Role of β-galactofuranose and β-glucan in *Aspergillus nidulans* hyphal cell wall ultrastructure and physical properties

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By

Biplab Chandra Paul

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FACULTY OF GRADUATE STUDIES AND RESEARCH

SUPERVISORY AND EXAMINING COMMITTEE

Biplab Chandra Paul, candidate for the degree of Master of Science in Biochemistry, has presented a thesis titled, *Role of β-galactofuranose and β-glucan in Asperillus Nidulans Hyphal Cell Wall Ultrastructure and Physical Properties*, in an oral examination held on May 2, 2012. The following committee members have found the thesis acceptable in form and content, and that the candidate demonstrated satisfactory knowledge of the subject material.

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ABSTRACT

The fungal cell wall is a first line of defence from the external environment or chemical treatments. Glucan, chitin and mannan are the main components of the *Aspergillus nidulans* hyphal cell wall. The sugar β-galactofuranose is a minor component of the cell wall and thought to be responsible for cross-linking of the other cell wall components, therefore responsible for maintaining cell wall structural integrity. We investigated the role of β-galactofuranose on the structure and physical properties of the hyphal cell wall. Based on its unique capacity to image live samples, atomic force microscopy was used to determine both the ultrastructure and physical properties of the hyphal cell wall. Five different knock-out strains of *Aspergillus nidulans* (*ugeAΔ, ugeBΔ, ugeAΔ,ugeBΔ* and *ugmΔ, ugeAΔ,ugmAΔ and ugtAΔ*) associated with β-galactofuranose synthesis were compared with the wildtype strain (AAE1). Atomic force microscopic imaging and force spectroscopy of the mutant and wild type strains suggest that a lack of galactofuranose reduces the integrity of cell wall components, where the surface subunits of *ugeAΔ* and *ugmΔ* are two-four fold larger than that of the wildtype (AAE1) respectively. The *ugeBΔ* strain shows similar sized subunits as the AAE1 strain, in contrast with the double mutant (*ugeAΔ,ugeBΔ*) which exhibits a fibrous cell surface structure. The *ugtAΔ* mutant strain, able to synthesize β-Galf but unable to incorporate the sugar into the cell wall, showed a similar surface structure to the double mutant *ugeAΔ,ugmAΔ*, with the largest surface subunits. The structural changes of the cell wall surface are accompanied by a change in
cell wall viscoelasticity, where the cell wall of the wild type strain is the most viscoelastic in comparison to that of mutant strains, and the lowest cell wall viscoelasticity can be attributed to the complete absence of β-Galf. Live and fixed cell walls of the ugmAΔ, ugtAΔ, ugeAΔ,ugeBΔ, and ugeAΔ,ugmAΔ strains had extremely low viscoelastic moduli, which we attributed to a limitation of the model used to accurately calculate viscoelasticity. Moreover, the impaired cell wall packing in mutant strains is consistent with greater surface hydrophilicity for mutant strains compared to wild type. We propose that the lack of galactofuranose disrupts the proper packing of cell wall components, giving rise to more disordered surface subunits and therefore greater deformability. Topographic images of glucanase- and laminarinase-treated wildtype strains suggest that glucan is at least one component of the cell surface subunits. Mutant strains which lack Galf were more susceptible to laminarinase treatment, which we attribute to deeper enzyme penetration into the more loosely packed cell walls.
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## LIST OF ABBREVIATIONS

<table>
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<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
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<tr>
<td>AGS</td>
<td><em>Aspergillus</em> glucan synthase</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>CCD</td>
<td>charge couple device</td>
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<td>Chs</td>
<td>chitin synthase</td>
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<td>CM</td>
<td>complete media</td>
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<td>dd</td>
<td>double distilled</td>
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<tr>
<td>DTM</td>
<td>dialysis tubing membrane</td>
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<tr>
<td>ECU</td>
<td>electronic control unit</td>
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<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide (FAD)</td>
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<tr>
<td>FS</td>
<td>force spectroscopy</td>
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<td>FWHM</td>
<td>full width at half maximum</td>
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<tr>
<td>Galf</td>
<td>galactofuranose</td>
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<td>GlfB</td>
<td>golgi localized transporter</td>
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<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
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<td>MPol</td>
<td>mannan polymerase</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
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<tr>
<td>ND</td>
<td>not determined</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reactions</td>
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<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
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<tr>
<td>STM</td>
<td>scanning tunnelling microscopy</td>
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<td>SYNA</td>
<td>Synaptobrevin</td>
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<td>Abbreviation</td>
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<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
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<td>U</td>
<td>enzyme units</td>
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<td>UDP</td>
<td>uridine diphosphate</td>
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<tr>
<td>Uge</td>
<td>UDP-glucose 4-epimerase</td>
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<tr>
<td>UGM</td>
<td>UDP-galactopyranose mutase</td>
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1. INTRODUCTION

1.1 Fungi

The environment contains diverse groups of fungi, which are all eukaryotes. Like animals, all fungi are heterotrophic organisms which can be classified as saprophytes and parasites (e.g. *Basidiomycetes*, *Ascomycetes*) (Moore-Landecker, 1990). Saprophytes (e.g. Rhizopus, Agaricus) use dead or decaying organic material as their source of nutrients, on the other hand parasites use living organisms to extract nutrients. Fungi absorb nutrients by secreting digestive enzymes into their surrounding environment which then break down macromolecules into simple forms for nourishment. Fungi can exist as both single celled organisms such as yeast, multi cellular organisms such as filamentous fungi or can be dimorphic. Yeast reproduces through the process of budding or mitosis, whereas filamentous fungi reproduce by sporulation. Multi cellular filamentous fungi form thread-like structures called hypha (plural; hyphae) that form an interweaving web called mycelium, which ultimately form fruiting bodies to produce spores. Hypha can be either binucleate, or multinucleate, each separated by a cross wall called a septum. However, aseptate filamentous fungal strains are also prevalent in the environment, for example a group of fungi called Zygomycetes (e. g. Rhizopus). The septa of different fungi vary from having single or multiple pores.
Our perception of the importance of filamentous fungus has drastically increased in recent years due to its prevalence as a causative agent of plant and human disease, and based on their enormous ecological and industrial importance.

1.2 *Aspergillus species*

The most widely distributed fungi in the natural environment are of the genus *Aspergillus*. *Aspergillus niger* and *Aspergillus oryzae* have significant industrial applications and there are twenty different species of *Aspergillus* which are responsible for a variety of allergic reactions and invasive diseases in animals and humans (Krishnan *et al.*, 2009). Among the invasive diseases, most are caused by the species *Aspergillus fumigatus*, which is closely related to *Aspergillus nidulans*. The well characterized sexual life cycle, highly developed genetic system, and availability of a full genome sequence make *A. nidulans* an excellent model system to understand a myriad of fungal processes. *A. nidulans* is a filamentous animal parasite that reproduces by sporulation, with a haploid nucleus that can be diploid prior to sexual reproduction.

1.3 Hyphal growth

Unlike the commonly studied unicellular budding yeast, *Saccharomyces cerevisiae*, filamentous fungi grow by extension of their apical region (Moore-Landecker, 1990) through the addition of new cell wall forming materials. This apical growth in *A. nidulans* produces an approximately 40 µm long basal cell with a hyphal diameter of 2-3 µm. Cell wall components are synthesized inside
the cell, packaged into vesicles and transported to the apical region. The vesicles arrive at the apical region of the hyphal tip where they then fuse with the plasma membrane and release their contents. The region of the hyphal tip where vesicles are distributed is called the Vesicle Supply Center (VSC) (Gierz, G. & Bartnicki-García, S. 2001) or Spitzenkörper (Reyanaga-Pena et al., 1997). Microtubules and actin filaments both play an important role in the transport of vesicles to the hyphal apex, however the exact mechanism of this process remains to be determined. Microtubules mediate long distance transport of vesicles to the VSC and actin plays a role in the short distance transport of vesicles to the cell wall surface (Steinberg. 2007). Fungi with disassembled microtubules, induced by an anti-microtubule agent or mutants lacking kinesin, exhibit defects in Spitzenkörper stability, protein secretion and impairment in intracellular vesicular transport (Howard, 1981). Taheri-Talesh et al., (2008) fluorescently tagged the cell membrane protein synaptobrevin homolog (SYNA), a component of the export vesicles, to demonstrate that the cell wall components and hydrolytic enzymes for nutritional substrate degradation are released from the cell by exocytosis and that SYNA is recycled from the plasma membrane through endocytosis (Figure 1). Thus exocytosis and endocytosis each play an important role in the formation of the fungal cell wall. Endocytosis also plays an important role in cell polarity, cell wall reconstruction and acquisition of nutrients from the exterior (Higuchi et al., 2005).
The extension of the cell wall at the hyphal tip is termed polarized growth to recognize its vectorial direction of extension. Extension is initiated where the membrane is rich in sterol and sphingolipid (membrane rafts), and therefore can be stained with filipin, a protein localized to membrane rafts, which determine sites of polarized growth in various fungal strains (Alvarez et al., 2007). The orientation of microtubules play an important role in polarized growth, with their plus end oriented toward the hyphal tip region, merging at one point (Schuchardt et al., 2005). Various models have been developed for polarized hyphal growth through tip extension, including the Steady-State model developed by Wessels, (1988) and the Vesicle Supply Center model of Bartnicki-García (Bartnicki-García et al., 1989). According to the steady-state model, cell wall materials are continuously synthesized at the apex, and are rigidified into a mature wall as the hyphae grow. However, the apex contains a steady-state amount of plastic cell wall materials, so that the hyphae can continue extension (Wessels, 2002). Bartnicki-García’s model states that hyphal growth is mediated by a continuous supply of vesicles, created in the Golgi apparatus, and their transport to a linearly advancing source or VSC. Subsequently, vesicles are free to migrate any random direction and eventually fuse with the cell membrane (Gierz, G. & Bartnicki- García S., 2001).
Figure 1. Growth of *A. nidulans* through extension of the apical region, the site of cell wall synthesis. The apex consists of apical actin clusters and vesicles that contain materials for cell wall synthesis. Vesicles containing components for cell wall synthesis are carried to the apical region by microtubules, where the plus end of the microtubules disassemble and transfer the vesicle to actin fibers which carry the vesicle to the tip and facilitate their fusion with the plasma membrane. The vesicle components are then released to the exterior, and enzymes therein incorporate sugars and proteins into the growing cell wall. Reproduced with permission from Taheri-Talesh *et al.*, 2008.
1.4 The *Aspergillus* cell wall

The fungal cell wall accounts for approximately 30% of the dry weight of the organism (Gastebois *et al.*, 2009), and is a dynamic organelle, consisting of a semi-permeable network of mannoprotein, chitin and glucan polysaccharides, which are remodelled continuously during fungal cell growth (Klis *et al.*, 2002). The fungal cell wall plays a pivotal role in protecting the cell from the external environment and provides sufficient mechanical strength to prevent cell damage from changes in osmolarity of the environment. The cell wall also maintains cell shape and facilitates attachment to biotic and abiotic surfaces. In spite of its important function, the fungal cell wall ultrastructure and biosynthesis have not been sufficiently studied (Klis *et al.*, 2006).

