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Aspergillus nidulans UDP-glucose-4-epimerase UgeA has multiple roles in wall architecture, hyphal morphogenesis, and asexual development

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ABSTRACT

Aspergillus nidulans UDP-glucose-4-epimerase UgeA interconverts UDP-glucose and UDP-galactose and participates in galactose metabolism. The sugar moiety of UDP-galactose is predominantly found as galactopyranose (Galp, the six-membered ring form), which is the substrate for UDP-galactopyranose mutase (encoded by *ugmA*) to generate UDP-galactofuranose (Galf, the five-membered ring form) that is found in fungal walls. In *A. fumigatus*, Galf residues appear to be important for virulence. The *A. nidulans* *ugeA*Δ strain is viable, and has defects including wide, slow growing, highly branched hyphae and reduced conidiation that resemble the *ugmA*Δ strain. As for the *ugmA*Δ strain, *ugeA*Δ colonies had substantially reduced sporulation but normal spore viability. Conidia of the *ugeA*Δ strain could not form colonies on galactose as a sole carbon source, however they produced short, multinucleate germlings suggesting they ceased to grow from starvation. UgeA purified from an expression plasmid had a relative molecular weight of 40.6 kDa, and showed *in vitro* UDP-glucose-4-epimerase activity. Transmission electron microscope cross-sections of wildtype, *ugeA*Δ, and *ugmA*Δ hyphae showed they had similar cytoplasmic contents but the walls of each strain were different in appearance and thickness. Both deletion strains showed increased substrate adhesion. Localization of UgeA-GFP and UgmA-GFP was cytoplasmic, and was similar on glucose and galactose. Neither gene product had a longitudinal polarized distribution. Localization of a UgmA-mRFP in a strain that resembled the *ugmA*Δ strain was cytoplasmic and lacked a longitudinal polarized distribution. The roles of UgeA in *A. nidulans* growth and morphogenesis are consistent with the importance of Galf, and are related but not identical to the roles of UgmA.

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1. Introduction

Fungi and protozoa are morphologically simple eukaryotes that are becoming increasingly important human pathogens. In 1939, *Aspergillus fumigatus* infections were considered to be 'so rare as to be of little practical importance' (reviewed in Latgé and Steinbach (2009)), whereas now *A. fumigatus* is considered to be a predominant opportunistic and also a primary human fungal pathogen (Fedorova et al., 2008). Diseases caused by fungi and protozoa are often therapeutically intractable due to their underlying metabolic similarities with animal systems, and systemic fungal infections have high mortality even with aggressive treatment. There are relatively few antifungal drugs, most targeting ergosterol and its biosynthetic pathway (amphotericin B, azoles, allylamines)

Abbreviations: CLSM, confocal laser scanning microscopy; Galf, galactofuranose; Galp, galactopyranose; GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein; SEM, scanning electron microscopy; TEM, transmission electron microscopy; UGE, UDP-glucose-4-epimerase; UGM, UDP-galactopyranose mutase.

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or more recently against fungal cell walls (echinocandins), but many of these are losing effectiveness due emerging fungal resistance (Cowen, 2008).

About 20% of *Saccharomyces cerevisiae* (Lesage and Bussey, 2006) and 12% of *Aspergillus nidulans* (Harris et al., 2009) gene function relates to cell wall formation and maintenance. The fungal cell wall comprises about 20% of its biomass. The fungal wall mediates interactions between the fungal organism and its environment, which is the host in the case of pathogenic species (Klis et al., 2007). Wall function is generally resilient to deletion of individual genes (de Groot et al., 2009) and to chemical stressors that trigger compensatory cell wall stress response pathways (Damveld et al., 2008). Fungal walls are remodeled during growth in culture (Momany et al., 2004) and in response to their environment (Gastebois et al., 2009; Hurtado-Guerrero et al., 2009). As with other human fungal pathogens, *A. fumigatus* forms biofilms in host tissue (Seidler et al., 2008; Loussert et al., 2010) that are more drug resistant than planktonic cultures (Seidler et al., 2008). Since fungal extracellular carbohydrates are not found in animal systems (Beverly et al., 2005), they and their biosynthetic pathways are drug development targets (Pederson and Turco, 2003).

Galf (the five-membered ring form of galactose) residues are found in the walls and extracellular carbohydrate sheaths of bacteria, protists, fungi, and plants, but not in animals (Beverly et al., 2005). The chemistry of Galf-containing polysaccharides is reviewed in Richards and Lowary (2009). As summarized in Fig. 1, UDP-Galf is formed from UDP-galactopyranose (UDP-Galp, the six-membered ring form) by UDP-galactopyranose mutase (UGM) (Beverly et al., 2005) prior to incorporation in the extracellular carbohydrate-containing compounds. Galf is essential in some prokaryotes (Nassau et al., 1996; Sanders et al., 2001; Beverly et al., 2005) and some protozoans (Roper et al., 2005; MacRae et al., 2006) but generally not so in fungi. Nevertheless, Galf is important for wildtype fungal growth, cell morphogenesis, wall architecture, and conidiation (Wallis et al., 2001; Damveld et al., 2008; El-Ganiny et al., 2008; Lamarre et al., 2009). Furthermore, Galf is important for pathogenesis in fungi (Bar-Peled et al., 2004; Perfect, 2005; Moyrand, 2007; Schmalhorst et al., 2008; Lamarre et al., 2009), and in protozoan parasites (Spath et al., 2000; Roper et al., 2005; MacRae et al., 2006).

