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A Single UDP-galactofuranose Transporter Is Required for Galactofuranosylation in *Aspergillus fumigatus*[*][S]

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Galactofuranose (Gal*f*) containing molecules have been described at the cell surface of several eukaryotes and shown to contribute to the virulence of the parasite *Leishmania major* and the fungus *Aspergillus fumigatus*. It is anticipated that a number of the surface glycoconjugates such as *N*-glycans or glycolipids are galactofuranosylated in the Golgi apparatus. This raises the question of how the substrate for galactofuranosylation reactions, UDP-Gal*f*, which is synthesized in the cytosol, translocates into the organelles of the secretory pathway. Here we report the first identification of a Golgi-localized nucleotide sugar transporter, named GlfB, with specificity for a UDP-Gal*f*. *In vitro* transport assays established binding of UDP-Gal*f* to GlfB and excluded transport of several other nucleotide sugars. Furthermore, the implication of *glfB* in the galactofuranosylation of *A. fumigatus* glycoconjugates and galactomannan was demonstrated by a targeted gene deletion approach. Our data reveal a direct connection between galactomannan and the organelles of the secretory pathway that strongly suggests that the cell wall-bound polysaccharide originates from its glycosylphosphatidylinositol-anchored form.

Galactofuranose (Gal*f*)[3] is an important constituent of the microbial cell surface (1). It occurs in structures essential for bacterial virulence or growth such as the *O*-antigen of the outer membrane lipopolysaccharide or the mycobacterial arabinogalactan (2). In eukaryotes, Gal*f* has principally been reported in glycoconjugates and polysaccharides of fungi and protozoan parasites (3), although distribution of the *glf* gene encoding the UDP-Gal*f* biosynthetic enzyme, UDP-galactopyranose mutase (UGM), suggests its presence in many lower eukaryotes (4, 5). Among fungi, the cell wall of the opportunistic pathogen *Aspergillus fumigatus* is one of the best studied. In this organism, Gal*f* has been found in the polysaccharides galactomannan (6), on glycoinositolphosphoceramides (7, 8) and in *N*- and *O*-linked glycans of glycoproteins (9–11). In *A. fumigatus*, as in the parasite *Leishmania major*, generation of a mutant devoid of Gal*f* resulted in attenuated virulence that highlights an important role of Gal*f* for eukaryotic pathogens (12, 13).

Little is known about the Gal*f* biosynthetic pathways in eukaryotes, although several enzymes involved in the synthesis of Gal*f* containing polysaccharide have been described in prokaryotes (see Ref. 14 for a recent review). Genetic and biochemical studies have shown that UDP-Gal*f* arising from the action of UGM is essential for galactofuranosylation in both prokaryotes and eukaryotes (2, 12, 13, 15, 16). This nucleotide sugar is the established or presumed donor substrate of specific galactofuranosyltransferases (GlfTs) comprising characterized bacterial β-GlfTs (14) and a family of putative β-GlfTs identified in the protozoan parasite *L. major* (17, 18). In addition, many eukaryotic and prokaryotic GlfTs such as the mycobacterial α-GlfTs are still unidentified. One of the putative eukaryotic transferases, known as LPG1, is involved in the biosynthesis of the lipophosphoglycan (LPG) of *L. major* and is localized in the Golgi apparatus (19). Similarly, enzymes involved in addition of the terminal Gal*f* to *N*-glycans, *O*-glycans, or glycolipids are assumed to be localized in this organelle. UDP-Gal*f* biosynthesis, however, occurs in the cytosol (12), which makes translocation of UDP-Gal*f* across the Golgi membrane necessary (Fig. 1).

Nucleotide sugar transporters (NSTs) are multitransmembrane proteins present in all kinds of eukaryotic organisms. They consist typically of 8–10 transmembrane α-helices linked by short loops. Further structural information is very limited because efforts to obtain crystals for x-ray structure determination have been hindered by the high hydrophobicity of NSTs. Many NSTs have been functionally characterized in biochemical assays measuring incorporation of radioactive nucleotide sugars into membrane vesicles. These experiments led to the development of a mechanistic model, in which NSTs work as antiporters that export a nucleotide sugar molecule in exchange for an equally charged nucleoside monophosphate molecule (20).

Because of their structural conservation, putative NSTs can be readily found by data base mining. In humans, they belong to the SLC35 (solute carrier 35) family (21), which comprise 10

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental data, Tables S1 and S2, and Figs. S1 and S2.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) FJ746723.

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[3] The abbreviations used are: Gal*f*, galactofuranose; UGM, UDP-galactopyranose mutase; NST, nucleotide sugar transporter; GlcA, glucuronic acid; GalNAc, *N*-acetylgalactosamine; SLC, solute carrier; GIPC, glycoinositolphosphoceramides; LPG, lipophosphoglycan; GlfT, galactofuranosyltransferase; GPI, glycosylphosphatidylinositol.

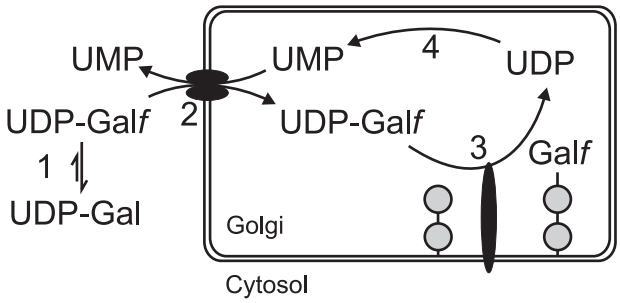


FIGURE 1. **Schematic model of galactofuranosylation.** UDP-Gal*f* is synthesized from UDP-Galactopyranose (UDP-Gal) (*1*) and translocated via an antiporter into the Golgi in exchange for UMP (*2*). Galactofuranosyltransferases transfer Gal*f* moieties from UDP-Gal*f* on various glycoconjugates (*3*) and UDP is converted to UMP by a nucleoside diphosphatase (*4*).

characterized members and 13 proteins whose function is currently unknown. Phylogenetic classification identified subfamilies that contain all of the characterized SLC35 proteins, but do not allow classification of most of the SLC35 proteins with unknown function (22). Furthermore, substrate specificity is hardly conserved within NST subfamilies, thus it is generally not possible to infer substrate specificity from the level of sequence identity or phylogeny.

NSTs are closely related to plastidic phosphate translocators that include translocators for triose phosphate, phosphoenolpyruvate, glucose 6-phosphate, and xylulose phosphate. Additionally, a variety of uncharacterized phosphate translocator-homologous proteins are found in plant and other organisms, including human (23). In this study, we describe the characterization of an *A. fumigatus* phosphate translocator-homologous protein with NST function and demonstrate its specificity for UDP-Gal*f*. The importance for *in vivo* galactofuranosylation is shown by a targeted gene deletion approach.

EXPERIMENTAL PROCEDURES

Materials—Radiolabeled nucleotide sugars were purchased from PerkinElmer Life Sciences (UDP-[^{3}H]Gal, UDP-[^{3}H]GlcNAc, and UDP-[^{14}C]GlcA), American Radiolabeled Chemicals (UDP-[^{3}H]GalNAc and [^{3}H]UMP), and GE Healthcare (UDP-[^{3}H]Glc). UDP-Gal*f* was chemically synthesized (24).

Strains, Medium, and Growth Conditions—For protein expression, *Saccharomyces cerevisiae* strain BY4741 in which the gene for the endoplasmic reticulum UDP-GlcNAc transporter, YEA4, had been deleted (*MAT*a; *his3D1; leu2D0; met15D0; ura3D0; YEL004w*::*kanMX4*; EUROSCARF, Frankfurt, Germany) was cultivated in SC minimal media (2% glucose, 1.7 g/liter Difco™ Yeast Nitrogen Base without amino acids and ammonium sulfate (BD Biosciences), 5 g/liter of ammonium sulfate) supplemented with L-histidine (50 mg/liter), L-methionine (50 mg/liter), and L-leucine (100 mg/liter).

A. fumigatus clinical isolate D141 was used in this study. For gene deletion purposes, a D141 strain deficient in non-homologous end-joining (AfS35) was used (25). Strains were grown at 37 °C on *Aspergillus* minimal medium containing 1% D-glucose as carbon source and 70 mM $NaNO_3$ as nitrogen source. Phleomycin was added for selection purposes at 30 mg/liter.

Bioinformatic Analyses—Transmembrane helix prediction was carried out using the ConPred II program (26). For plant proteins, predicted transmembrane domains were obtained from the ARAMEMNON data base (27). BLAST searches were performed using default parameter values with the low complexity filter switched off.

