Aspergillus nidulans galactofuranose biosynthesis affects antifungal drug sensitivity

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A R T I C L E  I N F O
Article history:
Received 15 May 2012
Accepted 14 August 2012
Available online 16 October 2012

Keywords:
Aspergillus nidulans
Galactofuranose
UDP-galactopyranosyluronic mutase
UDP-glucose/galactose-4-epimerase
UDP-galactofuranose transporter
alcA promoter

A B S T R A C T
The cell wall is essential for fungal survival in natural environments. Many fungal wall carbohydrates are absent from humans, so they are a promising source of antifungal drug targets. Galactofuranose (GalF) is a sugar that decorates certain carbohydrates and lipids. It comprises about 5% of the Aspergillus fumigatus cell wall, and may play a role in systemic aspergillosis. We are studying Aspergillus wall formation in the tractable model system, A. nidulans. Previously we showed single-gene deletions of three sequential A. nidulans GalF biosynthesis proteins each caused similar hyphal morphogenesis defects and 500-fold reduced colony growth and sporulation. Here, we generated ugeA, ugmA and ugtA strains controlled by the alcA(p) or niiA(p) regulatable promoters. For repression and expression, alcA(p)-regulated strains were grown on complete medium with glucose or threonine, whereas niiA(p)-regulated strains were grown on minimal medium with ammonium or nitrate. Expression was assessed by qPCR and colony phenotype. The alcA(p) and niiA(p) strains produced similar effects: colonies resembling wild type for gene expression, and resembling deletion strains for gene repression. GalF immunolocalization using the L10 monoclonal antibody showed that ugmA deletion and repression phenotypes correlated with loss of hyphal wall GalF. None of the gene manipulations affected itraconazole sensitivity, as expected. Deletion of any of ugmA, ugeA, ugtA, their repression by alcA(p) or niiA(p), OR, ugmA overexpression by alcA(p), increased sensitivity to Caspofungin. Strains with alcA(p)-mediated overexpression of ugeA and ugtA had lower caspofungin sensitivity. GalF appears to play an important role in A. nidulans growth and vigor.

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1. Introduction

Human systemic fungal infections are an increasing threat (Fisher et al., 2012), particularly due to improved medical technology leading to larger populations of immune-compromised people. Systemic infections from a dozen or more fungi lead to morbidity despite aggressive anti-fungal drug treatment (Lass-Florl, 2009). Overall, Aspergillus is second only to Candida as a cause of invasive fungal infections (Erjavec et al., 2009). Humans have few anti-fungal drug options because fungi share many biochemical pathways with animals. A major difference between organisms in these kingdoms is that fungal cells have a carbohydrate wall. This wall protects the fungal cell from damage, and mediates the interaction between the fungus and its environment. If the wall is removed or weakened, the fungus cannot survive in natural environments (Bennett et al., 1985). About 90% of the fungal wall is composed of polysaccharides that are not found in human hosts (Latgé, 2007). Echinocandins are the only antifungal drugs in clinical use that block an aspect of fungal wall synthesis, formation of β-1,3-glucans that comprise about half of fungal cell wall polysaccharides in some species (Espinel-Ingroff, 2009). However, echinocandins are clinically effective only against Candida and Aspergillus; they must be used intravenously (Denning, 2003); and drug-resistant strains have already been identified (Walker et al., 2010). There is a pressing need to develop additional antifungal drugs (Mircus et al., 2009) particularly against novel aspects of fungal physiology. Gauwerky et al. (2009) suggest that minor wall components as well as virulence factors have the potential to be useful antifungal drug targets.
We are exploring an aspect of wall biosynthesis in Aspergillus nidulans, the galactofuranose (Galf) biosynthesis pathway. Galf is the five-membered ring form of galactose found in the cell wall of many microorganisms including fungi, but only the six-member ring form called galactopyranose is found in mammals. In microorganisms, Galf residues form the side chains of glycoconjugates including galactomannan. Galf biosynthesis genes encode UDP-glucose/galactose-4-epimerase (UgA) (El-Ganiny et al., 2010), UDP-galactopyranose mutase (UgM) (El-Ganiny et al., 2008), and the UDP-galactofuranose transporter (UgtA) (Afroz et al., 2011). Although none of these genes was essential for A. nidulans in culture, these deletion strains had aberrant wall maturation (Paul et al., 2011) and 500-fold reduced hyphal growth and sporulation rate.

Galf residues in human serum can be used to track a patient’s response to therapy for systemic Aspergillus infections (Bennett et al., 1985; Stynen et al., 1992; Shibata et al., 2009). Galf has been shown to be essential for the virulence of the pathogenic protozoan, Leishmania (Klecka et al., 2007). Galf could be a virulence determinant in Aspergillus fumigatus based on studies with a UgmA (also known as GfaA) deletion strain in a murine model (Schmalhorst et al., 2008). Recent evidence to the contrary (Heesemann et al., 2011) showed that A. fumigatus hyphae pretreated with monoclonal antibodies (mAbs) specific to hyphal Galf residues had similar virulence to untreated hyphae. Nevertheless, Galf metabolism could still be a useful target for anti-fungal drug development (Pederson and Turco, 2003) because it plays important although not fully-understood roles in fungal growth.

