**MNN5 Encodes an Iron-Regulated α-1,2-Mannosyltransferase Important for Protein Glycosylation, Cell Wall Integrity, Morphogenesis, and Virulence in *Candida albicans***

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The cell walls of microbial pathogens mediate physical interactions with host cells and hence play a key role in infection. Mannosyltransferases have been shown to determine the cell wall properties and virulence of the pathogenic fungus *Candida albicans*. We previously identified a *C. albicans* α-1,2-mannosyltransferase, Mnn5, for its novel ability to enhance iron usage in *Saccharomyces cerevisiae*. Here we have studied the enzymatic properties of purified Mnn5 and characterized its function in its natural host. Mnn5 catalyzes the transfer of mannose to both α-1,2- and α-1,6-mannobiose, and this activity requires Mn²⁺ as a cofactor and is regulated by the Fe²⁺ concentration. An mnn5Δ mutant showed a lowered ability to extend O-linked, and possibly also N-linked, mannans, hypersensitivity to cell wall-damaging agents, and a reduction of cell wall mannosylphosphate content, phenotypes typical of many fungal mannosyltransferase mutants. The mnn5Δ mutant also exhibited some unique defects, such as impaired hyphal growth on solid media and attenuated virulence in mice. An unanticipated phenotype was the mnn5Δ mutant’s resistance to killing by the iron-chelating protein lactoferrin, rendering it the first protein found that mediates lactoferrin killing of *C. albicans*. In summary, MNN5 deletion impairs a wide range of cellular events, most likely due to its broad substrate specificity. Of particular interest was the observed role of iron in regulating the enzymatic activity, suggesting an underlying relationship between Mnn5 activity and cellular iron homeostasis.

*Candida albicans*, an opportunistic human fungal pathogen, frequently causes life-threatening systemic infections in immuno-compromised patients (3, 35). The worldwide appearance of drug-resistant strains and the limited options of anti-*C. albicans* drugs underscore an urgent need to identify new antifungal targets for drug development. Several virulence traits have been described for this pathogen, such as the transition between yeast and hyphal forms of growth (3, 41, 57), the production of lytic enzymes (23), phenotype switching (42), adherence to host cells (9), and iron acquisition (37). Since the cell wall provides functions at the interface between microbial pathogens and host cells, it often critically determines the outcome of host-pathogen interactions. The general structure of the fungal cell wall is conserved, containing an inner layer of structural polysaccharides, glucans, and chitin and an outer layer enriched for mannoproteins (25). The highly glycosylated mannoproteins are involved in host cell adhesion, antigenicity, and modulation of host immune responses (10, 46, 54). Previous studies showed that mannoproteins of the outer layer mediate direct interactions of *C. albicans* with host cells (11, 47) and play important roles in pathogenesis (8, 48).

Studies with *Saccharomyces cerevisiae* have provided much of our current understanding of protein glycosylation in fungi. Protein glycosylation starts in the endoplasmic reticulum, where the first mannose is transferred to the OH group of a serine or threonine residue in O-linked glycosylation (45) or an oligosaccharide core structure is attached to the NH₂ group of an asparagine residue in N-linked glycosylation (26). Then the glycoproteins move to the Golgi apparatus, where the elongation of O-linked mannans and synthesis of complex N-linked glycans take place (13, 30). Many of the glycosylation steps in *S. cerevisiae* have been extensively characterized at the biochemical and genetic levels (7, 16), providing valuable knowledge for the understanding of protein glycosylation in *C. albicans*. However, to date, only a few *C. albicans* genes responsible for protein glycosylation have been studied in detail, including *MNT1* (8) and *PMT1*, -2, -4, -5, and -6 (36, 39, 48, 49). These genes are members of the *MNT* and *PMT* families specifically involved in the O-glycosylation pathway. Gene deletion experiments demonstrated that the O-linked glycans are important for virulence (8, 39, 48, 49). MNN9 was found to have a role in extending N-linked glycan outer chains and maintaining normal cell wall composition (44). MNN4 is required for mannosylphosphate transfer to the acid-labile N-mannan side chains. An mnn4Δ mutant exhibited drastically reduced cell wall mannosylphosphate content, but its virulence and interaction with macrophages were not affected (20). CalVRG4 and CalSRB1 encode proteins required for supplying the Golgi with the mannose donor GDP-mannose and are essential in *C. albicans*, indicating the importance of overall protein glycosylation to cell viability (33, 55). The Golgi GDP-Pase CalGDA1 has also been shown to be important in transporting GDP-mannose into the Golgi. A gda1 null mutant is viable but has defects in cell wall biogenesis, hyphal formation, and O-mannosylation (19). Some metal ions are known to regulate mannosyltransferase activity. Tkacz et al. (50) showed that manganese ions are an essential cofactor of Golgi-bound
mannonolyltransferases. CaPmr1 was recently found to pump Ca\(^{2+}\)/Mn\(^{2+}\) ions into the Golgi. A Capmr1 null mutant showed defects in both O- and N-glycosylation, growth dependence on supplemented calcium after entering stationary phase, and attenuated virulence (2).