Cloning of the glfB Gene—Total RNA was isolated from *A. fumigatus* mycelium and *glfB* mRNA was reverse transcribed into single-stranded cDNA using the JE28 primer. All primer sequences are provided in supplemental Table S1. The *glfB* coding sequence was then amplified by PCR (JE26/JE28) and cloned via BamHI/XbaI into plasmid vector pYEScupFLAG*K* (28).

A. fumigatus Mutant Generation—Cassettes constructed for targeted gene replacement are described in the supplementary data. Mutants were generated by polyethylene glycol-mediated fusion of protoplasts as described in Ref. 29. Transformants were grown on *Aspergillus* minimal medium plates containing 1.2 M sorbitol as osmotic stabilizer under appropriate selection conditions and singled out twice before further analysis. Accurate gene deletion and reconstitution were confirmed by Southern hybridization.

Protein Expression in Yeast, Subcellular Fractionation, and in Vitro Transport Assay—The *glfB* cDNA as well as the human UDP-Gal transporter cDNA (SLC35A2, isoform a) (30) were cloned into the plasmid vector pYEScupFLAG*K* (complementing uracil auxotrophy) for copper-inducible expression of N-terminal FLAG-tagged proteins in yeast. *S. cerevisiae* spheroplasts were transformed using the lithium-acetate method described by Invitrogen. Transformants were selected on SC minimal media without uracil. For protein expression, transformants were grown in 1-liter cultures until A_{600} reached 0.8–0.9, expression was then induced by addition of 0.5 mM $CuSO_4$ (final concentration) and culture was continued for 2 h at 30 °C.

Subcellular fractionation of yeast cells and *in vitro* transport assay were performed as previously described (28). Briefly, 50 μl of Golgi vesicle preparations (containing typically 80 μg of total protein) and 50 μl of 2 μM ^{3}H-labeled nucleotide sugar or [^{3}H]UMP (0.37 kBq/μl) in assay buffer (10 mM Tris-HCl, pH 7.0, 0.8 M sorbitol, 2 mM $MgCl_2$) were incubated for 30 s at 30 °C. For competition assays, the assay buffer contained in addition 100 μM unlabeled nucleotide sugar (31). Reactions were stopped by dilution with 1 ml of ice-cold assay buffer. The vesicle suspension was then filtered through a mixed cellulose ester membrane (MF™ membrane filters, 0.45 μm, Millipore, Bedford, MA). Vesicles adhering to the filters were washed three times with 2 ml of ice-cold assay buffer and the radioactivity retained on the membrane was measured by liquid scintillation.

Subcellular Fractionation of A. fumigatus—Subcellular fractionation of *A. fumigatus* largely followed a protocol described for *Aspergillus oryzae* (32). Briefly, mycelium (approximately 30 g, squeeze-dried) from 1 liter of an *A. fumigatus* FLAG-*glfB* overnight culture (inoculum 10^6 ml^{-1} conidia) was ground in a mortar with 1–1.5 volumes of sterile sand and 50 ml of lysis buffer (15% (w/w) sucrose, 10 mM HEPES/Tris, pH 7.4, 1 mM EDTA, supplemented with Complete EDTA-free Protease Inhibitors (Roche)). Debris was removed by filtration through Miracloth (Calbiochem) and rinsed further in 45 ml of lysis

buffer. Large particles (whole cells, nuclei, and mitochondria) were removed by two sequential centrifugation steps (30 min at 10,000 × *g* and 20 min at 27,000 × *g*) and microsomes were pelleted at 110,000 × *g* (45 min) at 4 °C. The microsomal pellet was suspended in 800 μl of lysis buffer, applied on a multistep sucrose gradient (1 ml of each 60, 50, 40, 38, 36, 34, 32, 30, 28, 26, 24, 20, 18, and 15% (w/w) sucrose in 10 mM HEPES/Tris, pH 7.4, and 1 mM EDTA) and centrifuged at 110,000 × *g* for 16 h at 4 °C. Fractions (approximately 0.5 ml) were collected from the bottom of the tube and kept at 4 °C until analyzed. Density was calculated from the sucrose concentration determined by a standard optical refractometer and the protein concentration was determined with the Protein BCA Assay (Pierce).

Organelle Marker Enzyme Assays—Cytochrome *c* oxidoreductase was used as endoplasmic reticulum marker (33) and assayed by adding 20 μl of each fraction to 80 μl of a solution containing 80 mM potassium phosphate, pH 7.5, 150 μM cytochrome *c* (oxidized, Sigma), 1.5 mM β-NADH, and 7.2 mM NaCN. A_{550} was recorded over 5 min in a microplate reader, and substrate turnover was calculated from the slope according to Lambert-Beer's law (path length, 0.30 cm; molar absorptivity, 21 mM^{-1} cm^{-1}). Three days after fractionation, the Golgi marker inosine diphosphatase was assayed as described (34), and phosphate content was determined with the malachite green method (35).

Western Blots—Cell wall glycoproteins and soluble polysaccharides were extracted from 30 mg of ground *A. fumigatus* mycelium by incubation in 1 ml of sample buffer (15% glycerol, 100 mM Tris-HCl, pH 6.8, 1.5% SDS, 0.25% β-mercaptoethanol, 0.025% bromophenol blue) for 12 min at 95 °C. 20 μl of the supernatant was separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The monoclonal antibody EB-A2 (39) conjugated to horseradish peroxidase from the Platelia *Aspergillus* Test (Bio-Rad) or horseradish peroxidase-coupled lectin concanavalin A (Sigma) was used in a 1:50 dilution or at 0.2 μg/ml with or without 200 mM α-methyl-D-mannopyranoside, respectively. Horseradish peroxidase activity was visualized by an enhanced chemiluminescence system (Pierce). For sucrose gradient analysis, 7.5 μl of each fraction were incubated for 10 min at 50 °C with 7.5 μl of sample buffer. Both sample buffer and the polyacrylamide gel contained 4 M urea. For FLAG tag detection, mouse anti-FLAG monoclonal antibody M5 (Sigma) was used in a 1:1000 dilution. IRDye 800CW-coupled secondary antibody enabled band visualization and intensity measurement by infrared scanning on an Odyssey system (Li-Cor, Lincoln, NE).

Purification and Analysis of Glycosylinositolphosphoceramides (GIPCs)—GIPCs were extracted from 0.5 g of mycelium and purified as previously described (13). Purified GIPCs were redissolved in 20 μl of MeOH. High performance thin layer chromatography and immunostaining with the monoclonal antibody MEST-1 were carried out as described (7) using 2 μl for immunostaining and 18 μl for orcinol/H_2SO_4 staining.

N-Glycan Analysis—*N*-Glycan preparation and separation were carried out as described previously (36). Glycoproteins from 12 ml (40 × 300 μl) of *A. fumigatus* culture supernatant were transferred to Immobilon P Multiwell plates (Millipore). After peptide:*N*-glycanase-mediated *N*-glycan release and 8-amino-1,3,6-pyrene-trisulfonic acid labeling *N*-glycans were separated on a capillary electrophoresis DNA Sequencer (ABI PRISM® 3100-*Avant* Genetic Analyzer, Applied Biosystems, Foster City, CA). Reference glycans were purchased from Dextra Laboratories (Reading, UK).

Growth Assay—For radial growth measurement, a 5-μl drop containing 10,000 *A. fumigatus* conidia in phosphate-buffered saline was placed in the center of an *Aspergillus* minimal medium agar plate. Plates were incubated at various temperatures and colony diameters were measured twice daily.