Regulated promoters can be used to explore the role (Waring et al., 1989) and requirement (Hu et al., 2007) for Aspergillus genes, typically using the alcA (Waring et al., 1989; Monteiro and DeLucas, 2010) and niiA (Monteiro and DeLucas, 2010) promoters whose expression is controlled by carbon source or nitrogen source, respectively. For example, repression of A. nidulans chitin synthase chsB in an alcA(p)-chsB strain caused slow growth and highly branched hyphae (Ichinomiya et al., 2002). Repression of alcA(p)-regulated A. fumigatus O-mannosyltransferase 2 (AfPtmt2) caused growth retardation, abnormal cell polarity, defective cell wall integrity, and reduced conidiation (Fang et al., 2010). Repression of alcA(p)-regulated protein kinase C (pkC) caused germination rate, hyphal growth and conidiation, as well as increasing hyphal wall thickness and sensitivity to wall-selective agents: Caspofungin, Calcifluor White, and Congo Red (Ronen et al., 2007). As expected, this strain was also more sensitive to the protein kinase inhibitor staurosporine although it responded like wild type to induction, and MM containing 10 mM ammonium tartrate (MMA) was used for repress. Media used for niiA(p)-regulation were supplemented with vitamin solution and pyridoxine. We were not able to find a medium for overexpressing niiA(p)-regulated genes.

2. Materials and methods
2.1. Strains, plasmids and culture conditions

Strains, primers and plasmids are listed in Suppl. Table A. A. nidulans strains were maintained on complete medium (CM) with supplements for nutritional markers as described in Kaminisky (2001). To manipulate alcA(p)-regulated gene expression, CM that otherwise contained 1% glucose was modified as follows: maximum repression used CM containing 3% glucose (CM3G); overexpression used CM lacking glucose but containing 100 mM threonine (CMT). For niiA(p)-regulated gene expression, minimal medium (MM) containing 70 mM NaNO3 (MMN) was used for induction, and MM containing 10 mM ammonium tartrate (MMA) was used for repression. Media used for niiA(p)-regulation were supplemented with vitamin solution and pyridoxine. We were not able to find a medium for overexpressing niiA(p)-regulated genes.

2.2. Construction of regulatable promoter strains

A promoter exchange module consisting of AfpyrG and alcA(p) was amplified from the plasmid, pAleA(p) (a gift of Loretta Jackson-Hayes, Rhodes College). This module was inserted immediately upstream of each Galf biosynthesis gene to create alcA(p)-regulated strains. A 1.2-kb DNA fragment containing AfniA(p) was amplified by PCR from genomic DNA of wild type A. fumigatus. This was used to create a niiA(p)-regulated promoter module, also with AfpyrG as the selectable marker. This construct was inserted immediately upstream of the coding sequence for each Galf biosynthesis gene to create niiA(p)-regulated strains (Suppl. Fig. A).

2.3. Transformation

 Constructs were transformed into A1149 protoplasts. Protoplasting and transformation followed procedures in Osmani et al. (2006) and Szewczyk et al. (2007). Long-term storage of competent protoplasts is described in El-Ganiny et al. (2010). Primary transformants were grown on selective medium containing 1 M sorbitol or 1 M sucrose as osmostabilizer. Spores from these colonies were streaked three times on selective medium prior to gDNA extraction. Genomic DNA from transformant spores (see below) was used as template for PCR to confirm the each manipulation (Suppl. Fig. A).

2.4. Isolation of genomic DNA from A. nidulans spores

Approximately 1 × 10^7 spores from individual 2 d old colonies were harvested in sterile distilled water, pelleted in 1.5 mL microfuge tubes, and aspirated to dampness. Tubes containing damp spore samples were sealed with lid-locks, floated in 100 mL water,
and microwaved at full power for 6 min. Then 35 μL of TE buffer (10 mM Tris–Cl, 1 mM EDTA, pH 8) was added to each tube. Tubes were vortexed briefly and put on wet ice for 5 min. The spore-DNA suspension was centrifuged at 16,000 g for 5 min to pellet debris. One microliter of supernatant was used as PCR template for ampli-
cons up to 4 kb long.

2.5. RT-PCR and qPCR

Gene expression studies compared levels of Galf biosynthesis genes in wild type, deleted, and alcA(p)-, and niiA(p)-regulated ugeA, ugmA and ugtA strains. Spores were inoculated in CM or induction or repression liquid media, and incubated at 28 °C with shaking at 250 rpm for 16 h. Mycelia were collected by centrifugation, frozen in liquid nitrogen, and lyophilized. Total RNA was extracted using an RNasy plant kit (Qiagen) following manufacturer’s instructions. RNA concentration was measured using a Nanodrop™, then diluted to 400 ng/μL. Genomic DNA elimination and reverse transcription used a QuantiTect reverse transcrip-
tion kit (Qiagen) following the manufacturer instructions (Cuero et al., 2003).