We previously cloned a \textit{C. albicans} gene encoding a Golgi \(\alpha_1,2\)-mannosyltransferase with highest sequence homology to \textit{S. cerevisiae} Mnn5 and Mnn2 and thus named it CaMNN5 (1). In budding yeast, Mnn2 and Mnn5 are responsible for the branching and extension of the side chains of N-linked man- nose oligomers (38). Deletion of \textit{MNN2} or \textit{MNN5} in \textit{S. cerevisiae} results in shortened \(\alpha_1,2\)-linked mannose branches, blocking the subsequent addition of mannosylphosphate (12). CaMNN5 was initially identified for its ability to support the growth of \textit{S. cerevisiae} mutants severely defective in iron acquisi- tion under iron-limiting conditions (1). Heterologous expression in \textit{S. cerevisiae} revealed that CaMnn5 could enhance iron uptake and coprecipitate with \(^55\text{Fe}\), an activity not found for any other fungal mannosyltransferases. These intriguing features of Mnn5 prompted us to further characterize its function and role in its natural host, \textit{C. albicans}.

### MATERIALS AND METHODS

#### Strains and growth conditions.

The \textit{C. albicans} and \textit{S. cerevisiae} strains used for this study are listed in Table 1. The strains were routinely grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose), at 30°C. Iron-depleted media were prepared by adjusting the iron concentration to 50 µM. Iron-depleted media were prepared by adjusting the iron concentration to 50 µM for growth and coprecipitation with \(^55\text{Fe}\), an activity not found for any other fungal mannosyltransferases. These intriguing features of Mnn5 prompted us to further characterize its function and role in its natural host, \textit{C. albicans}.

**Table 1. Yeast strains used for this study**

| Strain | Relevant genotype | Source or reference |
|--------|------------------|--------------------|
| \textit{S. cerevisiae} | | |
| CRY2a | | |
| ScWYBC8 | | |
| ScWYBC8.1 | | |
| ScWYBC9 | | |
| ScWYBC9.1 | | |
| \textit{C. albicans} | | |
| SC5314 | Wild type | 17 |
| CAI4 | ura3::imm434/ara3::::imm434 | 14 |
| SPCa2 | CAI4, except for pmt1::hisG/pmt1::hisG UR43 | 36 |
| SPCa4 | CAI4, except for pmt2::hisG/pmt2::himG UR43 | 36 |
| SPCa6 | CAI4, except for pmt4::hisG/pmt4::hisG UR43 | 36 |
| SPCa10 | CAI4, except for pmt5::hisG/pmt5::hisG UR43 | 36 |
| SPCa8 | CAI4, except for pmt6::hisG/pmt6::hisG UR43 | 36 |
| NGY25 | CAI4, except for mann1::hisG/mnn1::::hisG UR43 | 32 |
| NGY105 | CAI4, except for mann2::hisG/mnn2::::hisG UR43 | 32 |
| CaWYBC1 | CAI4, except for MNN5::::hisG UR43 | This study |
| CaWYBC1.1 | CAI4, except for MNN5::::hisG UR43 | This study |
| CaWYBC1.2 | CAI4, except for MNN5::::hisG UR43 | This study |
| CaWYBC2 | CAI4, except for mann5::::hisG UR43 | This study |
| CaWYBC2.1 | CAI4, except for mann5::::hisG UR43 | This study |
| CaWYBC2.2 | CAI4, except for mann5::::hisG UR43 | This study |
| CaWYBC2.3 | CAI4, except for mann5::::hisG UR43 | This study |
| CaWYBC2.4 | CAI4, except for mann5::::hisG UR43 | This study |
| \textit{P. pastoris} | | |
| KM71 | arg4 his4 aux1::ARG4 | In Vitrogen |
| KMvec | KM71, except for pPIC9::HIS4 | This study |
| KMMNN5 | KM71, except for pPIC9::MNO5 HIS4 | This study |

#### Expression and purification of GST-Mnn5 fusion protein.

The region of \textit{C. albicans} MNN5 encoding amino acid residues 211 to 597 was cloned into pGEX-4T-1 (Amersham) in frame with the glutathione S-transferase (GST) gene and transformed into \textit{Escherichia coli} strain BL21 (Amersham). Purification of the GST-Mnn5 fusion protein was performed by following standard protocols (15).

