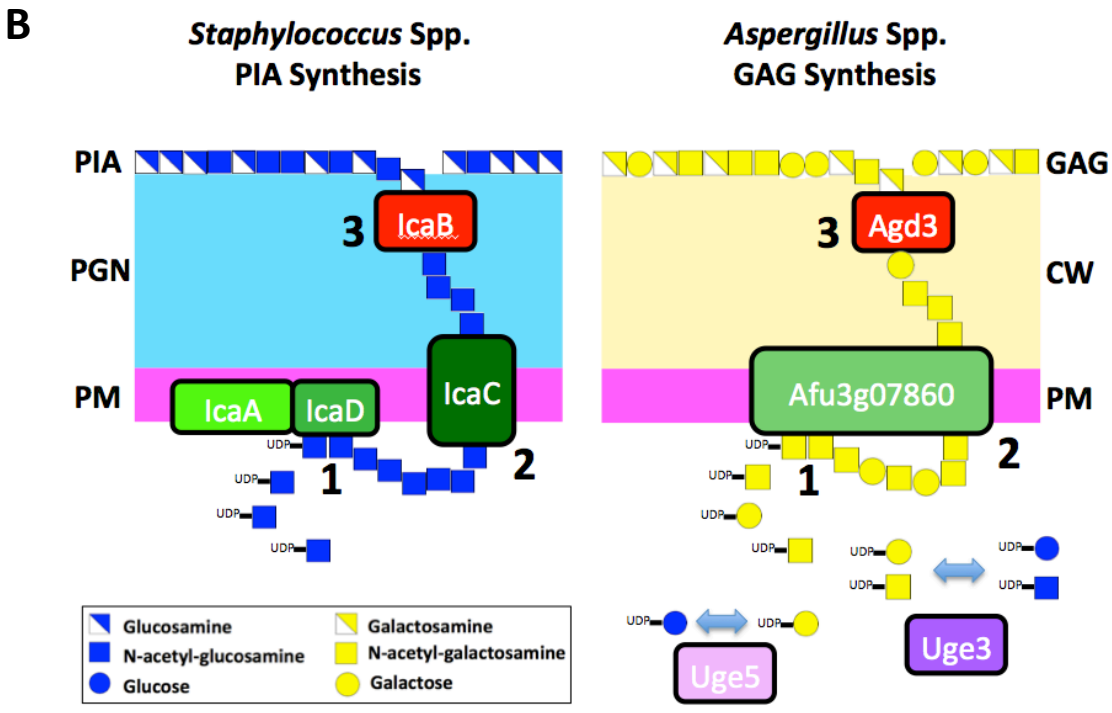
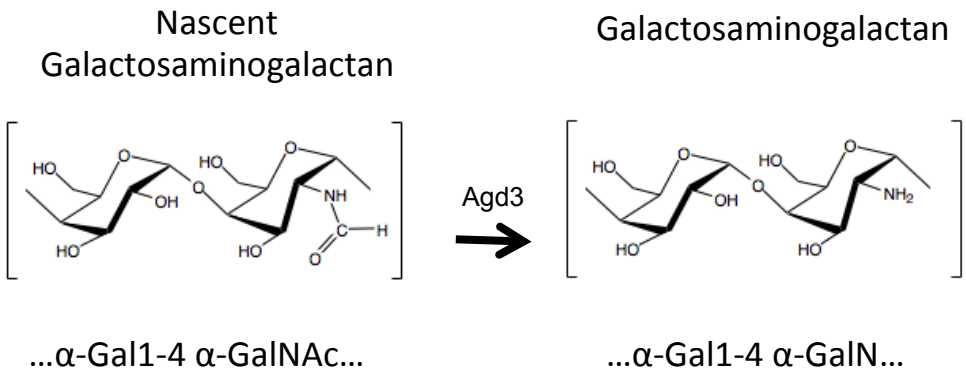
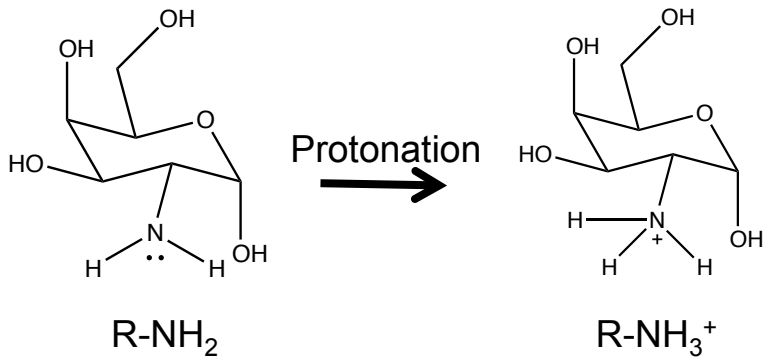

B
Staphylococcus Spp.
PIA Synthesis
Aspergillus Spp.
GAG Synthesis
PIA
PGN
PM
IcaB
IcaA
IcaD
IcaC
GAG
CW
PM
Agd3
Afu3g07860
UDP
1
2
3
Glucosamine
N-acetyl-glucosamine
Glucose
Galactosamine
N-acetyl-galactosamine
Galactose
Uge5
Uge3

Figure 2

A


Nascent
Galactosaminogalactan
Galactosaminogalactan
Agd3
…α-Gal1-4 α-GalNAc…
…α-Gal1-4 α-GalN…

B


Protonation
R-NH2
R-NH3+

C

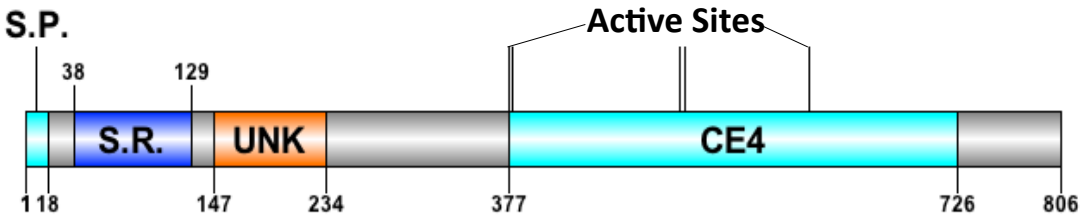

S.P.
Active Sites
38
129
S.R.
UNK
CE4
1
18
147
234
377
726
806

Figure 3

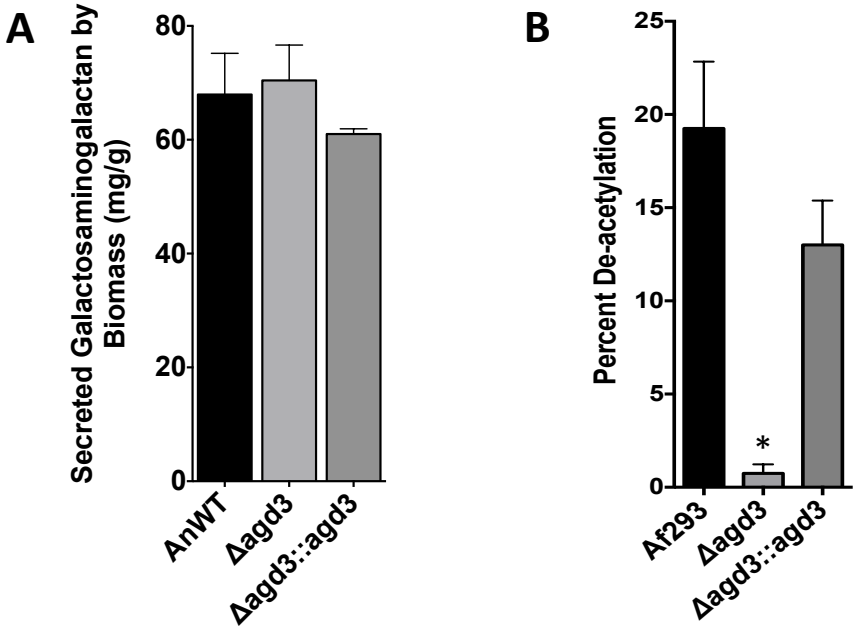

A
Secreted Galactosaminogalactan by Biomass (mg/g)
80
60
40
20
0
AnWT
Δagd3
Δagd3::agd3
B
Percent De-acetylation
25
20
15
10
5
0
*
Af293
Δagd3
Δagd3::agd3

Figure 4

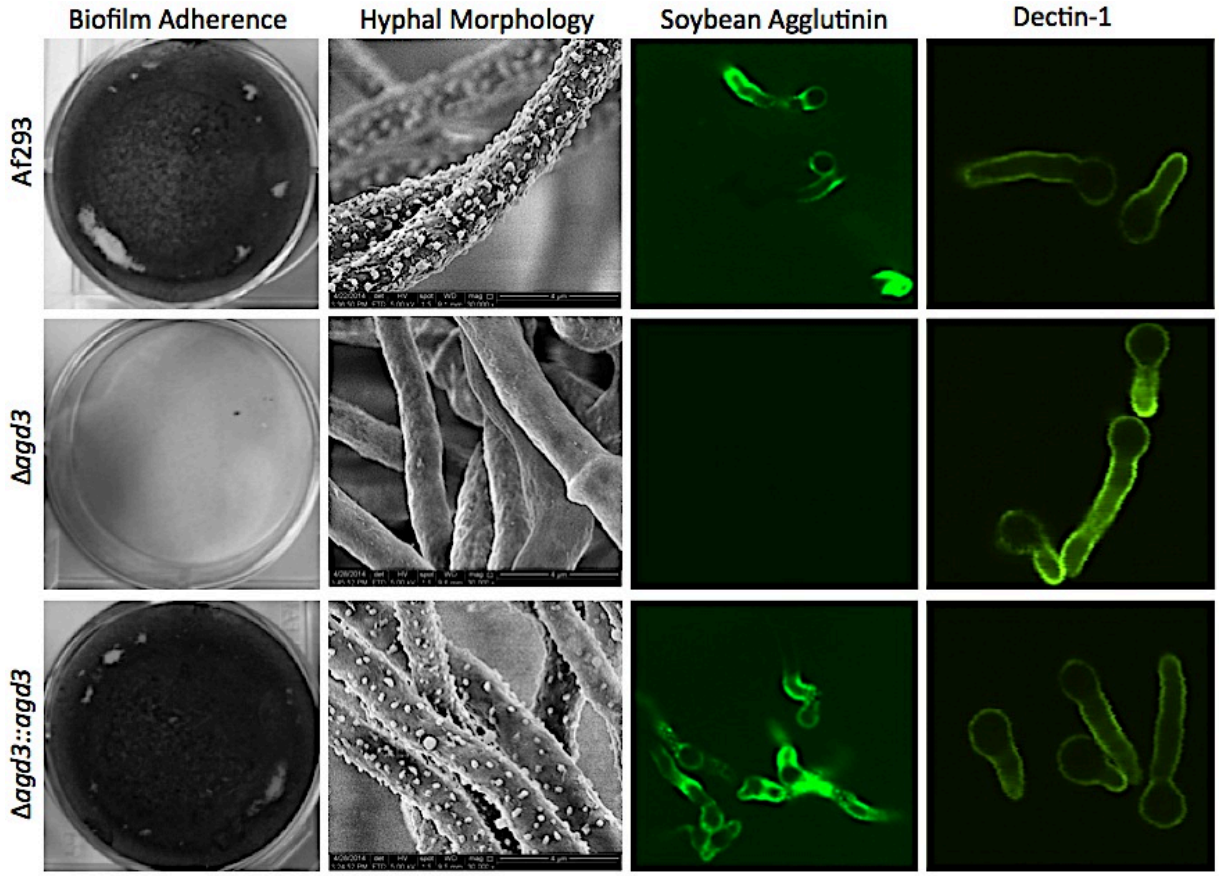

Biofilm Adherence
Hyphal Morphology
Soybean Agglutinin
Dectin-1
Af293
Δagd3
Δagd3::agd3

