Journal of Microbiology (2016) Vol. 54, No. 3, pp. 232–242
DOI 10.1007/s12275-016-6045-4
eISSN 1976-3794
pISSN 1225-8873

REVIEW

Recent advances in the understanding of the *Aspergillus fumigatus* cell wall

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(Received Jan 28, 2016 / Accepted Feb 2, 2016)

Over the past several decades, research on the synthesis and organization of the cell wall polysaccharides of *Aspergillus fumigatus* has expanded our knowledge of this important fungal structure. Besides protecting the fungus from environmental stresses and maintaining structural integrity of the organism, the cell wall is also the primary site for interaction with host tissues during infection. Cell wall polysaccharides are important ligands for the recognition of fungi by the innate immune system and they can mediate potent immunomodulatory effects. The synthesis of cell wall polysaccharides is a complicated process that requires coordinated regulation of many biosynthetic and metabolic pathways. Continuous synthesis and remodeling of the polysaccharides of the cell wall is essential for the survival of the fungus during development, reproduction, colonization and invasion. As these polysaccharides are absent from the human host, these biosynthetic pathways are attractive targets for antifungal development. In this review, we present recent advances in our understanding of *Aspergillus fumigatus* cell wall polysaccharides, including the emerging role of cell wall polysaccharides in the host-pathogen interaction.

Keywords: *Aspergillus fumigatus*, cell wall, glycobiology, biofilm, polysaccharide

Introduction

Aspergillus fumigatus is a ubiquitous, saprophytic mold commonly found in the soil and decaying organic matter. *A. fumigatus* produces large number of airborne asexual spores or conidia, which are inhaled by humans on a daily basis. Although these conidia are effectively cleared from the lungs of healthy individuals by innate immune defenses, in patients with impaired immunity, conidia can escape these defenses to germinate and form filamentous hyphae. These hyphae invade pulmonary tissues to produce a necrotizing pneumonia that, if untreated, can ultimately disseminate hematogenously to other organs. Invasive aspergillosis (IA) has increased in incidence in recent decades, in large part due to the increased number of patients receiving cytotoxic chemotherapy, corticosteroids and other immunosuppressive therapies (Kousha *et al.*, 2011). IA is associated with a high mortality rate, ranging from 30% to 100%, despite the use of currently available antifungal agents (Kousha *et al.*, 2011).

Although the invasive form of aspergillosis is the most urgent and life threatening, chronic forms of aspergillosis can also cause significant morbidity and mortality. Patients with underlying chronic pulmonary conditions such as asthma, cystic fibrosis, or chronic obstructive pulmonary disease have reduced airway clearance of *Aspergillus* spores due to deficient mucociliary activity and the production of excess amounts of pulmonary mucus (Kousha *et al.*, 2011; Huerta *et al.*, 2014). These patients can acquire fungal colonization of the airways, and a subset of these patients will develop a severe hypersensitivity reaction to fungal antigens termed allergic bronchopulmonary aspergillosis (ABPA). Individuals with pre-existing cavitary pulmonary disease can become colonized with *A. fumigatus*, resulting in the formation of non-invasive fungal balls clinically known as aspergilloma (Riscili and Wood, 2009). These individuals can develop severe hemoptysis requiring surgery (Zmeili and Soubani, 2007). Antifungal therapy for all of these chronic conditions has been disappointing, and the development of antifungal resistance on therapy is an emerging problem (Denning *et al.*, 2011).

The poor outcomes associated with *Aspergillus* infections have led to a search for novel treatments for these conditions, with recent efforts targeting the cell wall. The fungal cell wall is an attractive therapeutic target for several reasons. The cell wall is absent from mammalian cells; is the first point of contact with the host immune system; mediates defense against environmental stresses, and plays an important role in fungal growth and morphogenesis. Antibiotics targeting the bacterial cell wall such as vancomycin and the β-lactams have clearly demonstrated the power of cell wall-specific antibacterials; and the effectiveness of the cell wall-specific echinocandins targeting β-1,3-glucan in *Candida* species has shown that cell wall active agents can be effective against fungi. However, our limited understanding of the composi-

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tion and biosynthesis of the *A. fumigatus* cell wall has hampered the development of cell wall active antifungals that target this important fungus.

The composition of the *A. fumigatus* cell wall includes a variety of proteins, lipids, melanins, and other pigments. However, polysaccharides are the most abundant molecules within this structure (Bernard and Latge, 2001). These polysaccharides interact through covalent bonding and non-covalent interactions to form both rigid structures and loosely associated, amorphous matrices. The resulting structure is both dynamic and versatile, and plays a number of roles in the growth and protection of the fungal cell. The cell wall provides cellular structure, mediates adherence to various surfaces, acts as a molecular sieve allowing the selective passage of molecules into and out of the cell, and generates extracellular matrices that form the basis for a biofilm lifestyle. Although all fungi have cell walls, the composition and arrangement of cell wall polysaccharides varies by species. Even within the same species, the composition of the cell wall varies in response to growth conditions and during the different stages of the fungal life cycle. The synthesis of the cell wall polysaccharides is tightly regulated by a complex network of regulatory pathways, which we are just beginning to understand. In this work, we review the current understanding of the *Aspergillus fumigatus* cell wall components, with a focus on advances in our understanding of cell wall polysaccharide synthesis and the role of these glycans in host-pathogen interactions.