#### Preparation of polyclonal antibodies.

Two rabbits (New Zealand White) were purchased from the Sembawang Laboratory Animals Centre (Singapore). Each rabbit was injected with 500 µg purified Mnn5 emulsified in complete Freund’s adjuvant (Sigma). Booster injections using the same amount of antigen emulsified in incomplete Freund’s adjuvant (Sigma) were administered every 2 weeks. The rabbits were bled 10 days after the fifth injection and after each subsequent boost. Affinity purification of Mnn5-specific antibodies was performed using the purified GST-Mnn5 fusion protein coupled to glutathione-Sepharose 4B beads. The serum was incubated with 1 ml of the beads at 4°C for 3 h. The beads were then washed extensively with cold phosphate-buffered saline (PBS) (4°C), and the bound antibodies were eluted with ImmunoPure immunoglobulin G elution buffer (Pierce).

#### Western blot analysis.

Protein concentrations were determined by the Bradford assay (Bio-Rad). Samples for Western blot analysis were resolved by 10%
sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a Hybond-C nylon membrane (Amersham). The purified anti-Mnn5 antibodies described above were used as the primary antibody. An ECL Western blot detection kit (Pierce) was used to visualize the target protein.

**Mnn5 expression in Pichia pastoris and FPLC purification of Mnn5.** The expression of *C. albicans* Mnn5 using *P. pastoris* was done as previously described (1). To purify the secreted Mnn5 protein, the culture was centrifuged to pellet the cells, and the supernatant was concentrated by passage through a Centriplus spin filter (molecular size cutoff, 10 kDa) and washed three times with and then resuspended in a small volume (1/10 the original volume) of 20 mM Tris-HCl (pH 8) before being subjected to fast-performance liquid chromatography (FPLC) separation using a Mono-Q column (10 × 100 mm; Amersham). A KCl gradient, which was increased from 0 to 0.8 M from 0 to 50 min, was used to elute the protein. Fractions (4 ml/min) were collected for SDS-PAGE and Western blot analysis to locate Mnn5. The elution buffer of the fraction containing Mnn5 was replaced with 20 mM phosphate buffer (pH 6.0) by using a Centriplus filter as described above.

**Assay of Mnn5 enzyme activity.** The enzyme assay was carried out by using 10 μl (8 μg) of purified Mnn5 as the enzyme source in a buffer containing 50 mM Tris-Cl (pH 7.2), 10 mM MnCl₂, 0.23 μM GDP-[³²P]mannose (specific activity, 289 mCi/mmol), 20 mM 2- or 6-O-α-mannopyranosyl-α-mannopyranose acceptor (Sigma), and 5 μg/ml bovine serum albumin. Standard reactions were performed in 50 μl at 30°C for 60 min. To terminate the reaction, the mixtures were passed through 0.8 ml QAE-Sephadex anion-exchange resin to remove the unlabeled GDP-mannose. The labeled product was eluted with 0.8 ml water, and radioactivity was counted in a scintillation beta counter (Amersham).

**Gene deletion.** The MNN5 gene was deleted from CAI4 by using the LR clonase method (14), which is schematically illustrated in Fig. 3A. A pair of oligonucleotides (5'-CAATTGGCCGATTTACA-3' and 5'-AACATGCGTCTTCTTGA-3') was used to PCR amplify the region upstream of the coding sequences (coding region sites were added to the PCR primers when needed. Transformants were selected on histidine- or leucine-dropout medium according to the selection markers used. Correct deletion of each gene was verified by Southern blotting (data not shown).