RESULTS

Selection of a UDP-Galf Transporter Candidate Gene—BLAST searches of the *A. fumigatus* genome (37) with various characterized NST protein sequences identified 16 putative NST genes (supplemental Table S2). One of them (AFUA_3G12700) is adjacent to the recently identified *glfA* gene (AFUA_3G12690) encoding the UDP-Gal*f* biosynthetic enzyme UGM, and was thus considered a reasonable candidate because clustering of functionally related genes is sometimes observed in *A. fumigatus* (*e.g.* the siderophore genes *sidF*, *sidD*, and *mirB*) (38). This gene will be referred to as *glfB* because of its implication in galactofuranosylation, as demonstrated below. Interestingly, all fungi from the subphylum Pezizomycotina whose genome has been fully sequenced display a clear homolog of *glfB* clustered with *glfA*. When present in basidiomycota (*e.g. Cryptococcus neoformans*), these two genes are more distant and may be found on different chromosomes. In *A. fumigatus*, the predicted GlfB protein comprises 400 amino acids and shares up to 40% amino acid identity with uncharacterized *Arabidopsis* phosphate translocator homologs. The most similar protein with known function is the plant UDP-Gal transporter AtUDP-GalT1 (At1g77610) (39) that displays 21% identity with GlfB. The UDP-Gal transporters AtUDP-GalT2 (At1g76670) and AtNST-KT1 (At4g39390) (39, 40), and the uncharacterized SLC35C2 and SLC35E3 are more distantly related and show 14 to 19% identity with GlfB. Finally, the human GDP-Fuc transporter is the closest characterized transporter of the SLC35 family (12% identity). An alignment of GlfB with these sequences is presented in Fig. 2. This multiple sequence alignment underlines the conservation of two lysine residues (GlfB Lys-59 and Lys-294) that have been proposed to be involved in substrate binding (23, 41). GlfB was predicted to contain 11 transmembrane helices of which the first 10 aligned well with the predicted transmembrane helices of its homologs and other NSTs (fig. 2). We thus hypothesized that *glfB* encoded a NST and in line with its location in the genome speculated about a specificity for UDP-Gal*f*.

In Vitro Transport and Binding Assays—Uptake of radioactive nucleotide sugars by Golgi-enriched vesicles isolated from yeast expressing a putative NST is a method of choice to determine substrate specificity. Synthesis of radioactive UDP-Gal*f* has been reported (42) but is not commercially available and could thus not be directly tested in this *in vitro* assay system. Nevertheless, the transport of UDP-Gal, UDP-GlcNAc, UDP-GalNAc, or UDP-GlcA could be excluded. Indeed, the uptake of these nucleotide sugars by Golgi vesicles isolated from yeast cells expressing GlfB or mock trans-

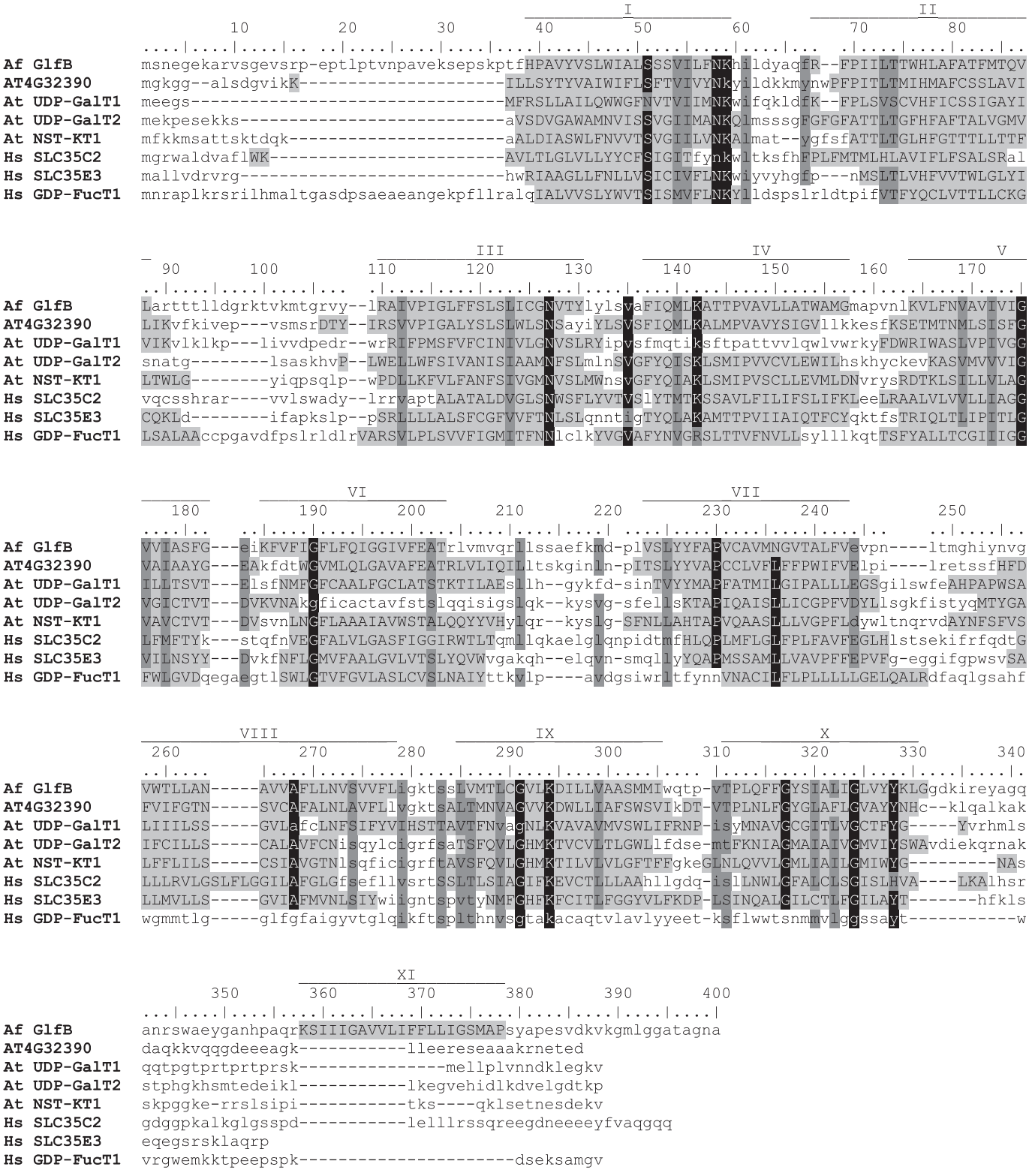


FIGURE 2. **Multiple sequence alignment of *A. fumigatus* (*Af*) GlfB and related proteins from *Arabidopsis thaliana* (*At*) and humans (*Hs*) with prediction of transmembrane helices.** The *Arabidopsis* uncharacterized protein encoded by the gene AT4G32390 and UDP-Gal transporter AtUDP-GalT1 are the closest GlfB homologs, whereas the plant UDP-Gal transporters AtUDP-GalT2 and AtNST-KT1, the human proteins SLC35C2 and SLC35E3, and the characterized GDP-fucose transporter (SLC35C1) are more distantly related. Conserved residues, *black shading*; similar residues, *dark-gray shading*; predicted transmembrane domains, *capital letters* and *light-gray shading*; predicted transmembrane domains for GlfB, *roman numbers*.

formed was virtually absent (background levels of 1.0 to 1.7 pmol mg^{-1} min^{-1}), whereas an endogenous UDP-Glc transport of ~7 pmol mg^{-1} min^{-1} verified the quality of the Golgi vesicle preparation. Additionally, transfection of CHO-Lec8 cells with GlfB resulted in Golgi expression of the protein but did not restore galactosylation of the surface glycoconju-

gates confirming absence of the UDP-Gal transport (data not shown).

NSTs are likely simple carrier proteins with a binding site alternating between both sides of the membrane (43). Presence of the antiport molecule (in this case UMP) at the *trans* (inside) or *cis* (outside) side of the membrane thus leads to stimulation or inhibition of the nucleotide sugar transport, respectively (43, 44). To assess UDP-Gal*f* binding, we thus evaluated the ability of unlabeled UDP-Gal*f* (45) to inhibit the uptake of radioactive UMP, the postulated counter substrate of GlfB (Fig. 3*A*). For this purpose, Golgi vesicles obtained from cells expressing either GlfB or the human UDP-Gal transporter were incubated with 1 μM [^{3}H]UMP and 100 μM unlabeled UMP, UDP-Gal*f*, UDP-Gal, or UDP-GlcNAc. In GlfB containing vesicles, the addition of UDP-Gal*f* resulted in an 80% inhibition of the [^{3}H]UMP transport and was thus comparable with addition of unlabeled UMP (88% inhibition). In contrast, UDP-GlcNAc and UDP-Gal slightly affected the UMP uptake indicating a limited binding of these nucleotide sugars to GlfB. Unfortunately, because UDP-Gal*f* is rather unstable, contamination of a UDP-Gal*f* solution by UMP is difficult to avoid (46) and was in this case estimated to 6–7% by high pressure liquid chromatography (47) (supplemental Fig. S1). This contaminating UMP explains the 50% decrease of [^{3}H]UMP uptake observed with vesicles expressing the UDP-Gal transporter because a comparable inhibition is observed with 8 μM unlabeled UMP (Fig. 3*B*). In contrast, inhibition of GlfB-mediated [^{3}H]UMP uptake by the contaminated UDP-Gal*f* solution was significantly higher than observed with 8 μM UMP ($p = 0.011$, *t* test), indicating that part of the inhibition observed is actually due to UDP-Gal*f* binding.