1.4.1 Chitin biosynthesis

Chitin, a significant component of the hyphal cell wall, is a linear polymer of β-1,4-N-acetylglucosamine synthesized by chitin synthases from the substrate UDP-N-acetylglucosamine. Most fungal cells have several chitin synthase genes, which are divided into two families and seven classes on the basis of sequence similarities (Gastebois *et al.*, 2009). *A. nidulans* and *A. fumigatus* both have nine chitin synthase (*chs*) genes which include: *chsA-G, csmA* and *csmB*. Mutants of *A. fumigatus* and *Neurospora crassa* missing *chsE* and *chs4*, showed significantly altered chitin synthesis and cell wall structure. *A. fumigatus* strains that do not express *chsE* and *chsG* show reduced chitin synthesis, which can be
compensated by an increase in α-1,3-glucan synthesis to maintain cell wall strength (Mellado et al., 2003). Lack of chitin impairs the structural integrity of the fungal cell wall and so is a prime target for the development of antifungal drugs.

1.4.2 Glucan biosynthesis

Monomeric units of the glucan chain are incorporated at the cytosolic side of the cell by membrane-bound glucan synthase complexes (Girard et al., 1984), while the chain is simultaneously extruded into the periplasmic region. The glucan synthase complex is composed of two different proteins subunits, the Rho1P regulatory subunit and Fks1P catalytic subunit (Beauvais & Latgé., 2001). Antifungal drugs such as echinocandins are used to treat fungal infections by targeting glucan synthase, rendering it inactive (Kartsonis et al., 2003).

The membrane bound glucan synthase enzyme synthesizes β-glucan through the polymerisation of UDP-glucose, processing up to 1500 monomeric subunits. The catalytic subunit of the enzyme is called FKS1. The Aspergillus glucan synthase (AGS) genes encode the enzyme necessary for the synthesis of α-1,3-glucan. Knocking out ags1 reduces the synthesis of α-1,3-glucan in A. fumigatus (Beauvais et al., 2005), whereas cells lacking both AGS1 and AGS2 show a change in both conidiation (formation of conidia) and polarity. Fifty to sixty glucose residues can be added to the hydroxyl group at the sixth carbon of glucose in a glucan chain to generate a branching point.
1.4.3 Galactomannan biosynthesis

Galactomannan is an abundant component of the A. fumigatus cell wall. Galactomannan is a branched polymer composed of a linear core of α-mannans with short side chains of β-1,5-galactofuranose (Gal\(f\)), and is a major antigen found in the blood serum of patients suffering from aspergillosis (Aquino., et al. 2007). The co-ordinated action of mannosyl and galactosyl transferases are required for the synthesis of galactomannan to link α-mannan and β-1,5-galactofuranose residues. The first step of mannan synthesis is the formation of a mannan chain with α-1,6 linkages, which then trigger the sequential action of two mannan polymerase (MPol) enzymes, MPol I and Mpol II (Stolz & Munro., 2002) which extend the mannan chain.

1.4.4 Biosynthesis and cell wall incorporation of galactofuranose

In mammals, galactose is found as the six membered ring galactopyranose (Gal\(p\)), whereas in bacteria and fungi galactose can be present as both the six membered and five membered ring form, β-galactofuranose (β-Gal\(f\)) (Pan et al., 2001). β-Gal\(f\) is an essential component of bacterial cell envelopes as it mediates virulence by contributing to the synthesis of the O-antigen, an outer membrane polysaccharide (Koplin et al., 1997), but the exact role of Gal\(f\) in A. nidulans remains unknown. Unlike glucan and chitin, β-Gal\(f\) is present in very low amounts and accounts for only 5% dry weight of the A. nidulans cell wall
β-Galf is also linked to other components including; a) polysaccharide galactomannan, b) proteins, which form the glycosylphosphatidylinositol (GPI), membrane-anchored glycoconjugate lipophosphogalactomannan (Costachel et al., 2005) and c) sphingolipids (Simenel et al., 2008). Galf is also a component of galactan, a chain of 35 β-Galf residues in Mycobacterium tuberculosis and the O-antigen of both Escherichia coli and Klebsiela pneumonia (Pan et al., 2001, Stevenson et al., 1996). Individual β-Galf residues can also be present in the O- and N-glycans of glycoprotein and glycosphingolipid of A. fumigatus and Schizophyllum commune, but cell wall polysaccharides also contain β-Galf (Leitao et al., 2003, Morelle et al., 2005). β-Galf can be secreted from the fungal cell and become incorporated in the host tissue, making it a good candidate for detecting Aspergillus infection (Mennink-Kersten et al., 2008). Moreover, β-Galf-containing glycoconjugates are potential allergens based on the lack of β-Galf in mammalian cells (de Lederkremer & Colli., 1995). The β-Galf biosynthetic pathway is an attractive target for designing both antibacterial and antifungal drugs. The galactosyltransferase enzyme in mycobacteria is involved in the polymerization of UDP-Galf during the synthesis of the galactan chain (Richards & Lowary., 2009). All Galf-containing polysaccharides are synthesized from the same building block, UDP-galactofuranose. In the fungal cell, the starting material for UDP-galactofuranose biosynthesis is UDP-glucose, which is converted to UDP-galactopyranose by UDP-glucose 4-epimerase (Uge) (Allard et al., 2001), and
then to UDP-Galf by UDP-galactopyranose mutase (Ugm) (Figure 2) (Nassau et al., 1996). Conversion of UDP-glucose to UDP-galactopyranose is a step of the Leloir pathway of galactose metabolism which is highly conserved from bacteria to humans (Holden et al., 2003). Thoden and Holden (2005) demonstrated that Ugm in S. cerevisiae, consists of a single polypeptide chain which has both galactose mutarotase and UDP-galactose 4-epimerase activity. Crystallographic studies of the enzyme show that Uge is a dimer, each subunit with an N-terminal and C-terminal domain connected by a Type II turn (Leu-357 to Val-360). The gene encoding Ugm was identified in E. coli in 1996, and more recently it was identified in both A. fumigatus and A. nidulans (Nassau et al., 1996). Eukaryotic and prokaryotic Ugm has only 20% homology, though both have sequence similarities at the active site of the enzyme. Sanders et al. (2001) solved the first crystal structure of Ugm isolated from E. coli, and also determined the crystal structure of Ugm bound to flavin adenine dinucleotide (FAD) in both the oxidized and reduced states. When Ugm is deleted in different strains, different effects are observed; in A. niger colony growth is altered, A. fumigatus exhibits a thinner cell wall whereas A. nidulans synthesizes a thicker cell wall.
Figure 2. Pathway for the synthesis of galactofuranose from UDP-Glucose in *Aspergillus nidulans*. UDP-glucose is converted to UDP-galactopyranose by UDP-glucose-4-epimerase (UgeA), which is converted to UDP-galactofuranose by UDP-galactopyranose mutase (UmgA) in the cytoplasm. UDP-galactofuranose is then transported by the UDP-Galf transporter (Ugt) to the site of cell wall synthesis and incorporated into the cell wall. Printed with permission from Paul et al. (2011).
Typically Galf is synthesized as nucleotide diphosphate sugars that act as the sugar group donor for glycosylation (Handford et al., 2006). UDP-Galf, a component of glycoproteins, further polymerizes the mannan components of the cell wall (Fontaine et al., 2003). UDP-Galf synthesized in the cytosol must first be transported to the endomembrane system for incorporation into the glycoconjugate or for polysaccharide synthesis, accomplished by nucleotide sugar transporters (NSTs) (Gerardy-Schahn et al., 2001) (Figure 3). Engel et al. (2009) identified a Golgi localized transporter (GlfB) in A. fumigatus and its counterpart in A. nidulans is called the UDP-Galf transporter (UgtA).

**Galactofuranose mutants**

The loss of galactofuranose in A. fumigatus alters the morphology and growth of the fungal cell and these strains display attenuated virulence in murine models of invasive aspergillosis. Moreover, mutant strains are more susceptible to antifungal drugs (Schmalhorst et al., 2008). El-Ganiny et al. (2008 & 2010) constructed mutant strains of A. nidulans lacking the ability to transport Galf or synthesize β-Galf from UDP-glucose by knocking out genes responsible for its transport and biosynthesis. The mutant strain lacking the ugmA gene did not display Galf on its surface when stained with a fluorescently labelled monoclonal antibody, EBA2, raised against A. fumigatus Galf-containing galactomannan. The mutant phenotype of the A. nidulans ugmA deleted strain (ugmAΔ) was remediated when grown on calcoflour white, unlike the ugmAΔ of A. niger, which is more susceptible to calcoflour (Damveld et al., 2008).
Figure 3. Schematic for galactofuranosylation and Galf transport in *Aspergillus fumigatus*. Synthesis of β-Galf from galactopyranose (1) followed by β-Galf transport into the Golgi apparatus by GlfB or Ugt (2). Inside the Golgi apparatus GlfB catalyzes galactofurnosylation (3) and finally UDP is converted to UMP (4). Reproduced with permission from Engel *et al.*, (2009).
The thickness of the *ugmAΔ* cell wall visualized by transmission electron microscopy (TEM) was significantly larger than wildtype, possibly the result of an increase in chitin or glucan synthesis (El-Ganiny et al., 2008). All the deleted strains (except *ugeBΔ*) are highly branched, have compact colony morphology and display little penetration into media. In contrast, the AAE1 (wild type) strain has extended colonies and submerged hyphae which grew into the medium.