**O-glycan analysis.** Total cellular O-glycans were analyzed as described previously (18), with a minor modification. To radioactively label mannans, the yeast cells growing in 2 ml of YP plus 0.5% sucrose were incubated with 1.85 MBq of [2-³²P]mannose (777 GBq mmol⁻¹) (Perkin-Elmer) at 30°C for 2 h. Cells were then pelleted, resuspended in 500 μl PBS, and lysed by bead beating as previously described (1). The cell wall was then pelleted by centrifugation and lyophilized. Equal amounts of the lyophilized cell walls from different strains were treated with 0.1 M NaOH for 24 h at room temperature for β-elimination of O-glycans. The reaction was terminated by adding HCl to a final concentration of 0.1 M, and the mixture was then centrifuged at 80,000 rpm using a TLA-100.2 rotor (Beckman) for 2 h at 4°C. ³²P-labeled glycans in the supernatant were analyzed as described previously (18), except that a lower concentration (100 μCi/ml) of [2-³²P]mannose was used. The reaction was terminated by adding HCl to a final concentration of 0.1 M, and the mixture was then centrifuged at 80,000 rpm using a TLA-100.2 rotor (Beckman) for 2 h at 4°C. ³²P-labeled glycans in the supernatant were subjected to thin-layer chromatography (TLC) on Silica Gel 60G plates (Merck) in ethyl acetate-butanol-acetic acid-water (3:4:2:5:4) with two ascents. The chromatograms were then treated with EN'HANCE reagent (Perkin-Elmer) and exposed to Kodak X-OMAT X-ray film at −70°C. To quantify each labeled species, a 1-cm by 0.5-cm area of the silica plate containing each species was cut out and subjected to scintillation counting of ³²P using a scintillation beta counter (Amersham).

### RESULTS

**Overexpression of MNN5 in *C. albicans*.** Since MNN5 expression can rescue the iron acquisition defects of an *S. cerevisiae ftr1Δ* mutant (1), we first wanted to determine whether MNN5 overexpression will do the same for a *C. albicans ftr1Δ* mutant. We used several relatively strong promoters, such as the *GAL1-10, ADH1*, and *MET3* promoters, to drive MNN5 expression from either a single copy integrated at the *RP10* locus or a multiple-copy plasmid (pABS1), but none of these constructs was able to alleviate the growth deficiency of the *C. albicans ftr1Δ* mutant (data not shown) under iron-limiting conditions. We then examined whether MNN5 expression in *C. albicans* cells is regulated by iron and found that neither the mRNA nor the protein level of MNN5 was affected by iron concentrations (data not shown).

**Mnn5 rescues the defect of an *S. cerevisiae mnn5Δ* mutant but not an *mnn2Δ* mutant.** Since CaMnn5 shares high sequence homology with two *S. cerevisiae* mannosyltransferases, Mnn5 and Mnn2, we wanted to know whether CaMnn5 can functionally complement an *S. cerevisiae mnn2Δ* or *mnn5Δ* mutant. Negatively charged mannophosphate units are known to be incorporated into N-linked oligosaccharides of mannoproteins, and thus defects in N-glycosylation are often associated with a decrease in the cell’s affinity for the cationic dye Alcian blue (31, 34). Both *mnn2Δ* and *mnn5Δ* mutant strains showed a significant loss of Alcian blue binding (Fig. 1) (39). When a copy of CaMNN5 driven by the *ADH1* promoter in a 2μm plasmid was transformed into the two mutants, Alcian blue binding was significantly improved in *mnn5Δ* but not in *mnn2Δ* mutant cells, suggesting that CaMnn5 may be the counterpart of *S. cerevisiae MNN5* and may provide similar functions in *C. albicans*.

**Mnn5 has α-1,2-mannosyltransferase activity.** To analyze the enzymatic activity of C. albicans Mnn5, we used a *P. pastoris* expression system to produce soluble, secreted protein.
Materials and Methods. [14C]GDP-mannose was used as the mannose donor, and α-1,2- and α-1,6-mannobiose were used as acceptors. Purified Mnn5 (8 μg) was used in each reaction. The same amount of bovine serum albumin was used as a negative control. The radioactive products were isolated for quantification in a scintillation counter, except with reaction buffers at different pHs. The same pattern was observed for the dependence of Mnn5 activity on pH, expressed as counts per minute (cpm)/mg of protein/h. The pH dependence of Mnn5 activity (b) was evaluated using the same protocol, except that the reaction buffer (pH 6.0) contained a 0.1, 1, or 10 mM concentration of one of the divalent metal ions, as indicated. (D) Ferrous iron regulates Mnn5 activity in the presence of Mn2+. The assay buffer (pH 6.0) contained 10 mM Mn2+ and was supplemented with different amounts of BPS and ferrous ammonium sulfate, as indicated at the bottom of the graph.