The cell wall of *A. fumigatus* conidia

The release of large number of small, highly hydrophobic, airborne conidia produced by asexual sporulation is the main mode of *A. fumigatus* dissemination. Up to thousands of conidia are inhaled by the average person every day, and are the first form of the fungus to interact with the host (Abad *et al.*, 2010). Their small size, typically 1–2 microns, allows them to penetrate deep within the airway system, where mucociliary clearance is less efficient (Dagenais and Keller, 2009; Abad *et al.*, 2010). The outer cell wall of conidia is covered by a layer of tightly organized proteins known as hydrophobins, which self-assemble on the surface of resting conidia to render them highly hydrophobic. RodA is the most important and well-characterized of these hydrophobins in *A. fumigatus* conidia (Paris *et al.*, 2003). Hydrophobins play a number of important roles in interaction of conidia with the host. These proteins enhance the adherence of conidia to macromolecules and inhibit innate immune responses to resting conidia (Dagenais and Keller, 2009; Abad *et al.*, 2010). RodA is poorly recognized by the innate immune system, and thus, can mask pathogen-associated molecular patterns (PAMPs) like β-1,3-glucan and α-mannose from recognition by the pattern recognition receptors (PRRs) dectin-1 and dectin-2, respectively (Steele *et al.*, 2005; Carrion Sde *et al.*, 2013). The ability of *A. fumigatus* to mask PAMPS is thought to prevent the development of potentially dangerous inflammatory responses within the lungs in response to the daily inhalation of resting conidia. Conidia produced by mutants deficient in RodA are more readily phagocytosed by macrophages *in vitro* (Paris *et al.*, 2003). RodA is rapidly shed as conidia swell and germinate, exposing PAMPs and permitting the recognition of organisms that have escaped the frontline innate defenses. Despite these multiple roles in immune interactions, there is conflicting evidence as to the significance of RodA in the pathogenesis of IA. Loss of RodA was not associated with a decrease in virulence in a mouse model of pulmonary IA (Thau *et al.*, 1994), however, in a murine model of corneal infection, loss of RodA resulted in attenuated virulence associated with reduced neutrophil recruitment (Carrion Sde *et al.*, 2013).

In addition to facilitating dispersal, the cell wall enhances the resistance of conidia to harsh environmental conditions such as abrupt changes in osmolarity and long periods of desiccation. Studies of the structure of the cell wall using electron microscopy have demonstrated the presence of two distinct layers of polysaccharides (Mouyna and Fontaine, 2009). Alkali fractionation studies have provided some insight into the composition of these two layers (Fontaine *et al.*, 2000; Latgé, 2010) (Table 1). The alkali-insoluble, inner cell wall fraction contains β-1,3-glucans (38%), galactomannan (26%), and chitin/chitosan (5.6%). Within the inner cell wall layer, membrane proteins anchor β-1,3-glucans, which then form branches and cross-link with chitin, galactomannan, and proteins to form a strong and rigid structure. Thus, the alkali-insoluble, inner cell wall layer is thought to provide structure and rigidity to the cell wall. The alkali-soluble, outer cell wall fraction contains α-1,3-glucans (14%), galactomannan (13%), β-1,3-glucans (5%), and chitin/chitosan (0.5%). In contrast to the inner cell wall glycans, the outer cell wall polysaccharides are non-covalently associated and form a much looser network of macromolecules.

The cell wall of *A. fumigatus* hyphae

Upon landing on favorable surface, resting conidia swell and enlarge to produce filamentous hyphae. These changes require remodeling and reshaping of the cell wall to accommodate alterations in size and shape. This is a considerable task given that the hyphal cell wall accounts for 20–40% of the dry weight of *A. fumigatus* mycelia (Gastebois *et al.*, 2009; Mouyna and Fontaine, 2009).

One of the major differences between the cell wall of hyphae and conidia is that hyphae produce an amorphous ex-

Table 1. Cell wall composition of *Aspergillus fumigatus*[a]

Polysaccharide	Conidia[b]		Hyphae[b]	
	Alkali-insoluble	Alkali-soluble	Alkali-insoluble	Alkali-soluble
α-1,3-Glucan	ND	14	ND	42
β-1,3-Glucan	38	5	30	ND
β-1,3/1,4-Glucan	NA	NA	3	ND
Chitin	1.7	0.3	13	ND
Chitosan	3.9	0.2	4	ND
Galactomannan[c]	26	13	5	1.4
Galactosaminogalactan	ND	ND	4	2.3

ND: not detected
NA: not available
[a]Adapted from Mouyna and Fontaine (2009)
[b]Composition in percent
[c]Combination of galactose and mannose

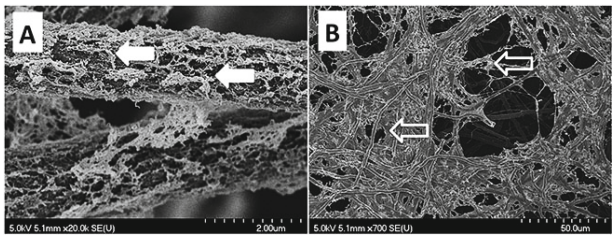

Fig. 1. Formation of the amorphous cell layer and extracellular matrix by hyphae of *Aspergillus fumigatus*. Scanning electron micrographs of *A. fumigatus* Af293 grown at 37°C for 24 h in phenol-red free RPMI 1640. (A) Hyphal surface morphology, and (B) Fungal biofilm formation. Solid arrows indicate cell wall decorations associated with the amorphous cell layer; open arrows indicated extracellular matrix.

tracellular matrix. Scanning electron micrographs have revealed that the hyphal surface is highly decorated (Fig. 1A) with extensive extracellular matrix (ECM) material (Fig. 1B). Immunoelectron microscopy studies have demonstrated the presence of the cell wall polysaccharides galactosaminogalactan, galactomannan and α-1,3-glucans within this matrix (Fontaine *et al.*, 2010; Gravelat *et al.*, 2013). These studies suggest that the relative abundance of these polysaccharides differs between the ECM and outer cell wall (Loussert *et al.*, 2010). The integrity of the extracellular matrix is dependent on the production of galactosaminogalactan, as mutants deficient in this polysaccharide are lacking the amorphous layer of the cell wall, as well as matrix production (Gravelat *et al.*, 2013; Lee *et al.*, 2015).

High-resolution transmission electron microscopy of the cell wall itself reveals the presence of two layers of contrasting electron density, as is seen in conidia (Fig. 2) (Fontaine *et al.*, 2000; Latgé, 2010). In hyphae, the alkali-insoluble, inner cell wall fraction contains β-1,3-glucans (30%), chitin/chitosan (17%), galactomannan (5%), galactosaminogalactan (4%), and β-1,3;1,4-glucans (3%), while the alkali-soluble, outer cell wall fraction contains α-1,3-glucans (42%), galactosaminogalactan (2.3%), and galactomannan (1.4%) (Table 1). Galactomannan and galactosaminogalactan are the only known cell wall polysaccharides found in both fractions/layers of the cell wall. As galactomannan is conjugated to β-1,3-glucans and chitin, it is anchored in the inner cell wall, and therefore, recovered in the alkali-insoluble fraction of the cell wall. How galactomannan is anchored within the outer cell wall is not known. Galactosaminogalactan is not known to be conjugated to other polysaccharides or cell wall proteins wall, and it is thought to be non-covalently, loosely associated throughout the cell wall. Thus, it would be predicted to be easily extracted by alkali extraction. The presence of galactosaminogalactan in the alkali-insoluble fraction, however, suggests that this glycan may also be anchored to an inner cell wall component.