The wall carbohydrate composition and architecture of *A. fumigatus* is the focus of intensive study, and was recently reviewed (Rementeria et al., 2005; Gastebois et al., 2009; Latgé and Steinbach, 2009). *A. nidulans* and *A. fumigatus* cell walls have similar but not identical carbohydrate composition (Guest and Momany, 2000). Although Galf is not essential in *Aspergillus* (Damveld et al., 2008; El-Ganiny et al., 2008; Schmalhorst et al., 2008), it clearly has important roles in wildtype growth and pathogenesis, and is a potential drug target for combination therapy. For example, deletion of *A. nidulans* *ugmA* (UDP-galactopyranose mutase), which is required for Galf synthesis and so for wildtype wall formation, causes compact colonial growth, abnormal hyphal wall structure and reduced conidiation (El-Ganiny et al., 2008).

In species ranging from *Escherichia coli* to human, UDP-glucose-4-epimerase (UGE, also called UDP-galactose-4-epimerase) catalyzes the inter-conversion of UDP-glucose and UDP-galactose (Fig. 1) (Allard et al., 2001; Holden et al., 2003). In various systems UGE is encoded by genes named *UGE*, *Gale*, *GAL10*. In *S. cerevisiae*, galactose metabolism (Leloir pathway) enzymes are encoded by *GAL1* (galactokinase), *GAL7* (galactose-1-phosphate uridylyltransferase), and *GAL10* (combined mutarotase and UDP-glucose/galactose-4-epimerase) (Douglas and Hawthorne, 1964). The *ScGAL10* epimerase function is essential for survival in the presence of traces of galactose, even if an alternate carbon source such as glycerol is available (Ross et al., 2004).

Some eukaryotes depend on UGE function for growth. *Trypanosoma cruzi* UGE (encoded by *TcGALE*) is the only source of galactose for its surface galactomannan synthesis, since its sole hexose transporter is unable to transport galactose (MacRae et al., 2006). *TcGALE* appears to be essential for *T. cruzi* survival in culture (MacRae et al., 2006). *A. nidulans* has 17 putative hexose transporters that are not yet fully characterized (Wei et al., 2004) and complex primary carbon metabolism (Flippin et al., 2009). Nevertheless, deleting UDP-galactopyranose mutase resulted in impaired cell growth, abnormal morphogenesis, and reduced sporulation (El-

Ganiny et al., 2008). Thus, the *A. nidulans* galactose metabolism pathway appears to be a chink in its metabolic armour.

A. nidulans *ugeA* (AN4727) encodes a UDP-glucose-4-epimerase. We present preliminary characterization of *A. nidulans* *ugeA* through analysis of the *ugeAΔ* deletion strain. Strains with single and double deletions of *ugeA* and *ugmA* produced phenotypically similar colonies. As expected, the *ugeAΔ* strain could not produce colonies on galactose as a sole carbon source, consistent with *in vitro* epimerase function of expressed UgeA.

2. Materials and methods

2.1. Strains and culture condition

Strains and plasmids are listed in Table 1, and primers in Supplemental data Table A. *A. nidulans* strains were grown as described in El-Ganiny et al. (2008). DNA manipulations and transformations followed procedures in Osmani et al. (2006) and Szewczyk et al. (2007).

2.2. Strain construction

Strain construction followed procedures described in El-Ganiny et al. (2008) based on Osmani et al. (2006) and Szewczyk et al. (2007). AN4727 (*ugeA*) was deleted from *A. nidulans* *nkuAΔ* strain A1147 using *AfpyrG* as a selectable marker (amplified from pAO18) to generate AAE4, and also from strain A1149 using the same marker to generate AAE5. Deletion of AN3112 (*ugmA*) from AAE5 used *AfpyroA* as a selectable marker (amplified from pTN1). Then *ugeA* was deleted from AAE5 to generate the [*ugeAΔ*, *ugmAΔ*] double deletion strain, AAE8. GFP-tagging constructs were amplified from pFN03. Deletions and tagging were confirmed by PCR using gDNA template as shown in Supplemental data Fig. A.

For long-term protoplast storage (Ken Bruno, *personal communication*), freshly made protoplasts were resuspended in STC buffer (1 M sorbitol, 50 mM Tris pH8, 50 mM CaCl₂) and adjusted to a concentration of at least 1.2×10^7 /mL. The protoplast-STC suspension was mixed with a solution of 40% (w/v) PEG₄₀₀₀, at 1:4 (v/v) PEG:protoplast-STC. DMSO was added to a final concentration of 7%. Aliquots of 200–300 μL protoplast-STC-DMSO suspension were frozen to -80°C . For transformation, the protoplast suspension was thawed on wet ice, mixed with DNA, and incubated for 20 min on wet ice. This was mixed with 1 mL of 40% (w/v) PEG₄₀₀₀, incubated at room temperature for 20 min, then spread on selective medium containing 1 M sucrose.