Figure 5

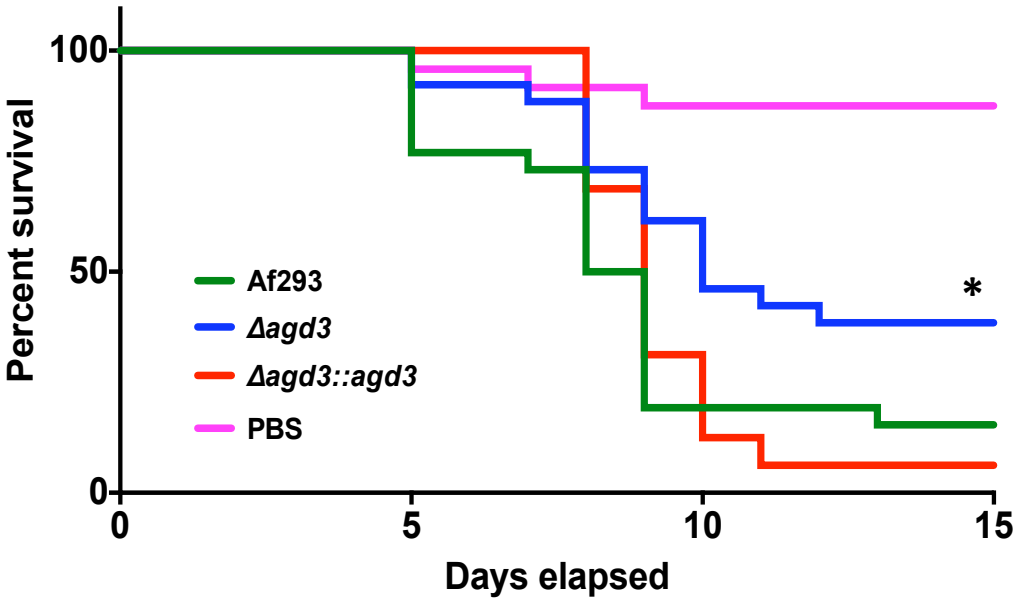

100
50
0
Percent survival
0
5
10
15
Days elapsed
Af293
Δagd3
Δagd3::agd3
PBS
*

Figure 6

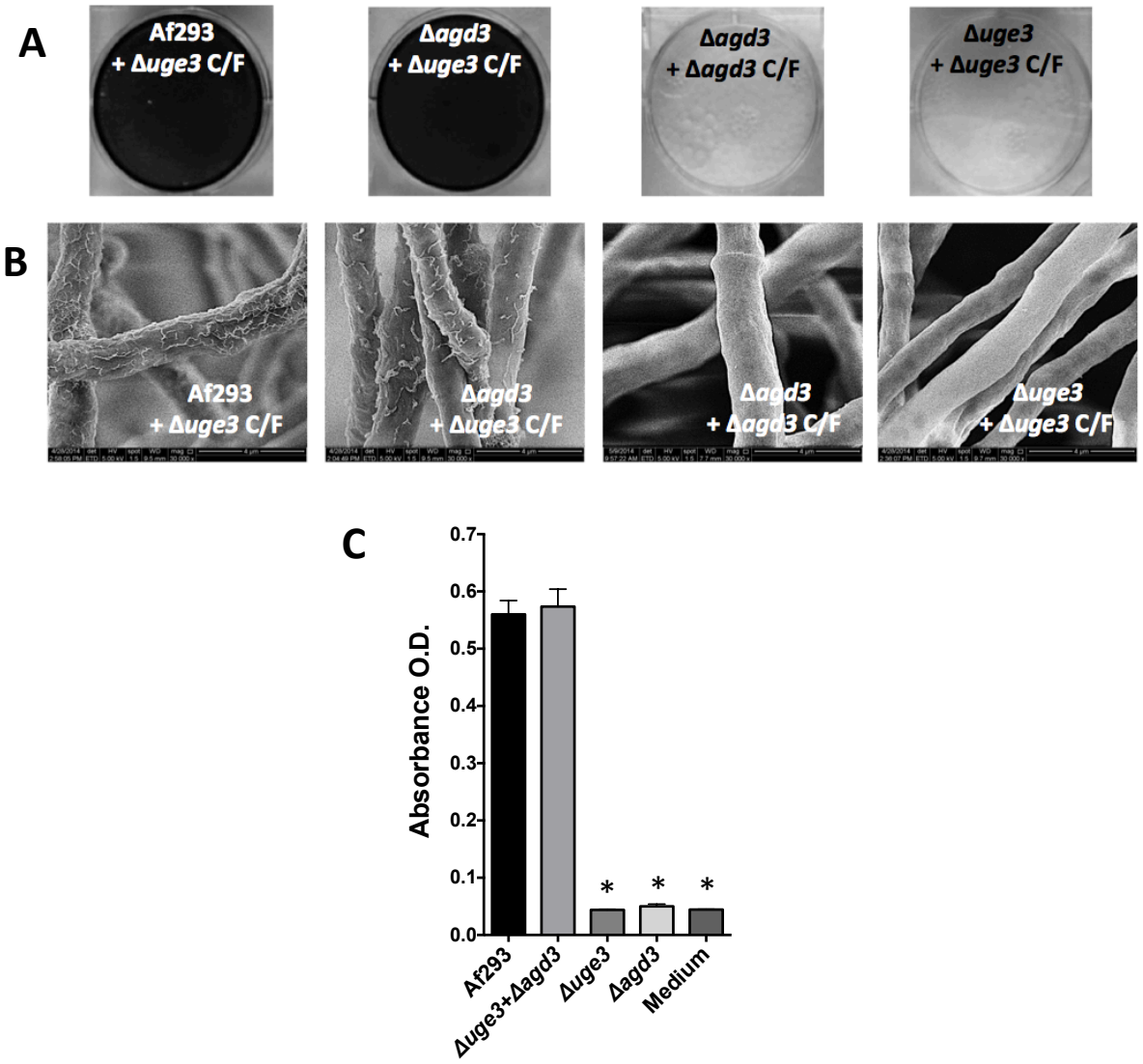
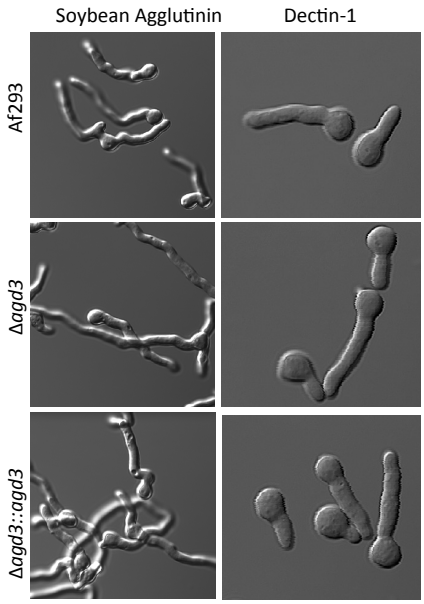

A
Af293
+ Δuge3 C/F
Δagd3
+ Δuge3 C/F
Δagd3
+ Δagd3 C/F
Δuge3
+ Δuge3 C/F
B
Af293
+ Δuge3 C/F
Δagd3
+ Δuge3 C/F
Δagd3
+ Δagd3 C/F
Δuge3
+ Δuge3 C/F
C
Absorbance O.D.
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
*
Af293
Δuge3+Δagd3
Δuge3
Δagd3
Medium

Figure S1



Preface to Chapter 5

In the previous chapters, we established that galactosaminogalactan is a virulence factor in *A. fumigatus*, and that the GalNAc/GalN component is required to mediate functions associated with galactosaminogalactan. However, given that other *Aspergillus* species also produce galactosaminogalactan, whether galactosaminogalactan production correlates to reported virulence in *Aspergillus* species remained unclear. In this chapter, we found that the GalNAc content of galactosaminogalactan correlated with reported virulence. Specifically, we found that *A. fumigatus* produced GalNAc-rich galactosaminogalactan, while less pathogenic species like *A. nidulans* produced GalNAc-poor galactosaminogalactan. When the GalNAc content of *A. nidulans* galactosaminogalactan was increased by heterologous expression of *A. fumigatus uge3*, this resulted in an increase in the cell wall bound fraction of this glycan. Increasing cell wall bound galactosaminogalactan increased adherence and biofilm formation of *A. nidulans* and enhanced virulence in a murine model of invasive aspergillosis. We discovered that the increase in virulence of the *uge3* expressing *A. nidulans* strain was due to an increase in resistance to NADPH oxidase-dependent neutrophil killing by extracellular antimicrobial peptides. These findings provide insight into the clinical observation that A. nidulans is recovered as a human pathogen only in patients with chronic granulomatous disease, a genetic deficiency in NADPH-oxidase.

CHAPTER 5: Galactosaminogalactan mediates virulence in *Aspergillus* species by enhancing resistance to NADPH oxidase-dependent neutrophil killing

Galactosaminogalactan mediates virulence in *Aspergillus* species by enhancing resistance to NADPH oxidase-dependent neutrophil killing

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ABSTRACT

Of the over 250 *Aspergillus* species, *Aspergillus fumigatus* accounts for up to 80% of human invasive infections. *A. fumigatus* produces galactosaminogalactan, an immunosuppressive exopolysaccharide composed of galactose and N-acetyl-galactosamine (GalNAc) that mediates adherence and is required for full virulence. We set out to investigate the role of galactosaminogalactan in the intrinsic virulence of *Aspergillus* species. Less pathogenic *Aspergillus* species produced galactosaminogalactan with a significantly lower GalNAc content than *A. fumigatus* and had lower levels of hyphae-associated galactosaminogalactan. Increasing the GalNAc content of galactosaminogalactan of the minimally pathogenic *A. nidulans* through overexpression of the UgeB or Uge3 epimerase increased the amount of hyphae-associated galactosaminogalactan, and augmented adherence and virulence to levels similar to *A. fumigatus*. The increased virulence of the overexpression strain was associated with enhanced resistance to NADPH oxidase-dependent neutrophil damage *in vitro* and was not seen in NADPH oxidase deficient mice.

INTRODUCTION

Invasive aspergillosis (IA) is the most common invasive mold infection in humans. In immunocompromised patients, the inhalation of airborne spores of *Aspergillus* species leads to a necrotizing fungal pneumonia that can disseminate hematogenously to the brain and other organs (1). Although the genus *Aspergillus* is comprised of over 250 members, *Aspergillus fumigatus* is responsible for more than 80% of invasive aspergillosis cases (2). Interestingly, the predominance of *A. fumigatus* as a cause human disease is not seen in patients with chronic granulomatous disease (CGD), a genetic disorder of the NADPH oxidase system that results in an impairment in production of the respiratory burst by phagocytes (3-5). In these patients, *Aspergillus nidulans* and other *Aspergillus* species are isolated with similar frequency to *A. fumigatus* (6). *A. nidulans* is rarely a cause of invasive disease outside of CGD, and in fact is used extensively as a model organism for the study of

eukaryotic cell biology. The disproportionate representation of *A. fumigatus* as a cause of invasive disease in patients without CGD, is not reflected in air or environmental sampling studies in which *A. fumigatus* accounts for only a minority of the total *Aspergillus* species recovered (7). Collectively, these observations suggest that *A. fumigatus* possesses unique traits that enhance its ability to cause human infection. Disruption of a number of putative virulence factors of *A. fumigatus* results in attenuated virulence of this species (reviewed in (7,8)). However, none of these factors have been demonstrated to confer increased virulence on less pathogenic *Aspergillus* species such as *A. nidulans*.

Recently, we and others reported that the secreted and cell wall exopolysaccharide galactosaminogalactan is required for normal virulence of *A. fumigatus* (9,10). Galactosaminogalactan is an α-1,4-linked linear heteroglycan composed of a variable combination of galactose and N-acetyl-galactosamine (GalNAc). Although the pathways governing galactosaminogalactan synthesis are not fully understood, two UDP-glucose 4-epimerases, Uge5 and Uge3, are required for galactosaminogalactan production. Uge5 mediates the conversion of UDP-glucose to UDP-galactose, while Uge3 is a bifunctional epimerase that can mediate both the interconversion of UDP-glucose to UDP-galactose and of UDP-N-acetylglucosamine to UDP-GalNAc (11). Deletion of Uge5 results in the production of galactosaminogalactan with a lower galactose content, while deletion of Uge3 completely abrogates galactosaminogalactan synthesis (10,11). Galactosaminogalactan plays a number of roles in host-pathogen interactions (9,10). This glycan mediates adherence to a variety of substrates, including host cells, and is required for normal biofilm formation (12). Also, galactosaminogalactan covers the surface of hyphae to conceal β-1,3-glucans from recognition by the pattern recognition receptor dectin-1 leading to decreased pulmonary inflammation (10). Purified galactosaminogalactan also induces NK cell-mediated apoptosis of neutrophils *in vitro* (13), and administration of purified galactosaminogalactan is immunosuppressive through the induction of IL-1RA production (14). Consistent with the pathogenic function of galactosaminogalactan, a galactosaminogalactan-deficient *A. fumigatus* mutant was found to have attenuated virulence in a mouse model of invasive aspergillosis (9,10). Galactosaminogalactan synthesis has been reported in other *Aspergillus* species, including *A. parasiticus* (15), *A. niger* (16), and *A. nidulans* (17), although the quantity and composition of

galactosaminogalactan from these species has not been compared. In light of the important role that galactosaminogalactan plays in the virulence of *A. fumigatus*, we investigated whether differences in galactosaminogalactan production or composition might contribute to the spectrum of virulence observed among *Aspergillus* species.

MATERIALS AND METHODS

Fungal Strains and Growth Conditions

A. nidulans strain A26 (Fungal Genetics Stock Center, Kansas City, MO, USA) was used as the parent wild-type strain for all molecular manipulations. Other strains used in this study include *A. fumigatus* strain Af293 (a generous gift from P. Magee, University of Minnesota, St. Paul, MN, USA), the *A. fumigatus* Δ*uge3* mutant (10), Δ*stuA* mutant (18) and clinical isolates of *A. flavus* and *A. niger* (obtained from the McGill University Health Center, Montreal, QC, Canada). Unless otherwise noted, *A. fumigatus* strains and *A. flavus* were maintained on YPD agar (Fisher Scientific), *A. niger* on potato-dextrose agar (Fisher Scientific), and *A. nidulans* strains on Aspergillus minimum medium agar (25) at 37°C. For growth in liquid medium, Brian medium (23) and phenol-free RPMI 1640 (Wisent) were used as indicated. All growth media for *A. nidulans* strains were supplemented with biotin (Fisher Scientific).

Molecular and Genetic Manipulations

Heterologous overexpression of *uge3* and endogenous overexpression of *ugeB* in *A. nidulans* was performed as previously described (19,20), with minor modifications. The open reading frames of *uge3* from pUge3-OX plasmid or *ugeB* from *A. nidulans* genomic DNA were amplified by PCR (Table1). The resulting PCR fragments with appropriate insertion sites were cloned downstream of the constitutive *gpdA* promoter in the previously constructed pGFP-Phleo plasmid (19,21) by excising the *gfp* ORF with restriction enzyme digest and replacing with either *uge3* or *ugeB* ORF using In-Fusion® HD Cloning Plus (Clontech, Inc.), following manufacturer's instructions, to yield plasmids

pUge3-OX or pUgeB-OX, respectively. DNA fragments were amplified by PCR using Uge3-gfp-F and Uge3-gfp-R for *uge3*, and UgeB-gfp-F and UgeB-gfp-R for *ugeB* (Table 1). *A. nidulans* A26 strain was used as the parental strain and transformed by spheroplasting as previously described (22). Gene expression was confirmed by real time RT-PCR (Table 1).

