Quantifying the importance of galactofuranose in *Aspergillus nidulans* hyphal wall surface organization by atomic force microscopy.

Running Title: The role of Gal in *A. nidulans* wall organization

Biplab C. Paul\textsuperscript{a,d}, Amira M. El-Ganiny\textsuperscript{b,c,d}, Mariam Abbas\textsuperscript{a}, Susan G. W. Kaminskyj\textsuperscript{b,e}, Tanya E. S. Dahms\textsuperscript{*a,e}

\textsuperscript{a}Department of Chemistry and Biochemistry, University of Regina, 6262 Wascana Parkway, Regina SK S2S 0A2, Canada.

\textsuperscript{b}Department of Biology, University of Saskatchewan, 112 Science Place, Saskatoon SK S7N 5E2, Canada.

\textsuperscript{c}Microbiology Department, Faculty of Pharmacy, Zagazig University, Egypt.

\textsuperscript{d}Contributed equally to the research.

\textsuperscript{e}Contributed equally to the writing.

\textsuperscript{*}Author to whom correspondence should be addressed. Email: tanya.dahms@uregina.ca; Telephone: 1 306 585 4246

Copyright © 2011, American Society for Microbiology and/or the Listed Authors/Institutions. All Rights Reserved.
Abstract

The fungal wall mediates cell-environment interactions. Galactofuranose (Galf), the five-member ring form of galactose, has a relatively low abundance in *Aspergillus* walls yet is important for fungal growth and fitness. *A. nidulans* strains deleted for Galf biosynthesis enzymes UgeA (UDP-glucose-4-epimerase) and UgmA (UDP-galactopyranose mutase) lacked immunolocalizable Galf, had growth and sporulation defects, and had abnormal wall architecture. We used atomic force microscopy and force spectroscopy to image and quantify cell wall viscoelasticity and surface adhesion of *ugeA*∆ and *ugmA*∆ strains. We compared them with a wild type (AAE1) and the *ugeB* deletion strain, which has wild type growth and sporulation. Our results suggest that UgeA and UgmA are important for cell wall surface subunit organization and wall viscoelasticity. The *ugeA*∆ and *ugmA*∆ strains had significantly larger surface subunits and lower cell wall viscoelastic moduli than those of AAE1 or *ugeB*∆ hyphae. Double deletion strains, [*ugeA*∆, *ugeB*∆] and [*ugeA*∆, *ugmA*∆], had more disorganized surface subunits than single deletion strains, and changes in wall surface structure correlated with changes in its viscoelastic modulus for both fixed and living hyphae. Wild type walls had the largest viscoelastic modulus, while those of the double deletion strains had the least. The *ugmA*∆ and particularly the [*ugeA*∆, *ugmA*∆] strains were more adhesive to hydrophilic surfaces than wild type, consistent with changes in wall viscoelasticity and surface organization. We propose that Galf is necessary for full maturation of *A. nidulans* walls during hyphal extension.
Introduction

The fungal wall supports and shields the hyphal cytoplasm, and mediates interactions between the cell and its environment. Fungal walls are typically about 30% of cell dry weight (7, 10), and a similar portion of the fungal genome is thought to contribute to cell wall biosynthesis and/or maintenance (11, 17). Fungal walls are composed of a variety of carbohydrate polymers (7, 11, 15), however, deleting many wall biosynthesis genes appears to be compensated by genetic redundancy and/or by induction of the cell wall integrity pathway (6, 7, 23).

The Aspergillus wall is reinforced by chitin fibrils, and has a matrix containing alpha- and beta-glucans, other sugars including galactomannans, and proteins. Galactofuranose (Gal\textsubscript{f}) is the five-member ring form of galactose that is found in the cell walls of Aspergillus (6, 7, 23), other fungi (reviewed in (15)), and certain other microbes (3). Deletion of UDP-galactopyranose mutase in several Aspergillus species has shown that Gal\textsubscript{f}, despite its relatively low abundance, is important for wild type fungal growth, cell morphogenesis, hyphal adhesion, wall architecture, and spore development (6, 8, 9, 14, 16, 25) and may mediate pathogenesis (1, 21-23).