Figure 2A (lane 2) shows that the culture supernatant of the P. pastoris strain expressing MNN5 contained a major 64-kDa protein that matches the predicted size of Mnn5 but was not present in the culture supernatant of the strain transformed with the empty vector (Fig. 2A, lane 3). To purify the protein, the supernatant was subjected to FPLC using a Mono-Q anion-exchange column and eluted with a KCl gradient. SDS-PAGE and Western blot analysis were performed to locate the fractions that contained Mnn5. The purified protein (Fig. 2A, lane 4) was subjected to a mannosyltransferase activity assay. Figure 2B (top) shows that the purified Mnn5 protein catalyzed the transfer of [14C]GDP-mannose to both α-1,6- and α-1,2-mannobiose, with similar activities. We also found that Mnn5 exhibited high activities at pH 6.0 and 9.0 under the assay conditions used (Fig. 2B, bottom).

Mnn5 requires Mn2+ and Fe2+ for activity. Several Golgi mannosyltransferases are known to require Mn2+ as an essential cofactor. Since iron was previously found to bind to Mnn5 (1), we tested whether it was required for the enzymatic activity of Mnn5 in the presence of Mn2+. Adding different amounts of the iron chelator BPS to the reaction buffer was found to inhibit the enzyme activity in a concentration-dependent manner (Fig. 2D). In the presence of 0.1, 0.2, and 0.4 mM BPS, the enzymatic activity was lowered to 50, 30, and 18% of the nonchelated control, respectively, suggesting that iron is required for the mannosyltransferase activity of Mnn5. This observation was further substantiated by the restoration of the enzymatic activity when Fe2+ was added to the buffer containing 0.4 mM BPS. Adding Fe2+ to the reaction buffer without BPS slightly enhanced the enzyme activity when the concentration of the added iron was within 2 to 10 μM, suggesting that the reaction mix initially contained sufficient iron. The ferrous iron chelator desferri-ferrichrome had no effect on Mnn5 activity (data not shown), suggesting that ferrous but not ferric iron is required. Adding other metal ions, such as Cu2+, Mn2+, and Mg2+, did not relieve the inhibition by BPS. Depleting Cu2+ by adding the chelator 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline-sulfonlic acid (BCS) also had no effect on the enzyme activity (data not shown). Taken together, the results indicate that Mn2+ is an essential cofactor for the mannosyltransferase activity of Mnn5 and that the activity is regulated by ferrous iron, consistent with its iron-binding ability detected in our previous study (1).

Construction of MNN5 deletion mutant. To investigate MNN5’s cellular functions in C. albicans, we used two different gene deletion cassettes, hisG-URA3-hisG and cat-URA3-cat, to sequentially delete the first and second copies of MNN5 from strain CAI4, as described previously (14, 22) (Fig. 3A). Figure 3B shows the strategy for reintroducing a wild-type MNN5 copy into the mutants by integration at the hisG locus. The genotypes of the heterozygous and homozygous mutants and reintegrants were verified by Southern blot analysis (Fig. 3C).

We determined the growth rate of the mnn5Δ mutant over a wide range of iron concentrations but did not observe a significant difference in comparison with the wild-type strain (data not shown). Also, the mnn5Δ mutant did not exhibit reduced 59Fe uptake under all conditions used (data not shown). The results indicate that unlike its function in the heterologous host S. cerevisiae, Mnn5 may not be involved in iron uptake in its natural host (see Discussion).
Study of Mnn5’s role in N-linked and O-linked glycosylation.

The *S. cerevisiae* mnn5/H9004 mutant is impaired in N-linked glycosylation. To test whether Mnn5 is involved in N-glycosylation, we performed an Alcian blue binding assay on mnn5/H9004 mutant cells. The assay showed drastically decreased binding of the dye to mnn5/H9004 cells compared to that for the wild-type cells (Fig. 4A). Approximately 90% of the dye coprecipitated with the wild-type cells, in contrast to only 20% with mnn5/H9004 mutant cells. Reintroducing a wild-type copy of MNN5 into mnn5/H9004 cells restored the dye binding activity. We previously showed that mutation of either one of the aspartic acid (D) residues to alanine (A) in the conserved DXD282-284 motif of Mnn5 totally abolished the mannosyltransferase activity (1). Reintroducing a mutated mnn5 gene (D282A or D284A) into the mnn5/H9004 mutant did not restore cell binding of the dye (Fig. 4A), indicating that Alcian blue binding ability is dependent on the enzyme activity of Mnn5. Taken together, the results show that the amount of cell surface mannosylphosphate is significantly reduced in the mnn5/H9004 mutant, which is most likely the consequence of blocked synthesis of N-linked mannan branches.