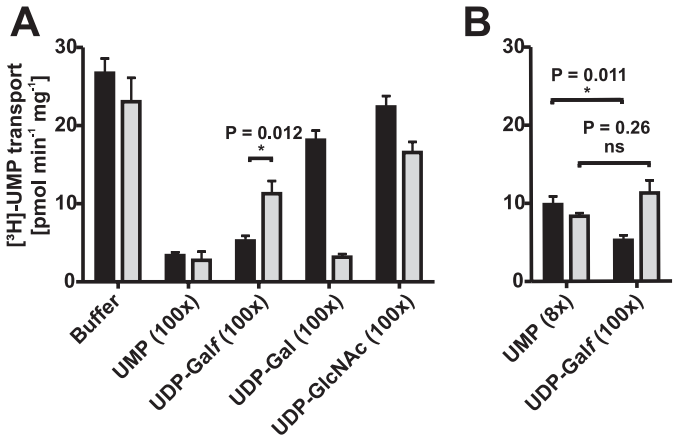


FIGURE 3. **UDP-Gal*f* binds to GlfB.** *A*, [^{3}H]UMP uptake by Golgi vesicles isolated from yeast expressing GlfB (*black bars*) or the human UDP-Gal transporter (*gray bars*) in the absence or presence of a 100-fold molar excess of unlabeled UMP, UDP-Gal*f* (containing 7% UMP), UDP-Gal, or UDP-GlcNAc. *B*, comparison of [^{3}H]UMP uptake in the presence of a 100 M excess UDP-Gal*f* containing 7% UMP or an 8 M excess UMP. Each value represents the average of three independent experiments with duplicate measurements. *ns*, not significant.

Deletion of glfB in A. fumigatus—To prove UDP-Gal*f* transport activity *in vivo*, evaluation of the effect of *glfB* loss on galactofuranosylation of glycoconjugates in *A. fumigatus* was undertaken. For this purpose a *glfB* deletion cassette was constructed by double-joint PCR (48) containing the selectable phleomycin resistance gene *ble* (49) flanked by up- and downstream regions of the *glfB* coding sequence. *A. fumigatus* wild type protoplasts were transformed with the linear cassette for exchange of the genomic *glfB* coding sequence for the phleomycin resistance gene by homologous recombination (Fig. 4*A*). Transformants were selected for phleomycin resistance and

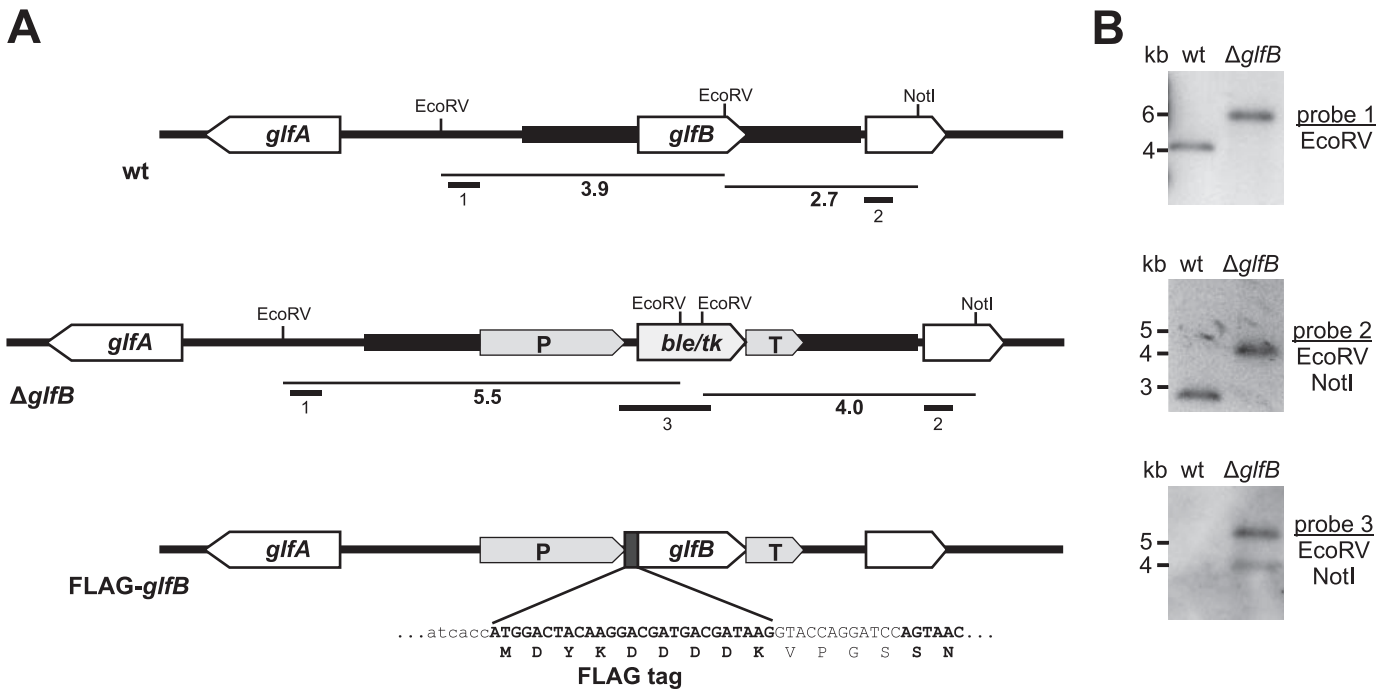


FIGURE 4. **Gene replacement of *glfB* in *A. fumigatus*.** *A*, strategy for the targeted replacement of *glfB* by the *ble/tk* selection marker cassette mediated by homologous recombination and subsequent re-introduction of the FLAG-tagged *glfB* coding sequence. The positions of probes (*1–3*) used for Southern blot hybridization along with the respective restriction fragments (size in kb) are indicated. *B*, Southern blots of genomic DNA digested with the indicated restriction enzymes and hybridized to three different digoxigenin-labeled probes. *wt*, wild type; *ble/tk*, phleomycin resistance/thymidine kinase fusion gene; *P*, *A. nidulans gpdA* promoter; *T*, *A. nidulans trpC* terminator; *glfA*, UDP-galactopyranose mutase open reading frame.