The A. nidulans gene products UgeA (UDP-glucose-4-epimerase) (8), and UgmA (UDP-galactopyranose mutase) (9) catalyze sequential steps in Gal\textsubscript{f} biosynthesis (Figure 1). The \textit{ugeA}\textsubscript{\Delta} and \textit{ugmA}\textsubscript{\Delta} deletion strains have similarly compact colonies, aberrant hyphal growth and reduced sporulation. The hyphal walls of these strains differ from wild type and with each other as visualized using transmission electron microscopy (TEM) (8).

Atomic force microscopy (AFM) imaging uses a fine-tipped probe mounted on a flexible cantilever to raster scan the surface of an object generating a topographic map. An approach-retract cycle of the AFM probe, called force spectroscopy (FS), can be used to calculate the viscoelastic modulus of the whole organism or its cell surface, and surface
adhesion. Previously we used AFM to show that *A. nidulans* cell walls of growing hyphal tips differ from those of mature regions (18), and to document changes associated with spore swelling, germination and the non-polarized hyphal growth of temperature sensitive mutants (19). Here, we compare the hyphal walls of wild type and a suite of Galf biosynthesis pathway deletion strains using TEM, AFM and FS to gain a better understanding of the role played by Galf in *Aspergillus nidulans* cell wall organization.

**Materials and Methods**

**Strains and culture conditions**

*Aspergillus nidulans* and *Escherichia coli* strains were grown as described in (8, 9) using media formulated as described in (13). Deletion strain construction followed procedures described in (8, 9) using *nkuA*Δ strains, plasmids and primers listed in supplemental Table SA. AN2951 (*ugeB*) was deleted from A1149 using *AfpyroA* as selectable marker (amplified from pTN1) to generate AAE9. Thereafter, *UGE* was deleted from AAE9 using *AfpyrG* as selectable marker (amplified from pAO18) to create the [*ugeA*Δ, *ugeB*Δ] double deletion strain AAE10. Construction of AAE8 [*ugeA*Δ, *ugmA*Δ] was described in (8).

**Confocal fluorescence and transmission electron microscopy (TEM).**

Samples were prepared for light microscopy as described in (9). Briefly, freshly harvested spores were grown on coverslips for 16 h at 28 °C in complete medium (CM) liquid, fixed and stained with Hoechst 33258 (for nuclei) Calcofluor (for cell walls), then imaged by confocal microscopy. Hyphal width and basal cell length were measured at septal positions in mature regions (~ 40µm from the tip) for 50 cells per strain using LSM examiner.
For TEM, wild type and gene deletion strains were grown on dialysis tubing laid over complete medium agar for 1 d at 28 °C, then fixed, embedded, and sectioned for as described previously (9). Hyphal wall thickness was measured on TEM cross-sections of ten hyphae per strain, typically three measurements per hypha, at places where the cell membrane was crisply focused.

**Atomic force microscopy.**

Samples for imaging fixed hyphae were prepared for AFM as previously described (18, 19). Briefly, conidia were germinated in liquid growth medium between two glass coverslips for 16 h. The top coverslip was carefully removed, and the hyphae were fixed with 3.7 % formaldehyde in 50 mM phosphate buffer, pH7.0, containing 0.2 % Triton X-100, followed by rinsing with distilled water and air drying. For live cell AFM imaging, hyphae were grown on dialysis tubing (Spectrapor, 12-14 kDa) overlaying agar medium. After 16 h growth, the dialysis tubing was transferred to a glass coverslip. Sterile Whatman #4 filter paper placed beneath the dialysis tubing was used to deliver liquid growth medium by capillary action, ~ 20 µL at a time.