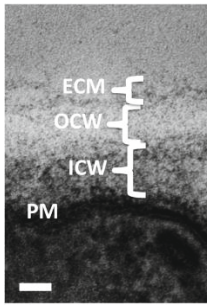

Fig. 2. Layers of the *Aspergillus fumigatus* cell wall. Transmission electron micrograph of cross-section of Af293 hyphae grown at 37°C for 24 h in phenol-red free RPMI 1640. Legend: ECM, extracellular matrix; OCW, outer cell wall; ICW, inner cell wall; PM plasma membrane. Scale bar, 20 nm.

Synthesis of cell wall polysaccharides

β-1,3-Glucan

This homopolymer of glucose residues linked by β 1 → 3 linkages (Fig. 3) is one of the major components of the hyphal cell wall. β-1,3-Glucan is synthesized by the β-1,3-glucan synthase complex which is composed of Fks1 (afu6g 12400), a glycosyltranferase (Beauvais *et al.*, 1993), and the regulatory subunit of Rho family of GTPases (Beauvais *et al.*, 2001).

Fks1 is localized on the plasma membrane, facing both the cytoplasmic and extracellular cell wall compartments. It attaches glucose residues from an intracellular pool of UDP-glucose substrates onto a growing chain of extracellular glucose polymer and extrudes the growing chain into the extracellular space. Inhibition of *fks1* by a nitrogen-regulated repressible promoter (Hu *et al.*, 2007) or by RNAi (Mouyna *et al.*, 2004), results in defective growth and swelling of the hyphae due to osmotic pressure. More recently, an *fks1* deletion mutant, Δ*fks1*, was generated and characterized (Dichtl *et al.*, 2015). Surprisingly, the Δ*fks1* mutant was viable, despite being completely devoid of β-1,3-glucan. The Δ*fks1* mutant displayed increased susceptibility to cell wall perturbing agents like calcofluor white and sodium dodecyl sulfate, suggesting marked dysregulation of cell wall synthesis. Loss of β-1,3-glucan was associated with the shedding of high levels of galactomannan into the culture supernatant, consistent with a model in which this glycan is anchored to β-1,3-glucan within the inner cell wall.

The echinocandin class of antifungals block the synthesis

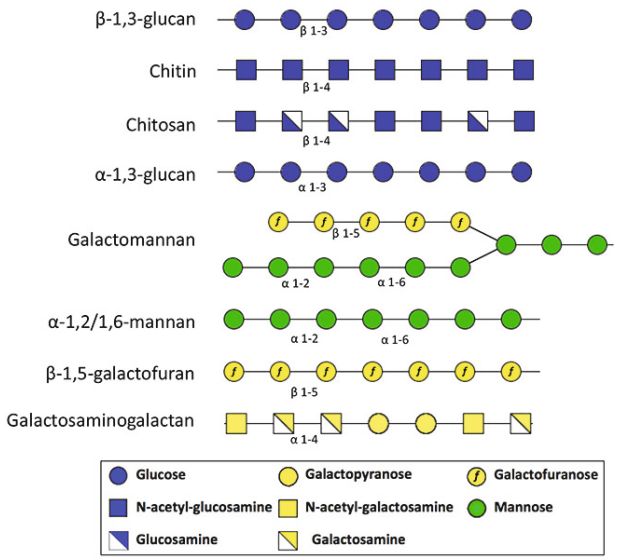

Fig. 3. *Aspergillus fumigatus* cell wall polysaccharides. Structural depiction of the cell wall polysaccharides showing linkages and monosaccharide components.

of β-1,3-glucan by non-competitively binding to Fks1 and are the only licensed antifungals that target the fungal cell wall (Chen *et al.*, 2011). Echinocandins are fungistatic against *A. fumigatus in vitro*, and exposure to these agents produces a phenotype similar to that seen in the Δ*fks1* mutant including hyphal swelling, as well as abnormal hyphal branching and extension (Chen *et al.*, 2011). The fungistatic, rather than fungicidal, effect of β-1,3-glucan inhibition by echinocandins or deletion of *fks1* is likely due to compensatory mechanism to maintain cell wall integrity. Exposure of *A. fumigatus* to echinocandins leads to increased chitin synthesis, and deletion of *fks1* results in a mutant with higher chitin and galactosaminogalactan production compared to the parental strain (Chen *et al.*, 2011; Dichtl *et al.*, 2015). These compensatory changes to the loss of a single cell wall polysaccharide highlight the complexity of cell wall biogenesis, and our need for a more complete understanding of the regulation of cell wall biosynthesis.

Rho proteins are small GTPases that have been implicated in sensing cell wall stressors and having a role in the maintenance of the cell wall integrity (Dichtl *et al.*, 2012). The genome of *A. fumigatus* contains 6 putative Rho genes, four of which have been characterized. Mutants with defects in *rho1* (afu6g06900), *rho2* (afu3g10340), or *rho4* (afu5g14060) are susceptible to various cell wall perturbing agents, implicating these Rho-GTPases in the regulation of cell wall synthesis (Dichtl *et al.*, 2012). Cellular localization studies using GFP-tagged Rho1 or Rho3 revealed that both localize to the hyphal tip, where the synthesis of β-1,3-glucan is most active (Dichtl *et al.*, 2010). Among the Rho proteins, Rho1 complexes with Fks1, suggesting that Rho1 is likely the key regulatory subunit of the β-1,3-glucan synthase complex (Beauvais *et al.*, 2001). The activation of Rho-GTPases requires the guanine exchange factor GEF. In *Saccharomyces cerevisiae*, two GEF proteins interact with Rho1 (Levin, 2011), however a single GEF ortholog, Rom2 (afu5g08550), interacts with Rho1 in *A. fumigatus* (Samantaray *et al.*, 2013). Consistent with this observation, inhibition of *rom2* expression results in a severe growth defect and enhanced susceptibility to various agents targeting the cell wall, including caspofungin (Samantaray *et al.*, 2013). It is therefore possible that a better understanding of the regulation of β-1,3-glucan synthesis may identify new targets that can improve the activity of echinocandins or other agents against *A. fumigatus*.