Mutant Characterization

Purification and analysis of galactosaminogalactan was performed as previously described (11). Hyphae were grown for 72h, and galactosaminogalactan was precipitated from culture supernatants with 2.5 volume ethanol. The GalNAc-rich insoluble fraction was collected by filtration on Nylon membrane and washed with 60% ethanol. Composition of galactosaminogalactan was determined by gas chromatography after derivatization to its alditol acetate, and quantified by hexose and hexosamine assays (9). Conidial size was measured by FACS analysis (BD LSR Fortessa) of conidia of each strain fixed with 4% paraformaldehyde. Biofilm adherence was assessed on 24h grown hyphae of indicated strains by rigorously washing and staining with 0.1% crystal violet for visualization, as previously described (10). For scanning electron microscopy (SEM), FITC-SBA lectin binding, and Fc-dectin-1 binding experiments, fungi were grown for the indicated times in phenol red-free RPMI at 37°C, 5% CO_2 incubation, fixed with either 2.5% gluteraldehyde or 4% PFA, and further processed (10). Briefly, for SEM, samples were sequentially dehydrated in ethanol, critical point dried, coated in Au-Pd, and imaged at 20,000X magnification (Hitachi, Inc). For FITC-SBA lectin or Fc-dectin-1 binding, samples were immunostained with FITC-SBA (Vector Labs, Inc) or Fc-dectin-1 (a generous gift from Dr. G.D. Brown, Aberdeen, UK) followed by fluorochrome labeled anti-human IgG, FCγ fragment specific (Jackson ImmunoResearch), respectively. For the caspase-3 activity assay, 9h grown hyphae of indicated strain were co-incubated with mouse bone marrow-derived macrophages and caspase-3 activity was measured using EnzChek® Caspase-3 Assay Kit (Invitrogen), as previously described (11).

Virulence Studies

Female Balb/c mice, 6-8 weeks old, were immunosuppressed with 5 doses of 10 mg of cortisone acetate per mouse (Sigma-Aldrich), administered intraperitoneally every other

day starting on day -4 relative to infection (23). For the survival study, groups of 20 mice were infected with either *A. nidulans* or An-Uge3, and groups of 10 mice were infected with either *A. fumigatus* or An-UgeB, intranasally, with $1x10^6$ conidia. A group of 8 mice was included as sham infection control. To prevent bacterial infection, enrofloxacin was added to the drinking water. For the fungal burden study, groups of 10 mice were infected with the respective strains or sham infection control. Mice were monitored for a period of 2 weeks for signs of illness and moribund animals were euthanized or were sacrificed 4 days after infection for determination of fungal burden and inflammation markers. At the time of sacrifice, BAL fluid was collected and then lungs were harvested and homogenized (10).

For histopathology studies, lungs from a subset of mice were fixed in formalin. Thin sections were stained with hematoxylin and eosin or periodic acid Schiff to visualize fungal elements. Caspase-3 immunohistopathology was performed using an anti-capsase-3 antibody. BAL fluid was analyzed for host immune cell recruitment markers by flow cytometry using anti-CD45 (BD Biosciences) for detection of total leukocytes and anti-Ly6G (BD Biosciences) for neutrophils. Analysis was performed using a FACS Canto System (BD Biosciences). Pulmonary fungal burden was measured by determining relative galactomannan levels by EIA (BioRad) using an internal standard, or fungal DNA content by real-time RT-PCR as previously described (10). Myeloperoxidase activity (Hycult Biotech), or cytokine production of TNF-α (eBiosciences, Inc.), IL-1RA (R&D Systems, Inc), or IL-1β (Sigma, Inc.) were measured by commercial EIA following the manufacturer's instructions. All procedures involving mice were approved by the Los Angeles Biomedical Research Institute Animal Use and Care Committee, and followed the National Institutes of Health guidelines for animal housing and care.

For virulence studies with neutropenic mice, Balb/c mice, 5-6 weeks old, were immunosuppressed with 250 mg/kg of cortisone acetate (Sigma-Aldrich) by subcutaneous injection on days -2 and +3, and intraperitoneally with 250 mg/kg of cyclophosphamide (Western Medical Supply, Inc.) on day -2 and 200 mg/kg on day +3 per mouse, relative to conidial challenge, as previously described (24).

For virulence studies in an NADPH oxidase-deficient host, *gp91^{phox}* deficient mice lacking functional NADPH oxidase were infected intranasally with $1x10^6$ conidia from either *A. nidulans* wild-type or An-uge3 strains. Survival was monitored over the course of

the infection. 8-12 week old male and female mice were used, in duplicate experiments for a total of 17 mice per infection group. All procedures involving mice were approved by the Montana State University Animal Care Facility.

Morphometric Analysis

Histopatholgy sections of lungs were stained with periodic acid-Shiff (PAS) stain, digitally scanned at up to 400X magnification. Fungal lesions were identified by visual inspection and lesion size and distance from airways were calculated using Spectrum® (Apio, Inc) software. To ensure unbiased data collection, readers were blinded to strain identity.

Susceptibility Experiments

Indicated fungal strains were grown at 37°C, 5% CO_2 for 6-9 h in Brian medium in a 96-well plate (Corning, Inc.). Dilution of hydrogen peroxide (Bioshop, Inc.) or cationic peptide LL-37 (Sigma, Inc.) were made in Brian medium for the indicated concentrations, and added to the germinating hyphae, with appropriate no treatment controls receiving only Brian medium. The hyphae were exposed to hydrogen peroxide for 12 hours, washed in PBS, and metabolic activity measured by reduction of tetrazolium reagent XTT (Bioshop, Inc.), as previously described (25).

Neutrophil Damage Experiments

Indicated fungal strains were grown at 37°C, 5% CO_2 for 6-9 h in either Brian medium or Iscove's Modified Dulbecco Medium (IMDM) (Life Technologies, Inc.) in a 24-well tissue culture treated plate (Corning, Inc.). HL-60 cells were grown in IMDM, and differentiated in DMSO (Bioshop, Inc.) and retinoic acid (Sigma, Inc.) as previously described (26). Differentiation was verified by flow cytometry (data not shown). Three-day differentiated HL-60 cells were added to wells with young hyphae and co-incubated for 12 h. Prior to co-incubation, differentiated HL-60 cells were primed in 100 nM fMLP (Sigma, Inc.) for 10 minutes prior to co-incubation. After 12 h co-incubation, wells were washed with PBS, and remaining differentiated HL-60 were lysed with distilled endotoxin-free water (Fisher, Inc.). To quantify killing, metabolic activity of fungi was measured by reduction of tetrazolium reagent XTT (Bioshop, Inc.), as previously described (25). To inhibit NADPH-

oxidase activity, differentiated HL-60 cells were incubated with 25 μM diphenyleneiodonium (Sigma, Inc.) for 1 h and washed twice in medium prior to co-incubation with fungi. For primary human neutrophil killing experiments, blood samples obtained from healthy donors were purified to obtain polymorphonuclear cells (PMN), as previously described (27). Briefly, white blood was separated using Ficoll gradation, followed by dextran sedimentation, and red blood cell lysis. The purified pellet containing PMNs was resuspended in the appropriate concentration. PMN hyphal killing assay was performed under the same experimental conditions as described above for hyphal killing assays using differentiated HL-60. To assess non-oxidative hyphal killing, 6-9 h grown young hyphae of indicated strains were incubated with PMN lysates from healthy human donors for 12 h, followed by XTT reduction assay, as previously described (25). PMN lysates were prepared by subjecting the cells through a freeze-thaw cycle, re-suspending in Brian medium pH 5.4 supplemented with 2% protease-free bovine serum albumin (Bioshop, Inc.), vigorously shaken by vortex for 60 seconds, spun by centrifugation at 17000 x *g* for 10 minutes, and supernatant were collected. The multiplicity of infection used for all assays, including PMN lysates, was 1:300 conidia to HL-60 cells or 1:100 conidia to PMN cell.

Statistical Analysis

For all mouse lung data analysis, unless indicated on the legend, the Krustal-Wallis rank analysis was applied for more than two group comparisons. For non-parametric pairwise comparison, or if significance result was observed in the Krustal-Wallis rank analysis, the Mann-Whitney U test was applied. For all other data analysis, statistical significance was determined by applying one-way ANOVA, partitioned with pairwise comparison, and Bonferroni correction applied where applicable. For pairwise comparison, Student's t test was applied. All statistical analyses were performed either with Prism® (GraphPad, Inc.) or SAS® (SAS Institute, Inc.) with significance determined at $p < 0.05$. For all statistical analysis, n.s. denotes statistically no significant differences found.

RESULTS

A. fumigatus galactosaminogalactan contains higher levels of GalNAc than galactosaminogalactan produced by other *Aspergillus* species.

To determine if different species of *Aspergillus* produce different levels of secreted galactosaminogalactan, we investigated two medically relevant species, *A. fumigatus* as well as *A. flavus*, which is the second most common *Aspergillus* isolate in IA patients (28). We also studied the less pathogenic *A. niger* and *A. nidulans*. When these organisms were grown under galactosaminogalactan-inducing conditions, there was no significant difference in the amount of galactosaminogalactan that was secreted into the medium by the different species (Figure S1A). However, scanning electron microscopy (SEM) of hyphae from each of these *Aspergillus* species demonstrated dramatic differences in the amount of galactosaminogalactan-associated decorations on the cell wall of hyphae (Figure 1A) (10). *A. fumigatus* displayed abundant cell wall-bound decorations, while the other species more closely resembled the previously described *A. fumigatus* Δ*stuA* mutant, which produces very low levels of galactosaminogalactan (Figure 1A) (10). To confirm that the alterations in cell wall morphology reflected changes in the amount of cell wall-associated galactosaminogalactan, the amount of cell wall bound galactosaminogalactan was examined by staining hyphae of each of the *Aspergillus* species with the GalNAc specific lectin, soybean agglutinin (SBA) (10,11,29,30). Staining of cell wall bound GalNAc by SBA was strongest with *A. fumigatus*, followed by *A. flavus,* while *A. niger* and *A. nidulans* exhibited minimal SBA binding (Figure 1B). Interestingly, the amount of GalNAc-rich galactosaminogalactan and cell wall decorations produced by these species paralleled their frequency of recovery from patients with invasive aspergillosis. Collectively, these results suggest that *Aspergillus* species exhibit significant differences in the amount of cell wall-associated galactosaminogalactan. Moreover, these differences correlate with the reported intrinsic virulence of these species. In light of these findings, *A. fumigatus* and *A. nidulans* were selected for further study as representatives of highly pathogenic and minimally pathogenic species of *Aspergillus* species that display significant differences in cell wall-bound galactosaminogalactan.

A. nidulans produces GalNAc-poor galactosaminogalactan and has impaired biofilm formation as compared with *A. fumigatus*

To test if the degree of cell wall-bound galactosaminogalactan could reflect differences in the composition of galactosaminogalactan produced by *A. fumigatus* and *A. nidulans*, monosaccharide analysis of secreted galactosaminogalactan from both species was performed by gas chromatography. Secreted galactosaminogalactan produced by *A. nidulans* contained significantly less GalNAc and more galactose as compared with *A. fumigatus* galactosaminogalactan (Figure 2A).

To test if the production of lower GalNAc-containing galactosaminogalactan by *A. nidulans* could affect the known properties of galactosaminogalactan, *A. nidulans* was compared with *A. fumigatus* with respect to its ability to form adherent biofilm and mask hyphal β-glucan exposure. *A. nidulans* was found to form less adherent biofilms (Figure 2B) as compared with *A. fumigatus*. In contrast, β-glucan binding by recombinant Fc-dectin-1 was not different between these two species (Figure 2C). These observations suggest that the degree of cell wall binding of galactosaminogalactan and the ability of galactosaminogalactan to mediate biofilm formation are dependent on the GalNAc content of galactosaminogalactan. In contrast, β-glucan masking may be GalNAc-independent, or require a lower amount of this hexosamine.

A. nidulans produces galactosaminogalactan with a lower GalNAc content due to low levels of expression of the glucose epimerase UgeB

The synthesis of the GalNAc component of galactosaminogalactan in *A. fumigatus* results from the activity of the UDP-glucose 4-epimerase, Uge3 (10,11). A search of the *A. nidulans* genome identified *ugeB* as the gene whose product had the closest homology to *A. fumigatus* Uge3 (85% amino acid identity). As with the *A. fumigatus uge3* gene (10), deletion of *ugeB* in *A. nidulans* resulted in a strain whose hyphae lacked detectable GalNAc by SBA staining (Figure S1B), suggesting that Uge3 and UgeB share the same function. Consistent with a previous report that *ugeB* expression is extremely low in *A. nidulans* (31), real-time RT-PCR demonstrated that the expression of *ugeB* in *A. nidulans* was significantly lower than the expression of *uge3* in *A. fumigatus* (Figure 2D), suggesting that the lower

GalNAc content of galactosaminogalactan produced by *A. nidulans* may be due to the lower expression levels of *ugeB*.

Overexpression of *uge3* or *ugeB* in *A. nidulans* increases the GalNAc content and cell wall binding of galactosaminogalactan, as well as enhances biofilm formation

To test the hypothesis that the low GalNAc content of *A. nidulans* galactosaminogalactan results from low expression of *ugeB*, strains of *A. nidulans* were constructed in which the *A. fumigatus uge3* or *A. nidulans ugeB* genes were expressed under the constitutively active *gpdA* promoter to produce strains An-Uge3 and An-UgeB, respectively (Figure 3A). Overexpression of either *uge3* or *ugeB* had no significant effect on total secreted galactosaminogalactan production (Figure S1C). However, increased expression of either gene resulted in an increase in the GalNAc content of secreted galactosaminogalactan (Figure 3B) to levels similar to that found in *A. fumigatus*. Even more dramatically, overexpression of either gene markedly increased the amount of cell wall bound galactosaminogalactan as detected by SBA lectin binding (Figure 3C) and SEM to levels indistinguishable from *A. fumigatus* (Figure 3D). These data suggest that the GalNAc content of galactosaminogalactan is important in determining the amount of galactosaminogalactan that binds to the hyphal cell wall.

Augmenting the GalNAc content of galactosaminogalactan in *A. nidulans* resulted in a marked increase in biofilm formation (Figure 3E), but had no effect on the size or germination of the conidia (Figure S1D-S1E) or hyphal growth rate (data not shown). Collectively, these data suggest that increasing GalNAc content of *A. nidulans* galactosaminogalactan by overexpressing either the native *A. nidulans ugeB* or *A. fumigatus uge3* gene resulted in a strain of *A. nidulans* that resembled *A. fumigatus* in vitro.

