Contribution of Galactofuranose to the Virulence of the Opportunistic Pathogen *Aspergillus fumigatus* \V^{

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The filamentous fungus *Aspergillus fumigatus* is responsible for a lethal disease called invasive aspergillosis that affects immunocompromised patients. This disease, like other human fungal diseases, is generally treated by compounds targeting the primary fungal cell membrane sterol. Recently, glucan synthesis inhibitors were added to the limited antifungal arsenal and encouraged the search for novel targets in cell wall biosynthesis. Although galactomannan is a major component of the *A. fumigatus* cell wall and extracellular matrix, the biosynthesis and role of galactomannan are currently unknown. By a targeted gene deletion approach, we demonstrate that UDP-galactopyranose mutase, a key enzyme of galactofuranose metabolism, controls the biosynthesis of galactomannan and galactofuranose containing glycoconjugates. The *gflA* deletion mutant generated in this study is devoid of galactofuranose and displays attenuated virulence in a low-dose mouse model of invasive aspergillosis that likely reflects the impaired growth of the mutant at mammalian body temperature. Furthermore, the absence of galactofuranose results in a thinner cell wall that correlates with an increased susceptibility to several antifungal agents. The UDP-galactopyranose mutase thus appears to be an appealing adjunct therapeutic target in combination with other drugs against *A. fumigatus*. Its absence from mammalian cells indeed offers a considerable advantage to achieve therapeutic selectivity.

The filamentous fungus *Aspergillus fumigatus* is the primary cause of invasive aspergillosis, an often fatal condition affecting people with a weakened immune system. Along with the immunocompromised population, the incidence of invasive aspergillosis is constantly growing, but therapy remains problematic. The sterol binding polycy anthericin B and the ergosterol biosynthesis inhibitor itraconazole have long been the drugs of choice for treatment of this infection, but because of their higher efficacy and lower toxicity, new triazoles, such as voriconazole or posaconazole, are supplanting these drugs (28, 33). Additionally, a novel class of antifungal agents called the echinocandins provides further options for treatment. These compounds inhibit the synthesis of \( \beta_1,3 \)-glucan, a major cell wall component, with resultant osmotic instability and lysis (12). Their minimal toxicity and synergistic activity with voriconazole and amphotericin B make them particularly attractive for combination therapy, although clinical validation is still awaited (33, 35). Despite these advances in therapy, invasive aspergillosis is often associated with significant morbidity and mortality, emphasizing the need for novel therapeutic strategies based on the fundamental knowledge of *A. fumigatus* pathogenesis.

The development of echinocandins illustrates the viability of targeting enzymes involved in cell wall biosynthesis and encourages the development of chitin synthesis inhibitors. Like glucan and chitin, galactomannan is an abundant component of the *A. fumigatus* cell wall (4). This polysaccharide, composed of a linear mannan core branched with short \( \beta_1,5 \)-linked galactofuranose (Galf) chains (22), is bound covalently to the cell wall \( \beta_1,3 \)-glucan, anchored to the lipid membrane by a glycosylphosphatidylinositol, or released in the environment during tissue invasion or growth in culture (3, 9, 14). Besides being an abundant component of the extracellular matrix, secreted galactomannans are used for serological diagnostic of invasive aspergillosis (1). The monosaccharide Galf has also been found in the N- and O-glycans of some glycoproteins as well as the glycosphingolipids of *A. fumigatus* (23, 29, 41, 47) and thus represents an important constituent of the cell wall of this fungus. Galf is otherwise infrequent in natural compounds but prevalent in pathogens. Moreover, since Galf is absent from higher eukaryotes and involved in the survival or virulence of various bacteria, the enzymes involved in the biosynthesis of Galf are considered attractive drug targets (32, 34).

Our understanding of Galf metabolism in eukaryotes is limited. Galf is most likely incorporated into cell surface components by specific galactofuranosyltransferases that use UDP-Galf as a donor. The work of Trejo and colleagues in the early 1970s already suggested the existence of an enzyme converting UDP-galactopyranose into UDP-galactofuranose involved in
the biosynthesis of the fungal cell wall (48). This enzyme, named UDP-galactopyranose mutase (UGM) and encoded by the glf gene, was described first for bacteria (17, 30, 50) and lately for several eukaryotic pathogens, including A. fumigatus (2, 5). UGM is to date the only characterized enzyme involved in the biosynthesis of galactofuranose-containing molecules in eukaryotes, whereas several galactofuranosyltransferases have been described for bacteria (15, 19, 27, 51). The identification of this enzyme, highly conserved among lower eukaryotes and present in many fungi, enables studies of the biological role of galactofuranose in these organisms. The present report highlights the role of galactofuranose in A. fumigatus growth and virulence.

MATERIALS AND METHODS

Strains, media, and growth conditions. A. fumigatus clinical isolate D141 (38) was used as the wild-type (wt) strain in this study. All strains were grown at 37°C on Aspergillus minimal medium (AMM) containing 1% glucose as the carbon source and 70 mM NaNO₃ as the nitrogen source (36), unless otherwise stated. Phloeo/tk blaster of pglfA was first replaced with the original A. fumigatus mycelium by incubation in 1 ml sample buffer (15% glycerol, 100 mM Tris-HCl, pH 6.8, 1.5% sodium dodecyl sulfate, 0.25% β-mercaptoethanol, 0.025% bromophenol blue) for at least 15 min on a rotating shaker. MeOH (3 ml) was then added to lower the density, and the mixture was centrifuged for 10 min at 2,000 g to remove insoluble material. Chloroform and H₂O were then added to the supernatant to obtain a biphasic system with an 8:4:3 ratio of CHCl₃/MeOH/H₂O. After centrifugation for 10 min, the glycolipids were eluted with 5 ml MeOH and dried under a stream of nitrogen. N-glycan analysis. N-glycans of secreted glycoproteins in the supernatant of an A. fumigatus liquid culture were analyzed after peptide N-glycosidase F (PNGase F)-mediated release and 8-amin-1,3,6-pyrenetrisulfonic acid (APTS) labeling by capillary electrophoresis, as recently described (20). Separation was carried out on a four-capillary DNA size-exclusion column (310 genetic analyzer; Applied Biosystems, Foster City, CA). Oligomaltose and bovine RNase B N-glycans (Prozyme, San Leandro, CA) served as references.