Quantitative real time PCR (qPCR) was performed in 96-well optical plates in an iQ5 real-time PCR detection system (Bio-Rad). Gene expression was assayed in triplicate in total volume 20 μL reactions containing cDNA at an appropriate dilution and SYBR green fluorescent (Qiagen). A no-template control was used for each gene. Actin was used as a reference gene (Bohle et al., 2007). Primers actF and actR (Suppl. Table A) were designed to amplify regions that included an intron in the actA genomic sequence. These primers generated a ~200 bp band from cDNA and ~400 bp band from gDNA, to detect genomic DNA contamination in the template. The qPCR amplification used the following conditions: 95 °C/15 min for one cycle, 95 °C/15 s, 55 °C/40 s and 72 °C/30 s for 40 cycles and final extension cycle of 72 °C/2 min. Melting curve analysis was done using the following cycle: 15 s at 65 °C with an increase of 0.5 °C each cycle to 95 °C. For FKS and ags I gene expression analysis, we used wild type (AAE1), ugmAΔ, and AfniA(p)–ugeA strains. The relative expression was normalized to actA (Upadhyay and Shaw, 2008) and calculated using the ΔΔCt method (Livak and Schmittgen, 2001). Three independent biological replicates were performed for each strain/medium.

2.6. Colony growth and sporulation

Colony characters were examined as described in El-Ganiny et al. (2008). Strains were grown for two generations on inducing or repressing media to control for possible dowry effects, then spores of wild type and regulated strains were streaked on induc-
tion or repression media and incubated for 3 d at 28 °C to give iso-
lated colonies. The diameter of 10 colonies/strain was measured to the nearest millimeter using a dissection microscope. The number of spores produced per colony was counted for four colonies/strain. Isolated colonies were vortexed in a microfuge tube containing 1 mL water, then samples of spores were counted with a hemocytometer.

2.7. Microscopy

Samples were prepared for confocal microscopy as described in El-Ganiny et al. (2008). Freshly harvested spores were grown on coverslips at 28 °C for 16 h in induction or repression media. Hy-
phae were fixed and stained with Hoechst 33258 (for nuclei) and Calcofluor (for cell walls). Samples were imaged using a Zeiss META510 as described previously (El-Ganiny et al., 2008). LSM im-
age browser software was used for measurements of hyphal width (at septa) and basal cell length (distance between adjacent septa).

Galf content of wild type, ugmA-GFP, ugmAΔ, and niiA(p)-ugeA hyphae was assessed using immunolocalization. Monoclonal anti-
Galf antibodies L10 and L99 were a generous gift of Prof. Frank Ebel (Univ Munich). Primary antibodies were used at full strength; TRITC-conjugated goat-anti-mouse was used at 1:10 dilution. Immunofluorescence procedures followed El-Ganiny et al. (2008) and samples were examined using confocal epifluorescence microscopy.

Transmission electron microscopy was used to measure hyphal wall thickness for alcA(p)-regulated colonies grown on dialysis tubing overlying CMT or CM3G. Samples were prepared as de-
scribed in Kaminskyj (2000). Hyphal wall thickness was measured where the cell membrane bilayer was crisply in focus. Typically three sites were measured for each of ten hyphae per strain/medium combination.

Scanning electron microscopy was used to examine conidiating wild type and alcA(p)-regulated colonies following the procedure in El-Ganiny et al. (2008). Strains were grown on dialysis tubing laid on CMT and CM3G for 3 d at 28 °C. Colonies were fixed by immersion in 1% glutaraldehyde, dehydrated in acetone, critical point dried (Polaron E3000, Series II), and gold sputter coated (Ed-
wards model 5150B). Samples were imaged with a JEOL 840A scanning electron microscope.

2.8. Drug sensitivity testing

Antifungal agents and solvents were obtained from the follow-

ing manufacturers: amphotericin B, terbinafine, itraconazole, di-
methyl sulphoxide (DMSO) (Sigma Chemicals); ethanol (VWR); caspofungin (a gift from Merck, now Schering-Plough). Stock solutions were prepared as follows: amphotericin B (20 mg/mL in DMSO), itraconazole (1.6 mg/mL in DMSO), terbinafine (1.6 mg/mL in 50% ethanol), and caspofungin (20 mg/mL in sterile water). Aliquots of stock solutions were stored at −80 °C.

Wild type and Galf-biosynthesis gene deletion strains were grown on CM; gene-regulated strains were grown on repression and expression media. Spore suspensions collected from 4 d old colonies were filtered through VWR 413 filter paper (average pore size 5 μm) to remove hyphae and conidiophores. Spores in the filtered suspension were counted using a hemocytometer and adjusted to 50,000 spores/μL.

Antifungal drug sensitivity testing used a disk-diffusion method modified from (Kontoyiannis et al., 2003; Lass-Florl, 2009), as de-
scribed in Afroz et al. (2011). Strains were grown for two rounds on the test media before being used for drug sensitivity studies, to compensate for potential spore dowry effects. For the drug sen-
sitivity test, 1 × 10^7 spores were mixed into 20 mL of 50 °C expres-
sion or repression agar and immediately poured in 9 cm diameter Petri plates. After the medium had solidified, sterile 6 mm paper disks were placed on the agar surface. Anti-fungal drug stock solu-
tions (described above) were micro-pipetted onto individual disks: 10 μL of terbinafine and itraconazole, and 20 μL of amphotericin B and caspofungin. Solvent control studies with DMSO and 50% eth-
anol showed no zone of inhibition. The plates were incubated at 28 °C and assessed after 48 h (Kiraz et al., 2009).