Increasing the GalNAc content of *A. nidulans* galactosaminogalactan increases the virulence of *A. nidulans*

To determine if the increase in cell wall bound GalNAc-rich galactosaminogalactan resulting from overexpression of either *uge3* or *ugeB* could result in increased virulence, corticosteroid-treated mice were infected intranasally with wild-type *A. fumigatus,* wild-type *A. nidulans*, An-Uge3, or An-UgeB. As expected, mice infected with wild-type *A.*

nidulans had a longer median survival than those infected with *A. fumigatus* (Figure 4A). Also, 25% of mice infected with *A. nidulans* survived to the end of the experiment, whereas none of the animals infected with *A. fumigatus* survived. In contrast, the median survival and overall mortality of mice infected with An-Uge3 or An-UgeB were similar to mice infected with *A. fumigatus*. Thus, increasing the GalNAc content of galactosaminogalactan in *A. nidulans* by increasing expression of a heterologous or endogenous GalNAc epimerase significantly enhanced the virulence of this minimally pathogenic *Aspergillus* species.

Increasing the GalNAc content of *A. nidulans* galactosaminogalactan resulted in increased tissue invasion

To examine the mechanisms underlying the increased virulence seen with overexpression of *uge3* or *ugeB* in *A. nidulans*, corticosteroid-treated mice were infected with *A. nidulans* or An-Uge3 and their lungs examined after 4 days of infection. The pulmonary fungal burden of mice infected with An-Uge3 was significantly higher than in mice infected with *A. nidulans*, as measured by galactomannan content, quantitative morphometric analysis of histopathology sections, or *Aspergillus* DNA content (Figures 4B, 4C, S1F). Histopathologic examination of lungs from these mice revealed striking differences in the degree of pulmonary invasion between *A. nidulans* (Figure 4D, top) and An-Uge3 (Figure 4D, bottom). Hyphae of *A. nidulans* were largely restricted to the airway lumen with minimal penetration into the pulmonary parenchyma. In contrast, hyphae of the An-Uge3 strain were markedly more invasive and penetrated significantly deeper into pulmonary tissues. Quantitative morphometric analysis of histopathology sections confirmed that hyphae of the An-Uge3 strain invaded much further from the airways than did the parent *A. nidulans* strain (Figure 4E). Importantly, the magnitude of tissue invasion by the An-Uge3 strain was much greater than the increase in total lesion size, 5-fold (Figure 4E) vs. 1.5-fold (figure 4C), respectively. In fact, in mice infected with the An-Uge3 overexpression strain, only 2 out of 51 lesions remained confined within the airway lumen compared to 18 out of 51 lesions from mice infected with *A. nidulans*. These results suggest that increasing the GalNAc content of galactosaminogalactan in *A. nidulans* increases the adherence and invasion of hyphae within pulmonary tissues.

Galactosaminogalactan has been reported to modulate immune responses through the masking of hyphal PAMPs, the direct induction of neutrophil apoptosis and the induction of IL-1RA production (9,10,14). Multiple parameters of the host inflammatory response were therefore analyzed in lungs of infected mice. No significant differences were observed between mice infected with *A. nidulans* or the An-Uge3 strain with respect to total leukocyte or neutrophil numbers in the bronchoalveolar lavage (Figure S2A-S2B); or total lung myeloperoxidase, TNF-α, IL-1β, and IL-1RA levels (Figure S2C-S2F). Further, histopathological examination of fungal lesions did not identify any differences in nuclear fragmentation surrounding fungal lesions suggestive of differences in apoptosis (Figure S3A), and immunohistochemical staining for caspase-3 did not demonstrate differences between lesions resulting from these two strains (Figure S3B). Taken together, these results suggest that changing the GalNAc content of galactosaminogalactan in *A. nidulans* does not alter virulence by altering the host inflammatory response.

Increasing cell wall associated galactosaminogalactan enhances resistance to neutrophil killing in vivo and in vitro.

Increasing the GalNAc content of galactosaminogalactan in *A. nidulans* markedly increased the amount of polysaccharide bound to the hyphal surface and was associated with increased fungal growth and invasion *in vivo*. In light of these findings, we hypothesized that cell wall-associated galactosaminogalactan might function analogously to a capsule and mediate resistance to neutrophil damage during infection. We therefore tested the virulence of *A. nidulans* and the An-Uge3 strain in a neutropenic model of invasive aspergillosis (Figure 5A). In the absence of neutrophils, no difference in virulence was observed between the strains, suggesting that the differences in virulence between these strains may reflect differences in susceptibility to neutrophil killing. We therefore examined the ability of differentiated HL-60 cells (a human granulocytic cell line) to damage hyphae of *A. fumigatus, A. nidulans* and the An-Uge3 strain. Consistent with our observations *in vivo*, *A. nidulans* was more susceptible to damage by HL-60 cells than *A. fumigatus* or the An-Uge3 strain (Figure 5B). Treating HL-60 cells with the NADPH oxidase inhibitor DPI abrogated this difference between strains (Figure 5B)

Increasing cell wall associated galactosaminogalactan enhances resistance to antimicrobial peptides.

Treating HL-60 cells with the NADPH oxidase inhibitor DPI abrogated this difference between strains (Figure 5B) suggesting that the higher levels of cell wall-bound GalNAc-rich galactosaminogalactan produced by both *A. fumigatus* and the An-Uge3 strain enhances the resistance to NADPH oxidase-dependent granulocyte damage. This enhanced susceptibility of *A. nidulans* compared to *A. fumigatus* and An-Uge3 to NADPH oxidase dependent-injury was also observed with primary human neutrophils (PMN) (Figure 5C). NADPH oxidase mediates production of toxic reactive oxygen species that can kill hyphae. Surprisingly however, *A. fumigatus* hyphae were found to be more susceptible to oxidative killing than *A. nidulans* (Figure 5D). These data suggest that cell wall associated galactosaminogalactan does not mediate resistance to oxidative killing by neutrophils.

In addition to mediating the production of toxic reactive oxygen species that can directly injure hyphae, NADPH oxidase activity has been reported to mediate antifungal host defense through inducing the release of antimicrobial peptides contained within neutrophil granules (32,33). Thus, to determine if increasing the amount of cell wall-bound galactosaminogalactan enhances the resistance to these non-oxidative neutrophil killing, the resistance of each of the *Aspergillus* strains to killing by primary human neutrophil lysates was determined (Figure 6A). As with live neutrophils, wild-type *A. nidulans* was more susceptible to damage caused by neutrophil lysates as compared to *A. fumigatus*. Similarly, overexpression of *uge3* enhanced the resistance of *A. nidulans* to neutrophil lysate damage to a level similar to that seen with *A. fumigatus*. To confirm if enhanced resistance to neutrophil lysates reflected resistance to neutrophil antimicrobial peptides, the susceptibility to the neutrophil antimicrobial peptide LL-37 (cathelicidin) was determined. Wild-type *A. fumigatus* and An-uge3 were found to be more resistant to killing by the antimicrobial peptide cathelicidin (LL-37) (Figure 6B). Collectively these data suggest that cell wall-bound GalNAc-rich galactosaminogalactan may enhance resistance to NADPH oxidase dependent neutrophil killing through increasing resistance to neutrophil granule contents rather than enhancing resistance to toxic reactive oxygen species.

To test if GalNAc-rich galactosaminogalactan mediated resistance to NADPH oxidase-dependent killing underlies the enhanced virulence of the An-Uge3 strain in vivo,

we compared the virulence of the overexpression strain to that of wild-type *A. nidulans* in *gp91^{phox}* deficient mice lacking functional NADPH oxidase. In contrast to its increased virulence in corticosteroid-treated BALB/c mice, the An-Uge3 strain was not more virulent than the wild-type *A. nidulans* parent strain in mice deficient in *gp91^{phox}* (Figure 6C). Collectively these data strongly suggest that the expression of GalNAc-rich galactosaminogalactan by *Aspergillus* mediates virulence through enhancing resistance of hyphae to NADPH oxidase-dependent neutrophil injury.

DISCUSSION

The unique virulence of *A. fumigatus* within the *Aspergillus* group is poorly understood. Most factors that influence *A. fumigatus* virulence have not been studied in other *Aspergillus* species. This study provides the first example of a virulence factor of *A. fumigatus* that is able to mediate enhanced virulence when expressed in a less pathogenic *Aspergillus* species. These data strongly suggest that the production of GalNAc-rich galactosaminogalactan contributes to the unique virulence of *A. fumigatus*, though it is likely that other factors unique to *A. fumigatus* can also contribute to virulence, perhaps under different conditions or in different hosts.

A direct correlation was observed between the amount of hyphae-associated galactosaminogalactan and the frequency of recovery of a given species from cases of invasive aspergillosis. Although this observation suggests that galactosaminogalactan plays an important role in the differences in intrinsic virulence in species other than *A. fumigatus* and *A. nidulans*, more work is needed to confirm this hypothesis. A larger screen of multiple clinical and environmental isolates will be required to establish the association between GalNAc-rich, hyphae associated galactosaminogalactan and intrinsic virulence. In addition, further mechanistic studies modulating galactosaminogalactan composition in these other species are required to confirm that the observations made in *A. nidulans* are applicable to other species that produce GalNAc-poor galactosaminogalactan.

It has been suggested that the larger spore size of *A. nidulans* leads to impaired penetration of this organism into the lower airways and more efficient clearance by the mucociliary elevator. However, the results of this study suggest that the size of *A. nidulans* conidia is not a major determinant of virulence. In corticosteroid-treated mice, increasing cell wall-bound GalNAc-rich galactosaminogalactan was sufficient to render *A. nidulans* as virulent as *A. fumigatus*, despite the fact that conidia of the An-Uge3 strain remained larger than those of *A. fumigatus*. These data suggest that the larger conidial size of *A. nidulans* is not a significant barrier to establishing disease. This observation is also consistent with clinical reports that while *A. nidulans* is a rare cause of invasive aspergillosis in general, it is a common cause of invasive aspergillosis in patients with CGD (4,6). Indeed, the results of these studies suggest that the low level of cell wall-bound GalNAc-rich galactosaminogalactan produced by *A. nidulans* may contribute to the unexplained observation that this species is only a common cause of invasive aspergillosis in patients with CGD. In patients with functional NADPH oxidase, *A. fumigatus* is more resistant than *A. nidulans* to NADPH-oxidase dependent killing by neutrophils due to the production of higher amounts of cell wall bound galactosaminogalactan. Thus, in these patients *A. fumigatus* is more pathogenic and is isolated more frequently. In contrast, in patients with CGD, the absence of functional NADPH oxidase negates this difference between these two *Aspergillus* species. Thus, in patients with CGD, *A. nidulans* is no longer disadvantaged relative to *A. fumigatus*, and is therefore recovered more frequently.

Galactosaminogalactan has been reported to mediate a number of functions *in vitro* that could influence virulence, including host cell adherence, the modulation of host immune responses and the induction of neutrophil apoptosis (10,13,14,34). While determining the contribution of each of these mechanisms to virulence is challenging, the present study provides some insights into this question. First, β-glucan masking was not different between *A. fumigatus*, *A. nidulans* wild-type and the *A. nidulans* overexpression strains, suggesting that β-glucan masking did not play a role in mediating the significant difference in virulence between these strains.. Further, increasing cell wall bound galactosaminogalactan increased *Aspergillus* virulence in the absence of any detectable difference in inflammation or immune response, including pulmonary IL-1RA levels and the induction of neutrophil apoptosis which have been previously reported to be induced

by soluble galactosaminogalactan (13,14). The failure to observe changes in these responses in the present study likely reflects the fact that these effects of galactosaminogalactan were seen with the administration of soluble galactosaminogalactan to mice. In the experiments reported here, the amount of total secreted galactosaminogalactan was not different between the three strains of *Aspergillus* and only the quantity of cell wall bound galactosaminogalactan differed between these strains.

Multiple lines of evidence suggest that increased cell wall associated galactosaminogalactan production augmented both resistance to neutrophil mediated injury and virulence in mice, but only in the presence of functional NADPH oxidase. These data suggest that the ability of cell wall bound galactosaminogalactan to mediate resistance to NADPH oxidase-dependent neutrophil killing is an important mechanism by which galactosaminogalactan promotes virulence *in vivo*.

The observation that cell wall bound galactosaminogalactan enhances resistance to both neutrophil lysates and live neutrophils suggests that galactosaminogalactan-mediated resistance to NADPH oxidase-dependent killing does not occur through the induction of neutrophil apoptosis or as a consequence of the production of toxic reactive oxygen species. These data are in agreement with a previous report suggesting that killing of *A. nidulans* by human neutrophils occurs predominately via a non-oxidative mechanism (35). Collectively, these data are consistent with a model in which cell wall bound GalNAc-rich galactosaminogalactan functions as an extracellular capsule to enhance resistance to neutrophil granule contents such as antimicrobial, analogous to bacterial capsular exopolysaccharide (36-38).

Why has *A. fumigatus* evolved this change in the composition of an exopolysaccharide and the resulting increase in cell wall associated galactosaminogalactan? As *A. fumigatus* is an environmental organism and only an incidental opportunistic pathogen of immunocompromised hosts, we speculate that selection for GalNAc-rich galactosaminogalactan was mediated through environmental pressures that are unique to *A. fumigatus*. One hypothesis is that the production of a capsule-like hyphal sheath could offer protection against competing microorganisms in the complex microbial environment of decomposing organic matter, where *A. fumigatus* is commonly found. Studies comparing the production and composition of

galactosaminogalactan in strains of different environmental origin may be helpful in shedding light on this question.

Modulating the composition of a single exopolysaccharide significantly enhanced the virulence of a relatively non-pathogenic *Aspergillus* species through enhancing resistance to neutrophil killing. This study highlights the importance of galactosaminogalactan as a key virulence factor of *A. fumigatus* and suggests that targeting this exopolysaccharide may be an effective antifungal approach.