TABLE 1. DNA oligonucleotides used in this study

| Oligonucleotide | Sequence ('5'→'3') | Description (restriction site) |
|-----------------|--------------------|-------------------------------|
| PS1             | ATAGGGGCGCGCCAACTGGGAGGCATTCA | 5' Flanking region pΔglfA reverse (NotI) |
| PS12            | TATACGGCGGCGCTCAAGGCATTCAGTTCC | 5' Flanking region pΔglfA sense (SacII) |
| PS3             | ATCCGTGCTAGATCGTCTGCA | 3' Flanking region pΔglfA sense (EcoRV) |
| PS4             | ATCCATGCATTATCTCTGAGGTCTCAG | 3' Flanking region pΔglfA reverse (ClaI) |
| PS66A           | TTACGGATCCATCCGACGTTT | Southern blot probe 1 sense |
| PS67A           | TGGGCTTGTGATGAAATGTTG | Southern blot probe 1 reverse |
| PS68A           | TCACGAGCTTGATCTGTAAA | Southern blot probe 2 sense |
| PS69A           | GTATGAACCTTCTCCCAATG | Southern blot probe 2 reverse |
| PS20            | AAGTTGCTGGTCTGATCCTCA | Southern blot probe 3 sense |
| PS21            | TCATGATGTTGTCGTCTCC | Southern blot probe 3 reverse |
| PS23A           | ATGCGGCTTGAGGCTGT | Site-directed mutagenesis glfA* sense (Xhol) |
| PS23r           | CACGGAACCTGAGGCGGCA | Site-directed mutagenesis glfA* reverse (XhoI) |
| PS28            | ATATGCGGGCGCAAAACAGGCGGAGT | 3' Flanking region pglfA reverse (NotI) |
| PS31            | ATATCCCAGGGATTGTTGTTGGTTGTTGATTG | 3' Flanking region pglfA* reverse (XmaI) |
| PS78            | CGTCTGATATCTGACCTTGTTGCTT | 18S rRNA gene fragment sense |
| PS79            | AACCTAGACTGATACATTITTTGACAG | 18S rRNA gene fragment reverse |
| Probe           | FAM-CCCGGCGAAGCCCCAACATCT-TAMRA | qPCR hybridization probe |

Restriction sites are underlined. TAMRA, 6-carboxytetramethylrhodamine.
pH 7.0 and supplemented with 2% glucose) to obtain the highest concentration to be tested. Nine serial 1:2 dilutions in double-strength RPMI 2% G were made, and to each dilution a volume of 100 μl of an A. fumigatus spore solution (2.5 × 10^7/ml in water) was added. Microtiter plates were incubated at 35°C, and fungal growth in each well was read out visually after 3 days and compared to growth in control wells that contained no antifungal.

Field emission scanning electron microscopy. For morphological studies and measurements of the cell wall thickness, A. fumigatus wt and ΔgflA mutant mycelia were fixed in 5% formaldehyde and 2% glutaraldehyde in cacodylate buffer (0.1 M cacodylate, 0.01 M CaCl₂, 0.01 M MgCl₂, 0.09 M sucrose, pH 6.9) for 1 h on ice. Samples were washed several times with cacodylate buffer and subsequently with TE buffer (20 mM Tris-HCl, 1 mM EDTA, pH 6.9) before dehydration in a graded series of acetone (10, 30, 50, 70, 90, and 100%) on ice for 15 min per step. Samples at the 100% acetone step were allowed to reach room temperature before another change in 100% acetone. Samples were then subjected to critical-point drying with liquid CO₂ (CPD 30; Balzers Union, Liechtenstein). Dried samples were then mounted onto conductive carbon adhesive tabs on an aluminum stub and sputter coated with a thin gold film (SCD 40; Balzers Union, Liechtenstein). For cell wall thickness measurements, mycelium was fractured by pressing another conductive carbon adhesive tab-covered stub onto the sample and separating both stubs immediately thereafter. Fractured hyphae were also made conductive by sputter coating with a gold film before examination with a field emission scanning electron microscope (Zeiss DSM 982 Gemini) using an Everhart Thornley SE detector and an in-lens SE detector at a 50:50 ratio at an acceleration voltage of 5 kV and at calibrated magnifications.

Mouse infection model. A low-dose mouse infection model of invasive aspergillosis for BALB/c mice which had been established previously (25) was essentially used. The immunosuppressive state was established by intraperitoneal injections of 100 mg cyclophosphamide (Endoxan; Baxter Chemicals)/kg of body weight on days −4, −1, 0, 2, 5, 8, and 11 and a single subcutaneous dose (200 mg/kg) of a cortisone acetate suspension (Sigma) on day −1. Groups of 20 mice were infected intranasally with 20,000 conidia of the wt strain, the ΔgflA strain, or the reconstituted gflA* strain on day 0. The control group received phosphate-buffered saline (PBS) only. Survival was monitored for 13 days after infection, and moribund animals were sacrificed. Incidence of severely reduced mobility, low body temperature, and breathing problems was defined as the moribundity criterion. Statistical analysis of survival data was carried out using a log rank test implemented in Prism 4 (GraphPad Software, San Diego, CA).