The extruded polymer of β-1,3-glucan, which can be up to 1,500 residues long (Beauvais *et al.*, 2001), can be processed and modified by several classes of enzymes. The most common modification involves cutting the extruding β-1,3-glucan chain and joining the fragment to an existing chain within the cell wall, thereby forming branching points or conjugating β-1,3-glucan to existing cell wall polysaccharides or glycoproteins. The enzymes mediating this activity include endo-β-1,3-glucanases (ENG), β-glucanosyltransferases (GEL), and the branching enzymes (BGT). ENG proteins are glycosylphosphatidylinositol (GPI)-anchored proteins that indiscriminately cleave internal residues of soluble β-1,3-glucans (Hartl *et al.*, 2011). Two of the six putative ENG proteins (Eng1 and Eng2) have been characterized. Single deletion mutants of *eng1* or *eng2* are unremarkable, suggesting that ENG proteins have redundant functions (Hartl *et al.*, 2011; Mouyna *et al.*, 2013). A double mutant of these two genes has not been constructed to date. GEL enzymes are glucanosyltransferases that mediate elongation of the β-1,3-glucan chain by cleaving a donor β-1,3-glucan chain and transferring the newly created reducing end to the non-reducing end of an acceptor β-1,3-glucan chain (Hartland *et al.*, 1996). Unlike the ENG enzymes, single deletions of three of the seven putative genes encoding for GEL proteins resulted in severe defects in growth and morphology. Deletion of *gel2* was associated with abnormal conidiogenesis and hypovirulence; deletion of *gel7* was associated with conidiation defects and a slight decrease in β-1,3-glucan; and deletion of *gel4* was lethal. BGT proteins are also glucanosyltransferases. Of the five putative BGT proteins, only Bgt1 and Bgt2 have been studied. Bgt1 activity produces a linear β-1,3;1,6-glucan chain, while Bgt2 produces a linear β-1,3-glucan with branched β-1,6-linked β-1,3-glucan (Mouyna *et al.*, 1998; Gastebois *et al.*, 2010). Single or double deletions of *bgt1* and *bgt2* had no effect on growth or resistance to cell wall stressors, likely due to compensatory activity of the other BGT enzymes (Gastebois *et al.*, 2010).

Recently, a glycosyltransferase required for the synthesis of β-1,3;1,4-glucan, Tft1, was identified and a deletion mutant was generated (Samar *et al.*, 2015). Interestingly, the Δ*tft1* mutant had no growth defects and displayed no increased susceptibility to cell wall antifungals nikkomycin or caspofungin. The Δ*tft1* mutant was not attenuated in virulence in a *Galleria mellonella* model of IA, suggesting that β-1,3;1,4-glucan is dispensable for fungal biology and virulence.

β-1,3-Glucan is also a PAMP that is recognized by the PRR dectin-1 that is expressed on dendritic cells and macrophages. Dectin-1 is a C-type lectin receptor (CLR) that mediates immune response to fungi, including secretion of proinflammatory cytokines like TNF-α and IL-1β (Romani, 2011; Gravelat *et al.*, 2013). Thus, a common strategy for fungus to evade immune detection is to minimize the exposure of β-1,3-glucan on its surface. In conidia of *A. fumigatus*, β-1,3-glucan is masked by hydrophobins and in hyphae by a variety of other polysaccharides as detailed below (Beauvais *et al.*, 2013; Gravelat *et al.*, 2013).

Chitin

Along with β-1,3 glucan, chitin is found within the inner cell wall and is an important structural polysaccharide. Chitin is a homopolymer of N-acetyl-glucosamine (GlcNAc) residues linked by β 1 → 4 linkages (Fig. 3). Chitin is present in many fungi, although the mechanisms of chitin biosynthesis and cell wall chitin content vary among fungal species (Lenardon *et al.*, 2010). While chitin only constitutes 1–2% of the *Saccharomyces cerevisiae* cell well, in filamentous fungi, chitin can reach 10–20% of the mycelial dry weight (Bartnicki-Garcia, 1968). Unlike β-1,3 glucan, chitin is synthesized through the action of multiple chitin synthases (CHS). Different species of fungi have varying number of CHS enzymes, which are categorized into seven different classes based on their amino acid sequences (Munro and Gow, 2001). In *A. fumigatus*, the chitin synthase machinery is comprised of eight CHS enzymes, encompassing all seven CHS classes (Gastebois *et al.*, 2009). The different classes of CHS are grouped into two families: Family I CHS members

(class I-III) require trypsin for activation *in vitro* while Family II CHS members (class IV-VII) do not. Structurally, the catalytic domain of Family I CHS is flanked on both sides by transmembrane regions, while Family II CHS contain only a single C-terminal transmembrane domain (Mellado *et al.*, 1996a; Roncero, 2002; Jimenez-Ortigosa *et al.*, 2012).

Single deletions of *chsA* (Class I), *chsB* (Class II), *chsC* (class III), *chsD* (class VI), *chsF* (class IV), or *csmB* (class VII) do not result in detectable defects in chitin synthesis or growth in *A. fumigatus*, suggesting that these CHS enzymes are dispensable or have redundant functions (Mellado *et al.*, 1996a, 1996b). However, deletion of *chsG* (class III) or *csmA* (class V, previously known as *chsE*), results in decreased chitin levels and impaired growth (Mellado *et al.*, 1996a; Aufauvre-Brown A *et al.*, 1997). Interestingly, the Δ*chsG* mutant displayed increased sensitivity to caspofungin treatment (Walker *et al.*, 2015), suggesting that ChsG may play a role in the development of echinocandin resistance (Walker *et al.*, 2015). Deletion of both *chsG* and *csmA* produced a mutant that was still viable, although which exhibited a severe growth defect and produced only half the amount of chitin in the cell wall found in the parental strain (Mellado *et al.*, 2003). This decrease in chitin was associated with an increase in cell wall α-1,3-glucan content, suggesting a compensatory link between chitin and α-1,3-glucan.

