# Inactivation of CaMIT1 inhibits Candida albicans phospholipomannan beta-mannosylation, reduces virulence, and alters cell wall protein beta-mannosylation

Céline Mille, Guilhem Janbon, Florence Delplace, Stella Ibata-Ombetta, Claude Gaillardin, Gerard Strecker, Thierry Jouault, Pierre-André Trinel, Daniel Poulain

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 46, Issue of November 12, pp. 47952–47960, 2004

*Printed in U.S.A.*

# Inactivation of *CaMIT1* Inhibits *Candida albicans* Phospholipomannan β-Mannosylation, Reduces Virulence, and Alters Cell Wall Protein β-Mannosylation*

Received for publication, May 18, 2004, and in revised form, September 3, 2004
Published, JBC Papers in Press, September 3, 2004, DOI 10.1074/jbc.M405534200

**Celine Mille‡§, Guilhem Janbon¶, Florence Delplace‖, Stella Ibata-Ombetta‡, Claude Gaillardin**, Gérard Strecker‖, Thierry Jouault‡, Pierre-André Trinel‡, and Daniel Poulain‡ ‡‡**

*From the ‡Laboratoire de Mycologie Fondamentale et Appliquée, Equipe Inserm E0360, Faculté de Médecine, Pôle Recherche, Place de Verdun, 59045 Lille Cedex, ¶Unité de Mycologie Moléculaire, Institut Pasteur, 28 Rue du Dr Roux, 75015 Paris, ‖Unité de Glycobiologie Structurale et Fonctionnelle, Université des Sciences et Technologies de Lille, Unité Mixte de Recherche 8576 CNRS, 59655 Villeneuve D'Ascq Cedex, and the **Laboratoire de Génétique Moléculaire et Cellulaire, Institut National Agronomique Paris-Grignon, 78850 Thiverval-Grignon, France*

**Studies on *Candida albicans* phospholipomannan have suggested a novel biosynthetic pathway for yeast glycosphingolipids. This pathway is thought to diverge from the usual pathway at the mannose-inositol-phospho-ceramide (MIPC) step. To confirm this hypothesis, a *C. albicans* gene homologue for the *Saccharomyces cerevisiae SUR1* gene was identified and named *MIT1* as it coded for GDP-mannose:inositol-phospho-ceramide mannose transferase. Two copies of this gene were disrupted. Western blots of cell extracts revealed that strain *mit1*Δ contained no PLM. Thin layer chromatography and mass spectrometry confirmed that *mit1*Δ did not synthesize MIPC, demonstrating a role of *MIT1* in the mannosylation of *C. albicans* IPCs. As *MIT1* disruption prevented downstream β-1,2 mannosylation, *mit1*Δ represents a new *C. albicans* mutant affected in the expression of these specific virulence attributes, which act as adhesins/immunomodulators. *mit1*Δ was less virulent during both the acute and chronic phases of systemic infection in mice (75 and 50% reduction in mortality, respectively). *In vitro*, *mit1*Δ was not able to escape macrophage lysis through down-regulation of the ERK1/2 phosphorylation pathway previously shown to be triggered by PLM. Phenotypic analysis also revealed pleiotropic effects of *MIT1* disruption. The most striking observation was a reduced β-mannosylation of phosphopeptidomannan. Increased β-mannosylation of mannoproteins was observed under growth conditions that prevented the association of β-oligomannosides with phosphopeptidomannan, but not with PLM. This suggests that *C. albicans* has strong regulatory mechanisms associating β-oligomannoses with different cell wall carrier molecules. These mechanisms and the impact of the different presentations of β-oligomannoses on the host response need to be defined.**