Preparation of genomic DNA from mouse lungs. Tissue homogenization was essentially performed using a tissue homogenizer. DNA was finally recovered in 200 μl of an A. fumigatus spore solution (2.5 × 10^7/ml in water) was added. Microtiter plates were incubated at 35°C, and fungal growth in each well was read out visually after 3 days and compared to growth in control wells that contained no antifungal.

Quantitative RT-PCR. A 18S rRNA primer pair (5′-ACACATTGGACATGATTGGAAA-3′ and 5′-TCTTTCGACCTGTGAGAAG-3′) and TaqMan Fast universal PCR master mix (Applied Biosystems) were used to obtain an 18S rRNA gene (GenBank accession number AF272879) fragment specific for A. fumigatus and a hybridization probe labeled with 6-carboxyfluorescein (FAM) (5′ end) and 6-carboxytetramethylrhodamine (3′ end) were designed using Primer Express software, version 3.0 (Applied Biosystems) (Table 1). qPCR reactions were performed with a 7500 Fast real-time PCR system instrument (Applied Biosystems) loaded with Microamp optical 96-well plates sealed with an optical adhesive cover (Applied Biosystems). Each qPCR reaction mixture (20 μl) contained 5 μl sample DNA, 250 nM dual-labeled hybridization probe, 500 nM primers, 250 μg/ml bovine serum albumin, and TaqMan Fast universal PCR master mix (Applied Biosystems). The latter contains hot-start DNA polymerase, deoxyribonucleoside triphosphates, and the fluorescent dye carboxyfluorescein (ROX) as a passive reference. Real-time PCR data were acquired using Sequence Detection software, v1.3.1. The FAM/ROX fluorescence ratio was recorded at every cycle, and a threshold cycle (Ct) value was assigned to each reaction product, defining the cycle number at which the FAM/ROX signal surpassed an automatically defined threshold. Ct values were corrected for differences in yield of genomic DNA by normalization to the DNA concentration of a control sample by calculating Ct/ln[DNAcontrol/DNAsample] or in RNA gene copy numbers was done as follows. Ct values of serial 1:10 dilutions containing 300 to 300,000 molecules (n) (calculated from Mn and DNA concentration determined by measurement of optical density at 260 nm) of a plasmid bearing the cloned A. fumigatus 18S rRNA gene were plotted against n to generate a calibration curve which was then used to assign an rRNA gene copy number to a given sample. Conidial equivalents were calculated from gene copy numbers by means of uninfected tissue samples that were spiked with defined numbers of conidia before tissue homogenization (7). Samples, controls, and standards were analyzed in triplicate.

RESULTS

Deletion and reconstitution of the gflA gene in A. fumigatus. To begin investigating the role of Galf in A. fumigatus biology, we deleted the gene encoding UGM (GenBank accession no. AJ871145) and named it gflA, following the recommendations for gene naming for Aspergillus. To do this, we generated a deletion plasmid containing the regions flanking the gflA coding sequence separated by the bifunctional selection cassette phleo/tk, which confers both resistance to phleomycin and sensitivity to FUDR (18). This construct was used to transform protoplasts of A. fumigatus clinical strain D141, which served as the wt, and phleomycin-resistant transformants were analyzed by Southern blotting using several digoxigenin-labeled probes (Fig. 1). One of the clones that had undergone the desired gene replacement (Fig. 1) was selected for further analysis and named ΔgflA.

The selected disruptant was further subjected to protoplast transformation with a large DNA fragment encompassing the gflA coding sequence which contained a single translationally silent nucleotide exchange that generated an XhoI restriction site. Gene replacement in the transformants resulted in the reconstitution of the gflA locus (Fig. 1) as detected by FUDR resistance and proven by Southern blot analysis for a selected clone named gflA* (Fig. 1B). The silent mutation introduced in the reconstituted strain allowed differentiation between the wt and the gflA* mutant, as demonstrated in Fig. 1B (top), and thus enabled us to rule out contamination by the wt strain. The reconstitution of the gflA locus ensures that any phenotype observed for the ΔgflA strain can be reverted and hence be securely attributed to the loss of the gflA gene.

Galf is absent from the A. fumigatus ΔgflA mutant. To confirm that deletion of gflA indeed altered the expression of Galf-containing glycoconjugates, aqueous mycelial extracts were tested for reactivity to the Galf-specific MAb EB-A2. This antibody recognizes preferably β1,5-linked Galf residues that are present in all forms of galactomannan (cell wall bound, membrane bound, and secreted) (42) as well as in some O-glycans (23). Moreover, a second binding epitope, Galf(β1,2)Man, which is part of galactofuranosylated N-glycans, has been postulated (29). Thus, EB-A2 can be used to detect galactomannan and galactofuranosylated glycoproteins simultaneously. Western blot analysis of wt and gflA* total mycelial extracts labeled with HRP-conjugated EB-A2 revealed a smear migrating around 40 to 80 kDa, in accordance with previous
findings (42). In contrast, the ΔglfA mycelial extract was not stained at all, indicating the absence of Galf in the galactomannan and glycoproteins of this mutant (Fig. 2A, left). ConA used as the loading control bound slightly better to the ΔglfA extract than to those of the wt and the glfA* mutant (Fig. 2A, right). The lack of Galf in the ΔglfA mutant might increase the accessibility of the mannan for ConA and thus could explain this finding.