Recently, using an innovative molecular approach (Hartmann *et al.*, 2010), Muszkieta *et al.* (2014) generated quadruple deletion mutants of the Family I (*chsACBG*) or Family II (*csmABchsFG*) CHS genes, as well as a mutant deficient in Family I and Family II CHS (*csmABchsFG*). Each of these quadruple mutants displayed significant cell wall and growth defects. Interestingly, the mutant deficient in both Family I and II CHS displayed the most severe defects in growth and cell wall, and produced the lowest level of chitin among these quadruple mutants (Muszkieta *et al.*, 2014). This mutant is also unable to produce viable conidia, suggesting that while members of either family of CHS enzymes can compensate for each other, members of both CHS family are required for proper conidiogenesis.

Mature chitin can be modified by the action of chitinases, which are enzymes that hydrolyze chitin. In yeast, these enzymes play an important role in cell separation during budding (Kuranda and Robbins, 1991; Dunkler *et al.*, 2005; Alcazar-Fuoli *et al.*, 2011). In *A. fumigatus*, 14 putative chitinases (CHI) have been identified and deletion mutants for each gene have been generated (Jaques *et al.*, 2003; Alcazar-Fuoli *et al.*, 2011). However, single deletions of all 14 genes, as well as, a quintuple mutant exhibited no detectable phenotype as compared to their parental strains, suggesting, once again, that there is likely compensatory activity among the enzymes in this class.

Until recently, host-pathogen dynamics associated with chitin in *A. fumigatus* were poorly understood due to the lack of functionally deficient mutants. The generation of the quadruple mutants with substantially reduced chitin synthase activity has allowed some initial characterization of the contribution of chitin to virulence. While, the Δ*chsACBG* mutant displayed reduced chitin synthase activity, there was no change in virulence in immunosuppressed mice (Muszkieta *et al.*, 2014). On the other hand, the Δ*csmABchsFG* mutant was more susceptible to caspofungin therapy in immunosuppressed mice as compared to wild-type *A. fumigatus* strains (Muszkieta *et al.*, 2014). Although the exact mechanism by which chitin influences virulence is not fully understood, in *Candida albicans*, human peripheral blood mononuclear cells were found to produce lower levels of TNF and higher levels of the anti-inflammatory cytokine IL-10 in response to infection with a chitin-deficient mutant as compared to wild-type *C. albicans* (Wagener *et al.*, 2014). Although no chitin deficient *A. fumigatus* mutant is currently available, it is likely that chitin plays a similar anti-inflammatory role in this fungus.

Chitin synthesis can be inhibited by various agents, most notably nikkomycin and polyoxins (Dahn *et al.*, 1976; Archer, 1977; Hector, 1993). Both are nucleoside-peptide metabolites naturally produced by *Streptomyces* species, and contain an UDP-N-acetyl-glucosamine moiety that acts as a competitive inhibitor of chitin synthase. Treatment with these agents inhibits chitin production, and resulting in osmotic lysis and fungal death (Krainer *et al.*, 1991). Nikkomycin has been widely studied, and while it is currently not licensed for clinical use, animal data suggests it is effective against *Histoplasma*, *Blastomyces*, and *Coccidioides* infections (Hector, 1993). Although nikkomycin is fungicidal against *A. fumigatus in vitro*, higher drug concentrations are required as compared to other fungi such as *Coccidioides posadasii* (Hector *et al.*, 1990; Hector, 1993). This may be attributed to the presence of multiple redundant CHS as outlined above.

More recently, combinatorial biosynthetic approaches have been used to generate hybrid analogs of nikkomycin and polyoxins in an effort to broaden the spectrum of chitin inhibitors (Li *et al.*, 2011). Considering the complex and highly redundant mechanism of chitin synthesis in *A. fumigatus*, it is likely that agents targeting multiple CHS will be needed for activity in this organism. Among hybrid analogs, polynik A and polyoxin P seem to show potent antifungal activity against *A. fumigatus* (Li *et al.*, 2011, 2012). Further study of these analogs, and others, may potentially lead to the development of new class of chitin-targeted antifungals with broad-spectrum coverage.

Chitosan

Chitosan is a derivative of chitin that is also found in the cell wall of many fungi, including *A. fumigatus*. Chitosan is synthesized by the deacetylation of chitin within the cell wall and extracellular matrix though the activity of chitin deacetylases (CDA). As the name implies, CDA enzymatically remove the acetyl group of the GlcNAc residues in chitin, resulting in glucosamine (GlcN) residues. In *A. fumigatus*, two genes encoding putative chitin deacetylases (afu4g09940, and afu6g10430) have been identified from genome annotations (Gastebois *et al.*, 2009). However, these genes remain uncharacterized in *A. fumigatus*, and therefore, the role of chitosan in growth and viability of this organism is unknown.

The degree of deacetylation confers differences in physical characteristics of the resulting glycan including solubility and rigidness (Arbia *et al.*, 2013). In recent years, chitosan has gained prominence due to its expanding industrial, pharmaceutical, and medical uses (No *et al.*, 2007). Coating catheters and other medical devices with chitosan-based ma-

trices has been found to prevent biofilm formation (Cobrado *et al.*, 2013). The mechanism by which chitosan mediates this antibiofilm effect remains uncharacterized.

As with chitin, chitosan can undergo hydrolysis by chitosanases. Four genes encoding predicted chitosanases are found in the *A. fumigatus* genome (afu3g14980, afu4g01290, afu8g00930, and afu6g00500). Among them, a single deletion mutant of *csnB* (afu4g01290) has been reported (Beck *et al.*, 2014). While no apparent growth or cell wall defects were observed, the mutant was unable to use chitosan as a carbon source, suggesting that CsnB is required for extracellular chitosanase activity and that hydrolysis of chitosan may be not essential.