2.3. Microscopy, cell morphometry, and sporulation

Cell morphometry was characterized by light microscopy as described in El-Ganiny et al. (2008). Briefly, spores were germinated on dialysis tubing overlying CM agar or on coverslips in liquid CM, grown for 16 h, fixed in formalin, stained with Hoechst 33258, and examined using confocal laser fluorescence microscopy (CLSM) using a Zeiss META 510. Spore production and viability were deter-

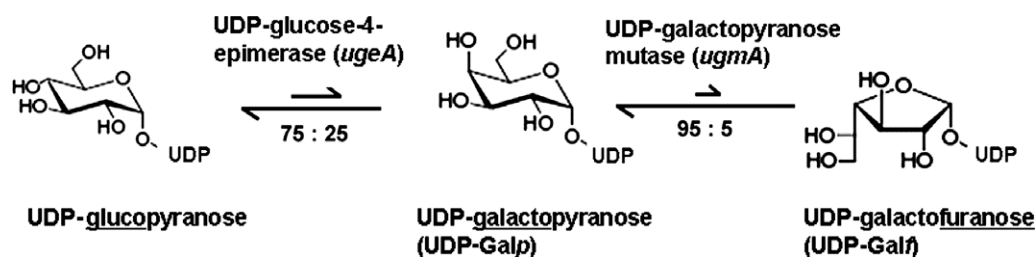


Fig. 1. UDP-galactofuranose synthesis in *Aspergillus nidulans*.

Table 1

Strains and plasmids used in this study.

<i>Aspergillus nidulans</i>	
A1147 ^a	<i>pyrG89; argB2; pabaB22; nkuA::argB; riboB2</i>
A1149 ^a	<i>pyrG89; pyroA4; nkuA::argB</i>
AAE1 ^b	<i>pyrG89::Ncpry4⁺; pyroA4; nkuA::argB</i>
AAE2 (<i>ugmAΔ</i>) ^b	<i>AN3112::AfpyrG; pyrG89; pyroA4; nkuA::argB</i>
AAE4 (<i>ugeAΔ</i>) ^c	<i>AN4727::AfpyrG; pyrG89; argB2; pabaB22; nkuA::argB; riboB2</i>
AAE5 (<i>ugeAΔ</i>) ^c	<i>AN4727::AfpyrG; pyroA4; nkuA::argB</i>
AAE6 (<i>ugmA-GFP</i>) ^c	<i>AN3112-GA₅-GFP + AfpyrG; pyrG89; pyroA4; nkuA::argB</i>
AAE7 (<i>ugeA-GFP</i>) ^c	<i>AN4727-GA₅-GFP + Af pyrG; pyrG89; pyroA4; nkuA::argB</i>
AAE8 (<i>ugeAΔ, ugmAΔ</i>) ^c	<i>AN4727::AfpyrG; AN3112::AfpyrG; pyrG89; pyroA4; nkuA::argB</i>
<i>Escherichia coli</i> ^d	
	BL21-gold (DE3)
Plasmids	
pAO18 ^a	S-TAG, <i>AfpyrG</i> , Kan ^R
pCR4-TOPO ^c	Kan ^R , amp ^R
pFNO3 ^a	GA5-GFP + <i>AfpyrG</i> , Kan ^R
pTN1 ^a	<i>AfpyrG</i> , Amp ^R
pHISTEV ^f	Modified pET30a, Kan ^R

^a Fungal Genetics Stock Center www.fgsc.net.^b El-Ganiny et al. (2008).^c This study.^d Novagen.^e Invitrogen.^f Liu and Naismith (2009).

mined as described previously. Hyphal widths and basal cell lengths were measured for 50 cells per strain. UgeA and UgmA were localized in growing hyphae of C-terminal GFP-tagged strains (Table 1).

SEM and TEM procedures were described previously (El-Ganiny et al., 2008). Hyphal wall thickness was measured on cross-sections of ten hyphae per strain where the cell membrane lipid bilayer was clearly resolved.

2.4. cDNA, recombinant UgeA production and activity

RNA was extracted using a Qiagen RNeasy kit, following manufacturer's instructions. Genomic DNA contamination was cleaned with DNaseI (Fermentas). cDNA was generated using an oligo-Dt primer and RevertAid TM M-MuLVRT (Fermentas) following manufacturer's instructions, and ligated into pCR4-TOPO. Constructs were confirmed by DNA sequencing at Plant Biotechnology Institute (PBI), Saskatoon. The *ugeA* cDNA was subcloned into the pEHISTEV vector (Liu and Naismith, 2009) after PCR amplification with *ugeAF* and *ugeAR* primers that contained *Nco*I and *Bam*H1 restriction sites, and *Nco*I and *Bam*H1 digestion. The resultant plasmid was confirmed by DNA sequencing, then pHISTEV-UgeA was transfected into BL21-gold (DE3) (Novagen) expression cells. These were grown in LB-media with kanamycin at 37 °C to OD 0.5, transferred to 15 °C, then induced with 0.2 mM IPTG to express the recombinant protein. The His₆-UgeA was purified from cell lysate of induced cultures using Ni²⁺-NTA affinity chromatography. Epimerase activity of the purified protein was assayed spectrophotometrically as described in Rosti et al. (2007) as conversion of UDP-galactose to UDP-glucose, and confirmed with HPLC (Waters) using CarboPac PA1 (Dionex Inc). UDP-galactose and UDP-glucose standards had retention times of 30.5 and 33.3 min respectively, when eluted with 0.2 M ammonium acetate buffer (pH 7.85).