Another single mutant developed by El-ganiny et al. (2010) by knocking out the UDP-galactopyranose epimerase A (*ugeA*) gene from *A. nidulans* was called *ugeAΔ*, and had similar morphological characteristics to *ugmAΔ*, but cell wall thickness half that of *ugmAΔ*. On the contrary, the UDP-galactopyranose epimerase B (*ugeB*) deletion strain, *ugeBΔ*, had wild type morphology, growth rate and sporulation (Paul et al., 2011), but the cell wall thickness was statistically greater than that of wild type. The predicted UgeB protein has approximately 38% amino acid sequence similarity with UgeA, and therefore it was initially termed an epimerase, but the exact function of this enzyme remains unknown (Figure 2; El-Ganiny, et al., 2010). Like the single mutant strains *ugeAΔ* and *ugmAΔ*, the strains lacking both *ugeA* and *ugeB* genes, *ugeA,ugeBΔ*, exhibited wider hyphae and had the thickest cell walls among all the strains in this study (Figure 4; Paul et al., 2011). Another double deleted strain *ugeAΔ,ugmAΔ* showed similar hyphal morphology to *ugmAΔ*, but a lower cell wall thickness than that of *ugmAΔ*. The hyphal cell also has altered morphology if Galf is not transported into the cell wall, despite its synthesis. Afrose et al.,
(2011) reported that the \textit{ugtA} gene is not essential for \textit{A. nidulans} survival, but the germination and growth of \textit{A. nidulans} is significantly altered in the absence of this gene. The \textit{ugtA}\Delta stains formed compact colonies similar to \textit{ugmA}\Delta strains (El-Ganiny \textit{et al.}, 2008; Paul \textit{et al.}, 2011). The hyphal width measured by confocal microscopy is greater than wild type (Figure 4) and the cell wall thickness is greater than some mutant strains but smaller than that of the \textit{ugeA}\Delta and \textit{ugmA}\Delta single mutant and \textit{ugeA}\Delta, \textit{ugeB}\Delta double mutant strains (Afroz \textit{et al.}, 2011).
Figure 4. Confocal microscopy images of strains examined in this study. All the strains were grown on coverslips for 16h at 28° C, fixed and stained with Hoechst 33258, and then examined by confocal microscopy (bar = 5 µm). Printed with permission from Paul et al. (2011) and Afrose et al. (2011).
1.4.6 Cell wall ultrastructure

Most of the available information regarding fungal cell wall structure emanates from chromatographic, nuclear magnetic resonance (NMR) or mass spectrometry (MS) studies of fractionated cell wall components (Yin et al., 2008). More than 90% of the fungal cell wall dry weight is composed of polysaccharides (Gastebois et al., 2009).

The inner most layer of the Aspergillus cell wall consists of chitin, a linear polymer of β-1,4- N-acetylglucosamine and the outer most layer is composed of glucan linked with glycoprotein (Bowman & Free., 2005). Chitin is present in the cell wall in relatively lower amounts, accounting for only 10-20% of the dry weight of filamentous fungi, such as Neurospora and Aspergillus (Fontaine et al., 2000a). The most significant component of the fungal cell wall is the branched β-1,3-glucan chain, making up approximately 60% of the cell wall dry weight. Glucan is readily soluble in alkali, and its non-reducing end acts as an attachment site for other polysaccharides. An NMR study by Fontaine et al. (2000a) revealed that glucan polysaccharides are branched through β-1,6 linkages to other β-1,3-glucans, which can also be linked to galactomannan; a polymer of α-mannan with short branched β-1,5-Galf (Latgé et al., 1994). This mannan backbone can also be branched by the addition of α-1,2-linked and α-1,3-linked mannose residues, and branch points are terminated with a Galf residue (Nakajima, et al., 1984). The β-1,3-glucan is linked β-1,4 to the cell wall chitin component, thought to line the plasma membrane.
Figure 5. Components of cell wall structure and their predicted organization. Major components of the fungal cell wall are glucan, chitin, mannan and proteins. Though the organization of cell wall components can vary from species to species, this is a hypothetical organization of fungal cell wall components based on studies of *Aspergillus* strains (Fontaine et al. 2000a; Bernard & Latgé., 2001 Paul et al., 2011). The β-1,3-glucan chain forms a framework that interconnects chitin, galactomannan and protein.
On the other hand, galactomannan is linked to chitin by β-1,6 linkages (Bernard & Latgé., 2001) and β-1,3-glucan is linked β-1,6 to the C-terminal of the glycosylphosphatidylinositol (GPI) anchored proteins situated on the cell membrane (Klis et al., 2006). The exact process and enzymes that mediate cross-linking remain unknown. There are other GPI anchored proteins in the cell wall that are thought to be responsible for catalytic activities (Popolo & Vai., 1999). Recent studies suggest that A. fumigatus has at least nine different GPI anchored proteins, common to yeast, for which most play an important role in cell wall morphogenesis (Li et al., 2007). These covalent linkages and branching cell wall components form fibrils, a three dimensional network of glucan, mannans and chitin.

1.4.7 Physical properties of the fungal cell wall

Through chemical analyses and molecular biology, the fungal cell wall has been studied extensively, but the ultrastructural, physical and mechanical properties of the cell wall have remained less well explored. The advent of atomic force microscopy (AFM) provided an excellent tool to study such properties of the fungal cell wall, both under fixed and live conditions (Ma et al., 2005; Ma et al., 2006, Dufrêne., 2010; Paul et al., 2011). Moreover AFM can be used to image the fungal cell surface of fixed and live cells at very high resolution. Zhao et al., (2005) characterized the viscoelastic properties of wild type Aspergillus nidulans and mutant with impaired chitin synthesis and Ma et al. (2005) extend these measurements to the cell walls of viable fungal hyphae.
1.5 Atomic force microscopy

Atomic force microscopy (AFM) evolved from scanning tunnelling microscopy (STM), which is a very popular tool for investigating a range of materials such as semiconductors, electronics, polymers, and biomaterials. The ability of AFM to image samples in liquid at high resolution has made it a popular tool of biologists to investigate live samples. Binning et al. (1986) introduced the AFM following the invention of the STM in 1982. The main difference between STM and AFM is that STM requires conducting samples whereas AFM can probe both conducting and non-conducting (biological) samples. The heart of the AFM instrument is the micro fabricated tip on the end a metal coated cantilever (Figure 5). Three piezoelectric micropositioners (X, Y and Z) maintain a constant distance (Z) between tip and sample as the tip is raster scanned (X, Y) over the sample surface (top design) and vice versa (bottom design). The optical lever, consisting of a red laser reflected from the top surface of the cantilever into a four quadrant photodetector, is the most common AFM detection method (David et al., 2010). Changes in tip-sample interactions give rise to cantilever deflection registered as a vertical or horizontal displacement in the photodetector. This signal is sent to the computer for data collection and to the electronic control unit (ECU) to manage the height (Z) of the AFM tip through a fast feedback loop (Figure 6). Thus, vertical and horizontal displacement of the red laser in the photodiode are registered as height (topography) and tip-sample interactions (lateral force), respectively. For rough samples such as microbial surfaces, lateral force images
are convoluted with topographic signals as the tip encounters large changes in the slope that are not manageable by the feedback loop, known as the “edge effect” (Nie et al., 1999; Qian et al., 2010). This phenomenon actually gives rise to improved image contrast for live samples (Ma et al., 2005). For a comprehensive review of AFM instrumentation, see Akiyama et al. (2010).

The resolution of traditional optical microscopy images is diffraction limited, but by using a very sharp AFM tip, it is possible to acquire images at sub-nanometer (nm) resolution. The application of AFM to investigate biological samples, from single cells to molecules, has grown rapidly within the last decade, with its enhanced popularity attributed to the ability to image live samples under ambient conditions. Along with topographic information about the sample surface, force spectroscopy (FS) can determine various physical properties of samples such as viscoelasticity, electrical and magnetic properties.