The radius of the zone of inhibition was calculated as

\[ \text{diameter (mm) with no visible growth} = \text{disk diameter (mm)/2} \]

Two measurements at orthogonal axes were taken for each disk on each plate; four plates were assessed for each strain and medium combination, and each experiment was repeated. These data were used for statistical analysis. Data are presented as indexed values with respect to wild type on CM (for deletion strains) and with re-

duct to wild type on the test medium (for regulated strains).
2.9. Data processing and analysis

Confocal images were processed using LSM examiner. Images were presented using Adobe Photoshop 7.1. Statistical analysis used Kruskal–Wallis and associated post hoc tests with R software: R 2.13 for kruskal.test, and R 2.9 for kruskalmc.test.

3. Results

We examined the roles of Galf biosynthesis gene products for anti-fungal drug sensitivity using conditional strains whose gene expression was under the control of the regulatable promoter, alcA(p) (Romero et al., 2003; Tribus et al., 2010). Previously we had deleted each of these genes: umgA (El-Ganiny et al., 2008); ugeA (El-Ganiny et al., 2010); ugtA (Afroz et al., 2011), to study the effects on A. nidulans morphogenesis, conidiation, and wall ultrastructure. We expected that gene repression would have similar effects to gene deletion, and that over-expression would have little effect. We used these strains to assess the role of Galf biosynthesis in sensitivity to anti-fungal drugs, especially Caspofungin that targets cell wall synthesis. For each section, we present umgA results in detail, followed by comparison with results for ugeA and ugtA. The alcA(p) data were confirmed using niiA(p)-regulation (Gauwerky et al., 2009; Monteiro and DeLucas, 2010).

3.1. Construction and validation of regulated strains

We replaced each endogenous Galf biosynthesis gene promoter with the alcA(p) and with the niiA(p) conditional promoters, using AfpyrG as the selectable marker (Szewczyk et al., 2007). Primary transformants were grown on CM or MM, respectively, containing 1 M sorbitol or 1 M sucrose as an osmotic stabilizer. Strain construction and confirmation is shown in Suppl. Fig. A.

To select media appropriate for regulation of Galf biosynthesis genes in A. nidulans, the alcA(p)-umgA strain was grown on CM containing a variety of carbon sources, and the colony morphologies were compared qualitatively. Media included CM with 100 mM threonine (CMT), 1% fructose plus 100 mM threonine, 1% ethanol, 1 M sorbitol or 1 M sucrose as an osmotic stabilizer. Strain construction and confirmation is shown in Suppl. Fig. A.

3.2. Repression of Galf biosynthesis genes causes compact colony growth and reduced sporulation

Growing the alcA(p)-umgA strain on CM3G to repress umgA produced growth and sporulation phenotypes similar to the ugtA

| Table 1 | Colony and hyphal characteristics\(^a\) of wild type and deletion strains grown on CM, and wild type and alcA(p)-regulated Aspergillus nidulans galactofuranose biosynthesis strains grown on over-expression (CMT) and repression (CM3G) media. First row of each column represents wild type and respective deleted strains grown on CM.