The role of chitosan in *A. fumigatus* infection is largely unknown. In *Cryptococcus neoformans*, chitosan-deficient mutants displayed defects in growth and attenuated virulence in a mouse model of infection (Baker *et al.*, 2007). In the pathogenic plant fungi, *Puccinia graminis* and *Uromyces fabae*, while the surface of invading hyphae is covered with chitosan, the surface of non-invading hyphae is not, suggesting that deacetylation of chitin may play a role in immune evasion or cellular invasion (El Gueddari *et al.*, 2002). Further studies elucidating the effects deacetylation of chitin on the physical and virulence-associated properties of chitosan, will be needed to establish its role in virulence of *A. fumigatus*.

Galactomannan

Mannose-containing polysaccharides are common cell wall components of many fungal species. In some organisms, such as *C. albicans*, mannans are conjugated to a wide variety of proteins, including those that mediate important functions such as hydrophobicity, adherence, and virulence (Masuoka and Hazen, 2004; Sandini *et al.*, 2007; Hall and Gow, 2013). In *A. fumigatus*, the principle mannose-containing cell wall polysaccharide is galactomannan. Unlike the homopolymers chitin and β-1,3-glucan, galactomannan is composed of mannose and galactofuranose (Gal*f*) chains. The mannan chain provides the backbone of galactomannan, with mannose residues linked in an α 1 → 2 or 1 → 6 fashion (Fig. 3) (Latgé *et al.*, 1991). This mannan backbone is decorated with β-1,3 and β-1,6 branching side chains of four to five β 1 → 5 linked Gal*f* residues (Fig. 3)(Latgé *et al.*, 1991, 1994).

The synthesis of the mannosyl chain of *A. fumigatus* galactomannan is poorly understood. In *S. cerevisae*, cell wall mannans originate from mannoproteins with extensive N- and O-glycosylations (Levin, 2005). The glycosylation of cell wall-bound mannoproteins starts in the endoplasmic reticulum and is mediated by the activity of multiple mannosyltransferases (Levin, 2005). As the mannoproteins progress through the *cis*-Golgi and through the plasma membrane, further extensions of the glycan chains occur (Nakayama *et al.*, 1992; Nakanishi-Shindo *et al.*, 1993). In *A. fumigatus*, the synthesis of galactomannan is also believed to occur within the Golgi. The transport of mannose into the Golgi is mediated by the GDP-mannose transporter, GmtA (Engel *et al.*, 2012). Deletion of *gmtA* resulted in a mutant devoid of galactomannan, and was associated with a severe weakening of the cell wall, and impaired growth and sporulation, confirming the importance of mannans in the cell wall of *A. fumigatus* (Engel *et al.*, 2012).

The identification of the mannosyltransferase that governs synthesis of the mannose chain of galactomannan has been unsuccessful to date. In *A. fumigatus*, four orthologs *of* the *S. cerevisiae* mannosyltransferase ScOch1 have been identified and characterized (Kotz *et al.*, 2010; Lambou *et al.*, 2010). Single and quadruple deletion mutants produced normal levels of mannans and displayed no other apparent cell wall-related defects, although heterologous expression of *och1* from *A. fumigatus* was able to complement a ScOch1-deficient mutant of *S. cerevisiae*. Thus, it is likely that other mannosyltransferases exist that can mediate the synthesis of the mannosyl chain of galactomannan.

Gal*f* is a five-member cyclic hexose found in many microbial pathogens but not in humans (Latgé, 2010). The synthesis of the galactofuranosyl chain of galactomannan begins in the cytosol where the pre-cursor of Gal*f*, UDP-galactopyranose (Galp), is converted from UDP-glucose from by the activity of the UDP-glucose 4-epimerases Uge5 (Lee *et al.*, 2014b). UDP-Galp is then transformed to UDP-Gal*f* by the UDP-galactose mutase, Ugm1 (encoded by *ugm1*, also known as *glfA*) (Schmalhorst *et al.*, 2008; Lamarre *et al.*, 2009; Lee *et al.*, 2014b). Cytosolic UDP-Galf is then transported into the *cis*-Golgi by the activity of the UDP-Gal*f* transporter GlfB (Engel *et al.*, 2009). The identification of a Gal*f*-specific transferase has not been reported. Deletion of any of these genes results in mutant strains devoid of galactomannan. However, single deletion mutants exhibit varying phenotypes, suggesting that they have functional roles other than the synthesis of galactomannan. For example, while the Δ*uge5* mutant has no defect in growth or branching, the Δ*ugm1* mutant exhibit decreased radial growth and aberrant branching (Lamarre *et al.*, 2009). Interestingly, scanning electron micrographs of both mutants show extensive hyphal surface decorations due to increased production of galactosaminogalactan (Gravelat *et al.*, 2013; Lee *et al.*, 2014b). Unlike the Δ*uge5* or Δ*ugm1* mutants, the Δ*glfB* mutant is sensitive to high temperature, 42°C and 47°C (Engel *et al.*, 2009). More importantly, the Δ*glfB* mutant was devoid of high-mannose type *N*-glycans bearing a single Gal*f* residue, suggesting that GlfB is required for proper galactofuranosylation of *N*-glycans in *A. fumigatus*.

The role of galactomannan in virulence remains unclear. The mannan component of this polysaccharide clearly plays an important role in maintaining cell wall structure (Engel *et al.*, 2012). Although mannan-deficient mutants have not been studied in mouse models, their marked growth defect *in vitro* would suggest that this polysaccharide is required for infection. The role of the galactofuranosyl side chain in virulence is less clear with conflicting reports from two different groups. Lamarre *et al.* (2009) reported no significant alterations in antifungal susceptibility or virulence in an *ugm1*-deficient mutant, while Schmalhorst *et al.* (2008) reported increased susceptibility to antifungal agents and attenuated virulence in an independently constructed *ugm1*-deficient mutant. Differences in strain background or design of the phenotypic characterization experiments may contribute to these differences, and a direct comparison of these mutants would be of interest.

There are currently no antifungal agents targeting galactomannan synthesis. However several unique aspects of

galactomannan have fostered an interest in elucidating the full biosynthetic pathway of this glycan, and developing inhibitors of its synthesis. Galactomannan is absent in humans, and is therefore a specific target for antifungal therapy. Galactomannan is the only polysaccharide that is synthesized in the Golgi and not exclusively through the action of plasma membrane-bound synthases. Thus, it is the only cell wall polysaccharide that shares glycosyltransferases involved in protein glycosylation (Jin, 2012). Finally, galactomannan detection is used in routine clinical as a diagnostic marker for *Aspergillus* infection, and understanding the regulatory and biosynthetic pathways governing the production of this glycan may guide strategies for better use of this diagnostic test.