3. Results

3.1. *A. nidulans* UDP-glucose/galactose-4-epimerase *ugeA*

The *A. nidulans* predicted amino acid sequence at the Broad Institute website was searched for resemblance to human UDP-

glucose epimerase (GalE, NCBI accession Np 000394.2 for protein). *A. nidulans* ANID4727.1 has 51% homology to GalE, and was named UgeA. Amino acid identity with characterized fungal UGEs is shown in Table 2. UgeA is predicted to be 372 amino acids long, with a molecular weight of 40.6 kDa. The *ugeA* coding sequence was predicted to have four exons and three introns. This was confirmed by isolating and sequencing the *ugeA* cDNA. The *ugeA* deletion strain was constructed and shown to be viable (Fig. 2A), but its hyphae were morphologically different from wild type (Fig. 2B). The [*ugeAΔ*, *ugmAΔ*] double deletion strain was also viable (Fig. 2C). Confirmation of the *ugeAΔ* deletions used PCR of gDNA from these strains as described previously (El-Ganiny et al., 2008) and shown in Supplemental data Fig. A.

3.2. Characterization of the *A. nidulans* *ugeAΔ* and [*ugeAΔ*, *ugmAΔ*] strains

Strains were grown for 16 h at 28 °C, then fixed, stained with Hoechst 33258 to visualize nuclei (and lightly contrast cell walls), and examined with CLSM (Fig. 2). The *ugeAΔ* and [*ugeAΔ*, *ugmAΔ*] hyphae resembled those of the *ugmAΔ* strain (El-Ganiny et al., 2008): wide and highly branched, with short basal cells and abundant nuclei (Table 2).

The *ugeAΔ* and [*ugeAΔ*, *ugmAΔ*] strains were streaked for single colonies, and grown for 3 d at 28 °C until they produced pigmented conidia. Isolated colonies were assessed for conidium production as described in El-Ganiny et al. (2008). The *ugeAΔ* and [*ugeAΔ*, *ugmAΔ*] strains had substantially reduced sporulation (Table 3), comparable to the *ugmAΔ* strain. Spore viability was not affected by deleting either or both of *ugeA* and *ugmA*.

Scanning electron microscope (SEM) images of 3 d-old *ugeAΔ* colonies showed they produced aerial hyphae but few conidiophores (Suppl. data Fig. Ba arrows and box). Most of these were morphologically aberrant (Suppl. data Fig. Bb). C conidium production of the *ugeAΔ* strain was scant, consistent with conidiation rates shown in Table 3.

Transmission electron microscope (TEM) images of hyphal cross-sections of wild type, *ugeAΔ*, and *ugmAΔ* hyphae are shown in Suppl. data Fig. Ca. The *ugeAΔ* hyphal walls were mostly electron-transparent and about twice as thick as wild type walls (Suppl. data Fig. Cb, Table 3). The *ugmAΔ* walls did not have a discrete electron-transparent inner layer (Suppl. data Fig. Cc). UgeA and UgmA appear have different functions in hyphal wall formation.

3.3. Localization of UgeA and UgmA using GFP tagging

Strains with UgeA-GFP and UgmA-GFP under the control of their wildtype promoters were examined with confocal fluorescence microscopy. UgeA-GFP and UgmA-GFP strains were morpho-

Table 2

Percent amino acid sequence identity of *Aspergillus nidulans* UgeA with selected fungal UDP-glucose-4-epimerases, and with the human and *A. fumigatus* sequences used in *ugeA* identification.

Organism	Gene name	UGEAp amino acid identity
<i>Aspergillus fumigatus</i>	<i>afu5g10780</i>	93
<i>Candida albicans</i>	<i>Gal10</i>	57
<i>Cryptococcus neoformans</i>	<i>UGE1</i>	47
<i>C. neoformans</i>	<i>UGE2</i>	45
<i>Homo sapiens</i>	<i>GalE</i>	51
<i>Kluyveromyces lactis</i>	<i>Gal10</i>	55
<i>Pichia stipitis</i>	<i>GALK</i>	61
<i>Saccharomyces cerevisiae</i>	<i>GAL10</i>	57
<i>Schizosaccharomyces pombe</i>	<i>uge1</i>	58
<i>S. pombe</i>	<i>gal10</i>	58