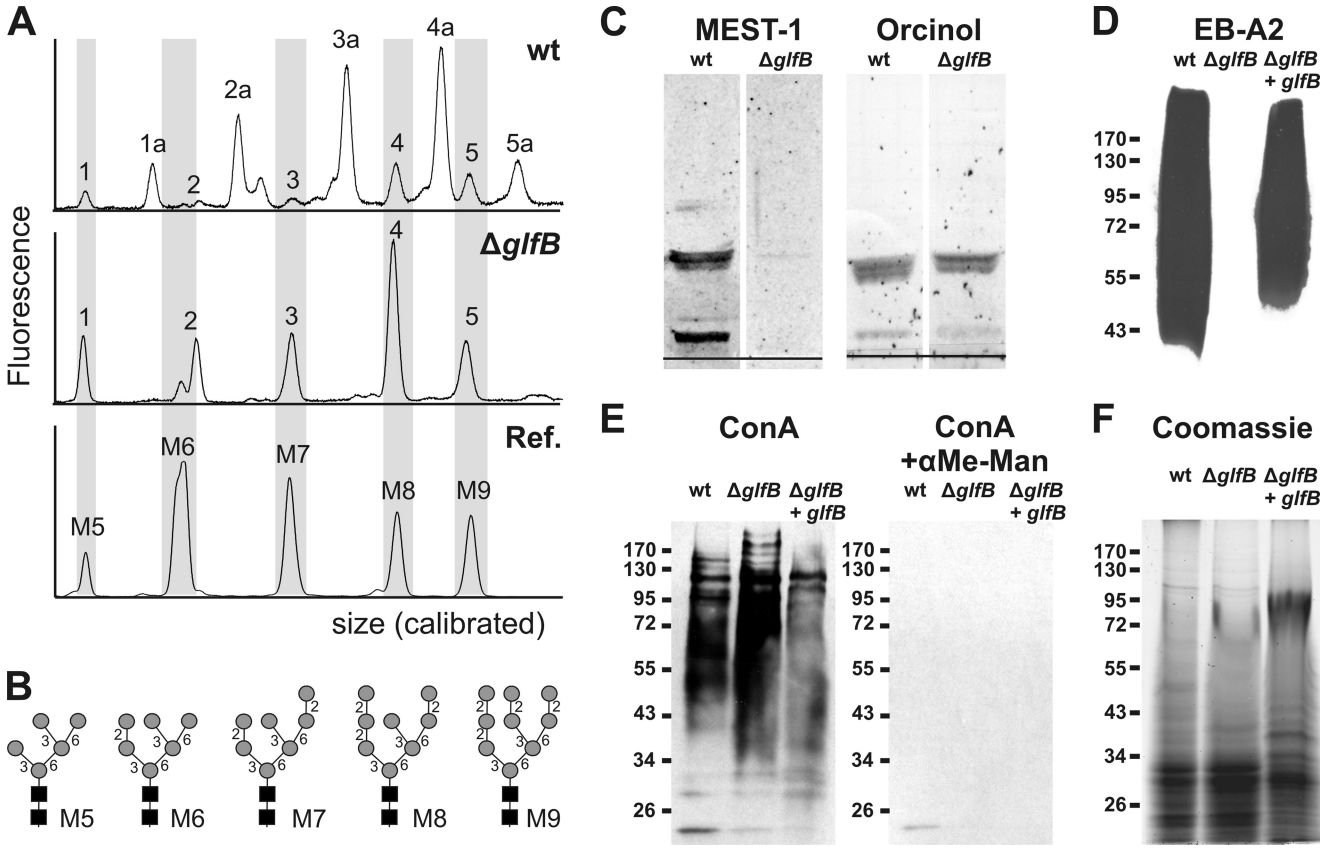


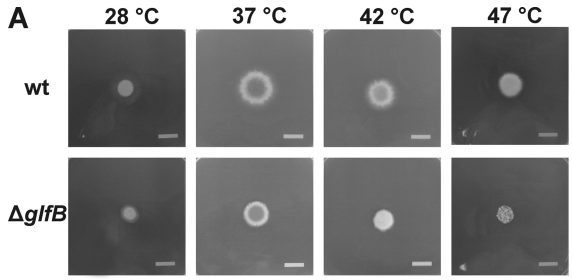
FIGURE 5. **The *A. fumigatus* Δ*glfB* mutant lacks Galf.** *A*, electropherograms of fluorescently labeled *N*-glycans enzymatically released from secreted glycoproteins of *A. fumigatus* wild type (*wt*) and the Δ*glfB* mutant. Commercial oligosaccharides (Dextra Laboratories) served as reference (*Ref.*). The *x* axis was calibrated to the fragment sizes of the GeneScan-500 ROX standard (Applied Biosystems). *B*, schematic structures of reference oligosaccharides. *Black squares*, *N*-acetylglucosamine; *gray circles*, mannose. *C*, GIPCs extracted from *A. fumigatus* mycelium, separated by high performance thin layer chromatography, and stained with either the Galf(β1–6/β1–3)-specific antibody MEST-1 (*left*) or with orcinol/H_2SO_4 (*right*). The *black line* indicates the loading spot. *D–F*, water-soluble extracts of *A. fumigatus* mycelium separated by SDS-PAGE, transferred onto nitrocellulose membrane, and stained with the Galf-specific monoclonal antibody EB-A2 (*D*), mannose-specific lectin concanavalin A (*ConA*) in the presence or absence of 200 mM α-methyl-D-mannopyranoside (*E*), or Coomassie G-250 as a loading control (*F*).

gene replacement was confirmed by Southern blot analysis (Fig. 4*B*). A single strain was chosen for further analysis and named Δ*glfB*.

Analysis of Galactofuranosylation in Δ*glfB*—Because *N*-glycosylation of proteins is known to take place along the secretory pathway and requires various NSTs, we first concentrated on this modification. Proteins from *A. fumigatus* culture supernatants were immobilized on polyvinylidene difluoride membranes and *N*-glycans were released by peptide:*N*-glycanase treatment. After labeling with the negatively charged fluorescent dye 8-amino-1,3,6-pyrene-trisulfonic acid, *N*-glycans were separated by capillary electrophoresis on a DNA sequencer (Fig. 5*A*). In the wild type electropherogram (*top panel*), the peaks labeled 1a to 5a can be assigned to high-mannose type *N*-glycans bearing a single Gal*f* residue (Gal*f*$Man_{5-9}GlcNAc_2$) as shown previously (10, 13). These Gal*f*-containing glycans were completely absent from the Δ*glfB N*-glycan electropherogram (*middle panel*) that exclusively displays nongalactofuranosylated *N*-glycans (peaks 1–5) co-migrating with $Man_{5-9}GlcNAc_2$ standards (*lower panel*, Fig. 5*B*). This finding demonstrates the requirement of GlfB for galactofuranosylation of *N*-glycans.

The contribution of GlfB to glycolipid biosynthesis was also analyzed by testing their reactivity to monoclonal antibody MEST-1 (50). This antibody reacts specifically with β1–6-Gal*f* found on several glycosphingolipids of *A. fumigatus* (Fig. 5*C*, *left*) (7). However, glycosphingolipids extracted from *A. fumigatus* Δ*glfB* mycelium and separated by high performance thin layer chromatography were not stained with MEST-1 indicating the absence of β1–6-linked galactofuranose in these molecules. As loading control, carbohydrates were stained with orcinol/sulfuric acid (Fig. 5*C*, *right*).

Finally we tested for reactivity of cell wall components toward the Gal*f*-specific monoclonal antibody EB-A2 (51). A tetrasaccharide of β1–5-linked Gal*f* has been described as the main epitope of EB-A2 (51). This structure is part of the cell wall polysaccharide galactomannan, which can be either linked to the cell wall β1–3/6-glucan (6) or to a GPI anchor (52). Also *N*-glycans with a single terminal Gal*f* have been reported to be recognized by EB-A2 (10). Aqueous extracts of wild type *A. fumigatus* mycelium separated on a polyacrylamide gel and transferred to a nitrocellulose membrane bound EB-A2 strongly, whereas in the case of the Δ*glfB* mutant, binding was totally absent (Fig. 5*D*). Importantly, episomal expression of the gene in the Δ*glfB* mutant restores EB-A2 binding excluding any influence of the deletion cassette on the adjacent *glfA* locus (Fig. 5*D*). In contrast, staining with the mannose-specific lectin concanavalin A appeared slightly stronger for Δ*glfB* than for wild



B

Temp. [°C]	Radial colony growth [µm/h] wt	ΔglfB	Ratio ΔglfB/wt
28	456 ±16	181 ±14	0.40 ±0.04
37	654 ±10	367 ±43	0.56 ±0.07
42	546 ±10	184 ±43	0.34 ±0.08
47	235 ±14	77 ±14	0.33 ±0.08

FIGURE 6. **The morphology and growth of *A. fumigatus* Δ*glfB* is altered.** *A*, colony morphology of *A. fumigatus* wild type (*wt*) and the Δ*glfB* mutant after 2 days of growth on minimal agar at various temperatures. *B*, absolute and relative growth rates obtained from colony diameter measurements (mean ± S.E, $n = 3$).