An Explorer™ AF microscope (Veeco http://www.veeco.com/) with a dry scanner (Veeco, model 5460-00) was used for contact mode imaging and force spectroscopy (FS), as previously described (18, 19). Hyphae were visualized by CCD camera (200 ×) and imaged first at low resolution (200 × 200 lines per scan). All topographic and lateral force data were collected from high-resolution images (500 × 500 lines per scan) of fixed and live (scan rate = 1 and 2 Hz, respectively) cells using Si₃N₄ probe tips (Veeco model #1520-00, k = 0.05 nN/nm, nominal resonance n = 17 kHz). Images represent typical results. AFM tip size and shape was calibrated using gold spheres according to (27).
**Force spectroscopy**

Cantilever spring constants, $k_c$, were determined prior to each force measurement using resonance frequencies according to (5). Tip-sample interaction was tracked by cantilever deflection as a function of Z piezo elongation during probe approach and retraction. For soft materials, meaningful FS comparisons often depend on the velocity of the surface approach (4). In this case, the approach velocity did not significantly affect the hyphal spring constant (data not shown), but this parameter was kept constant (100 nm/s) to facilitate comparison of viscoelastic moduli between samples. Repeated measurements of individual sites on mature walls gave consistent values, and images obtained before and after FS were unchanged (data not shown), indicating that the walls were not damaged during data collection. Values were averaged from force curves collected in triplicate at ten separate points on the surface of mature hyphal walls ($\geq 40 \mu m$ from the tip) for five hyphae per sample and typically three different samples.

Force approach curves measure the unit force (nN) required to indent a surface a given distance (nm), so the slope corresponding to the b-c segment of the approach cycle (refer to Figure 5A) was used to examine the relative cell wall elasticity. FS data were plotted as deflection (nA) versus distance (nm), converted to force (nN) versus distance (nm) curves using the piezo sensor response, and the slope of the line b-c (m) in nN/m used to determine the spring constant $k_w$ of the cell wall according to:

$$k_w = \frac{m_s k_c}{m_h - m_s}$$  \hspace{1cm} (1)
where $m_s$ is the sample slope and $m_h$ is the slope for a hard surface (mica). The value of $k_w$ was used for the subsequent determination of Young’s modulus according to the equation (28):

$$E \sim 0.80 k_w/h (R/h)^{1.5}$$  \hspace{1cm} (2)

where $E$ is the cell wall viscoelastic (Young’s) modulus, $R$ is the hyphal radius measured by either TEM or AFM, and $h$ is the thickness of the cell wall measured by TEM. Surface adhesion values were measured from the last segment of the retraction cycle (Figure 5A, segment e–f). If there is a chemical attraction between the sample and the Si$_3$N$_4$ AFM probe, which is hydrophilic, segment e–f will be a measure of its intensity in nN.

Data Processing and Analysis

AFM images were processed using horizontal levelling, with the maximal height adjusted for optimum contrast (SPMLab version 6.0 software, Veeco). Hyphal widths at mature regions (~ 40 \( \mu \)m from the tip) and surface feature dimensions from topography and lateral force images were measured at the FWHM of the peak height. AFM data are presented as mean ± standard deviation or as ranges of values. TEM and confocal data are presented as mean ± standard error of the mean. Differences in the mean subunit sizes of wild type and deletion strain hyphae were tested by a one-way ANOVA (InStat 3, GraphPad Prism). Standard errors propagated through equations 1 and 2 were calculated for viscoelastic moduli, and a Student’s $t$-test (two-tailed) was used to assess significant difference between values (InStat3, GraphPad Prism).
Results

Building on our previous experience using AFM to study *A. nidulans* hyphae (18, 19) we compared a suite of *A. nidulans* strains deleted for Gal biosynthesis enzymes UgeA and UgmA, the near-isogenic wild type strain AAE1, a strain deleted for an epimerase (UgeB) that did not affect hyphal morphogenesis, and double deletion strains \(u_{ge}A\Delta, u_{ge}B\Delta\) and \(u_{ge}A\Delta, u_{gm}A\Delta\). Double mutants were used to further explore the function of individual gene products.

Even enzymes that mediate a known biochemical function may have collateral defects in a deletion strain based on protein-protein interactions, for example if the protein is part of a scaffold for a multi-enzyme.