Similarly, the absence of Galf in ΔglfA glycolipids was shown by the absence of reactivity to the MAb MEST-1. This antibody, which recognizes β1,3- and β1,6-linked Galf residues (43), labeled several A. fumigatus GIPCs after separation by high-performance thin-layer chromatography, as previously shown (47), but did not label glycosphingolipids extracted from the ΔglfA mutant (Fig. 2B, left). The upper bands observed in Fig. 2B (left panel) might be attributed to GIPCs containing one or two Galf and two or three mannose residues, as recently described (41, 47). In addition, Simenel et al. (41) reported an unusual GIPC containing a Galf residue substituted at position 6 by a choline phosphate. The lower band present in the wt chromatogram could correspond to a similar GIPC. Staining of glycolipids by orcinol was used as the loading control (Fig. 2B, right). The simpler ΔglfA chromatogram is compatible with the absence of Galf-containing GIPCs. The uppermost band observed in the chromatogram most probably corresponds to Man(α1,3)Man(α1,2)Ins-P-Cer, while the band just beneath it could be attributed to Man(α1,2)Man(α1,3)Man(α1,2)Ins-P-Cer (47). The chromatograms obtained from the reconstituted glfA* mutant and the wt were indistinguishable (data not shown).

Additionally, N-glycans enzymatically released from A. fu-
migatus secreted proteins were analyzed by capillary electrophoresis after fluorescent labeling (8, 20). The profiles obtained are presented in Fig. 3A (panels 1 and 2). The peaks labeled 1, 2, 3, 4, and 5 present in both electropherograms comigrated with reference oligosaccharides M5 to M9 (Fig. 3A, panel 9, and 3B). Moreover, digestion of these N-glycans by Trichoderma reesei α1,2-mannosidase indicates that peaks 2, 3, 4, and 5 arise from substitution of oligosaccharide 1 with one to four mannose residues linked in α1,2 (Fig. 3A, panels 3 and 4). The profile obtained with wt N-glycans (Fig. 3A, panel 1) presents four additional peaks (labeled 1a, 2a, 3a, and 4a) that were absent from glfA N-glycans. The retention times of these peaks suggest that they arise from substitution of oligosaccharides 1 to 4 with a single Gal⁄ residue. The presence of a terminal nonreducing Gal⁄ residue in A. fumigatus N-glycans has been reported previously (9) and was demonstrated by hydrofluoric acid treatment of the N-glycans after T. reesei α1,2-mannosidase digestion (Fig. 3A, panels 5 and 6). This mild acid treatment, known to release Gal⁄, entirely converted oligosaccharide 1a into oligosaccharide 1 (Fig. 3A, panels 3 and 5). In contrast, hydrofluoric acid treatment did not change the profile of ΔglfA N-glycans digested with α1,2-mannosidase (Fig. 3A, panels 4 and 6).

Interestingly, the comparison of wt and ΔglfA N-glycans digested with T. reesei α1,2-mannosidase or jack bean mannosidase helps with positioning of the Gal⁄ residue. α1,2-Mannosidase treatment converted the oligosaccharides 2a, 3a, and 4a into 1a while the oligosaccharides 2, 3, and 4 generated 1 (Fig. 3A, compare panels 1 and 2 with panels 3 and 4). This indicates that the Gal⁄ residue does not protect any mannose residues from the exomannosidase digestion and thus does not substitute an α1,2-linked mannose (Fig. 3A, panels 3 and 4). Moreover, jack bean mannosidase digestion of wt N-glycans resulted in a major peak (peak 7), attributed to Gal⁄Man3GlcNAc2 from its retention time, in addition to Man3GlcNAc2 (peak 6), expected from digestion of high-mannose type N-glycans (Fig. 3A, panels 7 and 8). These experiments do not allow for the determination of the detailed N-glycan structures but suggest that they resemble the N-glycans of A. niger α-glucosidase and α-galactosidase (44, 45). More importantly, these experiments demonstrate the absence of Gal⁄ in the ΔglfA N-glycans.

**Loss of Gal⁄ alters morphology and growth of A. fumigatus.** The ΔglfA strain exhibited a marked growth defect on solid minimal media or complete media compared to the wt. This effect could be observed for a wide range of temperatures (Fig. 4) and was statistically different in all cases (P < 0.001; t test, n = 3). The most severe effect was found at 42°C, with a 75% reduction in radial growth (Fig. 4B). In parallel, ΔglfA conidiation was diminished by 90% at 37°C and was almost absent at 42°C. In contrast, the onsets and rates of germination of wt, ΔglfA, and glfA* conidia were similar. In minimal media at 37°C, the conidia of all strains started forming germ tubes at 3.2 h and reached 100% germination within 8 to 9 h (data not shown).

Scanning electron micrographs of intact mycelium, conidia, and conidia of Δglf/A did not reveal any obvious morphological differences when compared to wt. However, the observation of fractured mycelium revealed a marked reduction of the Δglf/A cell wall thickness (Fig. 5). Measurements indicated that the cell wall thickness of wt A. fumigatus varies from 85 to 315 nm, which is in good agreement with earlier findings (39). In contrast, Δglf/A cell wall thickness ranged from...
85 to 150 nm. The mean values (± standard deviations) of cell wall thickness obtained from 25 measurements were 227.5 nm (± 15.98 nm) and 109.7 nm (± 11.3 nm) for wt and ΔglfA hyphae, respectively. The cell wall of ΔglfA was thus approximately half the thickness of the wt cell wall.

**ΔglfA is more susceptible to drugs.** The structural cell wall defect caused by the Galf deficiency was accompanied by an increased susceptibility to several antifungal agents (Table 2). MICs determined by a broth microdilution test were slightly reduced for amphotericin B and caspofungin in the ΔglfA mutant. A more pronounced increase in susceptibility was seen for voriconazole (0.04 mg/liter for ΔglfA, compared to 0.3 mg/liter for the wt) and nikkomycin Z (63 to 125 mg/liter for ΔglfA and 500 mg/ml for the wt), suggesting an increased permeability of the cell wall caused by the loss of Galf. In contrast, the sensitivity to oxidative stress remained unchanged, as indicated by equal MICs for H2O2 in both wt and ΔglfA strains.