To determine whether the mnn5/H9004 mutant also has defects in O-glycosylation, we grew cells in the presence of [2-3H]mannose to label the mannose units of glycosylated proteins. The O-linked mannans were released by \(/\)-elimination and resolved by TLC. The \(/\)-elimination condition used was previously shown to completely release O-glycans from mannoproteins (18). An autoradiograph of the TLC plate (Fig. 4B) displays five major 3H-labeled molecules containing from one
to five mannose residues (M1 to M5), as previously shown by Herrero et al. (19). When the amounts of each 3H-labeled species were compared between the wild type and the mnn5Δ mutant, we found that the mnn5Δ mutant showed a markedly higher level of M1 and less M2, M3, and M5 than the wild type, suggesting an impaired ability of the mutant to extend O-linked mannan chains. The area on the TLC plate corresponding to each radioactive spot was cut out and subjected to quantification by scintillation counting of 3H (Fig. 4C). M1 was found to represent 16.6% of the total 3H count for all five species (M1 to M5) in the wild-type strain, whereas the percentage increased to 46.6% in the mutant, showing that a large fraction of the first O-linked mannose residue cannot be further extended in the absence of Mnn5. The radioactive signal at the origin of sample loading should largely represent N-glycans, which are resistant to β-elimination and remain attached to the proteins. Quantification of the 3H-labeled glycans at the origin showed a 50% reduction in the mnn5Δ mutant in comparison with the wild-type strain. These results indicate that Mnn5 is involved in extending O-linked, and likely also N-linked, mannans.

The mnn5Δ mutant exhibits markedly reduced sensitivity to LF. Lactoferrin (LF) is a mammalian iron-binding protein abundantly present in saliva, milk, and other exocrine secretions. It constitutes an important part of innate immunity, partly because its strong iron-chelating activity effectively restricts iron availability to invading pathogens. It is highly conserved in eukaryotes and is critical to innate immunity against various bacterial and fungal pathogens. Lactoferrin is abundant in saliva, milk, and other exocrine secretions and is known to inhibit the growth of several pathogens, including C. albicans, by chelating iron and depleting the iron pool available to the pathogen. Our results reveal that Mnn5 has a role in determining a cell’s sensitivity to LF, which depends on cellular iron homeostasis. Is the increased resistance to LF killing a unique phenotype of the mnn5Δ mutant or one shared by other mannosyltransferase mutants? To answer this question, we tested the LF sensitivity of a series of mannosyltransferase mutants, including pmt1Δ, pmt2Δ/PMT2, pmt4Δ, pmt5Δ, pmt6Δ, mnt1Δ, and mnt2Δ mutants (8, 32, 36). We found that only the mnn5Δ mutant exhibited increased resistance to LF killing (Table 2), indicating a unique role of Mnn5 in mediating LF killing of C. albicans.

The mnn5Δ mutant shows a weakened cell wall. For both S. cerevisiae and C. albicans, the deletion of genes encoding mannosyltransferases has been reported to cause defects in cell wall integrity. To determine whether the mnn5Δ mutant has a similar defect, we tested the sensitivity of the mnn5Δ mutant to lyticase, an enzyme normally used to remove the fungal cell wall. Exponential-phase cells grown in YPD were diluted to an OD600 of 0.6 in a hypo-osmotic solution containing 10 mM Tris and 0.2 mg/ml lyticase. The extent of cell lysis was monitored by measuring the OD600 of the cell suspension at timed intervals. Figure 6A shows that the mnn5Δ cell suspension had dropped from the increased resistance to LF killing a unique phenotype of the mnn5Δ mutant or one shared by other mannosyltransferase mutants? To answer this question, we tested the LF sensitivity of a series of mannosyltransferase mutants, including pmt1Δ, pmt2Δ/PMT2, pmt4Δ, pmt5Δ, pmt6Δ, mnt1Δ, and mnt2Δ mutants (8, 32, 36). We found that only the mnn5Δ mutant exhibited increased resistance to LF killing (Table 2), indicating a unique role of Mnn5 in mediating LF killing of C. albicans.
revealed that from 0.58 to 0.2. Microscopic examination of the cell suspension 0.52 by 1 h and dropped to 0.2 by 5 h. The heterozygous strains included in the assay: SC5314, CaWYBC1 (MNN5), SC5314, CaWYBC1 (MNN5), and CaWYBC2.2 (mnn5 Δ MNN5). Exponential-phase yeast cells of the strains tested were diluted to an OD

onto agar plates containing either 200 μg/ml Congo red or 200 μg/ml hygromycin B. The plates were incubated at 30°C for 3 days.