<table>
<thead>
<tr>
<th>Character</th>
<th>Expression (medium)</th>
<th>Wild type</th>
<th>umgA</th>
<th>ugeA</th>
<th>ugtA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony diameter (mm)(^b)</td>
<td>Neutral (CM, wild type, Δ)</td>
<td>17 ± 0.4(^f)</td>
<td>4 ± 0.2(^g)</td>
<td>5 ± 0.2(^d)</td>
<td>4 ± 0.2(^e)</td>
</tr>
<tr>
<td></td>
<td>Overexpression (CMT)</td>
<td>22 ± 0.5(^f)</td>
<td>17 ± 0.6(^g)</td>
<td>21 ± 0.5(^g)</td>
<td>16 ± 0.2(^f)</td>
</tr>
<tr>
<td></td>
<td>Repression (CMT3G)</td>
<td>16 ± 0.4(^d)</td>
<td>4 ± 0.2(^g)</td>
<td>5 ± 0.2(^d)</td>
<td>4 ± 0.1(^k)</td>
</tr>
<tr>
<td>Spores/colony (×10(^6))(^c)</td>
<td>Neutral (CM, wild type, Δ)</td>
<td>107 ± 2(^f)</td>
<td>0.2 ± 0.1(^k)</td>
<td>0.4 ± 0.1(^k)</td>
<td>1.3 ± 0.2(^d)</td>
</tr>
<tr>
<td></td>
<td>Overexpression (CMT)</td>
<td>12.4 ± 1(^f)</td>
<td>8.2 ± 1(^g)</td>
<td>10.8 ± 1(^h)</td>
<td>13.8 ± 1(^d)</td>
</tr>
<tr>
<td></td>
<td>Repression (CMT3G)</td>
<td>38 ± 1.1(^f)</td>
<td>0.6 ± 0.1(^k)</td>
<td>3.0 ± 0.6(^g)</td>
<td>3.8 ± 0.1(^k)</td>
</tr>
<tr>
<td>Hyphal width (μm)(^d)</td>
<td>Neutral (CM, wild type, Δ)</td>
<td>2.7 ± 0.4(^f)</td>
<td>3.6 ± 0.6(^g)</td>
<td>3.6 ± 0.1(^k)</td>
<td>3.5 ± 0.1(^f)</td>
</tr>
<tr>
<td></td>
<td>Overexpression (CMT)</td>
<td>3.1 ± 0.03(^f)</td>
<td>3.0 ± 0.06(^g)</td>
<td>2.2 ± 0.04(^d)</td>
<td>1.9 ± 0.05(^h)</td>
</tr>
<tr>
<td></td>
<td>Repression (CMT3G)</td>
<td>3.1 ± 0.05(^f)</td>
<td>3.7 ± 0.18(^g)</td>
<td>3.6 ± 0.1(^k)</td>
<td>3.4 ± 0.00(^d)</td>
</tr>
<tr>
<td>Basal cell length (μm)(^e)</td>
<td>Neutral (CM, wild type, Δ)</td>
<td>26.1 ± 1(^f)</td>
<td>15 ± 1(^g)</td>
<td>14 ± 1(^k)</td>
<td>16 ± 1(^k)</td>
</tr>
<tr>
<td></td>
<td>Overexpression (CMT)</td>
<td>27 ± 1.2(^f)</td>
<td>14 ± 0.2(^g)</td>
<td>22 ± 1.2(^h)</td>
<td>25 ± 0.5(^h)</td>
</tr>
<tr>
<td></td>
<td>Repression (CMT3G)</td>
<td>30 ± 1.7(^f)</td>
<td>17 ± 1.0(^g)</td>
<td>24 ± 1.1(^k)</td>
<td>16 ± 0.5(^k)</td>
</tr>
<tr>
<td>Cell wall thickness (nm)(^f)</td>
<td>Neutral (CM, wild type, Δ)</td>
<td>54 ± 2(^f)</td>
<td>204 ± 10(^d)</td>
<td>123 ± 4(^g)</td>
<td>183 ± 1(^f)</td>
</tr>
<tr>
<td></td>
<td>Overexpression (CMT)</td>
<td>36 ± 2(^f)</td>
<td>42 ± 1(^g)</td>
<td>39 ± 1(^k)</td>
<td>40 ± 1(^g)</td>
</tr>
<tr>
<td></td>
<td>Repression (CMT3G)</td>
<td>50 ± 2(^f)</td>
<td>82 ± 3(^d)</td>
<td>59 ± 2(^g)</td>
<td>83 ± 3(^f)</td>
</tr>
</tbody>
</table>

\(^a\) Values are presented as mean ± standard error. For colonies grown on CM, values for wild type and umgAΔ hyphae were taken from El-Ganiny et al. (2008); for ugeAΔ and ugtAΔ hyphae from Afroz et al. (2011). Cellular characteristics were compared using a Kruskal–Wallis test, and shown for each gene to be significantly different at P < 0.05. Differences for strains grown on CMT or CM3G were compared using a Kruskal–Wallis multiple comparison test. For each row, values followed by different letters (l–i) are significantly different at P < 0.05.

\(^b\) Ten colonies per strain/medium combination.

\(^c\) At least three colonies per strain/medium combination.

\(^d\) Fifty measurements per strain/medium combination. Hyphal width was measured at septa. Basal cell length was between adjacent septa.

\(^e\) Measured from transmission electron micrographs for at least seven cross-sectioned hyphae per strain/medium combination, typically at three places per hypha where the cell membrane bulayer was crisply in focus.
3.3. Deletion and repression of ugmA cause hyphal morphogenesis defects that correlate with loss of immunolocalizable wall Galf

We expected repression of Galf biosynthesis would reduce wall Galf content, as we had previously shown for ugmAΔ (El-Ganiny et al., 2008). Prof. Frank Ebel’s group (Univ. Munich) recently generated the L10 and L99 anti-Galf mAbs, (Heesemann et al., 2011) that have high affinity for hyphal wall Galf. They generously provided us with samples of each mAb. We had strong Galf immunolocalization in wild type phenotype A. nidulans hyphal walls using L10 (Fig. 4A, B and D), but less intense signal with L99 (not shown).

In addition to wall staining, the cytoplasm also contained L10-reactive material, consistent with UgmA-GFP localization being cytoplasmic (Fig. 4B and 4B’) and UDP-Galf being synthesized in the cytoplasm prior to Galf incorporation into wall compounds (El-Ganiny et al., 2010). Both ugmAΔ (Fig. 4C), and niiA(p)–ugmA strains grown on MMA repression medium (Fig. 4E), had wide, highly-branched hyphae. As expected, the ugmA deletion and repression strain hyphal walls lacked detectable wall Galf (Fig. 4C and E), as did their cytoplasm.