A series of studies has established that specific oligomannose sequences synthesized by *Candida albicans* are among the virulence attributes of this important opportunistic pathogen. These oligomannoses are linked through β-1,2 bonds that confer a unique spatial conformation (1) recognized by innate and acquired immunity of the host. In contrast to the ubiquitous α-linked mannose residues, β-1,2 oligomannosides induce protective antibodies (2), do not bind to C-lectins but to galectin-3 (3), trigger macrophages to produce TNF-α (4), and inhibit mouse gut colonization (5). In *C. albicans,* homopolymers of β-1,2 oligomannosides have been shown to be associated by phosphodiester bridges with phosphopeptidomannan (PPM),[1] commonly termed mannan, and to correspond to the acid-labile fraction of this molecule (6). Western blots of *C. albicans* cell wall extracts show that β-oligomannoside epitopes co-localize with α-oligomannoside epitopes on mannosylated proteins (7) and are electively expressed on low molecular weight antigens that have been characterized as phospholipomannan (PLM) (8). Besides PPM, PLM is the second *C. albicans* molecule in which the presence of β-1,2 oligomannosides has been demonstrated chemically (9). PLM appears as a member of the cell membrane MIPC family from which it diverges by the addition of mannose and phosphate linking long linear chains of β-mannosides (10). This large polysaccharide moiety confers hydrophilic properties on the molecule that allow it to diffuse into the cell wall (11). PLM is shed by *C. albicans* in contact with host cells (12) and, through β-oligomannoses of its polysaccharide moiety, induces TNF-α synthesis through a toll-like receptor-2-NFκB activation pathway (13). The same molecule is also at the center of the host cell response, but the mechanisms for this have not yet been elucidated. Finely tuned mechanisms induced by *C. albicans* PLM and involving the ERK pathway are among the non-adaptative events (14) but can transform *Saccharomyces cerevisiae* into a macrophage-surviving yeast through the induction of macrophage apoptosis (15).

In parallel, studies on β-1,2 oligomannoside expression in *C. albicans* have shown that the association of these sequences with different carrier molecules such as PPM, mannoproteins, and PLM is dependent on growth conditions like pH (16) and temperature likely to be encountered in host tissue (17). At low pH and high temperature, cell wall β-mannosides are not as-

* This work was supported by the Réseau Infection Fongique. The mass spectrometry facility used in this study is funded by the European Community (FEDER), the Région Nord-Pas de Calais (France), the CNRS, and the Université des Sciences et Technologies de Lille. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a grant from the Fondation pour la Recherche Médicale.

‡‡ To whom correspondence should be addressed. Tel.: 33-3-20-62-34-20; Fax: 33-3-20-62-34-16; E-mail: dpoulain@univ-lille2.fr.

[1] The abbreviations used are: PPM, phosphopeptidomannan; PLM, phospholipomannan; IPC, inositol-phospho-ceramide; MIPC, mannose IPC; $M(IP)_2C$, mannose-di-inositol-phospho-ceramide; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.

sociated with proteins but almost exclusively with PLM. Depending on the co-receptors involved, these differences in presentation could lead the same molecules to trigger different immune pathways.

Despite the recognized role of β-mannosides in *C. albicans* virulence, the enzymes responsible for the synthesis of β-mannosides and/or the genes encoding them have not yet been identified. In this study, elucidation of the structure of PLM and complete sequencing of the *S. cerevisiae* and *C. albicans* genomes enabled the identification of a candidate gene in the MIPC pathway, an analogue of *SUR1* in *S. cerevisiae* (18), which was named *MIT1* in *C. albicans*. Inactivation of this gene prevented PLM β-mannosylation. The constructed mutants exhibited normal growth and morphotypes but displayed reduced virulence. It was interesting to note that PPM β-mannosylation was altered in the mutant at neutral pH, whereas at low pH the mutant compensated for the absence of β-mannosides in PPM and PLM by β-mannosylating cell wall mannoproteins. These results suggest that the expression of β-1,2 oligomannosides, which are among the virulence attributes of *C. albicans*, is closely regulated in this species.

## EXPERIMENTAL PROCEDURES

*Strains and Medium*—The *C. albicans* strains used in this study are listed in Table I. Strains were grown on YPD-uridine medium (1% yeast extract, 2% peptone, 2% dextrose, 20 mg/l uridine) at 37 °C for 16 h. *S. cerevisiae* strain Su1 was included as a reference strain for the study of yeast-macrophage interactions (19). Synthetic dextrose was prepared as described previously (20). When required, 5-fluoroorotic acid was added to synthetic dextrose medium at a concentration of 1 g/liter. The bacterial strain *Escherichia coli* XL1-blue (Stratagene, La Jolla, CA) was used for propagation of all plasmids. All procedures for manipulating DNA were performed as described previously (21).