α-1,3-Glucan

Found exclusively in the outer cell wall of *A. fumigatus*, α-1,3-glucan is a homopolymer of glucose residues linked by α 1 → 3 linkages (Fig. 3). α-1,3-Glucan differs from β-1,3-glucan only in the orientation of its linkage; which are axial vs. equatorial, respectively. α-1,3-glucan is also present in the cell wall of other fungal species, including other clinically relevant fungi, where it plays an important role in cell wall morphology and virulence (Rappleye *et al.*, 2007; Reese *et al.*, 2007). In *Histoplasma capsulatum*, α-1,3-glucan masks β-1,3-glucans from immune recognition by dectin-1 (Rappleye *et al.*, 2007). In *C. neoformans*, α-1,3-glucan anchors the polysaccharide capsule and α-1,3-glucan-deficient strains are attenuated in virulence (Reese *et al.*, 2007).

The synthesis of α-1,3-glucan is mediated through the action of three synthases in *A. fumigatus*: Ags1, Ags2, and Ags3 (Henry *et al.*, 2012). Localization studies have shown that Ags1 and Ags2 are cell membrane proteins (Beauvais *et al.*, 2005), and Ags3 is also predicted to be cell membrane-bound. Deletion of either *ags1* or *ags2* but not *ags3* resulted in mutants with altered hyphal morphology and impaired conidiation. However only the deletion of *ags1* resulted in a detectable reduction in α-1,3-glucan levels (Beauvais *et al.*, 2005). These findings suggested that there is likely redundancy in the synthesis of α-1,3-glucan or compensatory activity between the three Ags proteins (Beauvais *et al.*, 2005). Deletion of all three *ags* genes resulted in a mutant that displayed normal growth and germination, but was completely devoid of α-1,3-glucan (Beauvais *et al.*, 2013). The triple mutant showed marked changes in cell wall organization of conidia, with increased exposure of β-1,3-glucans and presence of an extracellular amorphous glycoprotein matrix on the surface of conidia that covered the normal hydrophobin layer.

Recent studies have suggested that α-1,3-glucan plays an important role in the pathogenesis of IA (Beauvais *et al.*, 2013). Conidia that are deficient in α-1,3-glucan are more efficiently phagocytosed by murine alveolar macrophages, and the triple *ags* mutant is attenuated in virulence in neutropenic mice. Pulmonary histopathology studies revealed the absence of filamentous hyphae in the lungs of mice infected with the triple *ags* mutant, suggesting that the increased survival of these mice is due to enhanced immune recognition of the mutant conidia and/or impaired germination of the mutant strain *in vivo*.

Currently, there are no inhibitors of α-1,3-glucan synthesis. Given that deletion of all three α-1,3-glucan synthases was required to attenuate *A. fumigatus* virulence, development of antifungals targeting α-1,3-glucan synthesis must be broad enough to inhibit all three synthases. Another limitation to antifungal development targeting α-1,3-glucan synthesis is that the effects of blocking α-1,3-glucan synthesis is more pronounced in conidia than hyphae. Thus, α-1,3-glucan inhibitors may be restricted to the prevention of disease and be of limited use in the in the treatment of established infection, in which hyphae alone are present. Further study is required to further elucidate the role of α-1,3-glucan in the hyphal cell wall and its function during infection.

Galactosaminogalactan

Found in the extracellular matrix, as well as the inner and outer cell walls of *A. fumigatus* hyphae, galactosaminogalactan is a partially deacetylated glycan composed of galactose and N-acetyl-galactosamine (GalNAc) linked by α 1 → 4 linkages (Fig. 3). Within this heteropolymer, the galactose and GalNAc residues do not seem regularly arranged, giving rise to a highly variable polysaccharide (Mouyna and Fontaine, 2009). Galactosaminogalactan production has been reported in other *Aspergillus* species, including *A. nidulans* (Gorin and Eveleigh, 1970), *A. parasiticus* (Ruperez and Leal, 1981), and *A. niger* (Bardalaye and Nordin, 1976), as well as the non-*Aspergillus* fungus, *Bipolaris sorokiniana* (Pringle, 1981).

The synthesis of GAG is coordinated by the products of a five-gene cluster on chromosome three of *A. fumigatus*. Galactosaminogalactan biosynthesis begins with the conversion of UDP-galactose from UDP-glucose and of UDP-GalNAc from UDP-GlcNAc by the activity of the cytosolic epimerases Uge3 and Uge5 (Lee *et al.*, 2014b). Uge3 is a group 2 bifunctional epimerase that can mediate synthesis of both UDP-galactose and UDP-GalNAc, while Uge5 is a group 1 epimerase which mediates UDP-galactose synthesis. Uge5 is the only enzyme that has been linked to GAG synthesis that is not found within the GAG biosynthetic gene cluster (Lee *et al.*, 2014b). While Uge5 is the epimerase responsible for the majority of cellular UDP-glucose/galactose interconversion, in the absence of Uge5, the bifunctional activity of Uge3 is sufficient to produce UDP-galactose for the synthesis of galactosaminogalactan. In contrast, Uge3 activity alone is not sufficient to support the synthesis of galactomannan in the absence of Uge5 (Lee *et al.*, 2014b). Further studies delineating the downstream components of these two pathways are required to understand the mechanisms underlying this observation.

Linking of UDP-Gal and UDP-GalNAc and extrusion of the polymer through the plasma membrane is thought to be mediated by the large transmembrane glycosyl transferase Gtb3 (Afu3g07860). Following synthesis and extrusion of galactosaminogalactan by Gtb3, the emergent polymer undergoes partial deacetylation by the secreted and cell wall-associated deacetylase Agd3 (Afu3g07890), rendering the polymer polycationic. Fully acetylated galactosaminogalactan produced by the Δ*agd3* mutant does not adhere to the cell wall of *A. fumigatus* hyphae and is shed into the culture supernatant, suggesting that galactosaminogalactan associates

with the cell wall through charge-charge interactions (Lee *et al.*, 2014a). As with chitosan, it is possible that varying levels of galactosaminogalactan deacetylation may result in the formation of polysaccharides with distinct properties. Further studies investigating the physicochemical properties associated with deacetylation is needed to shed greater light into the role of Agd3 and the complexity of galactosaminogalactan synthesis.