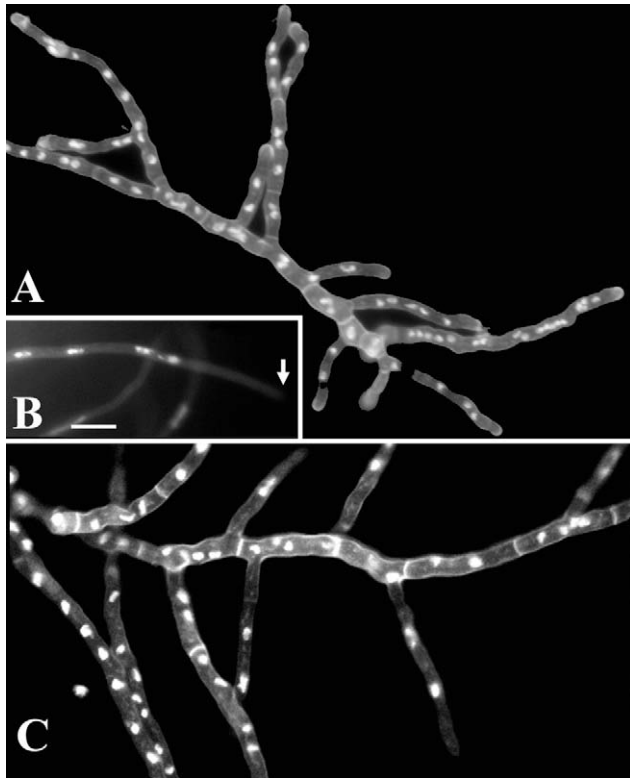


Fig. 2. Phenotype of: (A) *ugeAΔ*, (B) wild type and (C) [*ugeAΔ*, *ugmAΔ*] germlings grown on 1% glucose and stained with Hoechst 33258. Arrowhead in B indicates the end of the hyphal tip. Bar in B = 10 μm for all parts.

logically wildtype. Both the UgeA-GFP and UgmA-GFP fluorescence patterns were cytoplasmic, and both lacked a pronounced longitudinal gradient of abundance (Fig. 3). Subcellular localization of UgeA-GFP and UgmA-GFP was similar when the strains were grown on glucose and galactose (UgeA-GFP Fig. 3b, cf Fig. 3A and B; UgmA-GFP Fig. 3c1, cf Fig. 3C). In addition, a UgmA-RFP strain that had the *ugmAΔ* morphology presumably due to impaired UgmA function also had a non-polarized cytoplasmic distribution (Fig. 3c2).

3.4. Growth of *ugeAΔ* and *ugmAΔ* strains on glucose and galactose as sole carbon sources

Wildtype, *ugeAΔ*, and *ugmAΔ*, and double-deletion strains were grown on minimal medium containing 1% glucose or 1% galactose as sole carbon sources. All strains grew on glucose (Fig. 4A), and all grew faster at 37 °C (not shown). Wildtype hyphal and colony morphology was not substantially reduced by growth on galactose, although colony growth was sparser and sporulation was somewhat delayed. The *ugmAΔ* hyphal morphogenesis defect was partly