AFM can be operated in three different modes: (i) non-contact mode, (ii) contact mode, and (iii) tapping mode. Contact mode using cantilevers with low force constants provides the best contrast and resolution for live hyphae (Ma et al., 2005). In this mode, the tip remains in constant contact with the sample surface during scanning and the interaction force between the tip and sample is measured to extract information on the surface topography and physical properties (Fung et al., 2001).
Figure 6. Atomic force microscope with its various components. A sharp tip is mounted on a very thin cantilever. When the tip scans the surface of the sample, the cantilever deflects according to the sample features. Reflection of the laser from the back of the cantilever is detected by the photodiode in the vertical (topography) and horizontal (lateral force). The signal passes through the electronic control unit that houses the fast feedback control loop which alters the movement of the Z piezoelectric micropositioners. In this way, a constant distance is maintained between the sample and tip as it raster scans (X, Y) across the surface. The photodiode signal is also sent to the computer which converts the data into an image. Printed with permission from Sringer, USA.
FS uses an approach-retract cycle (Figure 7) of the AFM tip to measure precise tip-sample forces at any given point (Chandraprabha et al., 2010; Zhang et al., 2011) or in the case of force mapping, multiple points (Dufrêne & Hinterdörfer, 2008; Zhang et al., 2011). The approach curve (Figure 7, segment a – c) serves as a measure of indentation forces for the whole fungal cell, its cell wall or components thereof. Given an appropriate model, the force (nN) required to indent the entire cell or fungal cell wall by a given distance (nm) can be used to calculate viscoelasticity. Viscoelasticity, defined as the property of a substance exhibiting both viscous and elastic behavior upon deformation, can be related to whole cell turgor, or used to describe cell wall integrity. Complex models have been used to determine the relationship between indentation forces (nN/nm) and cell wall spring constants for fungi (Zhao et al., 2005). On the other hand, analysis of the tip retraction curve (Figure 7, segment e – f) is relatively straightforward for measuring adhesion forces (pN – nN), which relate to the surface chemistry of the probe and sample (Volle et al., 2008). For example, stronger adhesion forces between fungi and a hydrophilic tip indicates that the fungal surface has hydrophilic character (Ma et al., 2005). More sophisticated single molecule FS (Scheuring & Dufrêne, 2010) and chemical FS (Dufrêne & Hinterdörfer, 2008) studies use chemically modified tips to detect specific macromolecules in model systems or whole cells. FS, in general, has been used to study host–pathogen interactions, the nanomechanical properties of whole cells or components thereof, or finer details such as ligand-receptor interaction
forces and/or specific molecular distributions on microbial cell surfaces (Busscher et al., 2008).
Figure 7. Shown is a typical force curve for live *A. nidulans*. The approach cycle (a – c) is shown as a solid line and the retract cycle (d – g) appears as a dashed line. When the tip is far from the sample surface (a – b, f – g) there is no interaction force or deflection of the cantilever. The cantilever first deflects when the tip “jumps” into contact with the sample surface (b). The amount of force (nN) required to indent the sample surface a given distance (nm) is determined by the slope of b – c in the approach curve. This measure, when combined with an appropriate (organism-specific) model, can be used to determine cell wall viscoelasticity or whole cell turgor pressure. Cantilever deflection reverses when the tip begins to retract (d – e) from the surface, and the segment e – f represents overcoming the adhesion force between the AFM tip and the sample surface, at which point (f) there is no longer cantilever deflection. The difference between segment e – f is a measure of tip-sample adhesion (nN).
1.5.1 Application of AFM to fungi

Previous studies by our and other groups demonstrate the usefulness of AFM to probe the fungal cell wall structure and physical properties (Dufrêne 2010; Kaminskyj & Dahms, 2008; Ma et al., 2005; Ma et al., 2006). Ma et al. (2005) identified for the first time surface features of *A. nidulans*’ hyphae proposed to correspond to glucan triplices, an idea supported by more recent biophysical evidence (Lehtovaara & Gu, 2011). Investigation of the spore surface by AFM revealed the proteinaceous rodlet layer (Ma et al., 2005) in accordance with SEM studies (Dempsey et al., 2008). Ma et al., (2006) used AFM to study the structure of the spore and remodelling of the fungal cell wall during hyphal growth and branch formation. They further investigated the ultrastructural consequences of non-polarized growth, demonstrating for the first time active remodelling in a temperature-sensitive secretary pathway mutant (Ma et al., 2005). AFM has been used to study the structural alteration of *A. fumigatus* conidial surface during germination (Dague et al., 2008). In addition to surface ultrastructure, functionalized AFM tips have been used to measure the distribution of specific chemical groups throughout the yeast cell surface (Dague et al., 2007). AFM tips functionalized with monoclonal antibodies have been used to probe polysaccharides on cell wall surfaces of *Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae* (Alsteens et al., 2008). The latter study suggested that the longer extension of polysaccharides in *S. cerevisiae* may result from mannoprotein and polysaccharide stretching. It has also been possible to
measure the elasticity of individual carbohydrate molecules on the fungal cell wall by applying a small amount of force during retraction (Kaminskyj & Dahms., 2008). Moreover, whole cell wall elasticity can be determined using AFM without any tip modification (Ma et al., 2005). In this study the fungal cell surface ultrastructure and cell wall elasticity were studied as a function of Galf content.

1.6 Study objectives

The first aim of this work was to investigate the contribution of galactofuranose to the ultrastructure and physical properties of the A. nidulans cell wall. El-Ganiny et al. (2010) demonstrated that Galf plays a key role in hyphal morphology and colony growth using mutant strains, including a change in cell wall thickness. Such a change could be attributed to activation of a stress response pathway, which involves expression of the biosynthetic genes, such as chitinase, required for the synthesis of additional chitin (Damveld et al., 2008). I hypothesized that alterations in the mutant phenotype could also result from reduced cross-linking and impaired cell wall maturation.

The second aim of this study was to provide direct evidence for the localization of β-glucan components on the cell wall surface, and investigate the effect of glucanase on the cell wall in the presence and absence of β-Galf. Previous studies by our lab (Ma et al., 2005) discovered well-ordered 25 nm round-shaped features on the surface of A. nidulans, speculated to be glucan triple helices, but with no supporting biochemical evidence. I hypothesized that the
outer most subunits consist in part of glucan, and that glucanase-treated cells would display altered surface subunits, corresponding to a cell wall with lower viscoelasticity.
2. MATERIALS AND METHODS

In this section, unless otherwise stated all chemicals were reagent grade and purchased from Sigma-Aldrich, Canada. The water used for media preparation and culture handling was doubly distilled (dd) and that used for sample preparation was deionised (18MΩ, Nanopure, Barnstead, USA).

2.1 Hyphal strains

Wild type (AAE1) and all single (ugmAΔ, ugeAΔ, ugeBΔ and ugtAΔ) and double mutant strains (ugeAΔ,ugeBΔ and ugmAΔ,ugeAΔ) of A. nidulans used for these studies were kindly supplied by our collaborator, Dr. Susan Kaminskyj (University of Saskatchewan).

2.2 The growth and storage of Aspergillus nidulans conidia

The A. nidulans conidia were germinated and maintained in complete media (CM) containing the appropriate trace elements, vitamins and nitrate salts as originally described by Kafer (1977) and Harris et al. (1994). Briefly, one liter of the 20 × nitrate salts solution contains 120 g NaNO₃, 10.4 g KCl, 10.4 g MgSO₄·7H₂O, and 30.4 g KH₂PO₄. One hundred milliliters of trace element solution contains 2.2 g ZnSO₄·7H₂O, 1.1 g H₃BO₃, 0.5 g MnCl₂·4H₂O, 0.5 g FeSO₄·7H₂O, 0.17 g CoCl₂·6H₂O, 0.16 g CuSO₄·5H₂O, 0.15 g Na₂MoO₄·2H₂O, and 5 g Na₄EDTA. Complete medium (CM) was prepared by mixing 10 g D-glucose, 2 g Peptone, 1 g Yeast extract, 1 g Casamino acids, 1 mL trace elements, 1 mL vitamins and 20
mL nitrate salts with 800 mL water and then pH adjusted to 6.5 with KOH and the mixture diluted to 1 L. CM agar was prepared by adding 15 g of agar in 1 liter of CM liquid media. Conidia were grown by streaking mature cultures with an inoculating loop and then spreading the sample onto the surface of fresh CM agar in a sterile plastic petridish. Wild type stains form mycelia typically after 1-2 days, and start to produce conidia after 2-3 days, whereas mutant strains take 2-3 days for mycelial growth and 3-4 days for conidia formation. Cultures were stored for longer periods at 4 °C. AAE1 and ugeBΔ strains produced green colonies, whereas the other mutant strains (ugmAΔ, ugeAΔ, ugtAΔ, ugeAΔ,ugeBΔ, and ugmAΔ,ugeAΔ) produced white colonies. Contamination between strains could be easily detected by the production of different coloured colonies.

*Aspergillus nidulans* conidia were harvested by expelling 10 µL of dd water onto the surface of conidiating culture, and then recollecting the conidial suspension from the colony surface with a micropipette. A 1/10 dilution of conidia was prepared by adding 10 µL of *A. nidulans* conidial suspension to 90 µL of dd water in an Eppendorf tube (100 µL). The conidial suspension was mixed thoroughly by vortex and could be stored at 4 °C for weeks or months.

Newly harvested conidia suspended in dd water to make intermediate stock (5000 spores per µl) were counted using a Brightline Hemocytometer. Conidia were incubated at 37 °C for sixteen hours to initiate hyphal growth for wild type and all the mutant strains.
2.3 Sample preparation for AFM imaging and force spectroscopy

All strains were prepared in the same manner to facilitate comparison of ultrastructure and mechanical properties between wild type and mutant strains.

2.3.1 Preparation of fixed *Aspergillus nidulans*

A small amount of dust on the coverslip can produce image artefacts, and therefore clean coverslips were used as substrates for hyphae. Coverslips were cleaned by immersion in concentrated HCl (2 min), then rinsed with 200 µL deionised water, dried at room temperature (RT), immersed in piranha solution (5 mL H₂O₂ + 15 mL H₂SO₄; 1 h) washed with copious amounts of deionized water, immersed in methanol (2 min) and finally acetone (2 min). Cleaned coverslips were stored in dust-free containers.

Clean and sterile (autoclave 121°C, 20 min) glass coverslips were placed in a petridish (35 mm × 35 mm; Falcon Canada) and 200 µL of conidial suspension (5000 spores/µl CM) pipetted onto the surface. A second coverslip was carefully placed on top of the first to trap the spore suspension and to promote 2D hyphal growth which is desirable since AFM imaging requires a relatively flat sample surface (Ma *et al.*, 2005). CM liquid (2 mL) was added to the petridish surrounding, but not in contact with, the coverslips to prevent evaporation of the media-containing spore suspension, and the petri dish incubated (37 °C) long
enough to promote hyphal growth. After 16 h of growth, the top coverslip was removed and the remaining media wicked away from the bottom cover slip with a Kimwipe without touching the mycelia. After washing with 50 mM PBS (30.75 mL of 1 M K₂HPO₄ and 19.25 mL of 1 M KH₂PO₄ in 1 L 18 MΩ water), germlings were fixed (200 µL; 3.7% formaldehyde, 2% Triton-X, in 50 mM potassium phosphate buffer), rinsed with dd water, stored at RT and imaged within 2-3 days.