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FIGURE LEGENDS

Figure 1. Production of GalNAc-rich galactosaminogalactan correlates with reported virulence of *Aspergillus spp.*

(A) Scanning electron micrograph of hyphae of indicated species at 20,000X magnification. Arrows indicate surface decorations associated with cell wall bound galactosaminogalactan.

(B) Cell wall GalNAc staining with FITC-conjugated soybean agglutinin (SBA). SBA binding to mature hyphal mats of the indicated species was quantified by fluorometry.

For all panels: data are represented as mean +/- SEM. * indicates a significant difference between *A. fumigatus* and other species, $p<0.05$ by ANOVA.

Figure 2. *A. nidulans* produces GalNAc-poor galactosaminogalactan which is associated with non-adherence

(A) Galactose and GalNAc content of secreted galactosaminogalactan from either *A. fumigatus* or *A. nidulans* as identified by gas chromatography.

(B) Formation of adherent biofilms on tissue culture-treated polystyrene plates by *A. fumigatus* and *A. nidulans*. After washing, biofilms were visualized by staining with 0.1% crystal violet.

(C) Detection of β-1,3-glucan exposure on the surface of hyphae by immunostaining with Fc-dectin-1 antibody.

(D) Relative expression of *ugeB* and *uge3* in *A. nidulans* and *A. fumigatus,* respectively, during growth in Brian medium as measured by real-time RT-PCR. *tef1* from respective species was used as reference gene. PCR primer efficiency was verified, and was not different between species (Figure S1F).

For all panels: data are represented as mean +/- SEM. AfWT indicates *A. fumigatus*, and AnWT indicates *A. nidulans*. * indicates a significant difference between *A. fumigatus* and *A. nidulans*, $p<0.05$ by ANOVA.

Figure 3. Overexpression of *uge3* or *ugeB* in *A. nidulans* increases the GalNAc content of galactosaminogalactan and enhances the formation of adherent biofilms

(A) Relative expression of *uge3*

(B) in the An-Uge3 strain and *ugeB* in the An-UgeB strain compared to the expression level of *ugeB* in wild-type *A. nidulans* grown in Brian medium and as measured by real-time RT-PCR.

(B) GalNAc content of secreted galactosaminogalactan from the indicated strains as determined by gas chromatography.

(C) Cell wall GalNAc staining with FITC-conjugated soybean agglutinin (SBA). SBA binding to mature hyphal mats of the indicated strains was quantified by fluorometry.

(D) Scanning electron micrograph of hyphae of indicated species at 20,000X magnification. Arrows indicate surface decorations associated with cell wall bound galactosaminogalactan.

(E) Formation of adherent biofilms on tissue culture treated polystyrene plates by the indicated strains. After washing, biofilms were visualized by staining with 0.1% crystal violet.

For all panels: An-Uge3 indicates the *A. nidulans* overexpressing *uge3* strain*;* An-UgeB indicates the *A. nidulans* overexpressing *ugeB* strain*;* and AnWT indicates wild type *A. nidulans*.

Data are represented as mean +/- SEM and * indicates a significant difference between *A. nidulans*, and both overexpression strains, $p<0.05$ by ANOVA.

Figure 4. Overexpression of *uge3* in *A. nidulans* increases virulence with an associated increase in fungal burden and pulmonary tissue invasion

(A) Survival of corticosteroid treated Balb/c mice infected with the indicated conidial species and strains. N = 20 for *A. nidulans* or An-Uge3; N= 10 for *A. fumigatus* or An-UgeB; N = 8 for PBS sham infection. * indicates a significant difference in survival of *A. nidulans* compared with *A. fumigatus*, An-Uge3, and An-UgeB overexpression strains as determined by the Meir-Kaplan test.

(B) Pulmonary fungal burden measured by relative galactomannan content in the lungs of mice infected with the indicated strains, N = 10 for each strain

(C) Total fungal lesion size as determined by morphometric analysis of lung histopathology for the indicated strains.
(D) Pulmonary histopathology sections from mice infected with indicated strains and stained with PAS for visualization of fungi. The yellow dotted line indicates the limit of the airway used for morphometric analysis. White arrow indicates fungal elements outside the airway and invading into pulmonary tissues. Scale bar represents 100 μm (black) or 50 μm (white).
(E) Lesion invasion beyond the airway as determined by morphometric analysis of lung histopathology for the indicated strains.
For all panels: An-Uge3 indicates the *A. nidulans* overexpressing *uge3* strain; An-UgeB indicates the *A. nidulans* overexpressing *ugeB* strain; and AnWT indicates wild type *A. nidulans*.
For panel B, data are represented as median with interquartile ranges and * indicates a significant difference between *A. nidulans* and the An-Uge3 strain, $p<0.05$ by Mann-Whitney test.
For panels C and E, data are represented as mean +/- SEM and * indicates a significant difference between *A. nidulans* and the An-Uge3 strain, $p<0.05$ by ANOVA.
For morphometric analysis, 51 lesions from 4 mice were quantified for each strain.

Figure 5. Increasing cell wall associated galactosaminogalactan enhances resistance to neutrophil killing in vivo and to NADPH oxidase-dependent neutrophil killing in vitro.

(A) Survival of neutropenic mice infected with either *A. nidulans* or An-Uge3 conidia. N = 17 per infection group.
(B) Fungal killing measured by the level of metabolic activity determined by XTT reduction after 12 h co-incubation with differentiated HL-60 cells, and differentiated HL-60 cells pre-incubated with for 1 h in 25 μM diphenyleneiodonium (DPI) as NADPH oxidase inhibitor.
(C) Fungal killing measured by the level of metabolic activity determined by XTT reduction after 8 h co-incubation with human PMN, and human PMN pre-incubated with for 1 h in 25 μM diphenyleneiodonium (DPI) as NADPH oxidase inhibitor.

(D) Fungal killing measured by the level of metabolic activity determined by XTT reduction after 12 h co-incubation with 3.2 mM hydrogen peroxide.
For all panels: An-Uge3 indicates the *A. nidulans* overexpressing *uge3* strain; An-UgeB indicates the *A. nidulans* overexpressing *ugeB* strain; AnWT indicates wild type *A. nidulans*; and AfWT indicates wild type *A. fumigatus*.
For panels B-C, multiplicity of infection was 1:300 conidia to mammalian cells. All incubation was done at 37°C, 5% CO_2 in Brian medium, IMDM, or supplemented phenol-red free RPMI.
For panels B-D, data are represented as mean +/- SEM and * indicates a significant difference between *A. nidulans* and the An-Uge3 strain, $p<0.05$ by ANOVA.
For panel A, statistical analysis on survival was determined by the Meir-Kaplan test.

Figure 6. Increasing cell wall associated galactosaminogalactan enhances resistance to antimicrobial peptides.
(A) Fungal killing measured by the level of metabolic activity determined by XTT reduction after 12 h co-incubation with human PMN lysate.
(B) Fungal killing measured by the level of metabolic activity determined by XTT reduction after 12 h co-incubation with 32 μg/mL of LL-37.
(C) Survival of *gp91^{phox}* deficient mice lacking functional NADPH oxidase infected with either *A. nidulans* or An-Uge3 conidia. N = 17 per infection group.
For all panels: An-Uge3 indicates the *A. nidulans* overexpressing *uge3* strain; An-UgeB indicates the *A. nidulans* overexpressing *ugeB* strain; AnWT indicates wild type *A. nidulans*; and AfWT indicates wild type *A. fumigatus*.
For panels A and B, all incubation was done at 37°C, 5% CO_2 in Brian medium, IMDM, or supplemented phenol-red free RPMI. Data are represented as mean +/- SEM and * indicates a significant difference between *A. nidulans* and the An-Uge3 strain, $p<0.05$ by ANOVA.
For panel C, statistical analysis on survival was determined by the Meir-Kaplan test.

Figure S1. Additional characterization of mutant phenotypes

(A) Total secreted GAG normalized to biomass from culture supernatants of the indicated species grown in Brian medium.

(B) Cell wall GalNAc staining with FITC-conjugated soybean agglutinin (SBA). SBA binding to mature hyphal mats of the indicated strain was quantified by fluorometry.

(C) Total secreted GAG normalized to biomass from culture supernatants of the indicated species grown in Brian medium.

(D) Germination of A. nidulans wild-type or the An-Uge3 strain grown in Brian medium for the indicated time.

(E) Conidia size of the indicated strains quantified by flow cytometry determined by forward scatter detections. Color scheme is as follows: yellow is wild-type A. nidulans, cyan is the An-Uge3 strain, and red is the An-UgeB strain.

(F) Pulmonary Aspergillus DNA content from mouse infected with the indicated fungal strains. N=10 per group.

(G) Efficiency of primers for indicated genes relative to the expression of reference gene Tef1 in the respective Aspergillus species.

For all panels: Data are represented as mean +/- SEM and * indicates a significant difference between A. nidulans, and the An-Uge3 overexpression strain, $p<0.05$ by ANOVA.

Figure S2. Additional characterization of host response

(A-B) Leukocyte recruitment detected by FACS analysis of bronchoalveolar lavage fluid from mice infected with the indicated fungal strain using either (A) anti-CD45 for total leukocytes, or (B) anti-Lys6G for neutrophils.

(C) Pulmonary myeloperoxidase activity in mice infected with the indicated strains.

(D-F) Cytokine concentrations in lungs of mice infected with the indicated trains as determined by EIA (D) TNF-α, (E) IL-1β, and (F) IL-1RA.

For panels A-F: Data are represented as median with interquartile ranges and * indicates a significant difference between uninfected mice and mice infected with A. nidulans and the An-Uge3 overexpression strain, $p<0.05$ by Krustal-Wallis test.

Figure S3. Pulmonary histopathology to detect host apoptosis

(A-B) Pulmonary histopathology of mice infected with the indicated strains, stained with (A) hematoxylin & eosin or (B) immunostained with anti-caspase3.

No significant differences in nuclear fragmentation or caspase-3 detection were observed. Scale bar represents 50 μm (white) or 20 μm (black)

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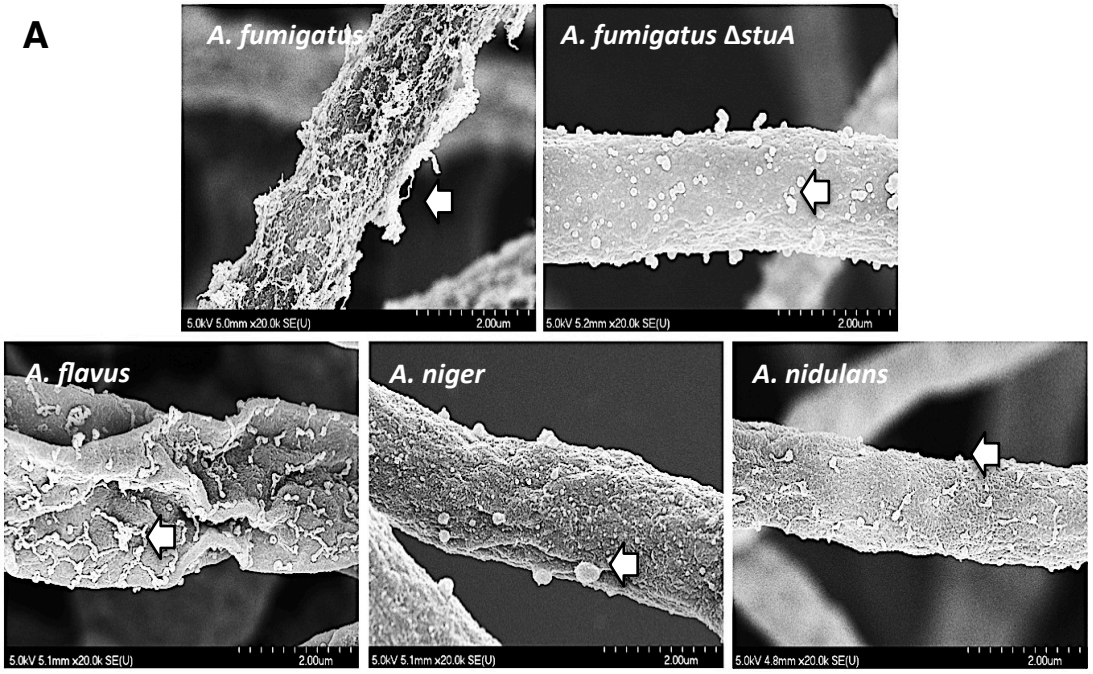
Table 1

Primer name	Target	Sequence
Uge3-gfp-F	pUge3-OX plasmid	CATCACCCCATGGATATCATGGACAGCACCAG
Uge3-gfp-R	pUge3-OX plasmid	CATCGCGGCCGCGATATCAGTAGATAACCCACT
UgeB-gfp-F	ugeB	CATCACCCCATGGATATCATGGATGGATAGTCCTCGAC
UgeB-gfp-R	ugeB	AAGTGGATCCACTAGTTCATAAAGTAACACCGCTAA
uge3-RT-FWD.F2	uge3	GCTGTTAGCCTCCCAGTACC
uge3-RT-REV.F2	uge3	GGACTTGGTCGTACCCCAT
AfTef1-RT-FWD	*A. fumigatus* tef1	CCATGTGTGTCGAGTCCTTC
AfTef1-RT-REV	*A. fumigatus* tef1	GAACGTACAGCAACAGTCTGG
AfgpdA-RT-FWD	*A. fumigatus* gpdA	GGCATTGTTGAGGGTCTCAT
AfgpdA-RT-REV	*A. fumigatus* gpdA	ACGTTGGAGGTAGGAACACG
ugeB-RT-FWD.Q2	ugeB	TTCAACTTGGGAACTGGGCG
ugeB-RT-REV.Q2	ugeB	CGATCTGGTAGCAACGGCAA
AnidTef1-RT-FWD	*A. nidulans* tef1	TCCAGACCCCCAAGTATGAG
AnidTef1-RT-REV	*A. nidulans* tef1	ACCGGAAGCGATGATAAGG
AngpdA-RT-FWD	*A. nidulans* gpdA	GGCATTGTTGAGGGTCTCAT
AngdpA-RT-REV	*A. nidulans* gpdA	ACGTTGGAGGTAGGAACACG