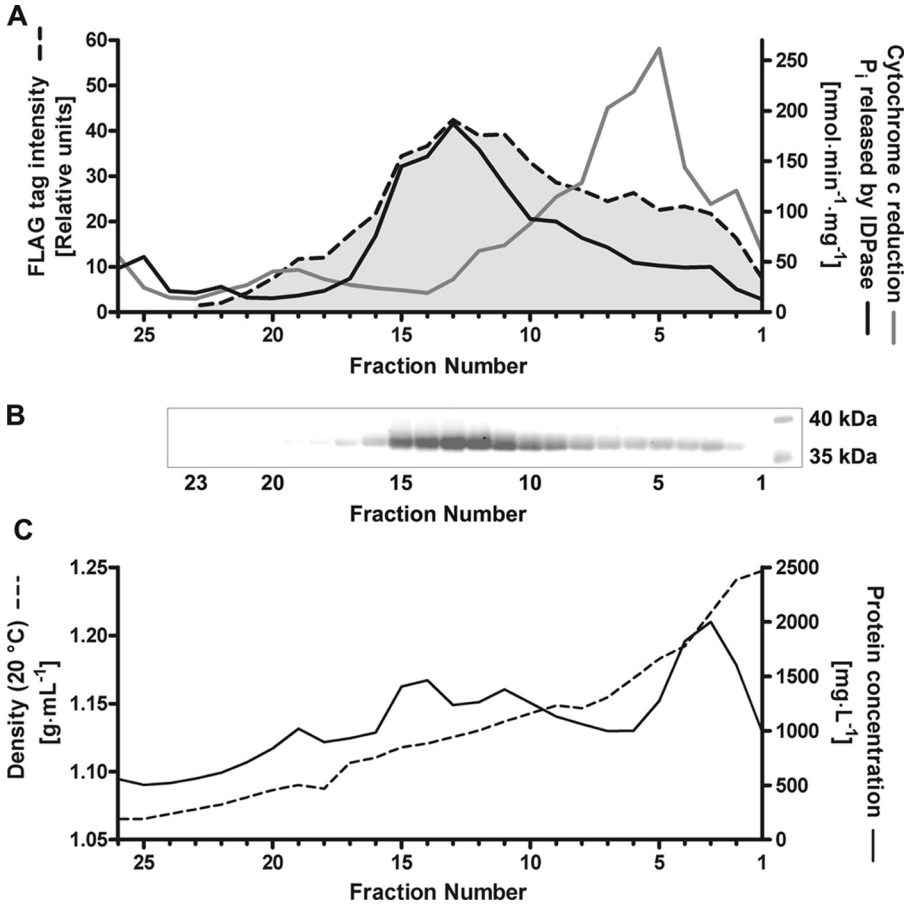


FIGURE 7. **Subcellular localization of GlfB in *A. fumigatus*.** *A. fumigatus* FLAG-*glfB* microsomes were separated by isopycnic centrifugation on a multistep sucrose gradient. Fractions were analyzed by Western blot and immunostained with an anti-FLAG antibody (*A* and *B*) and compared with marker enzyme activities for endoplasmic reticulum (cytochrome *c* oxidoreductase, *gray line*) and Golgi (inosine diphosphatase (*IDPase*), *black line*) (*A*). Protein concentration and density were determined (*C*).

type or the episomally complemented mutant, suggesting a higher exposure of cell surface mannan structures as previously observed with the Δ*glfA* mutant (Fig. 5*E*, *left*) (13). Specificity of this staining was demonstrated by α-methyl mannoside inhibition (Fig. 5*E*, *right*). Equal loading for EB-A2 and concanavalin A blots was verified by Coomassie staining of a gel run in parallel (Fig. 5*F*). Thus, the surface glycoconjugates of Δ*glfB* closely resemble those of the Gal*f*-deficient Δ*glfA* mutant (13), which suggests a complete loss of galactofuranosylation capacity in the Δ*glfB* mutant.

Growth and Thermotolerance of the Δglf B Mutant—Gal*f* deficiency has been shown to induce an altered culture morphology accompanied by a substantial growth defect in *A. fumigatus* (13). This effect was more pronounced at higher temperatures than the standard growth temperature of 37 °C indicating a decreased resistance to temperature stress. Indeed, radial colony growth of the Δ*glfB* mutant was found to be 45% slower than wild type at 37 °C and 66–67% slower at 42 or 47 °C (Fig. 6) in agreement with the observations for the Δ*glfA* mutant (13).

Localization of GlfB in A. fumigatus—Because of its involvement in protein and lipid glycosylation, GlfB was presumed to be localized in the Golgi apparatus. This localization was observed in transfected mammalian cells by immunofluorescence (supplemental Fig. S2). Moreover, we investigated localization of the GlfB transporter in *A. fumigatus* by isopycnic ultracentrifugation (Fig. 7). For this purpose, a cassette coding for FLAG-tagged GlfB placed under the *gpdA* promoter was inserted into the *glfB* locus (Fig. 4*A*). Presence of GlfB in fractions collected after ultracentrifugation of microsomes obtained from this transformant on a multistep sucrose gradient was then analyzed by Western blot using an anti-FLAG antibody (Fig. 7*B*). GlfB was found mainly in fractions 11 to 15 having the same buoyant density (1.12–1.14 g/ml) as Golgi vesicles detected by inosine diphosphatase activity (Fig. 7). In contrast, fractions displaying a high NADPH-cytochrome *c* oxidoreductase activity (endoplasmic reticulum marker) only contained low amounts of GlfB protein. These data strongly support Golgi localization and are in perfect agreement with a role of GlfB in protein and lipid glycosylation.

DISCUSSION

This report describes the first identification of a nucleotide sugar transporter with specificity for UDP-Gal*f*. The protein was called GlfB because of its implication in galactofuranose metabolism selected from its homology to other members of the NST family, its phylogenetic classification, as well as the location on chromosome 3 directly downstream of the *glfA* gene, which encodes the UDP-Gal*f* biosynthesis enzyme UGM (13). It is a 400-amino acid protein with 11 predicted trans-

membrane helices. This represents a distinctiveness of the GlfB protein because NSTs classically exhibit 8–10 predicted hydrophobic domains and follow an experimentally determined model in which both the N and C terminus are situated on the cytoplasmic side of the organelle (53).

Remarkably, filamentous fungi of the subphylum Pezizomycotina, including many human or plant pathogens, all seem to exhibit adjacent *glfA* and *glfB* genes and the presence of Gal*f* has been reported in many species of this subphylum. These genes are also found in a few basidiomycota such as the human pathogen *C. neoformans* but are absent from other fungi, notably yeasts. Thus, Gal*f* seems particularly important for filamentous fungi. Indeed several studies have already shown the role of this monosaccharide form for growth, hyphal morphology, and sporulation of the *Aspergillus* species (13, 54, 55). Hence we speculate that Gal*f* plays major roles in hyphal development and/or reproduction of all filamentous fungi of Pezizomycotina and thus has been maintained during evolution.

To address the function of a nucleotide sugar transporter, two approaches are commonly used. Complementation of a mutant strain or cell line not only provides a way to determine NST function *in vivo*, but also allows identification of the underlying gene(s) by expression of a cDNA library combined with sibling selection (39). Alternatively, NST substrates can be identified by measuring transport of radiolabeled nucleotide sugars into vesicles, either prepared from cell lysates or artificially reconstituted proteoliposomes (56, 57). Because of the unavailability of a UDP-Gal*f* transporter-deficient mutant or cell line and radioactive UDP-Gal*f*, we opted for targeted gene deletion of candidate genes in the opportunistic fungus *A. fumigatus.* This approach was enabled by the restricted number of candidate genes, the existence of a haploid stage that facilitates the isolation of clones by molecular techniques, and a comprehensive knowledge of the galactofuranosylated structures in this organism (11). Targeted replacement of the most promising candidate gene, *glfB*, led to the absence of Gal*f* bringing direct evidence of its involvement in Gal*f* metabolism.

The specificity of GlfB for UDP-Gal*f* was established by an indirect assay showing competitive inhibition of [^{3}H]UMP transport by GlfB with unlabeled UDP-Gal*f*. In addition, the transport of several other nucleotide sugars was excluded using a direct *in vitro* transport assay suggesting that GlfB is highly specific. In particular, absence of the UDP-Gal transport, which was confirmed by the inability of *glfB* to complement the CHO cell line Lec8, indicates that the transporter is able to discern the ring conformation of the monosaccharide. Vice versa, our data show that the human UDP-Gal transporter used as control in this study does not recognize UDP-Gal*f*. Similarly *L. major* UDP-Gal transporters LPG5A and LPG5B appear to be specific for the pyranic form of galactose because their concomitant deletion results in the synthesis of glycoconjugates containing Gal*f* but devoid of galactopyranose (58). The obvious difference in the three-dimensional structure of the furanic and pyranic rings certainly plays a role in the ability of these NSTs to differentiate the two cyclic forms. Initially NSTs were thought to be monospecific. However, several NSTs have now been shown to be multifunctional *in vitro* and usually recognize sugars activated with the same nucleotide that led to the assumption that the nucleotide part is a major player in recognition. With the exception of the Fringe Connection (59), which is thought to be a general UDP-sugar transporter, the specificity of NSTs is generally restricted to a few related nucleotide sugars demonstrating that the sugar part also plays a significant role in the recognition.