Characterization of *Aspergillus nidulans* ugeB

*Aspergillus nidulans* ANID2951.4 (which we named UgeB) shares 38% amino acid sequence identity with UgeA (8), and had been annotated as a UDP-glucose/galactose-4-epimerase (www.broadinstitute.org/annotation/genome/aspergillus_group/). The *ugeB* genomic sequence has a single exon that encodes a 428 amino acid peptide. The *ugeB* cDNA could not be amplified (three attempts), unlike *ugmA* (7) and *ugeA* (8). We deleted *ugeB* (Figure SA) as described in (9), and confirmed the deletion using PCR (Table SA, Figure SA). A \(u_{ge}A\Delta, u_{ge}B\Delta\) strain was generated and confirmed (Figure SB) as described in (8). The *ugmA\Delta* strain was described in (9) and the *u_{ge}A\Delta* and \(u_{ge}A\Delta, u_{gm}A\Delta\) strains were described in (8). UgeB was expressed in vitro using the genomic sequence, which does not contain introns, purified, and shown to convert UDP-galactose into a product that is not UDP-glucose, following the procedure shown in (8, and data not shown). This unknown product awaits conclusive identification.

The *ugeB* sequence was put under the control of the *AlcA* promoter and also tagged with red fluorescent protein (El-Ganiny and Kaminskyj, in preparation), then over-expressed by
culturing on CM containing 100 mM threonine (CMT). Under these conditions, pAlcA-ugeB-rfp was expressed, albeit weakly, in conidia and to a lesser extent in mature hyphae (Figure SC), but was not detectable in hyphal tips (data not shown).

Morphology of *Aspergillus nidulans* strains deleted for Galf biosynthesis genes

Confocal microscopy images showing the hyphal morphology of the suite of Galf biosynthesis deletion strains examined in this study (AAE1, ugeAΔ, ugeBΔ, [ugeAΔ, ugeBΔ], ugmAΔ, [ugeAΔ, ugemAΔ]) are shown in Figure 2. Strain morphometry is described in Table 1. Unlike the previously described ugeAΔ and ugmAΔ deletion strains, which had wide and highly branched hyphae and reduced sporulation (8, 9), the ugeBΔ strain had wild type morphology hyphae and growth rate, and abundant sporulation (Table 1; Figure 2; data not shown). The [ugeAΔ, ugeBΔ] strain was viable when grown on media containing glucose as carbon source, but it did not form colonies on media containing galactose as the sole carbon source (due to ugeAΔ), and its hyphae were wide and branched like those of ugeAΔ (Table 1; Figure 2).

Transmission electron micrographs of hyphal cross-sections showed that the walls of ugeAΔ and ugmAΔ strains were two-fold and four-fold thicker, respectively, than those of AAE1 (Table 1, Figure 3) (8, 9). Given the general correlation between hyphal morphology and wall thickness (e.g. 8, 9, 11, 12, 20, 24), we expected that the hyphal wall thickness of ugeBΔ might be similar to AAE1. Instead, the ugeBΔ strain hyphal walls were almost two-fold thicker than AAE1 (Table 1, Figure 3). Also unexpectedly, the [ugeAΔ, ugeBΔ] strain hyphal walls were about twice as thick as either single deletion strain, even thicker than those of the [ugeAΔ, ugmAΔ] strain (Table 1, Figure 3).

TEM images of the ugeAΔ (Supplementary Figure Cb in 8) and ugeBΔ (data not shown) strains grown in liquid shake culture accumulated debris, not observed for the same strains grown on dialysis tubing (Figure 3) or for the [ugeAΔ, ugeBΔ] strain (data not shown).
Atomic force microscopy imaging of wild type and Galf gene deletion strains.

We used AFM imaging to acquire high-spatial resolution information about the hyphal wall surfaces of two wild type and four Galf biosynthesis gene deletion strains. AFM imaging provides quantitative depth resolution, thus facilitating surface subunit measurements (18, 19).

Our previous work demonstrated that for the wild type strain, A28, the walls of hyphal tips and tips of lateral branches had matured by 3 µm behind the apex, at which point their surfaces resembled unbranched regions 20 µm and 40 µm behind the tip (18). To ensure that wall surfaces were mature for all strains, we chose analysis sites that were at least 40 µm behind the hyphal tips, expecting that wall maturation might be slower in the Galf biosynthesis gene deletion strains. AFM data can be collected from living or fixed cells (e.g. (18)). We present images of fixed hyphae for comparing wall surfaces amongst the suite of Galf biosynthesis gene deletion strains, since hyphal wall subunit size and distribution were similar to living cells but were more clearly defined (18).