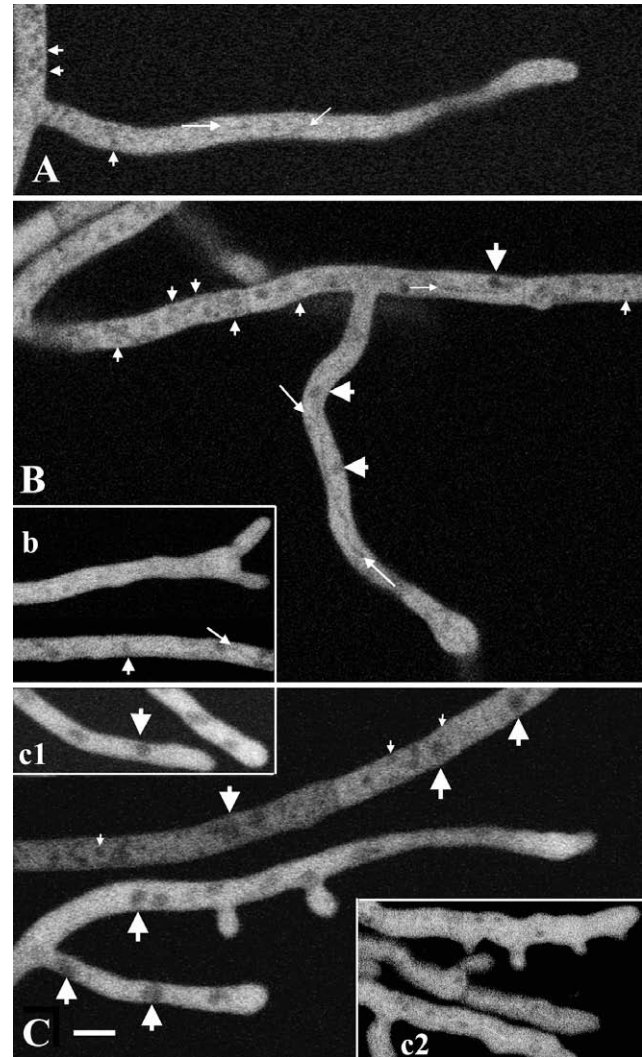


Fig. 3. UgeA (A, B) and UgmA (C) localization patterns on glucose and galactose. UgeA-GFP and UgmA-GFP were localized by confocal fluorescence microscopy in wild type phenotype hyphae. Both proteins were cytoplasmic, lacked pronounced longitudinal polarization, and had comparable localization patterns when grown on glucose (A–C) or galactose (insets b and c1). Areas of lower fluorescence intensity are indicated by small and large arrowheads and arrows. In contrast to the UgmA-GFP strain, which had wild type hyphae, a UgmA-RFP strain had *ugmAΔ* phenotype hyphae (inset c2), suggesting that UgmA function had been compromised, however this did not noticeably affect protein distribution. Bar in C = 5 μm, for all parts.

remediated on 1% galactose. The *ugeAΔ* spores produced germ tubes that contained at least four nuclei (not shown) but they were unable to form colonies. The [*ugeAΔ*, *ugmAΔ*] spores produced cylindrical and sparsely branched germ tubes that did not form colonies (Fig. 4A).

Table 3
Cell characteristics of *ugeAΔ*, *ugmAΔ*, and [*ugeAΔ*, *ugmAΔ*] and related strains.^a

	Hyphal width (μm)	Basal cell length (μm)	Nuclei/basal cell	Wall thickness (nm)	Conidia/colony (%)
Wildtype ^b	2.4 ± 0.0 ^e	26 ± 1 ^e	4.2 ± 1.1 ^e	54 ± 2 ^e	100
<i>ugeAΔ</i> ^c	3.6 ± 0.1 ^f	14 ± 1 ^f	3.2 ± 1.2 ^f	123 ± 4 ^f	4
<i>ugmAΔ</i> ^d	3.1 ± 0.1 ^{ef}	15 ± 1 ^f	3.6 ± 1.0 ^f	204 ± 10 ^g	2
<i>ugeAΔ</i> , <i>ugmAΔ</i> ^c	3.2 ± 0.4 ^f	16 ± 5 ^f	3.5 ± 1.1 ^f	– ^h	1

^a In any one column, values followed by different letters (e–g) are significantly different (ANOVA, $P < 0.05$).

^b AAE1 is near isogenic wildtype to the transformant parent strain (El-Ganiny et al., 2008).

^c This study.

^d AAE2 and *ugmAΔ* morphometry was originally presented in (El-Ganiny et al., 2008).

^h Data not available.