2.3.2 Sample preparation of live Aspergillus nidulans

Live hyphal samples for AFM imaging and force spectroscopy were prepared by inoculating a small amount (1 µL) of conidial suspension (5000/mL) onto a piece of dialysis tubing membrane (DTM; Spectra/Pore® MWCO-8000) overlaid on a petridish containing CM agar. Following growth (37 °C, 16 h) the DTM was attached to a coverslip with double sided tape. A paper wick (Watman, USA) was cut to size and inserted into the DTM tube to deliver media to the growing hyphae during imaging (Figure 8). The filter paper width was slightly smaller than that of the dialysis tubing, and slightly longer, such that part of the filter paper remained outside the membrane for media delivery. Media (~ 10 µL/30 min) was delivered to the sample to keep the hyphae viable. Since excess liquid on the DTM surface interferes with AFM feedback by creating extreme capillary forces, AFM imaging parameters sometimes needed to be adjusted more frequently.
Figure 8. Schematic of the sample set-up for live cell AFM imaging and force spectroscopy at the air media interface. Hyphae are actively growing on a dialysis tubing membrane under which media is delivered drop wise through the filter paper. Printed with permission from Springer, USA.
2.3.3 Enzymatic digestion of *Aspergillus nidulans* hyphae

Hyphae were grown on coverslips following the procedure described in section 2.3.2. The β-1,3-glucanase enzyme solution was prepared by adding 1 unit of enzyme (Sigma-Aldrich, USA) into 10 mL of phosphate buffer (30.75 mL of 1M K$_2$HPO$_4$ and 19.25 mL of 1M KH$_2$PO$_4$ in 1 L 18 MΩ water). KCl solution (100 mM) was added to the enzyme solution to protect fungal cells from osmotic shock. After 16 h growth, media was discarded β-1,3-glucanase enzyme solution (200 µL; 0.1 U/mL buffer), or buffer solution containing 100 mM KCl (200 µL) alone as a control, were added to coverslips and samples incubated (37 °C, 1, 2 and 7 h). To examine the effect glucanase on mutant and wildtype strains, hyphae were also treated with the less specific laminarinase enzyme (MP Biomedical, 0.5 U/mL buffer) for 0.5 h and 1 h, as were the associated buffer only control samples. Enzyme-and buffer-treated hyphae were then fixed according to the procedure described in section 2.3.1 or imaged live according to section 2.3.2.

2.4 Atomic force microscopy instrumentation

An Explorer™ AFM (TM Micro (now Bruker), US) was used for all the AFM studies. AFM is very sensitive to sonic and vibrational noise and so the instrument was housed on a platform made of lead-embedded plexiglass suspended by bungee cords in a wooden cabinet, insulated with soundproof foam (Sonex). The entire apparatus was housed on an optical table (Coherent...
Ealing Optics, UK) mounted on I-2000 pneumatic isolators (Newport Canada). A detailed description is given elsewhere (Snook, 2003; Ma et al., 2005). A dry AFM scanner (10 µm in Z and 100 × 100 µm in X and Y; model No. 540006; TM Micro, US) equipped with Si₃N₄ tips (Nominal spring constant: 0.05-0.5 nN/nm Tip radius: 10 nm; Veeco Metrology) was used to probe both fixed and live samples.

2.4.1 Atomic force microscopy imaging conditions

Samples were mounted on the AFM, and the hyphae first located by eye using the AFM charge couple device (CCD) camera and monitor. An area on the coverslip having little 3D growth and enough space between individual hyphae was selected for imaging. Any mycelium beneath the free end of the cantilever precluded AFM feedback control and so care was taken to avoid this scenario. All topographic and lateral force and error images of whole hyphae were acquired simultaneously in contact mode at a scan rate corresponding to half the scan size per second with resolution ranging from 100 × 100 – 500 × 500 pixels for any given image. Hypha were imaged (100 × 100 pixels; 100 × 100 µm) first at very low resolution (1 µm) to locate individual hyphae, a single hypha was selected and imaged (200 × 200 pixels; 80 µm × 80 µm) at medium resolution (0.4 µm) to locate a mature region, which was then imaged (500 × 500 pixels; 1.5 × 1.5 µm) at high resolution (3 nm). All hyphae were imaged at approximately the
same distance (40 μm) from the apex, taking into account the slower growth for mutant strains and so corresponding to the mature region for all strains.

Regions of interest for FS were chosen from the image directly after its acquisition, and the AFM mode switched while the tip remained in feedback to allow force curve measurement at any given point on the sample surface. Image and force data of the glass coverslip substrate, or DTM on a glass coverslip were obtained in a similar fashion.

2.4.2 Atomic force microscope calibration

The varying geometry and characteristics of AFM probes can cause image distortion and overestimation of the lateral size of small features since images represent a convolution of tip and sample features. Therefore, to minimize experimental error, AFM probes were calibrated using a standard semiconductor sample of known X, Y and Z dimension (Veeco, Model-10-10308-03). Contact mode images of standard semiconductor samples were collected at a very fast scan rate to ensure the highest possible resolution for this hard surface, and X, Y and Z dimensions of the features were measured from the topographic images. The measured Z value, expected Z value and previous values were used to calculate new values for the Z scanner from following equation:

\[
\frac{\text{Expected } Z}{\text{Measured } Z} \times \text{Old slope} = \text{New slope} \quad (1)
\]
Similarly, using the expected and measured X and Y values and previous standard voltage, the new standard piezo voltages for the X and Y scanner were calculated and input into the appropriate scanner file. For FS, the spring constant of the cantilevers were determined using the resonant frequency method as described by Cleveland et al. (1993). Detailed procedures for X, Y, Z piezo electric micropositioner calibration and spring constant determination can be found in the Explorer™ instrument operation manual.

2.4.3 Atomic force microscopy image processing and data analysis

All images were processed using SPMLab software (Version 5.01) to provide better image contrast and without altering the original data, unless topography was enhanced with error signals. The size of surface features was measured at the full-width at half-maximum (FWHM) height from image cross sections (Figure 9). AFM data of subunit size and roughness are presented as the mean ± standard deviation. A minimum of 5 different hyphae for each sample and 3 different samples were imaged by AFM. Subunit sizes were determined from both topography and lateral force images using a minimum of 60 subunits each. The difference in subunit size among wildtype and mutant strains were assessed using a one way ANOVA (InStat 3; GraphPad Prism).
Figure 9. Hyphal cross-section from a single line-scan of the AFM image.

Shown is a line measurement analysis of the $ugmA\Delta$ strain hyphal width, determined to be 3.7 µm at FWHM.
2.4.4 Calculation of surface roughness

After obtaining AFM images, surface roughness was calculated using the area analysis function of the SPMLab software (version 5.01) from 3 images of each sample for at least two different samples. Roughness was calculated based on the following equation:

\[ R_a = \frac{1}{n} \sum_{i=1}^{n} |Z_i - \bar{Z}| \]  \hspace{1cm} (4); 

where, \( \bar{Z} \) is the average height of the surface features, \( Z_i \) is the height of single surface features. Roughness is reported as mean ± standard deviation.

2.4.5 Force spectroscopy

Force-distance curves collected with the AFM have units of nA/nm, which are converted to nN/nm using by the force conversion factor (nN/nA) obtained from the instrument’s sensor response. Force curves (3 each) were taken at 10 different points on a single hypha, for a minimum of 5 hyphae per sample and typically 3 different samples. The slope of the approach portion (b-c in Figure 6) of the force vs. distance curve was used to determine \( K_w \) from the following equation:

\[ K_w = \frac{m_s K_c}{(m_h - m_s)} \]  \hspace{1cm} (2); 

where \( m_s \) is the slope of the force approach curve (Figure 6, segment b-c) for the sample, \( m_h \) is the slope of the force approach curve (Figure 6, segment b-c) for a
hard surface (i.e. glass coverslip), \( K_c \) is the spring constant of the cantilever determined from its resonant frequency.

The model developed by Zhao et al. (2005) used finite element analysis to determine the viscoelastic modulus (E) of the \( A. \) nidulans cell wall:

\[
E = 0.80 \left( \frac{K_c}{h} \right) \left( \frac{R}{h} \right)^{1.5}
\]  

(3);

where \( R \) is the hyphal radius measured by AFM, and \( h \) is the hyphal cell wall thickness measured by TEM.

The calculated viscoelastic moduli were presented as mean ± propagated error on the basis of equations 2 and 3.

2.4.6 Statistical analyses

Differences in surface subunit size, viscoelasticity and roughness among wildtype and mutant strains were assessed using a one way ANOVA (InStat 3; GraphPad Prism).
3. RESULTS

Previous work from our lab demonstrated that hyphal cell wall ultrastructure varies as a function of polarized growth. The apical region of the hyphal cell wall has larger and elongated subunits in comparison with mature regions, ≥ 3 µm away from hyphal tip (Ma et al., 2005). To ensure all hyphal cell walls were mature for comparison, regions 40 µm from the apex were chosen since the mutant devoid of Galf is known to mature at a slower rate than strains containing Galf. AFM can provide information about the ultrastructure of both fixed and live hyphal cell walls (Ma et al., 2005; Kaminskyj & Dahms, 2008); however for comparison of hyphal ultrastructure, fixed samples were used as they provide images with better defined features. Ma et al. (2005) reported that the ultrastructure of fixed and live hyphae have similar sized subunits and moreover, AFM and SEM of fixed, gold coated samples each show the same subunit size and shape. In the case of the ugmAΔ strain, fifty subunits were measured from both lateral force and topography images, demonstrating statistically identical subunit size, but with a lower standard deviation for those measured from lateral force images. The edge effect in the lateral force images provides clearer subunit delineations, allowing the user to better define subunit boundaries.