**ΔglfA displays attenuated virulence in a murine model of invasive aspergillosis.** The influence of the glfA deletion on the pathogenicity of *A. fumigatus* was assessed in a low-dose mouse infection model of invasive aspergillosis (25). Cyclophosphamide was used to induce neutropenia in female BALB/c mice, and a single dose of cortisone acetate was administered before intranasal infection with 20,000 *A. fumigatus* conidia. Neutropenia was maintained throughout the observation period of 13 days, and survival was recorded daily (Fig. 6A). Ninety percent of the animals infected with the wt did not survive beyond day 7 after infection, whereas half of the animals infected with ΔglfA were still alive on day 13. A log rank test of wt and ΔglfA survival data confirmed that the observed difference was statistically significant (*P* = 0.0004). The attenuation in virulence could clearly be attributed to the absence of glfA, since animals infected with the reconstituted glfA* strain showed a survival pattern nearly identical to that of the wt (no significant difference by the log rank test, *P* = 0.559). A histological examination of lung tissue from mice infected with the wt, ΔglfA, and glfA* strains 5 days after inoculation showed evident fungal growth surrounding bronchioles and tissue penetration (Fig. 7). For each strain, inflammatory cells were rarely observed at the sites of infection.

To correlate the delay in the onset and progression of mortality with a growth defect, the fungal burden in lungs of infected mice was determined by qPCR (Fig. 6B). Mice were treated and infected as described above. After 2, 4, and 6 days, animals were sacrificed and their lungs taken. DNA was isolated from homogenized lung tissue and fungal content determined by amplification of a part of *A. fumigatus* ribosomal DNA. As shown in Fig. 6B, growth of ΔglfA was restricted in vivo compared to that of the wt, which was in agreement with the slower growth observed in vitro.

**DISCUSSION**

The essential role of the β1,3-glucan in cell wall organization and growth of several pathogenic fungi has been the basis for the development of the echinocandins (11). Likewise, inhibitors of chitin biosynthesis are currently being explored as new antifungal drugs since chitin is an important structural element of the fungal cell wall (6). In contrast, although galactomannan is a major component of the cell wall and the extracellular matrix, the role of galactomannan had not yet been investigated, since the enzymes involved in its biosynthesis are unknown. Recently, we and others characterized the UGM of various pathogenic eukaryotes, including *A. fumigatus* (2, 5). In prokaryotes, like in the protozoan *Leishmania*, this enzyme is the only route to the formation of UDP-Galf, the donor substrate of galactofuranosyltransferases, and thus controls the biosynthesis of all Galf-containing molecules. Likewise, *A. fumigatus* UGM was found to be essential for the biosynthesis of galactomannan as well as some glycosphingolipids and glycoproteins. Like in other organisms (16, 32), deletion of the glfA gene resulted in the complete absence of Galf, as shown for instance by the absence of reactivity to the antibody EB-A2.

Besides demonstrating the lack of Galf in the ΔglfA mutant, our analyses provide useful structural information for *A. fumigatus* N-glycans. Treatment of wt secreted proteins with PNGase F released galactofuranosylated high-mannose type N-glycans. The size of the oligosaccharides and the presence of a single Galf residue are in agreement with previous studies of filamentous fungi (26, 29). Moreover, analysis of these oligo-

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**TABLE 2. MICs of various antifungal agents against *A. fumigatus* mutants, obtained from a broth microdilution assay**

| Genotype | AmB | Vor | Cas | NiZ | H2O2 |
|----------|-----|-----|-----|-----|------|
| wt       | 3.9 | 0.3 | 62.5| 218 | 500  |
| ΔglfA    | 2.0 | 0.04| 31.3| 218 | 218  |
| glfA*    | 2.0 | 0.3 | 62.5| 218 | 500  |

* Values for amphotericin B (AmB) and caspofungin (Cas) are MIC90 values, values for voriconazole (Vor) and nikkomycin Z (NiZ) are MIC50 values, and values for H2O2 are MIC100 values.
saccharides after digestion by jack bean or T. reesei α1,2-mannosidase helps with positioning of the Galf residue. These data and the comparison with high-mannose standards suggest that the N-glycans from A. fumigatus secreted proteins resemble those of A. niger α-d-galactosidase and α-d-glucosidase (44, 45, 49). These N-glycans might have arisen simply from trimming of the Glc3Man9GlcNAc2 precursor and substitution by a Galf residue. Aspergillus spp. indeed contain several α1,2-mannosidase genes, and trimming of high-mannose glycans has been shown previously (13, 52). Interestingly, Galf addition has been suggested to act as a stop signal for mannose addition, in analogy to the role proposed for the α1,3-linked terminal mannose in Saccharomyces cerevisiae (29, 49). However, preventing the addition of galactofuranose does not result in an increased size of the oligosaccharides. On the contrary, Man5GlcNAc2 is the main oligosaccharide found in the ∆glfA mutant, while GalfMan5GlcNAc2 is predominant in the wt.

Although glfA deletion has been shown to be lethal in Mycobacterium smegmatis (32), the in vitro viability of the A. fumigatus ∆glfA mutant is unsurprising, since Galf occupies a nonreducing terminal position in the molecules of this fungus. Hence, the absence of Galf does not perturb the basic organization of the cell wall, as would the absence of the underlying structures. Nevertheless, it resulted in marked alterations of the cell surface and a notably thinner cell wall, as revealed by electron microscopy. The basis of this drastic change is unclear and difficult to attribute to a particular cell wall component, since glycosylphosphatidylinositol/cell wall bound galactoman-

FIG. 6. (A) Survival of immunosuppressed mice infected intranasally with A. fumigatus wt (solid line), ∆glfA (dotted line), or glfA* (dashed line) strains and of uninfected mice (dotted and dashed line). Each group consisted of 20 animals. (B) qPCR determination of A. fumigatus burden (measured as conidial equivalents [eq.] [see Materials and Methods]) in lung tissue from immunosuppressed mice infected with wt (solid line) or ∆glfA (dotted line) strains. Each data point represents the mean value obtained from three to five animals. Error bars indicate standard errors of the means.