*Disruption of MIT1*—The two copies of the *MIT1* gene were deleted sequentially from strain CAI4 by the URA blaster method (22). The gene deletion strategy is shown in Fig. 3. Two ~1-kb DNA fragments specific to the *MIT1* upstream and downstream regions were amplified by polymerase chain reaction (PCR) using an HFPCR kit (Clontech) and the primer couples MIT1–5′5 (ATGTATGTGCTCATGGGTGCT), MIT1–5′3 (AGATCTGGAAACAGTTGGTGTTGTTGGG) and MIT1–3′5 (AGATCTGGATCCGGTTGTCATCATCTTCCTCGT), MIT1–3′3 (GACACGATTCTTCCATGGCATC), respectively. Amplified fragments were then cloned in a pGEM-t Easy cloning vector (Promega), resulting in plasmids pAL35 and pAL36, respectively. The BglII-SphI fragment of pAL36 was then cloned at the BglII-SphI sites of the pAL35 plasmid to give plasmid pAL39. Finally, plasmid pAL41 was obtained by inserting the BglII-BamHI fragment of the pCUB-6 plasmid (22) and contained the *hisG-URA3-hisG* gene at the BglII-BamHI sites of pAL39. *mit1*-Δ::*hisG-URA3-hisG* was released from the plasmid by SpeI digestion and used to transform CAI4 by the lithium acetate method described previously (23). Clones of *MIT1*/*mit1*-Δ::*hisG-URA3-hisG* were formed on synthetic dextrose plates, and cells were spread onto 5-fluoroorotic acid plates to isolate *MIT1*/*mit1*-Δ::*hisG* clones. A second round of transformation was performed to delete the second allele by the same method. Homologous integration of the cassette was checked by Southern blotting using DNA extracted from each strain and digested with SpeI. Released fragments were revealed with a *MIT1*-specific probe (pAL38/NotI; see below).

*Reintroduction of MIT1 into Mutant Cells*—The complete *MIT1* open reading frame and ~1-kb 5′ and 3′ regions were amplified by PCR using an HFPCR kit (Clontech) and the primer couples MIT1–5′5 and MIT1–3′3. The amplified fragment was cloned in a pGEM-t Easy cloning vector (Promega), resulting in plasmid pAL38. Plasmid pAL51 was obtained by inserting the NotI-NotI fragment of pAL38 into the SmaI site of plasmid pRC3915 (24) after fill in of the cohesive ends. Before transformation of the *mit1*-Δ::*hisG*/*mit1*-Δ::*hisG* strain, the plasmid was cut with SphI to target integration at the *LEU2* locus.

*Microscopic Observations*—Hyphae formation was induced on 5% horse serum, 2% agar, and on Spider medium (1% nutrient broth, 1% mannitol, 0.2% $K_2HPO_4$, 2% agar). Chlamydospore formation was analyzed in medium containing 17 g/liter corn meal agar (Difco), 0.33% Tween 80, 2% agar. Growth was performed at 28 or 37 °C for 2–5 days. For sensitivity assays to chemical agents, 5 μl of serial 1:10 dilutions of an overnight culture were spotted onto YPD agar (YPD medium, 2% agar) containing either 25 or 50 mM $CaCl_2$, or 10, 20, or 50 μg/ml calcofluor white, or 0.001, 0.01, or 0.05% sodium dodecyl sulfate (SDS). Plates were incubated at 37 °C for 1 day.

*Whole-cell Extraction*—Strains were grown at 37 °C on YPD-uridine at pH 6.0 or 2.0 (the pH was adjusted from 6.0 to 2.0 by adding 6 N HCl; Ref. 25) and were extracted by alkaline extraction under reducing conditions (26). Briefly, cells were incubated on ice in 1.85 M NaOH and 5% β-mercaptoethanol. Proteins and glycoconjugates were then extracted in SDS for 5 min at 100 °C. Extracts were adjusted to the same protein concentration and analyzed by SDS-PAGE (27) on a 5–20% acrylamide gel slab. Membranes were probed with monoclonal antibody 5B2 specific for β-1,2 oligomannoside epitopes (26, 28) (diluted 1:2000) and then incubated with a 1:2000 dilution of alkaline-phosphatase-conjugated anti-rat IgM (Southern Biotechnology Laboratories, Birmingham, AL).