We attempted to localize alpha-glucan using the monoclonal antibody, MOPC-104E, which had been described by Futagami et al. (2011) for dot-blot studies in A. nidulans. Our intent was to determine if alpha-glucan content or distribution was affected when Galf synthesis was reduced or absent. However MOPC-104E did not react for immunofluorescence localization. It is possible that the extractions required for cell wall ghost preparation exposed an epitope that is not accessible in whole cells.

3.4. Galf biosynthesis affects α-glucan synthase and β-glucan synthase expression

Both deletion and repression of Galf biosynthesis in A. nidulans are associated with increased hyphal wall thickness (Table 1; El-Ganiny et al., 2008), albeit to different levels. Damveld et al. (2008) had previously shown that A. niger strains with defective ugmA homologues had increased wall α-glucan content. We compared the expression level of α-glucan synthase (ags1) in wild type, ugmAΔ, and AfniiA(p)–ugmA strains growing on MMN and MMA (Fig. 5A). As expected, AfniiA(p)–ugmA grown on MMN (induction) had similar expression to wild type. Compared to the wild type
Fig. 2. Colony morphology of wild type and conditional promoter-regulated strains of ugmA, ugeA, ugtA. In each of A and B, the colonies are the same age. (A) Wild type, alcA(p)-regulated, and ugmAΔ strains grown on complete medium containing 100 mM threonine (CMT) or 3% glucose (CM3G) as sole carbon sources, for gene overexpression or repression, respectively. (B) Wild type and mkn(p)-regulated strains grown on minimal medium containing nitrate (MMN) or ammonium (MMA) for gene expression or repression, respectively. (For interpretation to colours in this figure, the reader is referred to the web version of this paper.)

Fig. 3. Scanning electron micrographs showing: colony (A), and conidiophore (B) phenotype for wild type and alcA(p)-regulated ugmA, ugeA, and ugtA strains grown on CMT for overexpression and CM3G for repression. (C) Colony and conidiophores of ugmAΔ on CMT and CM3G. On CMT media, the alcA(p) strains have abundant conidiophores with long chains of spores similar to wild type strain. On CM3G media, alcA(p)-ugmA, alcA(p)-ugeA, alcA(p)-ugtA produced many fewer conidiophores similar to ugmAΔ strain. Scale bar is 100 μm for A and C (upper panel) and 10 μm for B and C (lower panel).
parental strain, *ags1* expression was more than 4-fold higher in a *ugmA*Δ strain, or in the *AfniA*Δ(*p*)-*ugmA* on MMA (repression). We also compared the expression level of β-glucan synthase (*fks*) in wild type, *ugmA*Δ, and *AfniA*Δ(*p*)-*ugmA* strains growing on MMN and MMA (Fig. 5B). *AfniA*Δ(*p*)-*ugmA* grown on MMN (induction) had almost similar expression to wild type. Compared to the wild type parental strain, *fks4* expression was about 2.5-fold decreased in a *ugmA*Δ strain, or in the *AfniA*Δ(*p*)-*ugmA* on MMA (repression).

3.5. Repression of Galf biosynthesis increases sensitivity to Caspofungin

Sensitivity to anti-fungal drugs of wild type and gene deletion strains on CM, *alcA*(*p*)-regulated strains on CMT and CM3G, and *AfniA*Δ(*p*)- regulated strains on MMN and MMA, was tested using a disk-diffusion drug-sensitivity assay. We compared Caspofungin (blocks wall β-1,3-glucan synthesis) with drugs that target ergosterol (Amphotericin B) and ergosterol biosynthesis (Itraconazole

![Fig. 4. Galactofuranose immunolocalization in *Aspergillus nidulans* hyphae using the L10 monoclonal antibody. (A) Wild type, (B) UgmA-GFP, (C) ugmAΔ, (D) *AfniA*Δ(*p*)-*ugmA* grown on induction medium, (E) *AfniA*Δ(*p*)-*ugmA* grown on repression medium. Lower case letters are corresponding transmission images. B' shows UgmA-GFP distribution, which is cytoplasmic, as expected. B' shows a merge of B and B'. Bars = 10 µm.](image-url)
and Terbinafine). In addition, both 5-fluocytosine and Ninkomycin Z were assessed for their effect on wild type and Gal biosynthesis gene deletion strains. Neither of these latter drugs inhibited colony growth, but Ninkomycin Z delayed germination by several hours, causing spores to swell considerably before germ tube establishment (data not shown).