FIG. 6. The mnn5Δ mutant is hypersensitive to lyticase, Congo red, and hygromycin B. (A) Lyticase sensitivity assay. The following strains were included in the assay: SC5314, CaWYBC1 (MNN5/mnn5Δ), CaWYBC1,2 (MNN5/mnn5Δ MNN5), CaWYBC2 (mnn5Δ), and CaWYBC2.2 (mnn5Δ MNN5). Exponential-phase yeast cells of the strains tested were diluted to an OD

Mutant strain showed normal hyphal development in all the liquid inducing media used but no or poor hyphal growth on some solid inducing media, such as RPMI, serum, Spider, and embedding media (data not shown). Injection with SC5314 killed all animals within 1 week, whereas 80% of the mice injected with the mnn5Δ mutant survived the full 35-day course of observation. The mice injected with the reintegrant strain showed a mean survival time of 10 days (Fig. 8), again indicating a gene dosage effect. The results show that MNN5 is required for the virulence of C. albicans in this infection model.

DISCUSSION

CaMnn5 shares nearly equal sequence similarities with S. cerevisiae Mnn5 and Mnn2, but our complementation test showed that CaMNN5 restored Alcian blue binding to the cell surface of mnn5Δ but not mnn2Δ mutant cells, suggesting that CaMNN5 may be the functional counterpart of MNN5. Camnn5Δ and mnn5Δ mutants share a range of similar defects, such as a reduction of cell surface binding of Alcian blue and hypersensitivity to cell wall-damaging agents. The C. albicans mnn5Δ mutant exhibited defects in O-linked glycosylation, as β-elimination of O-linked glycans yielded a dramatic increase in the mannose monomer (M1) and a decrease in mannose oligomers (M2, M3, and M5).
glycosylation, as evidenced by the decreased Alcian blue binding and the amount of β-elimination-resistant glycans (Fig. 4). In addition to the defects commonly associated with mutants of mannosyltransferase genes in yeast, the mnn5Δ mutant also exhibited some phenotypes unique to C. albicans, such as a markedly reduced sensitivity to LF killing, a lack of hyphal growth on solid media, and attenuated virulence in mice. Of particular importance, Mnn5 activity was found to be regulated by iron, and manifestation of some of the phenotypes of the mnn5Δ mutant appears to be affected by the cellular iron status.

Since MNN5 was first cloned for its unanticipated ability to support S. cerevisiae growth in the presence of high concentrations of iron chelators, we were interested in knowing whether it may perform similar functions in C. albicans. Surprisingly, overexpression of MNN5 in C. albicans did not enhance cell growth, and the mnn5Δ mutant did not exhibit any growth defects under iron-limiting conditions. This discrepancy might be due to the different functions manifested by Mnn5 in the two different biological systems. The growth-enhancing activity of C. albicans Mnn5 found in S. cerevisiae under iron-limiting conditions appeared to be largely, if not entirely, attributable to the novel iron-binding ability of the protein, because the growth enhancement did not require mannosyltransferase activity but was abolished when the putative iron-binding sites were mutated (1). On the other hand, the effect of CaMnn5’s mannosyltransferase activity on budding yeast may be quite limited, possibly because of the redundant functions provided by host mannosyltransferases such as Mnn5. Consistently, overexpression of either MNN2 or MNN5 in S. cerevisiae also had no discernible effect (1). Nevertheless, the iron-dependent growth-enhancing activity of CaMnn5 found in S. cerevisiae revealed a novel property of the protein, namely, iron binding. In this study, we established that the mannosyltransferase activity of C. albicans Mnn5 is regulated by iron, being activated at low and inhibited at high iron concentrations (Fig. 2D). Also, the observation that high concentrations of BPS (100 to 400 μM) were needed to significantly reduce the enzymatic activity of purified Mnn5 suggests a high affinity of the protein for iron, consistent with the previously observed coprecipitation of 55Fe with Mnn5 (1). It is an attractive possibility that Mnn5 may regulate certain cell functions by altering protein glycosylation in response to the cellular or environmental iron status. In further support of this hypothesis, we observed a parallel between the mnn5Δ mutant’s resistance to LF killing and the significantly enhanced LF resistance of wild-type C. albicans cells grown under iron-limiting conditions. Iron is an essential nutrient for almost all organisms. Keeping free iron away from microbial pathogens is an important element of host innate immunity. Thus, tactics for the efficient acquisition and use of iron constitute virulence factors for many microbial pathogens, including C. albicans (37). At the moment, we do not know whether the iron regulation of Mnn5 is of specific importance for C. albicans survival in the host. Although the mnn5Δ mutant is much less virulent than the wild-type strain, this could be the result of many compromised cellular functions. The isolation of MNN5 mutants that have lost the re-

FIG. 7. The mnn5Δ mutant is defective in hyphal growth on some solid media. Yeast cells of strains SC5314 and CaWYBC2 (mnn5Δ) were inoculated onto several hypha-inducing solid media, as indicated, and incubated at 37°C for 3 days.