Contact mode AFM imaging simultaneously collects topography and lateral force information. The latter represents a convolution of topography and tip-sample interactions for rough samples, thus producing relief images with more clearly defined edge features (18).

High resolution images of fixed wild type (AAE1) and Galf biosynthesis gene deletion strain hyphae (Figure 4) show distinct differences in their surface subunit size (Table 1) and packing. AAE1 hyphae had small rounded subunits with a consistent size and even packing. In contrast, both the $ugeA\Delta$ and $ugmA\Delta$ strain hyphae had substantially larger and more variable-sized hyphal wall surface subunits than AAE1, and also had more disorganized subunit packing. The hyphal wall of the $ugeB\Delta$ strain most closely resembled that of AAE1, but with slightly elongated subunits. The [$ugeA\Delta$, $ugeB\Delta$] hyphal surface was notable in that its surface appeared fibrillar in the topographic images, hence maximum subunit sizes were not measured. Thus, both UgeA and UgeB appear to be important for wild type hyphal wall surface
formation. The \([ugeA\Delta, ugmA\Delta]\) strain hyphal wall surface subunits were similar in size to \(ugmA\Delta\). Taken together, each member of the suite of Galf deletion strains produced a distinctive wall phenotype.

**Cell wall viscoelasticity and adhesion of wild type and Galf biosynthesis gene deletion strains**

Viscoelasticity is the property describing materials such as hyphal walls, which exhibit both viscous (fluid-like) and elastic mechanical properties. Cell wall spring constants measured by FS (Figure 5A, segment b-c) were used to calculate their viscoelastic modulus for both fixed and live hyphae of AAE1 and the suite of Galf gene deletion strains (Table 1). Cell wall viscoelastic moduli for the single deletion strains, UgeA\Delta and UgeB\Delta, were significantly lower than that of AAE1 (Table 1). Viscoelastic moduli of \(ugmA\Delta\) and the double deletion strains \([ugeA\Delta, ugeB\Delta]\) and \([ugeA\Delta, ugmA\Delta]\) were at least an order of magnitude smaller than AAE1 (Table 1).

Notably, viscoelastic moduli of fixed hyphal walls were typically three-fold higher than that of live ones (Table 1). Hyphal wall viscoelasticity for the suite of Galf deletion strains exhibited the same trend for fixed and live hyphae.

The Si₃N₄ AFM probes used in this study have hydrophilic surfaces. We used the e-f segment of the FS curve (Figure 5A) to quantify adhesion between \(A. nidulans\) wall surfaces and the AFM tip during the retraction phase. AAE1 wall surface adhesion to Si₃N₄ is shown in (Table 1). The \(ugmA\Delta\) and \([ugeA\Delta, ugmA\Delta]\) hyphae had significantly (p < 0.05) stronger adhesion to the hydrophilic tip than wild type hyphae.
4. Discussion

Our most notable finding is that perturbing *A. nidulans* cell wall maturation by deleting genes in the Galf biosynthesis pathway, has profound effects on wall surface subunit size and packing that are directly associated with cell wall viscoelasticity. This is despite the fact that none of these genes is essential for growth in culture. Previously, we used AFM and FS to show for the first time that growing hyphal tips of a wild type *A. nidulans* strain, A28 (18), had wall surface characteristics that were consistent with long-accepted models of wall deposition and maturation (2, 26) that had yet to be quantitatively tested.

El-Ganiny et al. (8, 9) had shown using molecular biology, fluorescence microscopy and TEM that the *ugeA*Δ and *ugmA*Δ deletion strain hyphal morphogenetic defects appeared to be correlated with a lack of immunolocalizable wall Galf and to aberrant hyphal wall architecture. Now, we have used high spatial resolution AFM imaging and FS to directly quantify wall defects in this suite of Galf biosynthesis gene deletion strains and to compare them to wild type strains.