3.1 Cell wall ultrastructure of wild Type and mutant strains

AFM data shows that the wild type (AAE1) and the single mutant ugeBΔ have a similar hyphal width, whereas the ugeAΔ strain has wider hyphae (Figure 10),
consistent with the hyphal width measured by confocal microscopy (El-Ganiny et al., 2008). The fixed or dehydrated cell wall of mature wild type A. nidulans (AAE1) consists of rough, round shaped subunits as shown in Figure 10. Unlike the wild type strain, the cell walls of most mutant strains had slightly elongated and larger subunits. AAE1 and ugeBΔ have statistically similar (p <0.05) subunit sizes of 35 ± 5 nm and 39 ± 8 nm (n=60) respectively, however the cell wall of ugeBΔ is two times thicker than that of wild type. El-Ganiny et al., (2010) demonstrated that the cell wall thickness of ugeAΔ is 104 ± 10 nm, which is similar to that of ugeBΔ but their surface features varied significantly. The cell wall surface of ugeAΔ consisted of 63 ± 10 nm subunits, which is significantly larger (p < 0.05 ) than that of both wild type and ugeBΔ strains. The hyphal width measured from the AFM image of the mutant lacking UDP-galactopyranose mutase called ugmAΔ was 3.1 ± 0.2 µm, significantly larger (p < 0.05) than that of the wild type strain (AAE1), but significantly smaller (p < 0.05) than that of another single mutant ugeAΔ. Moreover, the surface subunits of ugmAΔ strains were also statistically larger (p < 0.05) than that of wild type and the two other single mutants, ugeBΔ and ugeAΔ. The ugmAΔ surface subunits were 108 ± 35 nm, statistically larger (p < 0.05) than those of wild type and ugeBΔ, and larger than any strain investigated in this study (Table 1).
Figure 10. Hyphal structure and the corresponding cell wall surface topography images of A) wildtype, B) UGEΔ and C) UGEΔ strains. The top row shows contact mode AFM images of fixed hyphae (15 ×15 µm, 300 × 300 pixels and Z= 1.5 to 2.0 µm) and the bottom row shows images of the corresponding hyphal cell wall surface at high resolution (1.3 × 1.3 µm, 500 × 500 pixels and Z= 40 to 60 nm). The arrow indicates a surface subunit.
Table 1: Hyphal width, surface subunit size, viscoelastic modulus and adhesion of wild type and mutant strains.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Hyphal width$^a$ (µm) (mean ± SD)</th>
<th>Subunit maximum dimension (nm) (mean ± SD)</th>
<th>Viscoelastic modulus (MPa) (mean ± SD)$^b$ of</th>
<th>Adhesion (nN) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fixed hyphal wall</td>
<td>Live hyphal wall</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type (AAE1)</td>
<td>2.4 ± 0.2</td>
<td>35 ± 5</td>
<td>211 ± 15</td>
<td>82.3 ± 12.9</td>
</tr>
<tr>
<td>$ugeA∆$</td>
<td>3.5 ± 0.1</td>
<td>63 ± 10</td>
<td>99 ± 48</td>
<td>24.6 ± 13.7</td>
</tr>
<tr>
<td>$ugeB∆$</td>
<td>2.6 ± 0.4</td>
<td>39 ± 11</td>
<td>74 ± 22</td>
<td>22.5 ± 8.6</td>
</tr>
<tr>
<td>$ugeA∆ugeB∆$</td>
<td>3.5 ± 0.4</td>
<td>ND$^c$</td>
<td>38 ± 21</td>
<td>9.8 ± 5.1</td>
</tr>
<tr>
<td>$ugmA∆$</td>
<td>3.2 ± 0.1</td>
<td>108 ± 35</td>
<td>14 ± 2</td>
<td>3.1 ± 0.4</td>
</tr>
<tr>
<td>$ugeA∆ugmA∆$</td>
<td>3.4 ± 0.4</td>
<td>97 ± 23</td>
<td>0.05 ± 0.02</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>$ugtA∆$</td>
<td>3.5 ± 0.2</td>
<td>77 ± 8</td>
<td>7 ± 2</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$ There was no significant difference in the hyphal width for fixed or live hyphae measured by either confocal microscopy or atomic force microscopy (AFM).

$^b$ Standard deviations (SDs) were calculated from the standard errors propagated through equations 1 and 2. See Materials and Methods.

$^c$ ND, not determined. See Results.
The double mutant $ugeA\Delta,ugeB\Delta$ strain exhibits a similar hyphal width compared to that of $ugeA\Delta$, but wider than that of $ugeB\Delta$, measured by both confocal and AFM (Paul et al., 2011). The hyphal width of another double mutant strain, $ugeA\Delta,ugmA\Delta$, lacking both epimerase and mutase is similar to that of the $ugmA\Delta$ strain, but smaller than the double deleted strain $ugeA\Delta,ugeB\Delta$. The high resolution AFM images of the hyphal cell surface show that the surface ultrastructure of these mutant strains are different than that of the wild type and other $uge$ single mutants (Figure 11). The surface features of the $ugeA\Delta,ugmA\Delta$ double mutant is similar to the $ugmA\Delta$ strain, but with statistically larger ($p < 0.05$) subunit sizes than all mutants lacking the epimerase enzyme (UgeA or UgeB) (Figure 11). The double mutant $ugeA\Delta,ugeB\Delta$ has a fibrous surface structure which is entirely different compared to all other strains investigated in this study.
Figure 11. Hyphal structure and the corresponding cell wall surface topography images of mutant strains A) $ugm\Delta$, B) $ugeA\Delta,ugeB\Delta$ and C) $ugeA\Delta,ugm\Delta$. The top row shows contact mode AFM images of fixed hyphae (15 × 15µm, 300 × 300 pixels and Z = 1.5 to 2.0 µm) and the bottom row shows images of the corresponding hyphal cell wall surface at high resolution (1.3 × 1.3 µm, 500 × 500 pixels and Z = 50-70 nm).
Another single mutant *ugtAΔ* strain, lacking the Galf transporter shows similar morphology to the *ugeAΔ, ugmAΔ* double mutant. The hyphal width of the *ugtAΔ* strain was measured by AFM to be 3.5 ± 0.2 μm, which is similar to the hyphal width of *ugmAΔ* single and double mutants (Paul *et al.*, 2011), and consistent with confocal microscopy measurements (Afrose *et al.*, 2011). However, the *ugtAΔ* hyphae are wider than other single mutants such as *ugeAΔ* and *ugeBΔ* and wildtype strains. The cell surface of *ugtAΔ* appears to be similar to that of *ugmAΔ* and the *ugeAΔ, ugmAΔ* double mutant strains (Figure 12). The size of the surface subunits measured from AFM is 77 ± 8 nm, significantly larger than that of the wildtype strain (*p* < 0.05).
Figure 12. Hyphal structure and the corresponding cell wall surface topography images of ugtAΔ mutant strain. A) Shows contact mode AFM images of fixed hyphae (15 × 15µm, 300 × 300 pixels and Z = 1.3 µm) and B) shows images of the corresponding hyphal cell wall surface at high resolution (1.3 × 1.3 µm, 500 × 500 pixels and Z = 50-70 nm).
3.2 Physical properties of wild type and mutant strain cell walls

Force curves of both fixed and live hyphae were collected to observe the change in viscoelastic moduli between the wild type and various mutant cell walls. The viscoelastic modulus of the hyphal wall for fixed specimens was consistently larger than that of live hyphae (Table 1), for all strains; and the viscoelasticity of fixed hyphae was twice that of live hyphae but followed the same trend among wild type and mutant strains. Both ugeAΔ and ugeBΔ strains have similar viscoelastic module but statistically smaller (p < 0.05), approximately 4-fold, than that of the wild type strain (Table 1). The ugeAΔ,ugeBΔ double mutant had the lowest viscoelasticity among all the uge deleted strains. In the case of both fixed and live hyphae, the viscoelasticity of the cell wall for the ugeAΔ,ugmAΔ strain is dramatically lower than all other strains examined in this study. A recent study showed that the cell wall of ugtAΔ is thicker than the cell wall of other strains such as wildtype and ugeAΔ ugeBΔ strains (Afrose et al., 2011), however the viscoelastic modulus of its fixed hyphal cell wall is 7.0 ± 2.0 MPa, significantly (p < 0.05) smaller than that of wildtype or other mutant strains such as ugeAΔ and ugeBΔ.

3.3 Hydrophilic characteristics of wild type, ugeAΔ and ugmAΔ strains

The adhesion between the hydrophilic AFM tip and the hyphal cell wall surface depends on the hydrophilic character of the cell wall surface (Ma et al., 2005).
Adhesion values were measured for the wild type, and \textit{ugmA}Δ and \textit{ugeA}Δ,\textit{ugmA}Δ strains that exhibited the most dramatic change in their cell wall ultrastructure (Figures 9 and 10). Both the mutant strains exhibited statistically greater \((p < 0.05)\) adhesion force between the tip and surface than wild type (see Table 1), indicating that these two strains are more hydrophilic than the wild type strain.

### 3.4 Effect \(\beta\)-1,3-glucanase on the ultrastructure of \textit{A. nidulans}

Hyphae incubated with buffer solution at a series of time points showed that buffer solution did not alter the ultrastructure of the cell wall significantly (Figure 13). Thus, changes observed following enzyme treatment can be attributed to the action of the \(\beta\)-1,3-glucanase enzyme.