For clarity, Table 2 shows drug sensitivity values for Itraconazole and Caspofungin only, since in this assay Amphotericin B and

<table>
<thead>
<tr>
<th>Expression (medium)</th>
<th>Wild type</th>
<th>ugmA</th>
<th>ugeA</th>
<th>ugtA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Itraconazole</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutral (CM); wild type and Δ strains</td>
<td>1.0</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>alcA(p) Overexpression (CMT)</td>
<td>0.8</td>
<td>1.1</td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Repression (CM3G)</td>
<td>0.8</td>
<td>1.1</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>niiA(p) Expression (MMN)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Repression (MMA)</td>
<td>1.0</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Caspofungin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutral (CM); wild type and Δ strains</td>
<td>1.0</td>
<td>1.2</td>
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<td>1.7</td>
</tr>
<tr>
<td>alcA(p) Overexpression (CMT)</td>
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<td>1.3</td>
<td>0.5</td>
<td>0.7</td>
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<tr>
<td>Repression (CM3G)</td>
<td>1.0</td>
<td>1.5</td>
<td>1.5</td>
<td>1.8</td>
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<tr>
<td>niiA(p) Expression (MMN)</td>
<td>1.0</td>
<td>1.0</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Repression (MMA)</td>
<td>1.0</td>
<td>1.6</td>
<td>1.6</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* Drug sensitivity was measured using a disk diffusion assay (see Fig. 6). For Caspofungin, sensitivity was the radius (mm) of the clear zone with no visible growth (see arrows on Fig. 6B). Mean ± SE of two measurements for each of four biological replicates were used for statistical analysis (not shown).

b Index sensitivity values are for each drug/strain combination compared to wild type on that medium. Wild type values are compared to CM with 1% glucose. Gene deletion strains are compared to wild type on CM. Regulated strains are compared to wild type on the same medium. Index values >1.0 are more sensitive than wild type. Index values that differed by >0.2 were based on data that were significantly different.

* See Section 2 for details regarding drug dosage and medium formulation.

4. Discussion

Deletion of any of three sequential-acting genes in the Gal biosynthesis pathway causes similar defects in *A. nidulans* hyphal morphogenesis and colony development (El-Ganiny et al., 2008, 2010; Afroz et al., 2011) and in wall surface structure, force compliance, and adhesion (Paul et al., 2011). Gal forms the side chains of many glycoconjugates (Latgé, 2007, 2009) but its role(s) in wall formation and maturation are still far from understood. We expected that wild type levels of Gal biosynthesis would be important for Caspofungin resistance because we had previously found that the walls Gal deletion strains were structurally compromised (Paul et al., 2011). To test this we compared the drug sensitivity of gene deletion and wild type with regulated Gal biosynthesis strains. This is the first study to explore the roles of Gal biosynthesis proteins on drug sensitivity of *A. nidulans* strains using the alcA and niiA promoters, and the first to examine the effect of regulating genes in the Gal biosynthesis pathway. We expected that the
products of these genes would have similar effects on sensitivity to antifungal compounds that targeted cell walls but not to those that targeted other aspects of fungal physiology. Consistent with this, we found increased echinocandin sensitivity for the deletion strains and repressed strains, but no significant change for compounds affecting ergosterol or its biosynthesis.

4.1. Altering Galf biosynthesis gene expression level affects wall formation, colony growth, and development

The phenotypes of strains deleted or repressed for Galf biosynthesis pathway genes were generally comparable. Repression of alcA(p)–ugmA with 3% glucose (CM3G) and of niiA(p)–ugmA with ammonium (MMA) both caused colony and hyphal defects comparable to the ugmAΔ strain (El-Ganiny et al., 2008). Consistent with our RT-PCR and qPCR results, this is strong evidence that our regulatable promoter strains were functioning as expected. Similar results have been found for regulated histone deacetylase (rpΔA) controlled by alcA(p) and xylP(p) (Tribus et al., 2010), and repression of protein kinase C (pkA) on hyphal growth, wall thickness, and antifungal compound sensitivity (Ronen et al., 2007).

Gene-deletion and gene-repression phenotypes were qualitatively similar in most respects. However, for each of the Galf biosynthesis genes considered in this study, alcA(p)-repressed strains sporulated better than the comparable deletion strains, and their hyphal walls were half the thickness of the respective deletion strains. These anomalies prompted us to repeat our studies on all three genes using niiA(p)-regulation which is repressed by ammonium. Results for colony growth and development regulated by the niiA(p) were comparable to those for alcA(p), consistent with the phenotypes were caused by changes in gene expression level. Overexpression of alcA(p)–ugmA on CMT also caused a hyper-branching phenotype that slightly reduced colony growth rate. Taken together, repression vs deletion morphology, and (over)expression vs wild type phenotypes were generally similar.

In A. niger, mutation of ugm1 is associated with an increase in wall alpha-glucan content (Danveld et al., 2008). We tested whether there was a comparable relationship between Galf and alpha-glucan in A. nidulans using qPCR to compare α-glucan synthase (ags1) levels. Both the ugmAΔA and alcA(p)–ugmA strain grown on repression medium had about fourfold increases in ags1 expression. The MOPC104E mAb has previously been used for an α-glucan dot blot assay, however, to date we have been unable to replicate these results at least with immunofluorescence. The function of Aspergillus wall α-glucan is not yet well understood (He and Kaminskyj, in preparation). Notably, a triple AGS mutant in A. fumigatus was found to lack α–1,3-glucans (Henry et al., 2011). This triple deletion strain had a wild type phenotype on solid medium, suggesting that α–1,3-glucans may be dispensable for A. fumigatus vegetative growth (Henry et al., 2011) at least under these conditions.