Synthesis of galactosaminogalactan also requires the participation of the membrane-bound protein Sph3 (Afu3g07900), as an Sph3-deficient mutant was devoid of galactosaminogalactan. Sph3 is a member of a novel glycoside hydrolase family, GH133, and recombinant Sph3 showed galactosaminogalactan-specific hydrolysis activity (Bamford *et al.*, 2015). The mechanism whereby this hydrolase participates in galactosaminogalactan synthesis remains undefined.

Recent studies have suggested that galactosaminogalactan contributes to virulence of *A. fumigatus* in a number of ways. Deletion of *uge3* results in a mutant devoid of galactosaminogalactan and is associated with defects in biofilm adherence, increased exposure of β-1,3-glucans, and attenuated virulence in leukopenic mice (Gravelat *et al.*, 2013). More recently, cell wall-associated GAG was reported to mediate resistance to neutrophil extracellular traps (NETs) (Lee *et al.*, 2015). This resistance to NETS is likely a consequence of electrostatic repulsion between largely positively charged histones and cationic antimicrobial peptides within NETs, and the polycationic nature of deacetylated GAG.

Studies using purified fractions of galactosaminogalactan have found that these preparations can induce natural killer (NK) cell-dependent apoptosis of neutrophils (Robinet *et al.*, 2014), and the production of IL-1RA from peripheral blood mononuclear cells (Gresnigt *et al.*, 2014). Treatment of mice with this preparation of GAG increased the virulence of *A. fumigatus* during invasive infection (Fontaine *et al.*, 2011) and reduced inflammation in murine models of dextran-sulfate induced colitis and allergic aspergillosis (Fontaine *et al.*, 2011; Gresnigt *et al.*, 2014). Thus, purified fractions of galactosaminogalactan may be useful as an immunomodulatory agent for the therapy of inflammatory conditions (Latge *et al.*, 2014).

The multiple roles of GAG in the pathogenesis of IA suggest that this glycan may be a promising antifungal target. While there are currently no antifungals targeting the synthesis of galactosaminogalactan, we recently reported that low concentrations of the recombinant hydrolase domain of Sph3 can digest both purified and cell wall-bound galactosaminogalactan (Bamford *et al.*, 2015). Further *in vitro* and *in vivo* studies are required to define the utility of this, and other glycoside hydrolases, in the treatment of IA.

Interplay between cell wall components: synthesis and regulation

Molecular approaches to study the biosynthetic pathways of cell wall polysaccharides have revealed the presence of regulatory mechanisms that govern the composition of the cell wall during cell wall stress or perturbation. For example, blocking the synthesis of α-1,3-glucan by triple deletion of all *ags* genes was associated with an increase in cell wall β-1,3-glucan and chitin content (Henry *et al.*, 2012; Beauvais *et al.*, 2013), and the loss of β-1,3-glucan in the Δ*fks1* mutant resulted in increased production of chitin and galactosaminogalactan (Dichtl *et al.*, 2015). These compensatory relationships are complex and may not be symmetrical. For example, while loss of galactomannan in the Δ*ugm1* mutant was associated with an increase in β-1,3-glucan and galactosaminogalactan, the absence of galactosaminogalactan in the Δ*uge3* mutant had no effect on the production of β-1,3-glucan or galactomannan (Lamarre *et al.*, 2009; Gravelat *et al.*, 2013). While these observations provide challenges for the study of deletion mutants, the body of evidence from these studies helps shape our understanding of broad mechanisms involved in cell wall integrity, and may provide better guidance in designing and developing new cell wall-targeting agents.

Multiple mechanisms may contribute to these compensatory changes among cell wall polysaccharides. Changes in cell wall composition may reflect active regulation of cell wall synthetic elements through elements of the cell wall integrity pathway, including calcium signaling through the calcineurin pathway (Cramer *et al.*, 2008; Soriani *et al.*, 2008); mitogen-activated protein kinases (MAPK), including MpkA and Hog1 (Valiante *et al.*, 2008; Jain *et al.*, 2011; Dichtl *et al.*, 2012); or heat shock protein (Hsp90) (Lamoth *et al.*, 2012). Additionally, changes in the cell wall composition may reflect substrate shunting in which the accumulations of sugar substrates from a blocked biosynthetic pathway can be utilized by another pathway for the production of a different cell wall polysaccharide. These two mechanisms are not exclusive, as it is likely that substrate shunting contributes to the activation of regulatory pathways as the organism detects shifts in intracellular pools of sugars and alters enzyme expression or activity in response to this perturbation in metabolism. The study of these types of changes in metabolic activity and their effect on substrate shunting will be challenging, particularly in models of *A. fumigatus* infection. The development of advanced tools and techniques will be required to further elucidate the role of substrate shunting and the cell wall integrity pathway in cell wall homeostasis during *A. fumigatus* infection.

The way forward

The *A. fumigatus* cell wall is a dynamic organelle whose complexity we are only now beginning to unravel. Despite the importance of the cell wall in fungal growth, and its absence from human cells, only a single class of antifungals, the echinocandins, have been developed that target this important fungal structure. Studies investigating the dynamics of the cell wall response to echinocandins and compensatory changes in glycan composition observed in cell wall mutants have helped shed light on the complexity of cell wall synthesis. The results of these studies may provide insight into the development of better cell wall-targeting agents, or inform the rationale choice of combination therapies. The identification of novel cell wall components, and a better understanding of their biosynthetic pathways and role in fungal pathogenesis will be critical in developing novel cell wall active therapies.

Acknowledgements

This work was supported by the following grants to DCS: Canadian Institutes of Health Research grants 13337 and 236182; Cystic Fibrosis Canada grant 3019; and the National Institute of Health grant # R01AI073829. DCS is the recipient of a Chercheur-Boursier award from the Fonds de Recherche Quebec en Sante.

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