Preliminary data following digestion of \textit{A. nidulans} hyphae with the \(\beta\)-1,3-glucanase enzyme shows a significant alteration of the cell surface structure and hyphal viability after 7 h (Figure 14). Although, the change in surface features after one and two hour enzyme treatments were not dramatic, structural changes are clearly visible. The \(\beta\)-1,3-glucanase treated cell surfaces show subunit sizes of \(58 \pm 12\) nm, statistically larger \((p < 0.05)\) than that of the control sample (see Table 1), which may in part be due to the degradation of glucans and/or an alteration of sugar packing. Preliminary data with another \(\beta\)-1,3-glucanase enzyme (data not shown), having suboptimal activity at 37 °C showed
indentations in the surface subunits after 2 and 7 h, more direct evidence that the subunits contain glucan components.
Figure 13. Effect of buffer on the *A. nidulans* hyphae and cell wall surface ultrastructure. Images A, B and C in the top row show contact-mode AFM topography images of fixed hyphae that had been incubated with buffer over 0 h, 1 h and 2 h respectively (size $13 \times 13 \mu m$ and $300 \times 300$ pixels) and the bottom row are corresponding topography images of the hyphal cell wall surface at high resolution ($1.5 \times 1.5 \mu m$ and $500 \times 500$ pixels).
Figure 14. Effect of β-1,3- glucanase enzyme on *A. nidulans* hyphae and corresponding cell wall surface structure. Images A, B and C in the top row are of whole fixed hyphae after enzyme digestion for 1 h, 2 h, and 7 h respectively (size 13 × 13 µm and 300 × 300 pixels) and the bottom row represents corresponding images of the hyphal cell wall surface at high resolution (1.5 × 1.5 µm and 500 × 500 pixels).
3.5 Effect of the laminarinase enzyme on cell wall ultrastructure

Hyphae incubated (0.5 and 1 h) with buffer containing nitrate salts showed no significant change in cell surface ultrastructure (Figure 15 A-C, G-I). On the other hand, incubation of hyphae with laminarinase (0.5 h and 1 h) alters the surface ultrastructure of the wildtype, and both mutant strains (Figure 15 D-F, J-L) since at higher enzyme units it was capable of degrading more glucan polymer than the more specific glucanase enzyme. The cell wall surface features of the ugeAΔ strain were significantly larger after both treatment times with the enzyme, compared to those incubated with only buffer (Table 2). The cell surface features of both ugmAΔ and wild type strains were no longer well defined after 1 h of enzyme treatment. The cell surface ultrastructure of the ugmAΔ strain was the most susceptible to the laminarinase, with significant changes in cell surface ultrastructure observed after only 0.5 h (Figure 15E). Though the ugeAΔ strain has defined surface features, their size is significantly (p < 0.05) larger (88nm ± 20 nm) than that of the wild type hyphal cell wall surface.

After 0.5 h of enzyme treatment surface roughness of all three strains remained the same as their control samples, but was dramatically reduced even after 1 h of enzyme treatment (Table 3). AEE1 has the roughest surface, based on surface subunit size and height.
Figure 15. Effect of laminarinase treatment on cell wall of AAE1, ugeAΔ and ugmAΔ. Wild type (A, G) ugmAΔ (B, H) and ugeAΔ (C, I) hyphal wall structure when incubated 0.5 h (A-C) and 1 h (G-I) with buffer containing 20 × nitrate salts. Wild type (D, J), ugmAΔ (E, K) and ugeAΔ (F, L) hyphal wall structure when treated with laminarinase enzyme for 0.5 h (D-F) and 1 h (J-L). The size of the images is 2 µm × 2 µm and resolution 500 × 500 pixels. All AFM contact mode topography images were processed shadowing effect.
Table 2. Subunit size of the *Aspergillus nidulans* hyphal cell wall surface following laminarinase digestion.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Subunit Size after 0.5 h (nm) (mean ± SD)</th>
<th>Subunit Size after 1 h (nm) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Laminarinase</td>
<td>control</td>
</tr>
<tr>
<td>AAE1</td>
<td>49 ± 13</td>
<td>44 ± 10</td>
</tr>
<tr>
<td><em>ugeAΔ</em></td>
<td>69 ± 9.9</td>
<td>63.6 ± 13</td>
</tr>
<tr>
<td><em>ugmAΔ</em></td>
<td>103 ± 15</td>
<td>86 ± 12</td>
</tr>
</tbody>
</table>

<sup>a</sup> SD, standard deviation.

<sup>b</sup> ND, not determined. See results.
Table 3. Roughness of the *Aspergillus nidulans* hyphal cell wall following laminarinase digestion.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Roughness after 0.5 h (nm) (mean ± SD(^a))</th>
<th>Roughness after 1 h (nm) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Laminarinase</td>
<td>Control</td>
</tr>
<tr>
<td>AAE1</td>
<td>10 ± 3*</td>
<td>9.9 ± 1.7</td>
</tr>
<tr>
<td>ugeAΔ</td>
<td>11 ± 2</td>
<td>11.9 ± 0.6</td>
</tr>
<tr>
<td>ugmAΔ</td>
<td>12 ± 3</td>
<td>13.6 ± 2.4</td>
</tr>
</tbody>
</table>

\(^a\) SD, standard deviation.
4. DISCUSSION

This study has demonstrated that the deletion of genes responsible for the synthesis and transport of Galf has a profound effect on cell surface ultrastructure and physical properties. El-Ganiny et al. (2010) and Afrose et al. (2011) demonstrated that deletion of these genes increased cell wall thickness and hyphal width, which we hypothesized, would be reflected in cell wall ultrastructure and physical properties.

4.1 β-Galf maintains wildtype surface morphology

High resolution AFM imaging of wild type and mutant strains was used to characterize subtle changes in surface features, overlooked by other characterization methods, as a result of removing and/or reducing β-Galf in the cell wall. The larger subunit size of mutant strain cell wall surfaces can be attributed to either an increased synthesis of other cell wall components or reduced cell wall cross linking. Consistent with the surface ultrastructure data and latter hypothesis, Ma et al. (2005) observed larger subunit sizes on the surface of immature regions of the hyphal cell wall, consistent with hyphal apices not yet fully matured. Based on their growth rate (~ 250 nm/min) and their distance (<3 μm) from the apex, cross-linking enzymes at the apex have not had time to fully cross link the cell wall (Ma et al., 2005). Having observed the same trend in terms of surface features for the wild type versus mutant strains, I hypothesized that the change in surface feature arrangement might be attributed
to a lack of cross-linking in the cell wall. In the context of the mutants, this would mean that β-Galf may play a vital role in the maturation process through cross-linking various components of the cell wall. In the case of the ugi single deletion strains, β-Galf synthesis could be compensated by other genes, whether ugiB has epimerase or another function. This gene might help to keep metabolic balance of the Leloir pathway, hence reducing metabolic stress. Therefore, we might expect β-Galf to be present in the cell wall of these mutant strains to some extent and able to facilitate cross-linking. On the contrary, the ugmAΔ, ugiAΔ,ugiBΔ and ugiAΔ,ugiMΔ strains have no redundant enzymes with which to potentially compensate, thus eliminating β-Galf completely along with potentially other changes and leading to the more dramatic changes in surface ultrastructure. The most affected strain was ugiAΔ,ugiBΔ, in which the cell wall surface subunits disappeared, replaced with a fibrous cell wall structure. Unlike the other β-Galf biosynthetic mutants, ugiTAΔ has the enzymes required for β-Galf biosynthesis but lacks the transporter, preventing β-Galf from being incorporated into the growing cell wall. Thus, in this mutant, β-Galf is completely unable to facilitate cell wall maturation, or glycosylate proteins that play important roles in cross-linking either directly or indirectly. The results of this study show that Galf is required to maintain wild type cell wall surface ultrastructure potentially through directly participating in cross linking other cell wall components or indirectly through glycosylation of proteins required to catalyze cross liking reactions.
4.2 Galf maintains A. nidulans cell wall properties

The physical characteristics of the cell wall will also depend on the maturation and packing of cell wall components, and since the cell surface ultrastructure was altered in all the mutants, a significant change in cell wall viscoelasticity for most of the strains was expected. The higher viscoelasticity of chemically cross-linked cell walls in comparison with those of live cells is the first direct evidence that viscoelasticity correlates with cross-linking in fungal cell walls. Lower viscoelastic moduli for all the mutant strains implies that the cell wall components are not properly cross linked nor are they as tightly packed as wild type. We see a similar effect for apical regions (Ma et al., 2005), where there has been inadequate time to properly cross link cell wall sugars, lending further support to the idea that the lower viscoelasticity of the mutant strains relates to impaired cross linking and therefore cell wall maturation. Therefore, Galf is essential for not only wild type cell surface ultrastructure but also to maintain viscoelasticity of the cell wall. Viscoelastic properties are not only important to protect hyphal cell from shear or osmotic shock from the environment, but are also required for polarized growth and branching (Bowman & free 2006).

The non-linear relationship between viscoelasticity and hyphal dimensions for the \( ugmA\Delta,\ ugtA\Delta \) and \( ugeA\Delta, ugmA\Delta \) strains, show that the Zhao model is not sufficient to explain cell wall viscoelasticity for those strains. We used the model developed by Zhao et al. (2005), which calculates the indentation of the cell wall assuming it to be a continuous layer of cell wall material. They used finite
element analysis to develop the model from FS data of fixed A. nidulans, so the plot of viscoelastic modulus versus hyphal dimension should be linear. A plot of viscoelastic modulus versus hyphal dimension (Figure 16) showed a good fit to the model for wild type and the ugeΔ single mutant strains, but a poor fit for the less well ordered cell wall of ugmΔ, ugtΔ and the double deletion mutants. In these cases, the difference in viscoelasticity can be attributed to a combination of cell wall composition and organization of its components.
Figure 15. A plot of *A. nidulans* hyphal cell wall viscoelasticity as a function of hyphal dimension. Viscoelasticity was calculated according to Equation 3 and hyphal dimension is represented by \((R/h)^{1.5}\) (Zhao *et al.*, 2005). The plot shows that the extremely low viscoelasticity for certain mutant strains does not correlate with hyphal dimension. \(R^2\)-values for linear regions were 0.99 for both fixed and live hyphae.
Based on the more, or completely disordered cell wall surface subunits for the
ugmAΔ, ugtAΔ and ugeAΔ,ugmAΔ hyphal walls, the lower viscoelasticity will be
partially attributable to penetration of the AFM tip into or between the cell wall
subunits or depressions for these mutant strains (Figure 17). The AFM tip has a
nominal radius of about 10 nm, which is significantly smaller than the subunits of
strains with more compromised cell walls, so it is likely that the AFM tip is
piercing or passing between individual subunits during force curve measurement.
This idea is further supported by the higher adhesion forces between the cell wall
surface and the AFM tip, which can indicate that a larger area of AFM tip is
interacting with cell wall components and/or it is piercing individual subunits
and/or the mutant subunits are more hydrophilic. If some mutants have less well
cross linked of cell wall components, the outer surface of the cell wall may be
softer allowing more AFM tip penetration, but extensive biochemical studies are
needed to provide direct evidence for a change in cross-linking pattern.
Figure 17. Schematic representation of the interaction between the AFM tip and the surface of the hyphal wall. For a) wild type, ugeAΔ, ugeBΔ and ugeAΔ,ugeBΔ strains, in which the entire cell wall is deformed, and b) ugmAΔ, ugeAΔ,ugmAΔ and ugtAΔ strains for which the AFM tip penetrates or slips between individual surface subunits.
Hyphal cell wall maturation depends on the bond formation and cross-linking between various components of the cell wall. Sugars are cross-linked through their available hydroxyl (OH) groups with proteins, and other components of the cell wall. As a result of cross-linking between glucan and chitin, which makes an ether linkage, water is lost (Cabib, 2009). Ma et al. (2005) also observed that the cell wall at the apical region of the hyphae is more hydrophilic than mature regions. Since, mutant strains of hyphae also have a more hydrophilic surface character, this provides further evidence that the mutant strain cell walls have not properly matured.