**Galf is required for wild type *Aspergillus nidulans* hyphal wall formation**

This AFM study is the first to make quantitative measurements of cell wall surface subunit features, wall viscoelasticity and adhesive properties of *A. nidulans* strains that had been deleted for enzymes having roles in Galf biosynthesis. Strong but circumstantial data in El-Ganiny et al (9) showed that *A. nidulans* strains lacking UgmA had defective hyphal morphogenesis, colony growth, and spore development deficits that correlated with lack of immunodetectable wall Galf. Using TEM cross sections, El-Ganiny et al (9) showed that hyphal walls of the *ugmA*Δ strain were more than four times the thickness of AAE1 and had poorly consolidated surfaces suggesting that Galf may play a role in wall organization.
Previously, we showed using AFM imaging that growing tips of wild type *A. nidulans* had ellipsoidal surface subunits that were larger and more variable in size than the round subunits found 3 µm or further back (Fig 3. in 18). Consistent with the decrease in subunit size and improved organization as a function of maturation, we showed an increase in surface hydrophobicity (Fig. 6D in 18) attributed to decreased exposure of sugar hydroxyl groups. El-Ganiny’s study (9) suggested that the *ugmA* ∆ strain walls were weaker than wild type since this phenotype was partially remediated by growth on 1 M sucrose.

Our present study shows that both *ugeA* ∆ and *ugmA* ∆ strains had substantially larger surface subunits than AAE1. In contrast, the *ugeB* ∆ strains, which had wild type colony phenotype and growth rate, had subunit sizes very similar to AAE1. Thus, it appears that *A. nidulans* surface subunit size is inversely correlated with cell wall maturation in A28 (18) and a suite of deletion mutants in the Galβ biosynthetic pathway. Our data also show that wall surface organization correlates with wall viscoelastic moduli, and that these data are mirrored by the thickness and surface layer characteristics visualized in hyphal cross sections using TEM.

Viscoelastic moduli of fixed and living cell walls had a strong positive correlation, demonstrating the value of comparing fixed strains, thus reducing data collection time. The viscoelastic moduli of fixed hyphal walls were consistently larger, revealing the relationship between chemical cross-linking of the cell wall and its viscoelasticity. The data offer insight into the enzymatic cross-linking of hyphal wall components as an integral step in wall maturation, where cross-linking likely contributes to wall integrity by increasing viscoelasticity.

The A4 (28), A28 (18) and AAE1 (current work) are morphological wild type strains. The cell wall viscoelastic modulus of the fixed AEE1 strain was lower than that determined for fixed, rehydrated A4 by Zhao and coworkers (28). Although both studies used the same method to determine cantilever spring constants (5), it is only an estimate and can account for the
difference in viscoelastic moduli. The viscoelastic modulus of live AEE1 cell walls (Table 1) was lower than that reported previously for A28 (115 ± 31 MPa; 18). However, since the latter study compared viscoelastic moduli in different regions along single hyphae, cantilevers were not calibrated.

We used Zhao’s model (18), which assumes the indentation of a contiguous layer (cell wall) surrounding a large cylinder (hyphae; Figure 5B), to calculate cell wall viscoelastic moduli. A plot of the dimensionless unit Eh/k_w versus (R/h) \(^{1.5}\) (data not shown) suggests this model fits AEE1, ugeAΔ and ugeBΔ strains, better than it does the poorly ordered walls of the ugmAΔ, [ugeAΔ ugeBΔ] and [ugeAΔ ugmAΔ] strains. Differences can at least in part be attributed to the composition and organization of cell wall components (Figure 5B), whereas viscoelastic moduli for ugmAΔ, [ugeAΔ ugeBΔ] and [ugeAΔ ugmAΔ] strains suggests the AFM tip may penetrate the loosely packed cell wall surface. The Si\(_3\)N\(_4\) AFM tips used in this study have tips that are about 5 nm wide (18). The subunits of the ugmAΔ and [ugeAΔ ugmAΔ] strains are 20-fold larger (Table 1), so the AFM tip could possibly pierce an individual subunit. The surface of the [ugeAΔ ugeBΔ] strain has a fibrillar appearance (Figure 5C), so its interaction with the AFM tip could be unlike the other deletion strains we studied.

