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Aspergillus nidulans galactofuranose biosynthesis affects antifungal drug sensitivity

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ABSTRACT

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 (Gal*f*) 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* Gal*f* 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. Gal*f* immunolocalization using the L10 monoclonal antibody showed that *ugmA* deletion and repression phenotypes correlated with loss of hyphal wall Gal*f*. 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. Gal*f* appears to play an important role in *A. nidulans* growth and vigor.

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 anti-fungal drug targets.

Abbreviations: Af, *Aspergillus fumigatus*; *alcA*(p), alcohol dehydrogenase I promoter; CM, complete medium; CM3G, complete medium with 3% glucose; CMT, complete medium with 100 mM threonine; Gal*f*, galactofuranose; gDNA, genomic DNA; mAb, monoclonal antibody; *niiA*(p), nitrite utilization promoter; MM, minimal medium; MMA, minimal medium ammonium; MMN, minimal medium nitrate; qPCR, quantitative real time PCR; UDP, uridine diphosphate; UGE, UDP-glucose/galactose-4-epimerase; UGM, UDP-galactopyranose mutase; UGT, UDP-galactofuranose transporter.

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We are exploring an aspect of wall biosynthesis in *Aspergillus nidulans*, the galactofuranose (Gal*f*) biosynthesis pathway. Gal*f* 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, Gal*f* residues form the side chains of glycoconjugates including galactomannan. Gal*f* biosynthesis genes encode UDP-glucose/galactose-4-epimerase (UgeA) (El-Ganiny et al., 2010), UDP-galactopyranose mutase (UgmA) (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.

Gal*f* 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). Gal*f* has been shown to be essential for the virulence of the pathogenic protozoan, *Leishmania* (Kleckza et al., 2007). Gal*f* could be a virulence determinant in *Aspergillus fumigatus* based on studies with a UgmA (also known as GlfA) 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 Gal*f* residues had similar virulence to untreated hyphae. Nevertheless, Gal*f* 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 (Af*pmt2*) 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 (*pkcA*) reduced germination rate, hyphal growth and conidiation, as well as increasing hyphal wall thickness and sensitivity to wall-selective agents: Caspofungin, Calcofluor 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 amphotericin B and voriconazole (Ronen et al., 2007). Binder et al. (2010) showed that an *alcA*(p)–*pkaA* strain grown on repression medium was hypersensitive to PAF, an antifungal protein. Taken together, expression-regulated strains have been used successfully to study cell wall formation in *Aspergillus* species.

Expression-regulated strains have also been used to identify new cell wall destabilizing compounds (Mircus et al., 2009) and to study cell wall integrity in the presence of anti-fungal agents. Fortwendel et al. (2009) used *alcA*(p)-regulated *A. fumigatus* strains defective in cell wall integrity pathway genes (*rasA*, *cnaA* and *crzA* strains) to show that low cell wall integrity correlated with echinocandin sensitivity. Jiang et al. (2008) created *A. fumigatus* strains conditionally expressing *alcA*(p)-regulated GDP-mannose pyrophosphorylase. These strains had defective cell walls, impaired polarity maintenance, reduced conidiation, and increased sensitivity to wall targeting chemicals. Regulating gene expression is a powerful adjunct to deletion analysis for exploring gene function.

Here, we describe studies on the function of Gal*f* biosynthesis genes using strains whose endogenous promoter was replaced by the alcohol dehydrogenase promoter *alcA*(p) (Ichinomiya et al., 2002; Waring et al., 1989) which is highly induced by alcohols, especially threonine, and is repressed by glucose (Romero et al., 2003; Tribus et al., 2010). As a precaution against potential complications from using a sugar-repressed promoter to study the carbohydrate-rich wall, we repeated key experiments with these genes regulated by *niiA*(p), which is regulated by nitrogen source (Monteiro and deLucas, 2010). Under gene repression conditions with both promoters, the regulated strain colonies phenocopied their respective deletion strains. Notably, all the deletion strains were significantly more sensitive to the wall-targeting drug Caspofungin, as were the repressed strains. Our results suggest that the role of Gal*f* biosynthesis plays multiple roles in fungal growth, wall architecture, and drug sensitivity, and as such may be a useful drug target.

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 Kaminskyj (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 $NaNO_3$ (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 Af*pyrG* and *alcA*(p) was amplified from the plasmid, p*alcA*(p) (a gift of Loretta Jackson-Hayes, Rhodes College). This module was inserted immediately upstream of each Gal*f* biosynthesis gene to create *alcA*(p)-regulated strains. A 1.2-kb DNA fragment containing Af*niiA*(p) was amplified by PCR from genomic DNA of wild type *A. fumigatus*. This was used to create a *niiA*(p)-regulatable promoter module, also with Af*pyrG* as the selectable marker. This construct was inserted immediately upstream of the coding sequence for each Gal*f* 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,