Figure 1


A
A. fumigatus
A. fumigatus ΔstuA
A. flavus
A. niger
A. nidulans
5.0kV 5.0mm x20.0k SE(U)
2.00um
5.0kV 5.2mm x20.0k SE(U)
2.00um
5.0kV 5.1mm x20.0k SE(U)
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2.00um

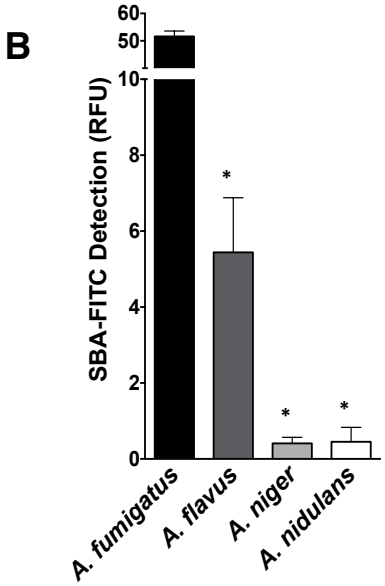

B
SBA-FITC Detection (RFU)
60
50
10
8
6
4
2
0
*
*
*
A. fumigatus
A. flavus
A. niger
A. nidulans

Figure 2

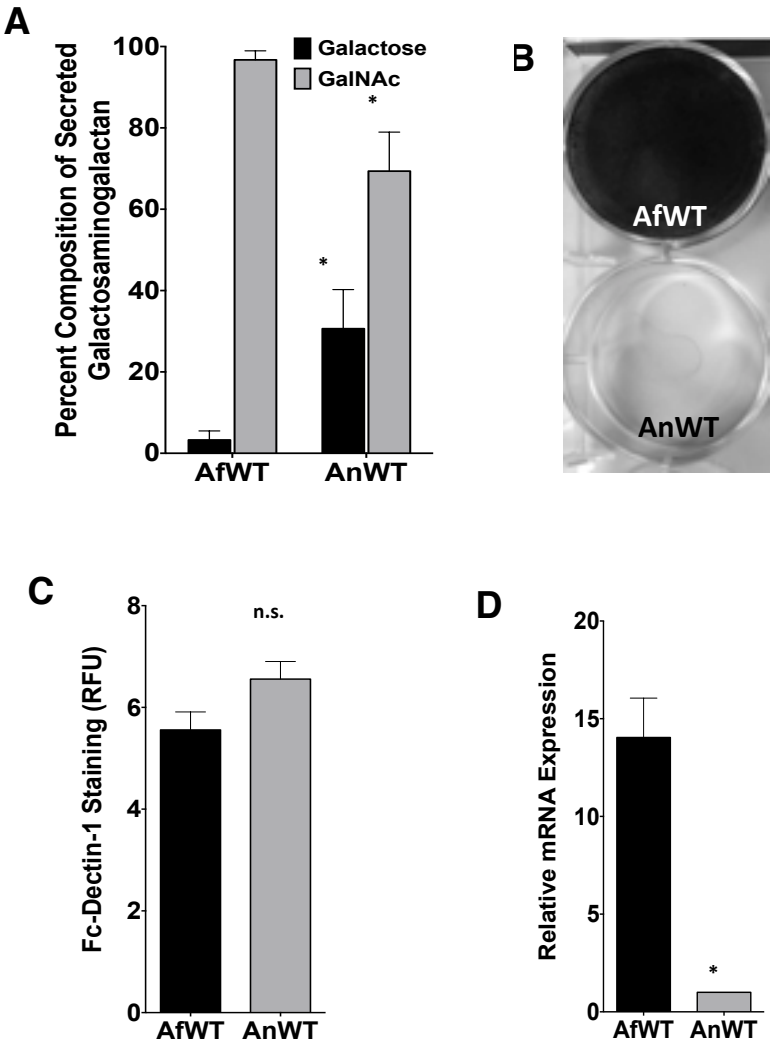
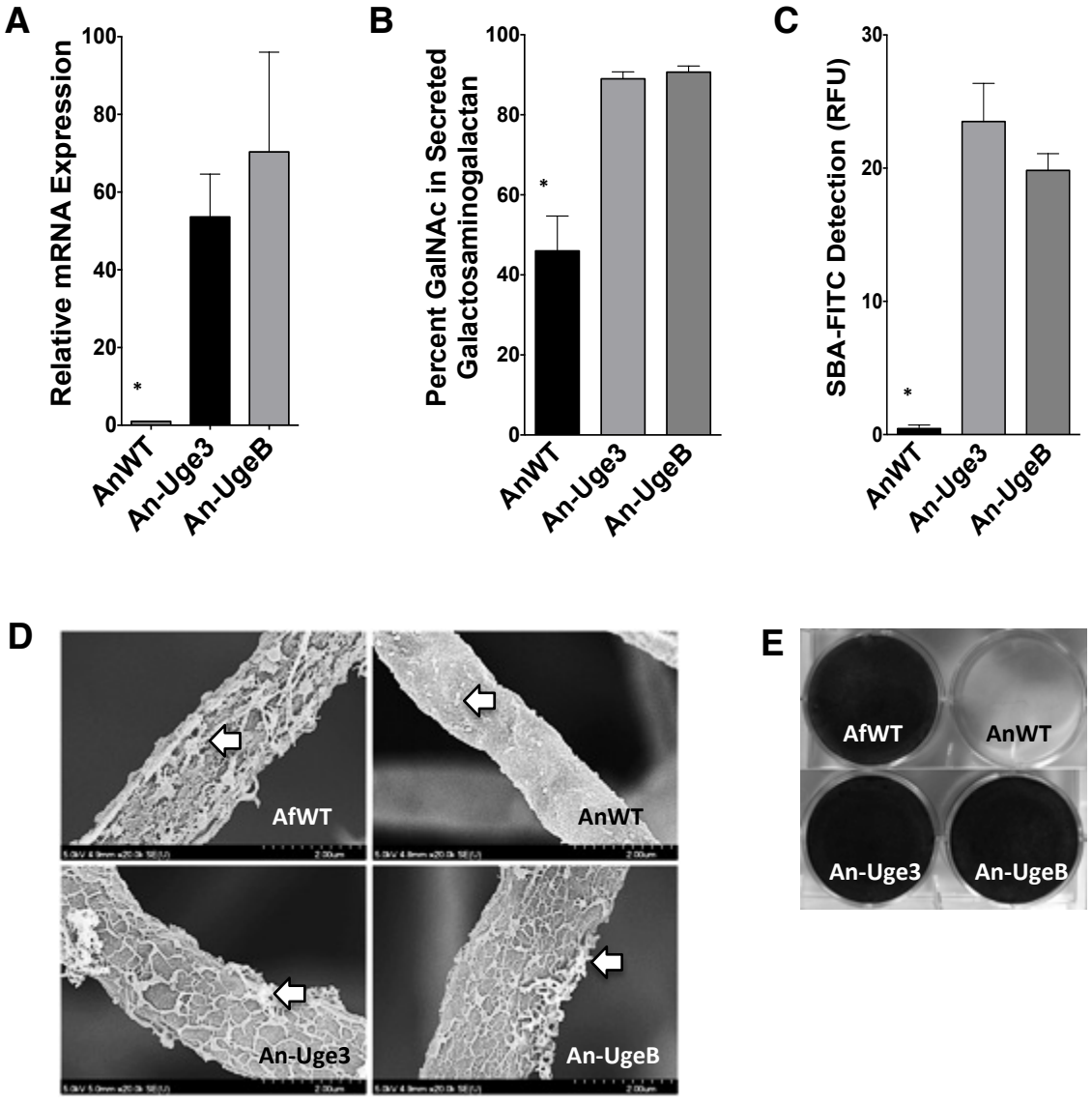

A
Percent Composition of Secreted Galactosaminogalactan
100
80
60
40
20
0
Galactose
GalNAc
*
*
AfWT
AnWT
B
AfWT
AnWT
C
Fc-Dectin-1 Staining (RFU)
8
6
4
2
0
n.s.
AfWT
AnWT
D
Relative mRNA Expression
20
15
10
5
0
*
AfWT
AnWT

Figure 3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Relative mRNA Expression
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80
60
40
20
0
*
AnWT
An-Uge3
An-UgeB
B
Percent GalNAc in Secreted Galactosaminogalactan
100
80
60
40
20
0
*
AnWT
An-Uge3
An-UgeB
C
SBA-FITC Detection (RFU)
30
20
10
0
*
AnWT
An-Uge3
An-UgeB
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AnWT
An-Uge3
An-UgeB
E
AfWT
AnWT
An-Uge3
An-UgeB

Figure 4

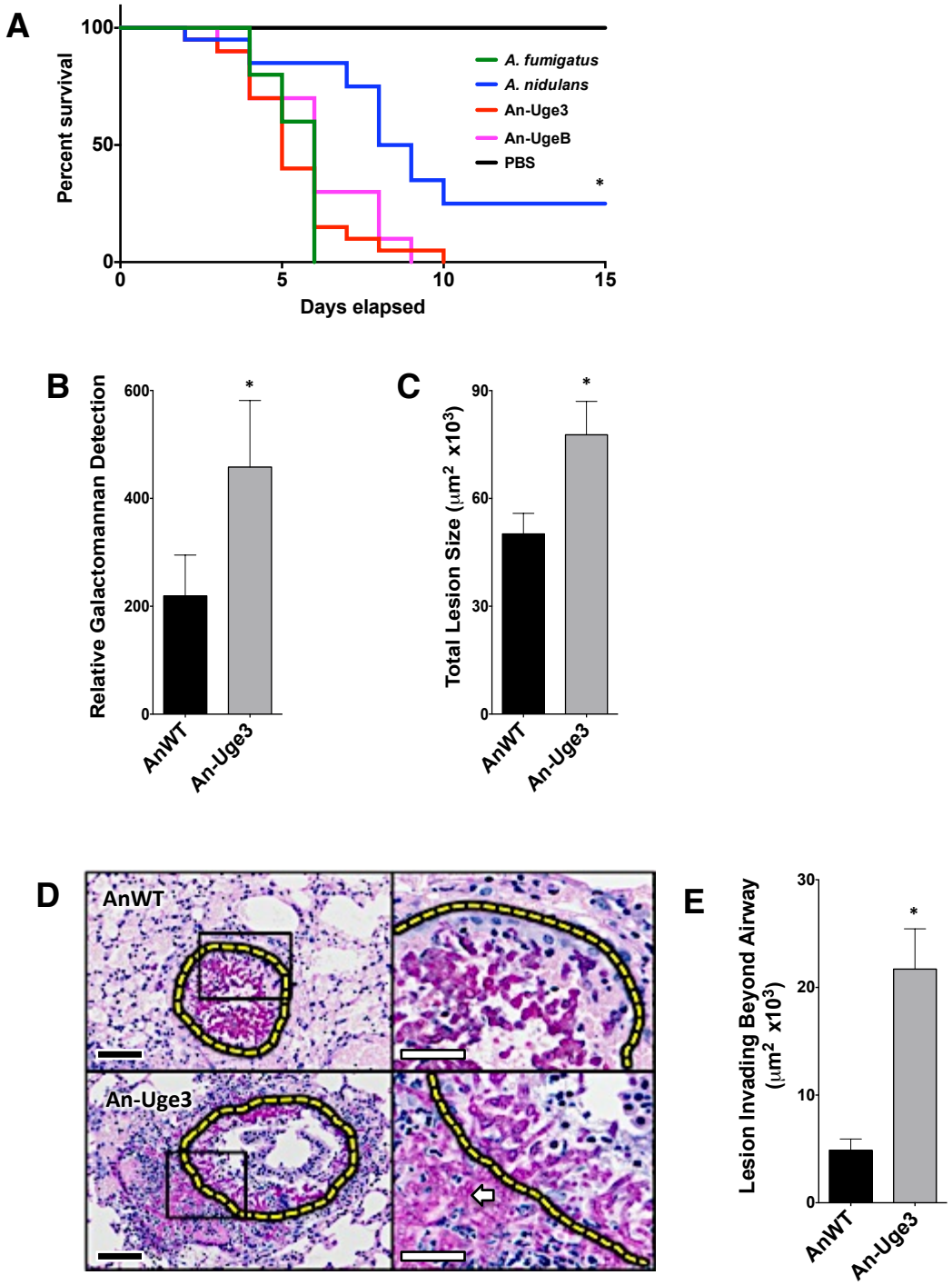

A
100
50
0
Percent survival
0
5
10
15
Days elapsed
A. fumigatus
A. nidulans
An-Uge3
An-UgeB
PBS
*
B
Relative Galactomannan Detection
600
400
200
0
*
AnWT
An-Uge3
C
Total Lesion Size (μm² x10³)
90
60
30
0
*
AnWT
An-Uge3
D
AnWT
An-Uge3
E
Lesion Invading Beyond Airway (μm² x10³)
30
20
10
0
*
AnWT
An-Uge3