Galf appears to mediate Aspergillus nidulans hyphal wall surface and hyphal adhesion

Lamarre et al (14) suggested that hyphal wall Galf plays a role in A. fumigatus hyphal wall surface properties, which they showed qualitatively by the accumulation of material on Afugm1Δ hyphal walls using SEM, and by hyphal adhesion to substrates including glass and plastic coverslips, latex beads and epithelial respiratory cells.

We quantified the adhesion between the hydrophilic Si\(_3\)N\(_4\) AFM tip and walls of living AAE1, ugmAΔ and [ugeAΔ, ugmAΔ] hyphae. The increasing hyphal wall surface disorder in
"ugmAΔ" and \([ugeAΔ, ugmAΔ]\) strains correlates with effects on hyphal wall viscoelasticity and adhesion. The loose packing of hyphal walls surfaces observed by AFM imaging of Galf mutants would expose polar groups normally masked during wall maturation, increasing hydrophilic character of the wall surface and resulting in greater adhesion. Consistent with Lamarre et al (14) the "ugmAΔ" and \([ugeAΔ, ugmAΔ]\) strains tended to adhere to microscope coverslips compared to AAE1 and ugeBΔ (data not shown). Adhesion values between the Si₃N₄ tip and "ugmAΔ" and \([ugeAΔ, ugmAΔ]\) walls were comparable to those previously reported for A28 hyphae at growing tips, where the wall is newly deposited and not yet mature (~ 9 nN, (18)). Thus, surface subunit size, wall viscoelasticity, and wall adhesion to hydrophilic surfaces show consistent trends for wild type and Galf gene deletion strains.

Schmalhorst et al. (23) provided data to suggest that the \(A. fumigatus\) glfAΔ (homologous to \(A. nidulans\) ugmA (9)) strain had attenuated virulence in a murine model for systemic aspergillosis. However, scanning electron microscopy of cross-fractured glfAΔ hyphal walls were half the thickness of wild type \(A. fumigatus\) walls, the opposite trend to our study that deserves further attention.

By combining gene deletion characterizations with TEM, AFM, and FS, we have shown that Galf is important for \(Aspergillus\) hyphal wall maturation. However, despite the strong correlation between our results and those of Lamarre et al (14) we are not yet able to determine the likely location(s) of Galf-containing molecules in \(Aspergillus\) walls. Indeed, Latgé’s 2010 model (16) discusses these molecules without indicating many details relating to their deployment in the three-dimensional wall architecture.

We have shown that perturbing wildtype Galf cell wall deposition has substantial effects on the surface ultrastructure and viscoelasticity of the \(Aspergillus nidulans\) cell wall. Galf appears to have crucial and multiple roles in \(Aspergillus\) hyphal cell wall maturation and
integrity. Studies addressing potential roles of mannose in \textit{A. nidulans} wall structure and adhesive properties, and potential roles of Galf in pathogenicity are underway.

\textbf{Acknowledgements}

This research was supported by Natural Science and Engineering Research Council of Canada Discovery grants to TESD and SGWK, a Canadian Institutes of Health Research/Regional Partnership Program grant to SGWK, and by an Egyptian Ministry of Higher Education grant to AME. BCP was partially supported by the Department of Chemistry and Biochemistry in the Faculty of Science, University of Regina.
References