4.3 Cell wall properties and composition

Increased cell wall thickness, lower viscoelasticity and a dramatic change in cell wall surface morphology could be attributed to either or both the direct consequences of reduced cross-linking or the indirect effect of activating the cell wall integrity (CWI) signalling pathway. β-Galf is attached to the mannan components of the cell wall through a β-1,3-linkage forming galactomannan (Latgé et al. 1994), furthermore, galactomannan can be attached to the non-reducing end of β-1,3-glucans, The non-reducing end of β-1,3-glucan can also be linked with glycoprotein or a GPI anchored protein (Fontain et al., 2002), for which β-Galf plays an important role in glycosylation (Bowman & Free., 2006). Therefore, we speculate that the absence of β-Galf may perturb the linkage between mannan and glucan as well as glucan and glycoprotein. Our viscoelastic data (see Table 1) demonstrate that chemical fixation was unable to recover wild
type viscoelasticity for mutant hyphal cell walls. This observation is consistent the
cross-linking of primary amines by formaldehyde, which would include chitin and
protein, but which would exclude a major component of the cell wall, including
glucan and mannan polysaccharides. Thus, this data provides indirect evidence
that the cell wall maintains viscoelasticity through cross linking among proteins
and polysaccharides, possibly mediated by β-Galf.

In addition to the direct effect glycosylation has on the cell wall, it can indirectly
affect the physical properties and ultrastructure of the A. nidulans cell surface.
Glycosylation is required for the proper function and localization of proteins;
therefore the absence of β-Galf can lead to the inactivation or impaired
localization of cell wall proteins. If cross-linking enzymes are glycosylated
proteins, then the mutant strains would be rendered devoid of enzymes
responsible for cross-link formation (Schmalhorst et al., 2008). Therefore, the
cross-linking required by glucan or chitin would be impaired, rendering the cell
wall more pliable than that of the wild type strain.

Moreover, mutant strains unable to synthesize the UgeA enzyme may activate
the stress response pathway through the metabolic imbalance created by
accumulation of UDP-glucose inside the fungal cell. El-Ganiny et al., (2010)
demonstrated that ugeAΔ strains are unable to grow in media in which galactose
is the sole carbon source. The reaction catalyzed by UgeA is a part of the Leloir
pathway for galactose utilization, and its absence halts the pathway and hence
galactose metabolism. This metabolic stress could be remediated by increased
chitin or glucan synthesis. Li et al., (2007) found that alteration of GPI anchored proteins increased glucan and chitin synthesis through stress response pathways, and deletion of genes responsible for the synthesis of cell wall components are capable of directly activating these pathways (Damveld et al., 2008). Therefore, the deletion of β-Galf biosynthetic enzymes may lead to constitutive activation of the stress response pathway, resulting in induced expression of several genes that remodel the fungal cell wall. If this were the case, we would expect over expression of certain genes induced by the CWI pathway.

Previous studies introduced the idea that the round shaped features on hyphal surfaces may represent triplexes of branched glucans (Ma et al., 2005), and biochemical studies show that glucan is the most abundant component of the A. nidulans cell wall (Bowman & Free., 2005). The surface structure previously imaged by scanning electron microscopy (SEM) did not resolve fine features of the cell wall molecular architecture, which are better determined by transmission EM through immunogold labelling (Reviewed in Osumi, 1997). The significantly larger subunits of the β-1,3-glucanase treated cell surfaces compared to that of control sample (see Table 1), can be attributed to the degradation of glucans and/or an alteration of sugar packing. An alternate explanation is that mannan and proteins linked to glucan components of the cell wall are rearranged, altering cell surface ultrastructure and surface subunit size.
4.4 Relationship between Galf and glucan cell wall components

The cell surface features of *ugmAΔ*, *ugeAΔ* and the wild type strain were no longer well defined following glucanase treatment, again attributable to degradation of the glucan polymers. Therefore, glucan likely reaches the cell surface, disrupting surface subunit packing when digested. Similar results were observed when wild type hyphae were treated with the more specific β-1,3-glucanase enzyme. The cell surface ultrastructure of the *ugmAΔ* strain was the most susceptible to laminarinase, with significant changes in cell surface ultrastructure observed after only 0.5 h incubation. The latter result reflects the absence of β-Galf in the cell walls of the *ugmAΔ* strain, allowing greater accessibility of the enzyme to cell wall β-glucans and a possible role β-Galf in cross-linking.

A previous study by El-Ganiny *et al.* (2010) proposed that the redundant genes in *A. nidulans* might be able to compensate β-Galf biosynthesis in the mutants. The AFM study of the *ugeAΔ* strain demonstrated that cell wall surface features are smaller than the *ugmAΔ* strains, with a greater viscoelasticity compared to other mutant strains such as *ugmAΔ*, *ugeAΔ,ugeBΔ* and *ugeAΔ,ugmAΔ* (Paul *et al.*, 2011). Therefore, the *ugeAΔ* strain cell wall subunits are more tightly packed than that of *ugmAΔ* strains, and thus the glucan components of the cell wall will be less accessible to the enzyme. However, after 1 h of enzyme treatment, all strains including wild type showed a significant change in their surface features, despite statistically identical surface roughness for all three strains compared
control samples following 0.5 h of enzyme treatment (Table 3). Taken together, this provides evidence that glucan polymers reside at or near the cell surface. AEE1 has the roughest surface, a calculation based on surface subunit size and height. The susceptibility of the ugmAΔ strains to laminarinase treatment must result from organizational changes in the cell wall components based on the absence of β-Galf. Thus, β-Galf plays an important role in the tight packaging of cell wall components (Paul et al. 2011).
5. CONCLUSIONS

This study has contributed to our understanding of the role of galactofuranose, an antifungal target, in the maturation of *Aspergillus* hyphal cells. Comparison of cell wall physical properties for fixed and live hyphal cells provides the first direct evidence of cross-linking effects on cell wall viscoelasticity and how trends among wild type and mutant strains are consistent for both fixed and live hyphae. Despite β-Galf being a minor component by weight of the cell wall, its absence has a dramatic effect on the packaging and maturation of cell wall components. Removal or reduction of this cell wall component gives rise to cell walls with larger subunits that are less well packed, and with lower viscoelasticity. The consistently higher viscoelastic moduli for chemically cross linked hyphal cell walls, whether wild type or mutant, shows that viscoelasticity can be directly related to cross-linking of cell wall components. The model used in this study was deemed inappropriate for accurately measuring the viscoelasticity of the *ugmAΔ* and *ugtAΔ* single mutant strains and *ugeAΔ,ugeBΔ* and *ugeAΔ,ugmAΔ* double mutant strain, each having less well packaged cell wall surface subunits.

Preliminary AFM data has provided insight into the organization and localization of cell wall polysaccharides, a study which is ongoing. Enzymes used to degrade glucan cell wall polymers highlight its importance in cell wall maturation, and comparison of wild type and mutant hyphae reinforces the idea that Galf is involved in the ultrastructural organization of *A. nidulans* cell wall components.
6. FUTURE STUDIES

From this study we can speculate that the alteration of viscoelasticity and ultrastructure may result from impaired cross-linking in the absence of β-Galf, but we currently lack complete biochemical evidence. The idea that the deletion of β-Galf biosynthetic genes may lead to an increase in chitin or glucan synthesis could be tested by quantitative-PCR analysis of the mutant strains. Further, both O-linked and N-linked protein glycosylation could be determined using a glycoproteomics approach. Glucanase treatment of wildtype hyphae supports the previous hypothesis that glucan extends to the cell surface, and this data would be complemented by studying the effects of proteases and mannan and chitin degrading enzymes on the hyphal cell surface structure. Single molecule force spectroscopic studies of A. nidulans protoplast regeneration using anti-glucan, anti-chitin and anti-mannan modified AFM tips during the gradual synthesis of cell wall components would provide the greatest degree of direct information on the arrangement of sugars in the hyphal wall.
7. REFERENCES


Cabib E (2009) Two novel techniques for determination of polysaccharide cross-links show that Crh1p and Crh2p attach chitin to both β-1,6- and β-1,3-glucan in the *Saccharomyces cerevisiae* cell wall. *Eukaryot. Cell* 8(11), 1626–1636


Figure A: Topographic and physical properties of glass coverslip surface.

The same glass coverslips were used for growing hyphae. They exhibit uniform physical properties throughout the surface.
Figure B. Topographic and physical properties of dialysis tubing membrane. This is the same dialysis tubing used for growing hyphae for live hyphal imaging. The exhibit heterogeneous elastic properties.
Figure C. The topographic image of standard sample used for calibrations of X and Y piezo scanner. The distance of the pitch was used for calibration.
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