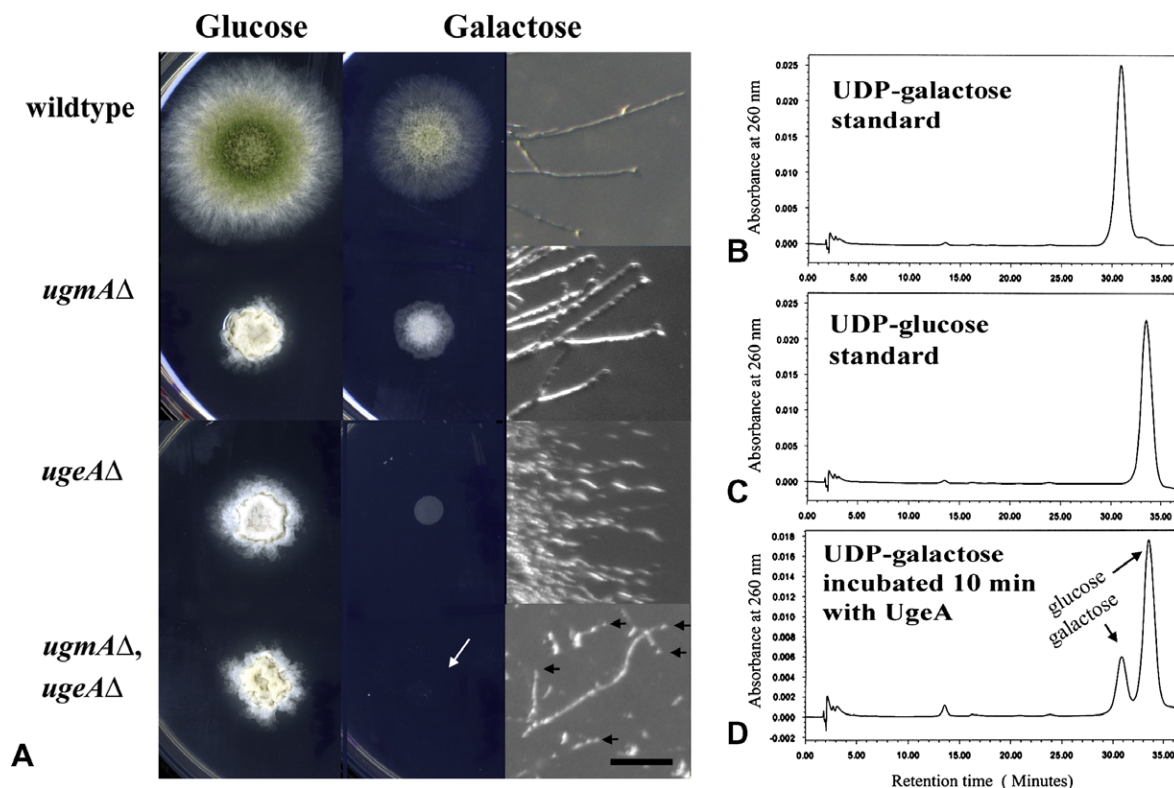


Fig. 4. *Aspergillus nidulans* UgeA converts UDP-galactose → UDP-glucose. (A) Growth of *A. nidulans* wild type, *ugmAΔ*, *ugeAΔ*, and double-deletion strains on minimal medium containing 1% glucose or 1% galactose, and micrographs of these strains grown on 1% galactose. Bar = 100 μ m for all micrographs. When germinated on galactose, the wild type hyphal morphology was unaffected although colony growth and sporulation was sparse; the *ugmAΔ* hyphal phenotype was partly remediated; the *ugeAΔ* strain produced wild type germlings but failed to form colonies; the [*ugeAΔ*, *ugmAΔ*] strain produced short wild type germlings (arrowheads). (B–D) UgeA converts UDP-galactose to UDP-glucose *in vitro*. B) UDP-galactose standard (peak 1, at 30.5 min). C) UDP-glucose standard (peak 2, at 33.3 min). (D) A 10 min incubation of UDP-galactose with UgeA gives a mixture of UDP-galactose:UDP-glucose = 25:75. Comparable results (not shown) are seen when incubating UDP-glucose with UgmA for 10 min.

Hyphal morphogenesis defects can frequently be remediated by growth on high osmolarity medium, which we also had shown for 10 μ g/mL Calcofluor. Wildtype, *ugeAΔ*, and *ugmAΔ*, and double-deletion strains were grown on minimal medium containing 1% glucose amended with 1 M sucrose, or with 10 μ g/mL or 30 μ g/mL Calcofluor. Colonies and hyphae grown on these media are shown in Supplemental data Fig. D and E, respectively. The 1 M sucrose partly remediated the hyphal morphology but not the colony growth phenotypes of the single and double-deletion strains. Growth and hyphal phenotypes of the single and double deletions strains were partly remediated on 10 μ g/mL calcofluor, but not on 30 μ g/mL Calcofluor.

In wildtype *A. nidulans*, Galf localizes to conidial and hyphal walls (El-Ganiny et al., 2008). As with the *ugmAΔ* strain described in El-Ganiny et al. (2008), we were unable to immunolocalize Galf in *ugeAΔ* strain hyphae or spores (not shown). This is consistent with the demonstrated function of UgeA, shown below (Fig. 4B–D).

3.5. *In vitro* enzymatic activity of UgeA

UgeA was expressed with an N-terminal His₆ tag, and purified using Ni²⁺-NTA affinity chromatography. SDS-PAGE electrophoresis on a 10% polyacrylamide gel showed that purified UgeA had an approximate molecular weight of 40.6 kDa (not shown) consistent with the expected translation product. UgeA was able to convert UDP-galactose to UDP-glucose *in vitro* and *vice versa*, as tested with coupled reactions. Enzyme activity was linearly related to the amount of UgeA in the reaction mix (not shown). Using either UDP-galactose (Fig. 4B) or UDP-glucose (Fig. 4C) as substrates for UgeA resulted in two peaks in a 75:25 mixture of UDP-glucose:UDP-gal-

actose (Fig. 4D). The data in Fig. 4D show the HPLC result with UDP-galactose as substrate. Similar results are seen with UDP-glucose as substrate (not shown).

4. Discussion

4.1. *A. nidulans* ugeA encodes a UDP-glucose-4-epimerase

The *A. nidulans* UgeA sequence was identified by its homology to human GalE (Daude et al., 1995). Galactose metabolism is mediated by enzymes in the Leloir pathway (Holden et al., 2003). These include galactose mutarotase (also called galactose-1-epimerase), galactokinase, galactose-1-phosphate uridylyltransferase, and UDP-glucose-4-epimerase. In fungi like *S. cerevisiae* and *Candida albicans*, the UDP-glucose-4-epimerase and mutarotase functions are both encoded by GAL10, which has two domains (Ross et al., 2004; Singh et al., 2007). Other eukaryotes including fungi like *Aspergillus* have separate mutarotase- and epimerase-coding genes. *A. nidulans* UgeA was predicted to be a UDP-glucose-4-epimerase by sequence comparison. The *A. nidulans* ugeA sequence has similar exon organization to that of the predicted *A. fumigatus* sequence afu5g10780, with which it shares 93% sequence identity. The phenotype of the *ugeAΔ* strain is closely related to that of the *ugmAΔ* strain (El-Ganiny et al., 2008), suggesting that UgeA is the source of the UDP-Galp that ultimately generates wall Galf residues needed for wildtype *A. nidulans* growth and development.