Table 1. Morphological characteristics, maximum dimension of surface subunits, and cell wall viscoelastic moduli of wild type and Gal/biosynthesis enzyme gene deletion strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hyphal width&lt;sup&gt;a&lt;/sup&gt; (µm) ± SE</th>
<th>Wall thickness&lt;sup&gt;b&lt;/sup&gt; (nm) ± SE</th>
<th>Subunit maximum dimension (nm) ± SD</th>
<th>Viscoelastic moduli of fixed hyphal wall (MPa)&lt;sup&gt;c&lt;/sup&gt; ± SD</th>
<th>Viscoelastic moduli of live hyphal wall (MPa)&lt;sup&gt;c&lt;/sup&gt; ± SD</th>
<th>Adhesion (nN) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype (AAE1)</td>
<td>2.4 ± 0.0</td>
<td>54 ± 2</td>
<td>35 ± 5</td>
<td>211 ± 15</td>
<td>82.3 ± 12.9</td>
<td>5.7 ± 1.6</td>
</tr>
<tr>
<td>ugeAΔ</td>
<td>3.6 ± 0.1</td>
<td>104 ± 10</td>
<td>63 ± 10</td>
<td>99 ± 48</td>
<td>24.6 ± 13.7</td>
<td>ND</td>
</tr>
<tr>
<td>ugeBΔ</td>
<td>2.5 ± 0.0</td>
<td>95 ± 11</td>
<td>39 ± 8</td>
<td>74 ± 22</td>
<td>22.5 ± 8.6</td>
<td>ND</td>
</tr>
<tr>
<td>ugeAΔ, ugeBΔ</td>
<td>3.5 ± 0.1</td>
<td>217 ± 62</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>38 ± 21</td>
<td>9.8 ± 5.1</td>
<td>ND</td>
</tr>
<tr>
<td>ugmΔ</td>
<td>3.1 ± 0.1</td>
<td>204 ± 10</td>
<td>108 ± 35</td>
<td>14 ± 2</td>
<td>3.1 ± 0.4</td>
<td>8.1 ± 0.3</td>
</tr>
<tr>
<td>ugeAΔ, ugmΔ</td>
<td>3.2 ± 0.4</td>
<td>162 ± 8</td>
<td>97 ± 23</td>
<td>0.05 ± 0.02</td>
<td>0.03 ± 0.01</td>
<td>17.3 ± 3.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> There was no significant difference in hyphal width for fixed or live hyphae measured either by confocal microscopy or AFM.

<sup>b</sup> Measured from TEM hyphal cross-sections. See Materials and Methods section.

<sup>c</sup> SD was calculated from errors propagated through equations 1 and 2. See Materials and Methods section.

<sup>d</sup> Not determined. See Results section.
Figure 1: Biosynthesis of GalF from UDP-Glucose. UDP-glucose is converted to UDP-Glucose by UDP-Glucose 4-epimerase (UgeA). In the reaction shown, UDP-glucose is converted to UDP-Galactopyranose (UDP-Galp) by UgeA. UDP-Galactopyranose is then converted to UDP-Galactofuranose (UDP-Galf) by UDP-Galactopyranose mutase (UgmA). These two enzymes are localized in the cytoplasm, and then UDP-galactofuranose is transported to the fungal Golgi equivalent, which is the site of incorporation into other cell wall components.
Figure 2. Appearance of the wild type and Galβ-biosynthesis deletion strain hyphae used in this study. Strains were grown for 16 h, then fixed and stained with Hoechst 33258 to visualize nuclei. Images are combined fluorescence and transmitted light. Bar = 5 µm (for all images).
Figure 3. Transmission electron micrographs of hyphal wall cross-sections of AAE1 and Galf-biosynthesis deletion strains used in this study. Bar = 100 nm (in A, wild type image, for all parts). Impressions regarding the relative hyphal diameter as assessed by curvature may be misleading, because some hyphal cross sections were not perpendicular to the hyphal axis. Images have been contrast adjusted to highlight wall structure; the cytoplasm is dark because wall carbohydrates stain poorly for TEM. Arrows indicate the outer boundaries of cell wall thickness measurements.
Figure 4: Atomic force microscopy images of wild type and GalF-biosynthesis deletion strains used in this study. Grey scale for lateral force images is ~ 2 nA and for topography images ranges from 50-70 nm. Bar in A = 200 nm, and is valid for all images.
Figure 5: A) Representative force curve shows tip approach (a-c) with jump into contact (b) and tip retraction (c-g). The slope of b-c was used to calculate sample viscoelastic modulus and segment f-e represents tip-sample adhesion. Schematic representation of the AFM tip (black) interacting with the hyphal surface (grey) of B) AEE1, ugeAΔ, ugeBΔ, in which the entire cell wall is deformed by tip indentation, compared to that of C) ugmAΔ, [ugeAΔ,ugeBΔ] and [ugeAΔ,ugmAΔ] in which the tip likely deforms the loosely packed, larger individual subunits or penetrates the space between.