Detailed analyses of *A. fumigatus* Δ*glfB* total extract and purified glycoconjugates revealed the complete absence of galactofuranose in *N*-glycans, glycolipids as well as galactomannan. It can be inferred from this result that GlfB is the only NST in *A. fumigatus* capable of UDP-Gal*f* transport. The Golgi localization of this transporter is moreover in perfect agreement with its implication in galactofuranosylation of *N*-glycans and glycolipids. The lack of Gal*f* in galactomannan was, however, less predictable because its biosynthesis is currently unknown. Recently, we demonstrated that the terminal sugar of this polysaccharide arises from UDP-Gal*f* synthesized in the cytoplasm by UGM (13). Galactomannan is either linked to the membrane by a GPI anchor, covalently bound to the cell wall β1,3/1,6-glucan, or secreted in the environment (6, 52). Because they present the same carbohydrate structure, it has been postulated that these three forms of galactomannan share a common biosynthetic pathway. By analogy to the biosynthesis of the *Leishmania* GPI-anchored polysaccharide LPG (19), we assumed that biosynthesis of the GPI-linked galactomannan takes place in the Golgi apparatus. The total absence of EB-A2 staining in the Δ*glfB* mutant, indicative of the absence of all forms of galactomannan, supports this location. Galactomannan would then be transferred to the β1,3/1,6-glucan from the GPI-anchored polymer as it has been proposed for some GPI-anchored proteins in ascomycetous yeasts (60). The secreted form would arise from enzymatic cleavage of surface galactomannan. Our data demonstrating the absence of galactofuran in the Δ*glfB* mutant establish a clear link between galactomannan including the cell wall bound form and the secretory pathway and thus strongly support this model. In contrast, the synthesis of α1–3-glucan, β1–3-glucan, and chitin seem to occur at the plasma membrane (61).

In agreement with the complete absence of Gal*f* in glycoconjugates and galactomannan, the growth phenotype of the *glfB* gene deletion mutant closely resembles the previously described *Galf*-deficient Δ*glfA* (13, 16). In the case of Δ*glfA*, the growth defect was correlated with a reduction of virulence (13). It can thus be extrapolated that *glfB* is most probably required for full virulence of the fungus. Another example of nucleotide sugar transporter implicated in pathogenicity and restricted to certain organisms is the Golgi GDP-Man transporter. Deletion of this NST led to avirulence of the parasite *L. major* (62) and is lethal in *S. cerevisiae*, *Candida albicans*, and *Candida glabrata* (63–65). In the latter organisms, Golgi mannosylation comprises *N*-glycan outer chain elongation, *O*-mannosylation of proteins, and GIPC biosynthesis. The *A. fumigatus* genome contains a clear GDP-Man transporter homolog (AFUA_5G05740) whose importance is difficult to predict. It has recently been shown that protein *O*-mannosylation is dispensable in this fungus but is required for cell wall stability and full virulence (66, 67). Yet the importance of *N*-glycan branching and elongation, and mannosylation of GIPCs, is currently

undetermined. The absence of Gal*f* is, however, not sufficient to abolish growth of *A. fumigatus* as shown previously (13, 16) and confirmed in this study. Even chitin, a polysaccharide that is believed to contribute to the rigidness of the fungal cell wall is not strictly essential for *S. cerevisiae* (68, 69). Therefore, therapeutic strategies directed against cell wall biosynthesis will likely have to address several targets to be successful.

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REFERENCES

1. Peltier, P., Euzen, R., Daniellou, R., Nugier-Chauvin, C., and Ferrières, V. (2008) *Carbohydr. Res.* **343,** 1897–1923
2. Pan, F., Jackson, M., Ma, Y., and McNeil, M. (2001) *J. Bacteriol.* **183,** 3991–3998
3. de Lederkremer, R. M., and Colli, W. (1995) *Glycobiology* **5,** 547–552
4. Beverley, S. M., Owens, K. L., Showalter, M., Griffith, C. L., Doering, T. L., Jones, V. C., and McNeil, M. R. (2005) *Eukaryot. Cell* **4,** 1147–1154
5. Bakker, H., Kleczka, B., Gerardy-Schahn, R., and Routier, F. H. (2005) *Biol. Chem.* **386,** 657–661
6. Latgé, J. P., Kobayashi, H., Debeaupuis, J. P., Diaquin, M., Sarfati, J., Wieruszeski, J. M., Parra, E., Bouchara, J. P., and Fournet, B. (1994) *Infect. Immun.* **62,** 5424–5433
7. Toledo, M. S., Levery, S. B., Bennion, B., Guimaraes, L. L., Castle, S. A., Lindsey, R., Momany, M., Park, C., Straus, A. H., and Takahashi, H. K. (2007) *J. Lipid Res.* **48,** 1801–1824
8. Simenel, C., Coddeville, B., Delepierre, M., Latgé, J. P., and Fontaine, T. (2008) *Glycobiology* **18,** 84–96
9. Leitao, E. A., Bittencourt, V. C., Haido, R. M., Valente, A. P., Peter-Katalinic, J., Letzel, M., de Souza, L. M., and Barreto-Bergter, E. (2003) *Glycobiology* **13,** 681–692
10. Morelle, W., Bernard, M., Debeaupuis, J. P., Buitrago, M., Tabouret, M., and Latgé, J. P. (2005) *Eukaryot. Cell* **4,** 1308–1316
11. Latge, J. P. (2009) *Med. Mycol.* **47,** Suppl. 1, S104–S109
12. Kleczka, B., Lamerz, A. C., van Zandbergen, G., Wenzel, A., Gerardy-Schahn, R., Wiese, M., and Routier, F. H. (2007) *J. Biol. Chem.* **282,** 10498–10505
13. Schmalhorst, P. S., Krappmann, S., Vervecken, W., Rohde, M., Müller, M., Braus, G. H., Contreras, R., Braun, A., Bakker, H., and Routier, F. H. (2008) *Eukaryot. Cell* **7,** 1268–1277
14. Richards, M. R., and Lowary, T. L. (2009) *Chembiochem.* **10,** 1920–1938
15. Novelli, J. F., Chaudhary, K., Canovas, J., Benner, J., Medinger, C., Kelly, P., Hodgkin, J., and Carlow, C. K. (2009) *Dev. Biol.* **335,** 340–355
16. Lamarre, C., Beau, R., Balloy, V., Fontaine, T., Hoi, J. W., Guadagnini, S., Berkova, N., Chignard, M., Beauvais, A., and Latgé, J. P. (2009) *Cell Microbiol.* **11,** 1612–1623
17. Zhang, K., Barron, T., Turco, S. J., and Beverley, S. M. (2004) *Mol. Biochem. Parasitol.* **136,** 11–23
18. Späth, G. F., Epstein, L., Leader, B., Singer, S. M., Avila, H. A., Turco, S. J., and Beverley, S. M. (2000) *Proc. Natl. Acad. Sci. U.S.A.* **97,** 9258–9263
19. Ha, D. S., Schwarz, J. K., Turco, S. J., and Beverley, S. M. (1996) *Mol. Biochem. Parasitol.* **77,** 57–64
20. Gerardy-Schahn, R., Oelmann, S., and Bakker, H. (2001) *Biochimie* **83,** 775–782
21. Ishida, N., and Kawakita, M. (2004) *Pflugers Arch. Eur. J. Physiol.* **447,** 768–775
22. Jack, D. L., Yang, N. M., and Saier, M. H., Jr. (2001) *Eur. J. Biochem.* **268,** 3620–3639
23. Knappe, S., Flügge, U. I., and Fischer, K. (2003) *Plant Physiol.* **131,** 1178–1190
24. Peltier, P., Guégan, J. P., Daniellou, R., Nugier-Chauvin, C., and Ferrières, V. (2008) *Eur. J. Org. Chem.* 5988–5994
25. Krappmann, S., Sasse, C., and Braus, G. H. (2006) *Eukaryot. Cell* **5,** 212–215
26. Arai, M., Mitsuke, H., Ikeda, M., Xia, J. X., Kikuchi, T., Satake, M., and Shimizu, T. (2004) *Nucleic Acids Res.* **32,** W390–W393
27. Schwacke, R., Schneider, A., van der Graaff, E., Fischer, K., Catoni, E., Desimone, M., Frommer, W. B., Flügge, U. I., and Kunze, R. (2003) *Plant Physiol.* **131,** 16–26
28. Ashikov, A., Routier, F., Fuhlrott, J., Helmus, Y., Wild, M., Gerardy-Schahn, R., and Bakker, H. (2005) *J. Biol. Chem.* **280,** 27230–27235
29. Punt, P. J., and van den Hondel, C. A. (1992) *Methods Enzymol.* **216,** 447–457
30. Kabuss, R., Ashikov, A., Oelmann, S., Gerardy-Schahn, R., and Bakker, H. (2005) *Glycobiology* **15,** 905–911
31. Muraoka, M., Miki, T., Ishida, N., Hara, T., and Kawakita, M. (2007) *J. Biol. Chem.* **282,** 24615–24622
32. Record, E., Asther, M., Moukha, S., Marion, D., Burlat, V., Ruel, K., and Asther, M. (1998) *Can. J. Microbiol.* **44,** 945–953
33. Lord, J. M., Kagawa, T., Moore, T. S., and Beevers, H. (1973) *J. Cell Biol.* **57,** 659–667
34. Green, J. R. (1983) in *Isolation of Membranes and Organelles from Plant Cells* (Hall, J. L., and Moore, A. L., eds) pp. 135–152, Academic Press, London
35. Baykov, A. A., Evtushenko, O. A., and Avaeva, S. M. (1988) *Anal. Biochem.* **171,** 266–270
36. Laroy, W., Contreras, R., and Callewaert, N. (2006) *Nat. Protoc.* **1,** 397–405
37. Nierman, W. C., Pain, A., Anderson, M. J., Wortman, J. R., Kim, H. S., Arroyo, J., Berriman, M., Abe, K., Archer, D. B., Bermejo, C., Bennett, J., Bowyer, P., Chen, D., Collins, M., Coulsen, R., Davies, R., Dyer, P. S., Farman, M., Fedorova, N., Fedorova, N., Feldblyum, T. V., Fischer, R., Fosker, N., Fraser, A., García, J. L., García, M. J., Goble, A., Goldman, G. H., Gomi, K., Griffith-Jones, S., Gwilliam, R., Haas, B., Haas, H., Harris, D., Horiuchi, H., Huang, J., Humphray, S., Jiménez, J., Keller, N., Khouri, H., Kitamoto, K., Kobayashi, T., Konzack, S., Kulkarni, R., Kumagai, T., Lafon, A., Lafton, A., Latgé, J. P., Li, W., Lord, A., Lu, C., Majoros, W. H., May, G. S., Miller, B. L., Mohamoud, Y., Molina, M., Monod, M., Mouyna, I., Mulligan, S., Murphy, L., O'Neil, S., Paulsen, I., Peñalva, M. A., Pertea, M., Price, C., Pritchard, B. L., Quail, M. A., Rabbinowitsch, E., Rawlins, N., Rajandream, M. A., Reichard, U., Renauld, H., Robson, G. D., Rodriguez de Córdoba, S., Rodríguez-Peña, J. M., Ronning, C. M., Rutter, S., Salzberg, S. L., Sanchez, M., Sánchez-Ferrero, J. C., Saunders, D., Seeger, K., Squares, R., Squares, S., Takeuchi, M., Tekaia, F., Turner, G., Vazquez de Aldana, C. R., Weidman, J., White, O., Woodward, J., Yu, J. H., Fraser, C., Galagan, J. E., Asai, K., Machida, M., Hall, N., Barrell, B., and Denning, D. W. (2005) *Nature* **438,** 1151–1156
38. Schrettl, M., Bignell, E., Kragl, C., Sabiha, Y., Loss, O., Eisendle, M., Wallner, A., Arst, H. N., Jr., Haynes, K., and Haas, H. (2007) *PLoS Pathog.* **3,** 1195–1207
39. Bakker, H., Routier, F., Oelmann, S., Jordi, W., Lommen, A., Gerardy-Schahn, R., and Bosch, D. (2005) *Glycobiology* **15,** 193–201
40. Rollwitz, I., Santaella, M., Hille, D., Flügge, U. I., and Fischer, K. (2006) *FEBS Lett.* **580,** 4246–4251
41. Gao, X. D., Nishikawa, A., and Dean, N. (2001) *J. Biol. Chem.* **276,** 4424–4432
42. Mariño, K., Marino, C., Lima, C., Baldoni, L., and de Lederkremer, R. M. (2005) *Eur. J. Org. Chem.* 2958–2964
43. Tiralongo, J., Ashikov, A., Routier, F., Eckhardt, M., Bakker, H., Gerardy-Schahn, R., and von Itzstein, M. (2006) *Glycobiology* **16,** 73–81
44. Capasso, J. M., and Hirschberg, C. B. (1984) *Biochim. Biophys. Acta* **777,** 133–139
45. Peltier, P., Daniellou, R., Nugier-Chauvin, C., and Ferrières, V. (2007) *Organic Lett.* **9,** 5227–5230
46. Marlow, A. L., and Kiessling, L. L. (2001) *Organic Lett.* **3,** 2517–2519
47. Lee, R., Monsey, D., Weston, A., Duncan, K., Rithner, C., and McNeil, M. (1996) *Anal. Biochem.* **242,** 1–7
48. Yu, J. H., Hamari, Z., Han, K. H., Seo, J. A., Reyes-Domínguez, Y., and