Figure 5

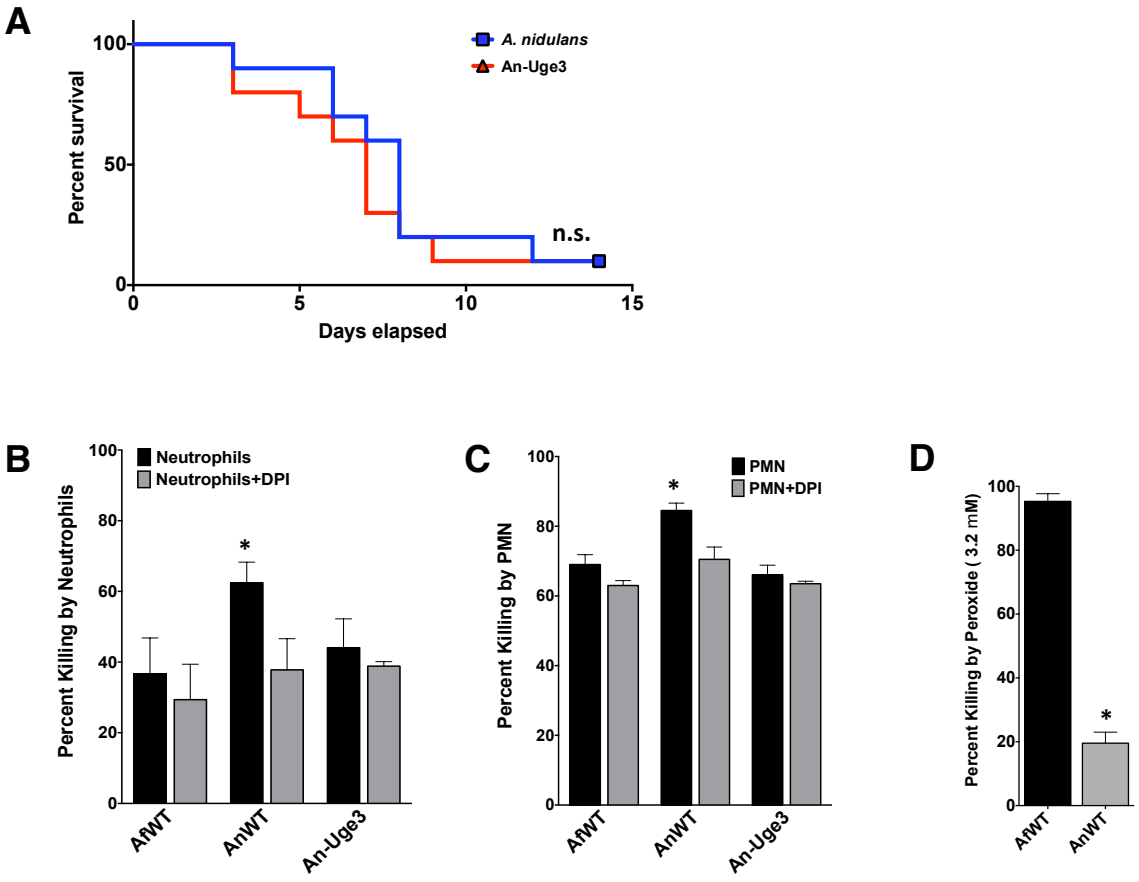

A
100
50
0
Percent survival
A. nidulans
An-Uge3
n.s.
0
5
10
15
Days elapsed
B
Neutrophils
Neutrophils+DPI
Percent Killing by Neutrophils
100
80
60
40
20
0
*
AfWT
AnWT
An-Uge3
C
PMN
PMN+DPI
Percent Killing by PMN
100
80
60
40
20
0
*
AfWT
AnWT
An-Uge3
D
Percent Killing by Peroxide (3.2 mM)
100
80
60
40
20
0
*
AfWT
AnWT

Figure 6

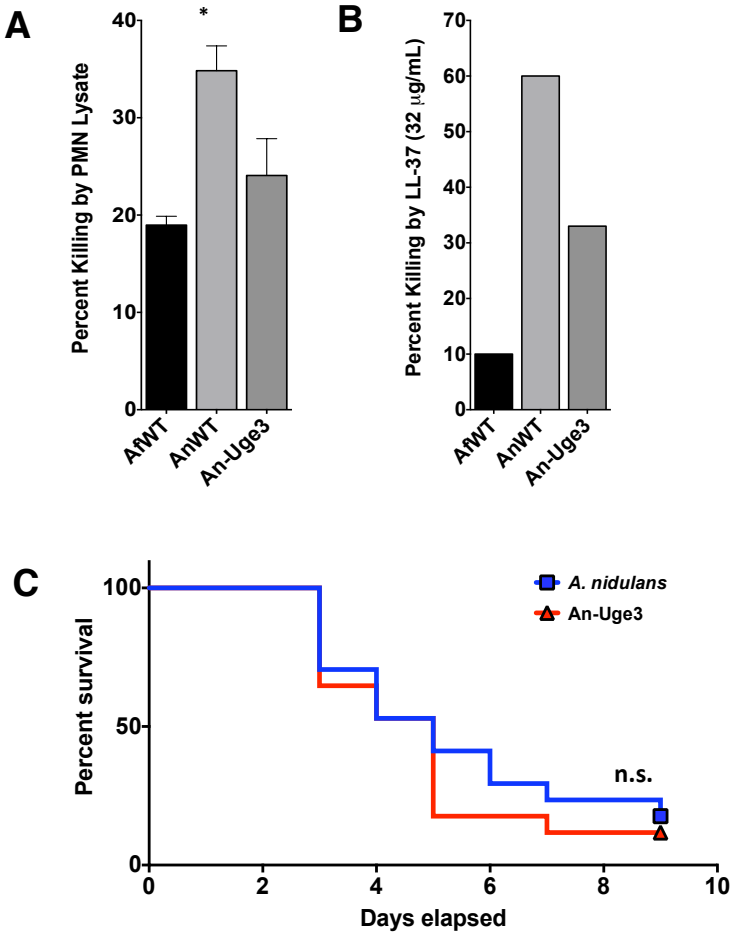

A
Percent Killing by PMN Lysate
40
30
20
10
0
*
AfWT
AnWT
An-Uge3
B
Percent Killing by LL-37 (32 μg/mL)
70
60
50
40
30
20
10
0
AfWT
AnWT
An-Uge3
C
Percent survival
100
50
0
A. nidulans
An-Uge3
n.s.
0
2
4
6
8
10
Days elapsed

Figure S1

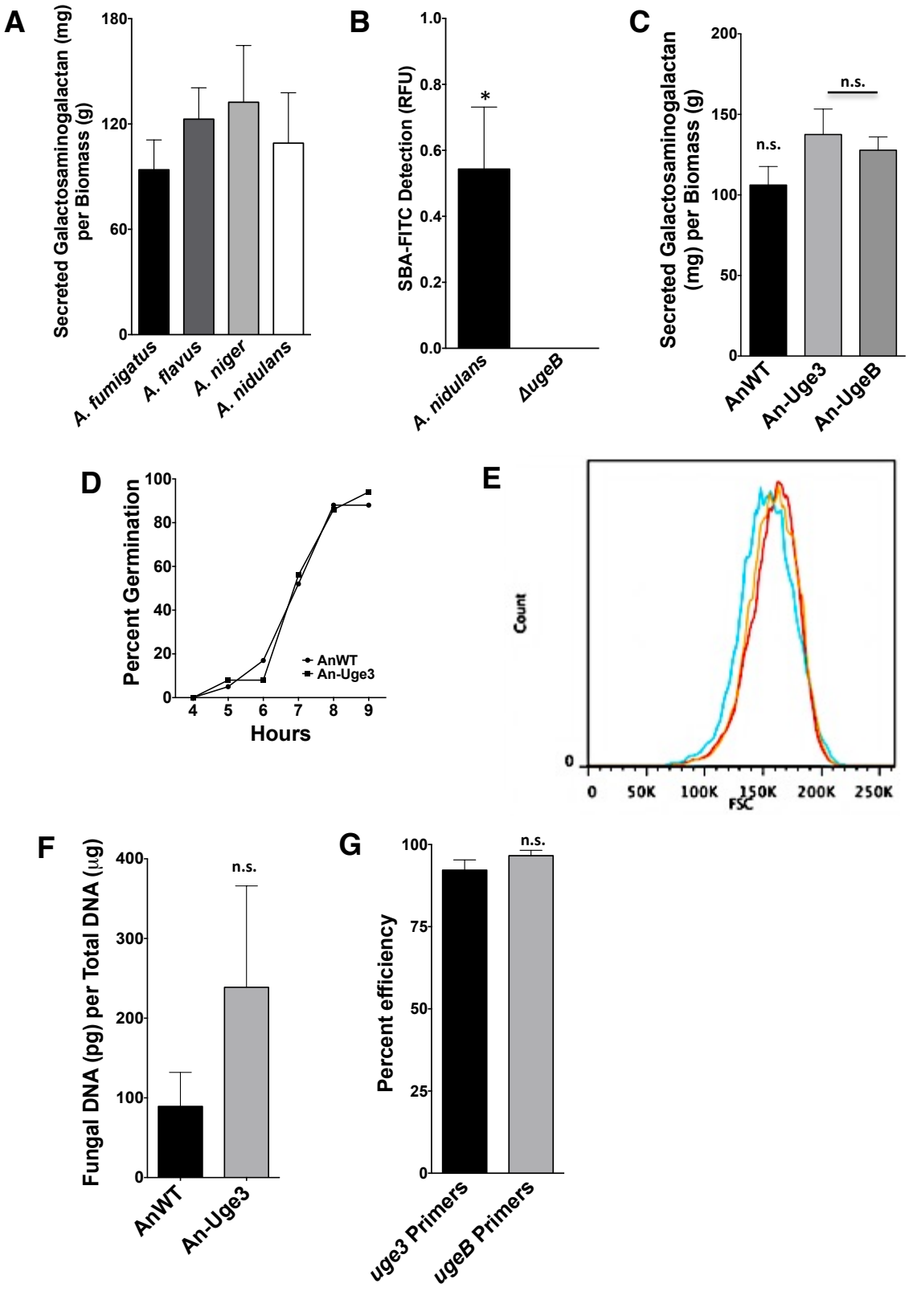

A
Secreted Galactosaminogalactan (mg) per Biomass (g)
180
120
60
0
A. fumigatus
A. flavus
A. niger
A. nidulans
B
SBA-FITC Detection (RFU)
1.0
0.8
0.6
0.4
0.2
0.0
*
A. nidulans
ΔugeB
C
Secreted Galactosaminogalactan (mg) per Biomass (g)
200
150
100
50
0
n.s.
n.s.
AnWT
An-Uge3
An-UgeB
D
Percent Germination
100
80
60
40
20
0
4
5
6
7
8
9
Hours
AnWT
An-Uge3
E
Count
0
0
50K
100K
150K
200K
250K
FSC
F
Fungal DNA (pg) per Total DNA (μg)
400
300
200
100
0
n.s.
AnWT
An-Uge3
G
Percent efficiency
100
75
50
25
0
n.s.
uge3 Primers
ugeB Primers

Figure S2

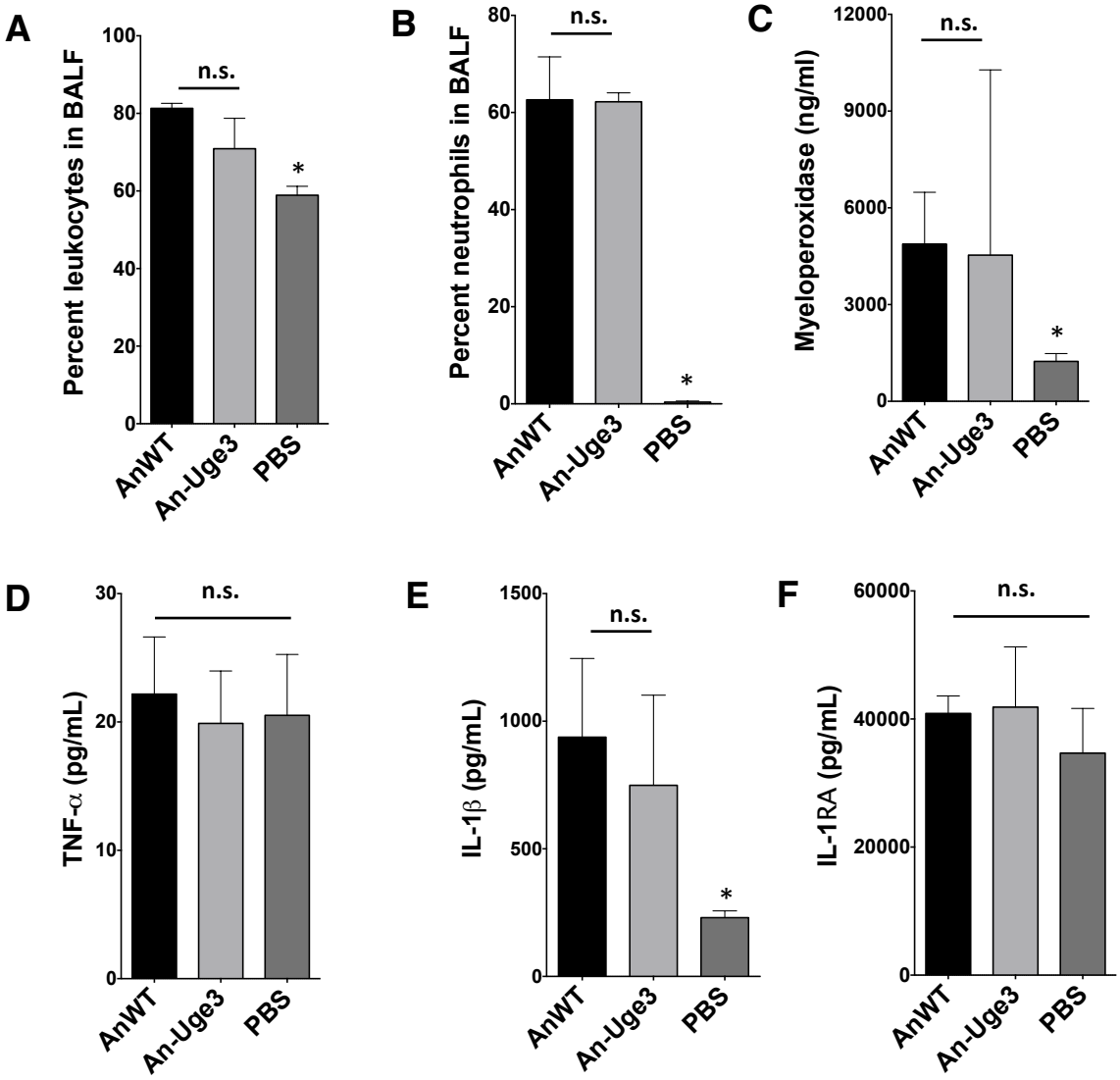
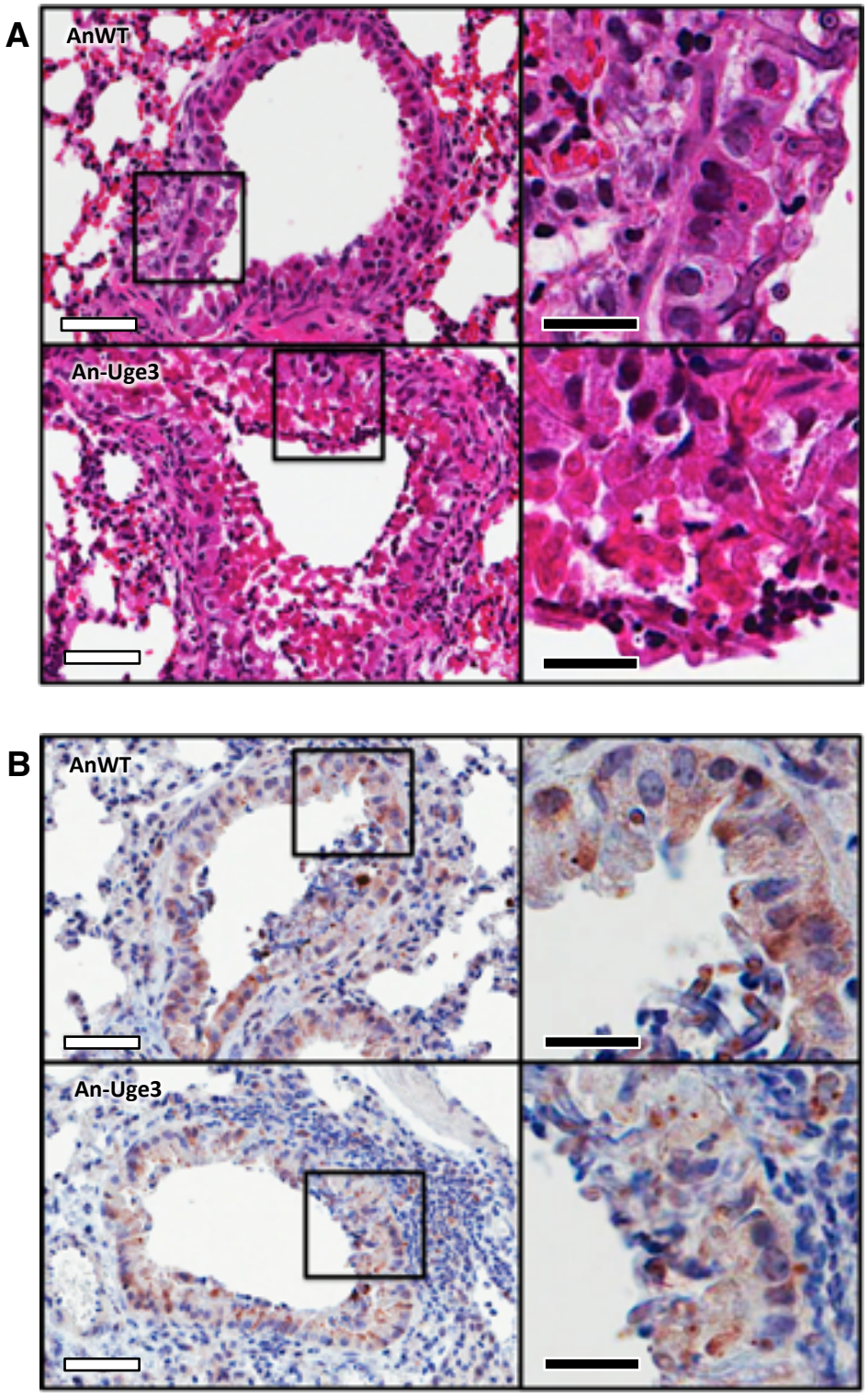