4.2. Altering Galf biosynthesis-gene expression level affects Caspofungin sensitivity

We compared sensitivity of wild type, Galf biosynthesis-gene deletion strains, and Galf biosynthesis-regulated strains for their sensitivity to Caspofungin compared to drugs that target fungal membranes via ergosterol or ergosterol-biosynthesis. In this study, sensitivity to Itraconazole was typical of other membrane-targeting drugs, and was not related to Galf biosynthesis gene presence or function. Therapeutically, Aspergillus species are reported to be resistant to Itraconazole (Erjavec et al., 2009), making it a useful control treatment. Statistical analysis compared average radii of growth inhibition, presented as an index of sensitivity with respect to wild type.

All of the gene deletion strains were significantly more sensitive than wild type to Caspofungin, consistent with our previous AFM studies on the deletion strains that showed defects in wall architecture and strength (Paul et al., 2011). For ugmAΔ strains regulated by alcA(p) and niiA(p), colonies grown in repression conditions were at least as Caspofungin-sensitive as the knockout strains. Since we showed that deletion or repression of ugmAΔ decreased fksA expression, this might contribute to Caspofungin sensitivity. Unexpectedly, colonies with over-expressed alcA(p)–ugmA were also significantly more sensitive to Caspofungin than wild type. This is consistent with the abnormal width and branching of overexpressed alcA(p)–ugmA hyphae, but the cause is not known.

Unlike ugmA, although both ugaA and ugtA, alcA(p)- and niiA(p)- repression caused Caspofungin hypersensitivity, the alcA(p)-overexpressed ugeA and ugtA strains were significantly less sensitive to Caspofungin. Consistent with this, increasing Mycobacterium ugmA expression was reported to increase resistance to isoniazid (Richards et al., 2009). Galf biosynthesis genes have both shared and divergent functions in cell wall metabolism.

4.3. Effect of gene deletion versus gene repression

We had expected that gene deletion and repression phenotypes would be similar for most aspects of colony and cell morphology, since net UgmA and UgtA activity should be substrate-limited and to our current knowledge the only role of Galf is in the cell wall. Contrast enhancement of the RT-PCR results in Fig. 1 showed that transcription was not abolished on gene repression media. Similarly gene over-expression was expected to have little effect due to substrate limitations, but found that Caspofungin sensitivity decreased for alcA(p)-overexpression of ugeA and ugtA. Transcript stability and translation of ugmA mRNA has not been explored in this study.

Galactose metabolism genes are clustered in Saccharomyces cerevisiae (Slot and Rokas, 2010). In A. nidulans the ugmA and ugtA coding sequences are AN3112 and AN3113, respectively, but the other genes in its Leloir pathway are not clustered. Gene product interaction is important for many pathways, e.g. signal transduction, reviewed in Keshet and Seger (2010). Surface topography and charge mediate protein–protein interactions. The surface of a selectable marker product will be unlike the gene product it replaces, so it cannot participate in wild type pathway interactions. In contrast, suppression of gene expression, even to 1% of wild type level as estimated for alcA(p)- and niiA(p)-ugmA repression in this study, could lead to a small amount of wild type protein. Consistent with this, some aspects of the repression morphometry phenotypes were less severe than the deletion strains. The alcA(p)-overexpression drug sensitivity phenotypes also suggest that modulating wild type gene product abundance might have subtle effects.

In sum we have shown that the alcA(p)- and niiA(p)-regulated control of Galf biosynthesis provides novel information about the role of this pathway in A. nidulans. In general, the gene repression and gene deletion phenotypes were comparable for colony growth and morphology, and overexpressed strains resembled wild type. That this was not always so, suggests possible interactions with other aspects of cell wall synthesis or maintenance. Notably, deletion and repression phenotypes were significantly more sensitive to Caspofungin, suggesting that as proposed in Paul et al. (2011), their walls are significantly less robust. We remain hopeful that an inhibitor of this pathway may be therapeutically beneficial.

Note added in proof

He and Kaminskyj (in preparation) used a modified the MOPC-104E immunostaining protocol and have demonstrated robust α-glucan localization in A. nidulans hyphal walls.
Acknowledgments
S.G.W.K and D.A.R.S are pleased to acknowledge shared funding from the Canadian Institutes of Health Research Regional Partnership Program and individual funding from the Natural Sciences and Engineering Research Council of Canada Discovery Grant program. A.M.E. thanks the Egyptian Ministry of Higher Education for her Fellowship. K.A. thanks the University of Saskatchewan (UofS) for a UGS postgraduate scholarship. S.A. thanks UofS for a Graduate Equity scholarship and a UGS. We thank Merck (now Schering-Plough) for their gift of Caspofungin. Fungal Genetics Stock Center archives fungal strains and related resources. We thank Prof. Frank Ebel, University of Munich for the gift of L10 and L99 monoclonal anti-Gal antibodies, and Dr. Loretta Jackson-Hayes, Rhodes College, Memphis TN for the alcG(p) plasmid. We thank Dr. Peta Bonham-Smith and Chad Stewart (UofS Biology) for assistance with the qPCR and Tom Bonli, UofS Geological Sciences, for SEM technical assistance.

Appendix A. Supplementary material
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2012.08.010.

References