Scazzocchio, C. (2004) *Fungal. Genet. Biol.* **41,** 973–981
49. Krappmann, S., Bayram, O., and Braus, G. H. (2005) *Eukaryot. Cell* **4,** 1298–1307
50. Suzuki, E., Toledo, M. S., Takahashi, H. K., and Straus, A. H. (1997) *Glycobiology* **7,** 463–468
51. Stynen, D., Sarfati, J., Goris, A., Prévost, M. C., Lesourd, M., Kamphuis, H., Darras, V., and Latgé, J. P. (1992) *Infect. Immun.* **60,** 2237–2245
52. Costachel, C., Coddeville, B., Latgé, J. P., and Fontaine, T. (2005) *J. Biol. Chem.* **280,** 39835–39842
53. Eckhardt, M., Gotza, B., and Gerardy-Schahn, R. (1999) *J. Biol. Chem.* **274,** 8779–8787
54. Damveld, R. A., Franken, A., Arentshorst, M., Punt, P. J., Klis, F. M., van den Hondel, C. A., and Ram, A. F. (2008) *Genetics* **178,** 873–881
55. El-Ganiny, A. M., Sanders, D. A., and Kaminskyj, S. G. (2008) *Fungal. Genet. Biol.* **45,** 1533–1542
56. Puglielli, L., and Hirschberg, C. B. (1999) *J. Biol. Chem.* **274,** 35596–35600
57. Segawa, H., Soares, R. P., Kawakita, M., Beverley, S. M., and Turco, S. J. (2005) *J. Biol. Chem.* **280,** 2028–2035
58. Capul, A. A., Barron, T., Dobson, D. E., Turco, S. J., and Beverley, S. M. (2007) *J. Biol. Chem.* **282,** 14006–14017
59. Goto, S., Taniguchi, M., Muraoka, M., Toyoda, H., Sado, Y., Kawakita, M., and Hayashi, S. (2001) *Nat. Cell Biol.* **3,** 816–822
60. Kollár, R., Reinhold, B. B., Petráková, E., Yeh, H. J., Ashwell, G., Drgonová, J., Kapteyn, J. C., Klis, F. M., and Cabib, E. (1997) *J. Biol. Chem.* **272,** 17762–17775
61. Klis, F. M., Ram, A. F. J., and De Groot, P. W. J. (2007) in *Biology of the Fungal Cell* (Howard, R. J., and Gow, N. A. R., eds) pp. 97–120, 2nd Ed., Springer, Berlin
62. Späth, G. F., Lye, L. F., Segawa, H., Sacks, D. L., Turco, S. J., and Beverley, S. M. (2003) *Science* **301,** 1241–1243
63. Dean, N., Zhang, Y. B., and Poster, J. B. (1997) *J. Biol. Chem.* **272,** 31908–31914
64. Nishikawa, A., Mendez, B., Jigami, Y., and Dean, N. (2002) *Yeast* **19,** 691–698
65. Nishikawa, A., Poster, J. B., Jigami, Y., and Dean, N. (2002) *J. Bacteriol.* **184,** 29–42
66. Zhou, H., Hu, H., Zhang, L., Li, R., Ouyang, H., Ming, J., and Jin, C. (2007) *Eukaryot. Cell* **6,** 2260–2268
67. Wagener, J., Echtenacher, B., Rohde, M., Kotz, A., Krappmann, S., Heesemann, J., and Ebel, F. (2008) *Eukaryot. Cell* **7,** 1661–1673
68. Roy, S. K., Chiba, Y., Takeuchi, M., and Jigami, Y. (2000) *J. Biol. Chem.* **275,** 13580–13587
69. Schmidt, M. (2004) *Microbiology* **150,** 3253–3260