A
Percent leukocytes in BALF
100
80
60
40
20
0
n.s.
*
AnWT
An-Uge3
PBS
B
Percent neutrophils in BALF
80
60
40
20
0
n.s.
*
AnWT
An-Uge3
PBS
C
Myeloperoxidase (ng/ml)
12000
9000
6000
3000
0
n.s.
*
AnWT
An-Uge3
PBS
D
TNF-α (pg/mL)
30
20
10
0
n.s.
AnWT
An-Uge3
PBS
E
IL-1β (pg/mL)
1500
1000
500
0
n.s.
*
AnWT
An-Uge3
PBS
F
IL-1RA (pg/mL)
60000
40000
20000
0
n.s.
AnWT
An-Uge3
PBS

Figure S3



CHAPTER 6: Conclusions and General Discussions

Although galactosaminogalactan has been described in fungal species decades ago (1-4), the synthesis, function, or role of this polysaccharide in virulence has not been studied. The goal of this thesis was to elucidate the biosynthetic pathway and biologic functions of galactosaminogalactan in *Aspergillus* species. In the experiments described here, we identified galactosaminogalactan as an adhesin and virulence factor; elucidated the first step in its biosynthesis; established deacetylation as a required post-synthetic modification; and identified a relationship between the quantity of cell wall bound galactosaminogalactan and virulence among *Aspergillus* species. One of the most significant findings of this thesis is that galactosaminogalactan mediates resistance to NADPH oxidase-dependent extracellular neutrophil killing. This important finding provides an explanation for the clinical observation that *A. nidulans* infections are observed only in individuals with chronic granulomatous disease, a hereditary disorder of NADPH oxidase.

In Chapter 2, we presented two key findings. First, we identified that galactosaminogalactan is required for hyphal adherence and normal biofilm production. Importantly, galactosaminogalactan is the only hyphal adhesin in *A. fumigatus* that has been identified to date. Additionally, blocking galactosaminogalactan synthesis, by deletion of the UDP-glucose 4-epimerase *uge3* also dramatic altered the cell wall morphology. The Δ*uge3* mutant had a smooth, naked hyphal morphology, in contrast to the parental strain, which was heavily decorated. This type of morphology is similar to reports of mutant bacteria deficient in the synthesis of exopolysaccharide (5), suggesting the possibility that there may be similarities between the fungal and bacterial exopolysaccharide systems.

Galactosaminogalactan was also required in *A. fumigatus* to cloak β-1,3-glucans from immune recognition. Unmasking of β-1,3-glucans increased both in vitro and in vivo inflammatory responses. Similar masking of β-1,3-glucans from dectin-1 recognition has been reported in *A. fumigatus* conidia, in masking is mediated by the hydrophobin RodA, as well as in other fungal species, including *Histoplasma* and *Candida*, where α-glucans or mannans on the outer cell wall serve the same function (6-8). Thus, masking of cell wall β-1,3-glucans seems to be a widely used strategy by fungi to evade inflammatory immune responses, and may be a useful target for future antifungal therapies.

Our initial studies demonstrated that deletion of *uge3* resulted in a mutant devoid of galactosaminogalactan. However, the exact function of Uge3 and the role of other UDP-glucose 4-epimerases in galactosaminogalactan synthesis were unknown. To better understand this process we expressed and purified recombinant Uge3 and constructed mutants deficient in the other two putative epimerases found within the genome: Uge4 and Uge5. Only Uge3 and Uge5 were expressed, and galactose catabolism was found to be dependent on the production of at least one of these two proteins. This study is the first comprehensive study of sugar epimerases in *A. fumigatus* and yielded some interesting insights into galactose-containing polysaccharide synthesis. A surprising finding of this study was the inability of Uge3 to support the synthesis of galactomannan in the absence of Uge5, despite the fact that Uge3 can produce sufficient galactose to support galactosaminogalactan synthesis. This unexpected finding could not be explained by compartmentalization of epimerase activity since we found that both Uge3 and Uge5 are localized in the cytoplasm. Thus, substrate shunting alone would not explain this phenomenon. It is possible that a regulatory mechanism blocked galactomannan synthesis in favor of galactosaminogalactan, although futures studies are required to confirm this hypothesis.

Work in this thesis has also discovered that synthesis of the primary galactose-GalNAc chain of galactosaminogalactan is not sufficient to produce functional galactosaminogalactan. To produce galactosaminogalactan that can mediate adherence and modulate virulence, post-synthetic modification of the primary polysaccharide is required. Deacetylation of GalNAc into galactosamine through the action of the deacetylase Agd3 confers a positive charge under acidic conditions, turning nascent galactosaminogalactan into a cationic polysaccharide. This modification permits charge-based interactions such as the adhesion of the polysaccharide to fungal hyphae, and to host cells and other substrates.

Another significant finding was that Agd3-mediated deacetylation of galactosaminogalactan occurs in the extracellular space, as demonstrated by the use of culture supernatants to reconstitute deacetylation of galactosaminogalactan in a fungal-free system. Given that intracellular drug penetration and drug efflux are major limitations to the development of an antifungal therapeutic (9), the extracellular nature of Agd3 and

the requirement of deacetylation for galactosaminogalactan function suggests that this enzyme may be an attractive drug target.

Collectively the findings of Chapter 2, 3, and 4 suggest a similarity between galactosaminogalactan synthesis, modification, and function with bacterial exopolysaccharides like the polysaccharide intercellular adhesin (PIA) produced by a number of bacteria including *E. coli* and *S. epidermidis* (10). This polysaccharide consists of β-1,6 linked N-acetyl glucosamine residues that are synthesized by the coordinated action of the products from bacterial operons that consist of a glycosyltransferase, hydrolase, transporter, and deacetylase. A similar set of enzymes is encoded within the galactosaminogalactan biosynthetic gene cluster identified in our studies. As with galactosaminogalactan, partial deacetylation of PIA by a deacetylase is required to modify the nascent polysaccharide into a cationic polysaccharide that adheres to the bacterial surface. Further, as with *A. fumigatus*, loss of these exopolysaccharide deacetylases attenuates the virulence of multiple bacteria including *Bacillus anthracis* (11), *Streptococcus iniae* (12), *S. epidermidis* (13), and *Enterococcus faecalis* (14).

Sequence analysis shows no homology between the bacterial operons and the members of the galactosaminogalactan gene cluster. This observation, combined with the similarities in the synthetic pathway and the production of functionally similar but compositionally distinct polysaccharides suggests that these systems have developed through convergent rather than divergent evolution. Given that these microbes are under similar environmental pressures, it is perhaps understandable that they developed similar tools to adapt and survive.

The role of galactosaminogalactan in pathogenesis were further explored in Chapter 5. Our first major finding was that while other *Aspergillus* species produce galactosaminogalactan, the ratio of GalNAc to galactose, and by extension that of galactosamine to galactose, is important and in direct correlation to virulence. Increasing the GalNAc/GalN content of galactosaminogalactan increased the amount of cell wall bound polysaccharide and enhanced the virulence of the relatively non-pathogenic *A. nidulans*. This is a significant finding since to date, candidate virulence factors of *A. fumigatus* have only been studied by gene deletion in *A. fumigatus*. The studies reported here represent

the first successful heterologous expression of a candidate virulence factor in a less virulent *Aspergillus* species to study pathogenesis.

The second major finding of these studies was the identification of a role of galactosaminogalactan in mediating resistance to NADPH oxidase-mediated neutrophil killing. Interestingly this resistance was not mediated through enhancing resistance to NADPH oxidase-mediated oxidative killing. Rather, our data suggest that increasing the amount of cell wall bound, cationic galactosaminogalactan in *A. nidulans* confers resistance to cationic antimicrobial peptides released in the context of neutrophil extracellular traps (NETs) – a process mediated by NADPH oxidase. This observation is highly clinically relevant as the differential susceptibility of *A. fumigatus* and *A. nidulans* to NETs provides an explanation for the observation that *A. nidulans* is a cause of invasive aspergillosis only in individuals with chronic granulomatous disease (15). These patients suffer from a hereditary disorder of NADPH oxidase and are unable to kill *A. nidulans* via NET formation.

In addition to the functions of galactosaminogalactan presented in this thesis, others have described an anti-inflammatory role of galactosaminogalactan. In one study, administration of purified galactosaminogalactan induced the production of IL-1 receptor antagonist (IL-1RA) in human peripheral blood mononuclear cells (PBMC) and in mice (16). By increasing the production of IL-1RA, galactosaminogalactan effectively blocked IL-1 signaling. This anti-inflammatory property of galactosaminogalactan was explored as a potential therapeutic agent to treat chronic inflammatory illness, and was shown in an experimental murine model of colitis, that administration of galactosaminogalactan decreased the severity of disease. In another study, purified galactosaminogalactan was shown to induce neutrophil apoptosis (17). Purified galactosaminogalactan increased intracellular levels of reactive oxygen species in neutrophils, which in turn, activated NK-cells to produce apoptosis-promoting signal in neutrophils (18).

Although, these studies have shown that galactosaminogalactan mediates an anti-inflammatory response in the host by either blocking pro-inflammatory signaling or suppressing cellular response, it not clear whether the anti-inflammatory properties of galactosaminogalactan reported in these studies are relevant during infection. Given that these studies were conducted using secreted galactosaminogalactan that was then purified, and administered it at a concentration that may not be physiologically relevant, these

findings need to be directly verified in a model of invasive aspergillosis. It is also possible that these studies may have identified phenotypes that are mediated by secreted, rather that hyphal associated galactosaminogalactan, as these eperiments were performed by administering a soluble fraction of this exopolysaccharide.

In conclusion, the work presented in this thesis has contributed to the growing body of knowledge in cell wall polysaccharides and their relevance to pathogenesis. We mapped the early steps of galactosaminogalactan biosynthesis and provided a thorough survey of sugar epimerase in *A. fumigatus*. Additionally, we have demonstrated that the production of cationic polysaccharide is a common strategy used by microorganisms across domains of life to address similar challenges. The studies described in this thesis have identified key roles for galactosaminogalactan in virulence, including mediating adherence, the masking of PAMPs, and resistance to host antimicrobial peptides and NETs. Importantly, we provided greater insights into understanding the mechanism underlying the unique susceptibility of individuals with chronic granulomatous disease to *A. nidulans* infection.

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