FIG. 7. Periodic acid-Schiff-stained lung sections of mice infected with wt, ∆glfA, or glfA* A. fumigatus strains. Fungal colonies appear purple/red. Infected sites are typically surrounded by areas of necrotic tissue but show no or hardly any infiltrating leukocytes. Bar, 100 μm.
nan, N-glycans, O-glycans, and GIPCs are affected by UDP-Galf deficiency. In other fungi, the loss of terminal sugar residues has sometimes been associated with reduced cell wall strength. For instance, a Schizosaccharomyces pombe mutant deficient in cell wall galactosylation displays morphological changes, attenuated growth, and a 25 to 35% reduction in cell wall thickness (46).

The structural changes originating from the glfA deletion are associated with slower growth, indicating that Galf plays an important role in Aspergillus fumigatus morphogenesis. The temperature-sensitive growth defect at a higher temperature displayed by the ΔglfA mutant is reminiscent of that observed for the ΔAIPmt1 mutant, a mutant characterized by reduced O glycosylation (53). Interestingly, an influence of GlfA deficiency on the growth rate was also observed for ΔglfA mutants of Aspergillus nidulans (F. H. Routier, unpublished data) and Aspergillus niger (10). Conversely, glfA deletion had no effect on the in vitro growth of Leishmania parasites (16), highlighting that the role of Galf cannot be translated to every Galf-containing organism.

The ability to thrive at 37°C is a characteristic of human pathogens that has been shown to correlate with virulence potential in the case of A. fumigatus (31). Consequently, mutations that affect the growth of fungi at mammalian body temperature are commonly associated with attenuated virulence (40). In this study, we observed slower growth of the A. fumigatus ΔglfA mutant in vitro but also in vivo by using qPCR. In agreement with this observation, the mutant was clearly attenuated in virulence, showing a delay in both the onset and the progression of mortality when tested in a low-dose mouse infection model of invasive aspergillosis. An altered immune response caused by the different cell wall structure of the ΔglfA mutant may also contribute to the attenuation in virulence. However, there is no difference in adherence and uptake of wt and ΔglfA conidia by murine bone marrow-derived dendritic cells or in production of tumor necrosis factor alpha or interleukin-10 by infected murine bone-marrow derived macrophages were observed (K. Kotz, F. Ebel, and F. H. Routier, unpublished data).

The value of echinocandins in invasive aspergillosis treatment resides in their synergistic effects with azoles and amphotericin B. Similarly, chitin synthesis inhibitors demonstrate synergy with echinocandins and azoles (24). These synergistic effects that offer new options for combination antifungal therapy are most likely due to greater cell wall permeability. We did note an increase in susceptibility of the ΔglfA mutant to several antifungal agents, notably to voriconazole. However, in the liquid culture conditions classically used for antifungal susceptibility testing, the fungus is not surrounded by an extracellular matrix. This extracellular matrix that delays the penetration of drug is rich in galactomannan (3) and is probably altered in the ΔglfA mutant, as suggested by the compact appearance of colonies on agar plates. In vivo, a greater increase in susceptibility of the ΔglfA mutant to drugs would therefore be expected. Besides the attenuated virulence, this suggests that inhibitors of UGM might be useful in antifungal therapy. The absence of Galf biosynthesis in mammals would represent a considerable advantage for the development of antifungal drugs with selective toxicity.