Hydropathy analysis of *C. neoformans* UGE1 and UGE2 (Moyrand et al., 2008) suggested these proteins each have a single membrane-spanning domain near their N-terminal. CnUGE1-RFP and CnUGE2-GFP distribution were associated with the cell membrane

at 37 °C, but only CnUGE2-GFP was cell membrane-associated at 25 °C (Moyrand et al., 2008). Hydrophathy analysis of *A. nidulans* UgeA shows that it lacks a predicted membrane-spanning domain. The cellular distribution of UgeA-GFP was cytoplasmic, like that of UgmA-GFP, and consistent with our expectation of their metabolic function. The lack of a longitudinal abundance gradient for UgeA-GFP and UgmA-GFP is consistent with the length of hypha required to support growth of the tip, and with relatively long-distance intracellular transport of wall-building components.

The UgeA-GFP and UgmA-GFP distributions were similar when wild-type morphology tagged strains were grown on glucose and galactose. Also, a UgmA-mRFP strain that appeared to have impaired UgmA function, since grew with the *ugmAΔ* hyphal phenotype, had a non-polarized cytoplasmic distribution. Thus, localization of these gene products did not appear to be affected nutrition or by hyphal morphogenesis.

4.2. Galactose → glucose conversion in *A. nidulans* uses UgeA

Wildtype *A. nidulans* strains are able to grow on galactose as a sole carbon source. *A. nidulans* has 17 putative hexose transporters (Wei et al., 2004) and a primary carbon metabolism that permits nutritional flexibility (Flipphi et al., 2009). However, the *ugeAΔ* strain did not form colonies when grown on galactose. Galactose was toxic to an *S. cerevisiae* *GAL10Δ* (epimerase-mutarotase) strain (Mehta et al., 1999). Although *A. nidulans* *ugeAΔ* spores were unable to form colonies when grown on galactose, they produced multinucleate germlings so they were probably exhausting their endogenous reserves, rather than succumbing to toxicity. *A. nidulans* appears to lack the ability to import nutritionally significant amounts of galactose from its surroundings, so UgeA is likely the source for glucose to support metabolism during growth on galactose. Consistent with these results, we have shown that expressed UgeA interconverts UDP-galactose and UDP-glucose *in vitro*, with an equilibrium in favor of UDP-glucose.

4.3. The phenotype of *ugeAΔ* and [*ugeAΔ*, *ugmAΔ*] strains

The *ugeAΔ* strain cell morphometry, hyphal branching pattern, and sporulation resembled that of *ugmAΔ* strains described previously (El-Ganiny et al., 2008). The colony growth and hyphal morphogenesis defects of the single and double-deletion strains were partially remediated by high osmolarity and by low levels of Calcofluor, consistent with results shown previously for the *ugmAΔ* strain (El-Ganiny et al., 2008). Cytoplasmic UDP-Galp is needed to produce UDP-Galf for the *A. nidulans* hyphal wall. In solution, Galp and Galf are naturally in equilibrium (95:5) so growing the *ugmAΔ* strain on galactose would have provided low-abundance Galf, which could account for the partial remediation.

The organelle appearance and distribution in TEM cross-sections of *ugeAΔ* and *ugmAΔ* hyphae resembled wildtype strains. The *ugeAΔ* hyphal walls were more than twice as thick as wildtype strains, consistent with hyphal morphogenesis and wall defects in the *A. nidulans* *hypA1^{TS}* mutant (Kaminskyj and Boire, 2004). Compared to the *ugeAΔ* strain, the *ugmAΔ* hyphae had a relatively poorly consolidated and more electron dense inner wall layer. Differences in the appearance of *ugeAΔ* and *ugmAΔ* strain walls, despite related functions of UgeA and UgmA, and similar colony phenotypes for the *ugeAΔ* and *ugmAΔ* strains, is consistent with the complexity of fungal cell wall formation. Lamarre et al. (2009) showed that some of the Galf in the outer layer of the *A. fumigatus* wall is in the galactomannan, and that *AfUGM1Δ* strains have exposed mannose on their cell wall surface that contributes to a morphologically altered and more adhesive wall surface. Consistent with this, *A. nidulans* *ugmAΔ* and *ugeAΔ* hyphae adhered more firmly to glass coverslips than do wildtype strains.

The electron dense material on the surface of *ugeAΔ* walls seen with TEM may be material that adhered during liquid shake culture. Taken together, Galf residues appear to have roles in multiple aspects of *A. nidulans* hyphal wall structure and function, consistent with a large body of work on *A. fumigatus* (reviewed in Latgé, 2009).

4.4. The Galf synthesis pathway is expected to be therapeutically useful target

We have shown that galactose metabolism mediated by UgeA and UgmA, which is required for deposition of Galf residues in the wall, is not essential in *A. nidulans*. However, deleting *ugmA* or *ugeA* seriously perturbs cell growth and sporulation, reducing fitness of these strains. With the exception of polyenes that effectively target ergosterol in fungal cell membranes but have substantial human toxicity, antifungal drugs that target single aspects of fungal metabolism have proven repeatedly to lack durability (Cowen, 2008). Inhibitors that target Galf synthesis are expected to be therapeutically useful in combination with established antifungals.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2010.03.002.

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