FIG. 8. The mnn5Δ mutant showed markedly reduced virulence. Strains SC5314, CaWYBC2 (mnn5Δ), and CaWYBC2.2 (mnn5Δ MNN5) were grown in GMM at 30°C to exponential phase. A 200-μl volume of 1 × 10⁶ cells of each strain in PBS was injected into each animal through the tail vein. Eight mice were used for each strain, and their survival was monitored daily for 35 days. The experiment was done twice, with similar results.
sponse to iron may help to answer this question in future. Nevertheless, for the purpose of drug development, Mnn5 may serve as a good target.

The ability of \textit{C. albicans} to switch from yeast to filamentous growth is important for pathogenicity (28, 57). The outer layer of the fungal cell wall is rich in mannan proteins, and mutations in genes responsible for constructing mannan structures have been reported to cause various degrees of filamentous growth defects in \textit{C. albicans} (44, 48, 49). For example, all \textit{pmt} mutations except \textit{pmt5} were reported to suppress hyphal morphology under standard inducing conditions (36). In this study, we found that the loss of \textit{MNN5} function blocked hyphal formation on several solid hypha-inducing media, but not in the corresponding liquid media. Although the reasons remain unclear, it might be that hyphal growth on and penetration into solid media are much more demanding on cell wall strength and integrity than growth in liquid media or that hyphal growth on solid medium requires certain cell surface properties which are lost in the mutant. Such conditional hyphal growth defects have been described for many \textit{C. albicans} mutants (4, 48, 49), underscoring the high degree of complexity of the signals that feed into the signal transduction pathways for filamentous growth. The impaired hyphal growth observed as a result of various mannosyltransferase gene mutations suggests that the defect might be a consequence of the loss of proper glycosylation of many cell surface proteins important for the construction of hyphal morphology. However, it remains possible that some members of the mannosyltransferase family may have more specific roles in regulating cell morphogenesis. Indeed, Timple et al. (48, 49) found that overexpression of genes encoding components involved in hyphal formation suppressed the hyphal growth defect of a \textit{pmt6} mutant, suggesting that \textit{Pmt6} may act upstream of the morphogenetic signaling pathways.

LF is a 77-kDa iron-binding protein present in milk and mucosal secretions. It is also released by specific granules of polymorphonuclear leukocytes during inflammation. LF has been ascribed many diverse biological functions, most of which are immunomodulatory or antibacterial (5, 51, 52). It inhibits microbial growth by effectively sequestering free iron from the environment. The protein and its N-terminal fragment (amino acids 1 to 11) are also known to have direct bactericidal and candidcidal activities (6, 21). However, the mechanisms of cell killing are not understood. Some studies have suggested that the peptide targets energized mitochondria, causing the production of ATP and reactive oxygen species. The reactive oxygen species may lead to a reduction in the cellular thiol level, whereas ATP interacts with the LF-interacting proteins in the cell envelope. These events are thought to collectively contribute to cell death (29). We demonstrated that the \textit{mnn5Δ} mutant lost sensitivity to LF killing and that this effect depends on the cellular iron status, rendering \textit{MNN5} the first gene identified that mediates the candidcidal activity of LF. \textit{Mnn5} is an intracellular protein and thus unlikely to be the direct target of LF. It is more plausible that Mnn5 modifies some cell surface proteins which are responsible for the interaction with LF or the peptide. The failure of enzyme-dead Mnn5 to restore LF sensitivity to the \textit{mnn5Δ} mutant supports this view. Importantly, among a total of eight \textit{C. albicans} mannosyltransferase mutants tested, only the \textit{mnn5Δ} mutant exhibited increased resistance to LF. In contrast, several mutants exhibited increased sensitivity to either Congo red, hygromycin B, or both. These observations strongly suggest that Mnn5 has a specific role in determining \textit{C. albicans} sensitivity to LF, while the functions of multiple mannosyltransferases are required for resistance to cell wall-damaging agents or certain antifungal compounds. Our finding provides important information for further elucidation of the mechanisms underlying the LF killing of \textit{C. albicans}. Given the potent candidcidal activity of LF, finding the LF target and designing compounds that may mimic LF activity could be a worthy strategy for developing new anti-\textit{C. albicans} therapies.

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