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REFERENCES
1. Aquino, V. R., L. Z. Goldani, and A. C. Pasqualotto. 2007. Update on the contribution of galactomannan for the diagnosis of invasive aspergillosis. Mycopathologia 163:191-202.
2. Bakker, H., B. Kleczka, R. Gerardy-Schahn, and F. H. Routier. 2005. Identification and partial characterization of two eukaryotic UDP-galactopyranose-<i>N</i>-acetate dehydrogenase. Biol. Chem. 386:567-661.
3. Beauvais, A., C. Schmidt, S. Guadagnini, P. Roux, E. Perret, C. Henry, S. Paris, A. Mallet, M. C. Prévost, and J. P. Latgé. 2007. An extracellular matrix glue structures the aerial-grown hyphae of <i>Aspergillus fumigatus</i>. Cell. Microbiol. 9:1147-1154.
4. Bernard, M., and J. P. Latgé. 2001. <i>Aspergillus fumigatus</i> conidial cell wall: composition and biosynthesis. Med. Mycol. 39(Suppl 1):9-17.
5. Beverley, S. M., K. L. Owens, M. Showalter, C. L. Griffith, T. L. Doering, V. E. Jones, and J. R. McNeil. 2005. Eukaryotic UDP-galactopyranose mutase (GLF gene) in microbial and metazoal pathogens. Eukaryot. Cell 4:1147-1154.
6. Borgia, P. T., and C. L. Dodge. 1992. Characterization of <i>Aspergillus nidulans</i> mutants deficient in O-galactosyl or gluco-galactosyltransferase. Mol. Microbiol. 6:125-283.
7. Bowman, J. C., G. K. Abruzzo, J. W. Anderson, A. M. Flattery, C. J. Gill, V. B. Pikounis, D. M. Schmatz, P. A. Liberator, and C. M. Douglas, 2001. Quantitative PCR assay to measure <i>Aspergillus fumigatus</i> burden in a murine model of disseminated aspergillosis: demonstration of efficacy of caspofungin acetate. Antimicrob. Agents Chemother. 45:3474-3481.
8. Callewaert, N., S. Gyseys, F. Molemans, and R. Contreras. 2001. Ultrasensitive profiling and sequencing of N-linked oligosaccharides using standard HPLC electrospraying equipment. Glycoconjugate J. 18:275-281.
9. Costache, C., B. Coddeville, J. P. Latgé, and T. Fontaine. 2005. Glycosphingosyldihydrolinositol-anchored fungal polysaccharide in <i>Aspergillus fumigatus</i>. J. Biol. Chem. 280:39835-39842.
10. Damveld, R. A., A. Franken, M. Arentshorst, P. J. Punt, F. M. Klis, C. A. van den Hondel, and A. F. Ram. 2008. A novel screening method for cell wall mutants in <i>N</i>-acetylglucosamine-2-acetate dehydrogenase. Antimicrob. Agents Chemother. 52:2643-26440.
11. Denning, D. W. 2002. Echinocandin: a new class of antifungal. J. Antimicrob. Chemother. 49:889-916.
12. Denning, D. W. 2003. Echinocandin antifungal drugs. Lancet 362:1142-1151.
13. Eades, C. J., and W. E. Hintz, 2000. Characterization of the class I alpha-mannosidase gene family in the filamentous fungus Aspergillus nidulans. Gene 255:23-34.
14. Fontaine, T., C. Simenel, G. Dubreucq, O. Adam, M. Delepierre, J. Lemoine, C. E. Vorgias, M. Diaquin, and J. P. Latgé. 2000. Molecular organization of the alkaline-insoluble fraction of <i>Aspergillus fumigatus</i> conidia wall. J. Biol. Chem. 275:27594-27607.
15. Guan, S., A. J. Clarke, and C. Whitfield. 2001. Functional analysis of the galactosyltransferases required for biosynthesis of <i>T</i>-galactan I, a component of the lipopolysaccharide O1 antigen of <i>Klebsiella pneumoniae</i>. J. Bacteriol. 183:319-325.
16. Kleczka, B. A. C. Lamerz, G. van Zandbergen, A. Wenzel, R. Gerardy-Schahn, M. Wiese, and F. H. Routier. 2007. Targeted gene deletion of <i>Leishmania major</i> UDP-galactopyranosyl mutase leads to attenuated virulence. J. Biol. Chem. 282:10498-10505.
17. Köpflin, R., J. R. Brisson, and C. Whitfield. 1997. UDP-galactofuranosyl precursor required for formation of the lipopolysaccharide O antigen of <i>Klebsiella pneumoniae</i> serotype O1 is synthesized by the product of the gene. J. Bacteriol. 179:117-133.
18. Kremer, L., L. G. Dover, C. Morehouse, P. Hitchin, M. Everett, H. R. Morris, A. Dell, P. J. Brennan, M. R. McNeil, C. Flaherty, K. Duncan, and G. S. Besra. 2001. Galactan biosynthesis in <i>Mycobacterium tuberculosis</i>. Identification of a bifunctional UDP-galactofuranosyltransferase. J. Biol. Chem. 276:26340-26440.
19. Lara, W., R. Contreras, and N. Callewaert. 2006. Glycome mapping on DNA sequencing equipment. Nat. Protoc. 1:397-405.
20. Lass-Främl, C., M. Cuenca-Estrella, D. W. Denning, and J. L. Rodríguez-Tudela. 2006. Antifungal susceptibility testing in <i>Aspergillus</i> spp. according to EUCAST methodology. Med. Mycol. 44:319-325.
immunological characterization of the extracellular glycoconjugates of Aspergillus fumigatus. Infect. Immun. 62:5424–5433.

23. Leitão, E. A., V. C. Bittencourt, R. M. Haido, A. P. Valente, J. Peter-Katalinic, M. Letzel, L. M. de Souza, and E. Barreto-Bergter. 2003. Beta-galactofurananecontaining O-linked oligosaccharides present in the cell wall and peptidoglycans of Aspergillus fumigatus contain immunodominant epitopes. Glycobiology 13:681–692.

24. Li, R. K., and M. G. Rinaldi. 1999. In vitro antifungal activity of nivolumycin Z in combination with fluconazole or itraconazole. Antimicrob. Agents Chemother. 43:1401–1405.

25. Liebmann, B., T. W. Mühleisen, M. Müller, M. Hecht, G. Weidner, A. Braun, M. Brock, and A. A. Brakhage. 2004. Detection of the Aspergillus fumigatus llsyne biosynthesis gene lls by encoding homoocamtase leads to attenuated virulence in a low-dose mouse infection model of invasive aspergillosis. Arch. Microbiol. 181:378–383.

26. Maras, M., I. van Die, R. Contreras, and C. A. van den Hondel. 1999. Filamentous fungi as production organisms of glycoproteins for bio-medical interest. Glycocon. J. 16:99–107.

27. Mikusová, K., M. Beláňová, J. Kordula´kova´, K. Honda, M. R. McNeil, S. Mahapatra, D. C. Crick, and P. J. Brennan. 2006. Identification of a novel galactosyltransferase involved in biosynthesis of the mycobacterial cell wall. J. Bacteriol. 188:6592–6598.

28. Mohr, J., M. Johnson, T. Cooper, J. S. Lewis, and L. Ostrosky-Zeichner. 2008. Current options in antifungal pharmacotherapy. Pharmacotherapy 28:614–645.

29. Morelle, W., M. Bernard, J. P. Debeauquis, M. Buitrago, J. P. Latgé. 2005. Galactomannanproteins of Aspergillus fumigatus. Eukaryot. Cell 4:1308–1316.

30. Nassau, P. M., S. L. Martin, R. E. Brown, C. Astumian, M. Monney, M. R. McNeil, and K. Duncan. 1996. Galactofuranose biosynthesis in Escherichia coli K-12: identification and cloning of UDP-galactofuranosyltransferase. J. Bacteriol. 178:1047–1052.

31. Paisley, D., G. D. Robson, and D. W. Denning. 2005. Correlation between in vitro growth rate and in vivo virulence in Aspergillus fumigatus. Med. Mycol. 43:397–401.

32. Pan, F., M. Jackson, Y. Ma, and M. McNeil. 2001. Cell wall core galactofuranan synthesis is essential for growth of the mycobacterium. J. Bacteriol. 183:3991–3995.

33. Patterson, T. F. 2006. Treatment of invasive aspergillosis: polyenes, echinocandins, or azoles? Med. Mycol. 44(Suppl 1):357–362.

34. Pedersen, L. L., and S. J. Turco. 2003. Galactomannan metabolism: a potential target for antimicrobial chemotherapy. Cell. Mol. Life Sci. 60:259–266.

35. Perea, S., G. Gonzalez, A. W. Fothergill, W. R. Kirkpatrick, M. G. Rinaldi, and T. F. Patterson. 2002. In vitro interaction of caspofungin acetate with vonocamine against clinical isolates of Aspergillus spp. Antimicrob. Agents Chemother. 46:3039–3041.

36. Pontecorvo, G., J. A. Roper, L. M. Hemmens, K. D. MacDonald, and A. W. Buttin. 1953. The genetics of Aspergillus niger. Adv. Genet. 5:141–238.

37. Punt, P. J., and C. A. van den Hondel. 1992. Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. Methods Enzymol. 216:447–457.

38. Reichard, U., S. Böttner, H. Eiffert, F. Stahl, and R. Rüchel. 1990. Purification and characterisation of an extracellular serine proteinase from Aspergillus fumigatus and its detection in tissue. J. Med. Microbiol. 33:243–251.

39. Reijula, K. E. 1991. Two common fungi associated with farmer’s lung: fine structure of Aspergillus fumigatus and Aspergillus umbrosus. Mycopathologia 113:143–149.

40. Rementeria, A., N. López-Molina, A. Ludwig, A. B. Vivanco, J. Bikandt, J. Ponton, and J. Garalar. 2005. Genes and molecules involved in Aspergillus fumigatus virulence. Rev. Iberoam. Microl. 22:1–23.

41. Simoncel, C., B. Coddeville, M. Delepierre, J. P. Latgé, and T. Fontaine. 2008. Glycosylinositolphosphoceramides in Aspergillus fumigatus. Glycobiology 18:64–96.

42. Stynen, D., J. Sarfati, A. Goris, M. C. Prévost, M. Lesourd, H. Kamphuis, V. Darras, and J. P. Latgé. 1992. Rat monoclonal antibodies against Aspergillus galactomannan. Infect. Immun. 60:2237–2245.

43. Suzuki, E., M. S. Toledo, H. K. Takahashi, and A. H. Straus. 1997. A monoclonal antibody directed to terminal residue of beta-galactofuranose of a glycolipid antigen isolated from Paracoccidioides brasiliensis: cross-reactivity with Leishmania major and Trypanosoma cruzi. Glycobiology 7:463–468.

44. Takayama, T., A. Kimura, S. Chiba, and K. Ajisaka. 1994. Novel structures of N-linked high-mannose type oligosaccharides containing alpha-D-galactofuranosyl linkages in Aspergillus niger alpha-D-glucosidase. Carbohydr. Res. 256:149–158.

45. Takayama, T., K. Kushida, K. Idonuma, and K. Ajisaka. 1992. Novel N-linked oligo-mannose type oligosaccharides containing an alpha-D-galactofuranosyl linkage found in alpha-D-galactosidase from Aspergillus niger. Glycocon. J. 9:229–234.

46. Tanaka, N., M. Konomi, M. Osumi, and K. Takegawa. 2001. Characterization of a Schizosaccharomyces pombe mutant deficient in UDP-galactose transport activity. Yeast 18:903–914.

47. Toledo, M. S., S. B. Levery, B. Bennion, L. G. Guimaraes, S. A. Castle, R. Lindsey, M. Momany, C. Park, A. H. Strain, and H. K. Takahashi. 2007. Analysis of glycosylinositolphosphorylceramides expressed by the opportunistic mycopathogen Aspergillus fumigatus. J. Lipid Res. 48:1801–1824.

48. Trejo, A. G., J. W. Haddock, G. J. Chittenden, and J. Baddiley. 1971. The biosynthesis of galactomannanresidues in galactocaroiose. Biochem. J. 122:49–57.

49. Wallis, G. L., R. L. Easton, K. Jolly, F. W. Hemming, and J. F. Peberdy. 2001. Galactomannan-oligomannose N-linked glycans of alpha-galactosidase A from Aspergillus niger. Eur. J. Biochem. 268:4134–4143.

50. Weston, A., R. J. Stern, R. E. Lee, P. M. Nassau, D. Monsey, S. L. Martin, M. S. Scherman, G. S. Besra, K. Duncan, and M. R. McNeil. 1997. Biosynthetic origin of mycobacterial cell wall galactomannanresidues. Tub. Lung Dis. 78:123–131.

51. Wing, C., J. C. Errey, B. Mukhopadhyay, J. S. Blanchard, and R. A. Field. 2006. Expression and initial characterization of WbbI, a putative D-Galf:alpha-D-Glc beta-1,6-galactofuranosyltransferase from Escherichia coli K-12. Org. Biomol. Chem. 4:3945–3950.

52. Yoshida, T., Y. Kato, Y. Asada, and T. Nakajima. 2000. Filamentous fungus Aspergillus oryzae has two types of alpha-1,2-mannosidases, one of which is a membrane enzyme that remove a single mannose residue from Man9GlcNAc2. Glycoconj. J. 17:745–748.

53. Zhou, H., H. Hu, L. Zhang, R. Li, H. Ouyang, J. Ming, and C. Jin. 2007. O-Mannosyltransferase 1 in Aspergillus fumigatus (AfPmt1p) is crucial for cell wall integrity and conidiom morphology, especially at an elevated temperature. Eukaryot. Cell 6:2260–2268.