*Phosphopeptidomannan Extraction*—PPM was extracted as described previously (29, 30) from cells grown in neutral or acidic YPD-uridine as described above. Briefly, cell pellets were suspended in 0.02 M citrate buffer and autoclaved at 125 °C for 90 min. Suspensions were harvested, and Fehling's solution was added to the supernatant to precipitate PPM. The PPM was then washed in methanol/acetic acid (8/1) and dried in a Speed Vac concentrator. Sugar concentrations were estimated by the sulfuric phenol colorimetric method (31). PPM was analyzed by SDS-PAGE as described above.

*Analysis of Sphingolipids*—Strains were grown on Sabouraud's dextrose agar (Sabouraud's dextrose broth (30 g/liter; Difco), agar (15 g/liter)) for 16 h at 37 °C, washed in phosphate-buffered saline, and treated with cold 5% trichloroacetic acid. After two washes in water, cells were extracted twice in ethanol/water/diethylether/pyridine/ammonia (15/9/5/1/0.018) (32) for 1 h at 60 °C. The supernatants were pooled and dried in a Speed Vac concentrator. Then each sphingolipid extract was treated successively with chloroform/methanol (10/10), chloroform/methanol/water (10/10/1), and chloroform/methanol/water (10/10/3). The different supernatants were pooled, analyzed by thin layer chromatography (TLC) on 10 × 5 cm silica gel 60 plates (Merck) using a chloroform/methanol/ammonia 4.2 N (9/7/2) solvent system and revealed with orcinol. The sphingolipid extracts were finally analyzed by mass spectrometry. Electrospray mass measurements were carried out in negative ion mode on a triple quadrupole instrument (Micromass Ltd, Altrincham, UK) fitted with an atmospheric pressure ionization electrospray source. A mixture of polypropylene glycol was used to calibrate the quadrupole mass spectrometer. The samples were dissolved in dimethyl sulfoxide and further diluted in methanol to obtain a final concentration of ~0.25 μg/μl. Solutions were infused using a Harvard syringe pump at a flow rate of 3 μl/min. The quadrupole was scanned from 400 to 2200 Da with a scan duration of 6 s and a scan delay of 0.1 s. The samples were sprayed using a 3.5-kV needle voltage, and the declustering cone was set at 70 V.

*Animal Model for Assessment of Virulence*—Strains were grown on YPD-uridine at 37 °C for 16 h and then suspended at a density of $10^6$ yeasts/ml in phosphate-buffered saline. 200 ml of each suspension were injected intravenously into the lateral tail vein of Swiss mice (7 weeks of age, female). The survival of each mouse was monitored until day 25 after injection.

*Yeast-Macrophage Interactions*—The mouse macrophage-like cell line, J774 (ECACC 85011428), which was derived from a tumor in a female BALB/c mouse, was cultured at 37 °C in an atmosphere containing 5% $CO_2$ in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (Valbiotech, Paris, France), 5 mM L-glutamine, 100 μg/ml streptomycin, and 50 μg/ml penicillin. Before each experiment, cells were gently scraped with a rubber policeman, distributed into 12-well culture plates at a concentration of $10^6$ cells/well, and incubated for 18 h to allow adherence. Plated cells were incubated with yeasts at a concentration of 20 yeasts/J774 cell. After 30 min of incubation, the cells were washed to remove unbound yeasts and prepared for either biochemical analysis or fungicidal assays.

*Fungicidal Assays*—J774 cells were incubated for 30 min at 37 °C with yeast cells, washed with Dulbecco's modified Eagle's medium, and then recultured at 37 °C for a further 90 min. The cultures were washed with Dulbecco's modified Eagle's medium, and endocytosed yeasts were released by lysing the J774 cells with sterile water. The yeast cells recovered were counted, and 100 individual cells in 1 ml of phosphate-buffered saline were plated onto Sabouraud's dextrose agar. After incubation for 24 h, the number of colony-forming units was determined.

*ERK1/2 Phosphorylation Analysis*—Stimulated cells were washed with 1 ml of ice-cold phosphate-buffered saline containing 1 mM $Na_3VO_4$ and 10 mM NaF. Cultures were extracted with 500 μl of boiling 2× concentrated electrophoresis sample buffer (1× 125 mM Tris-HCl, pH

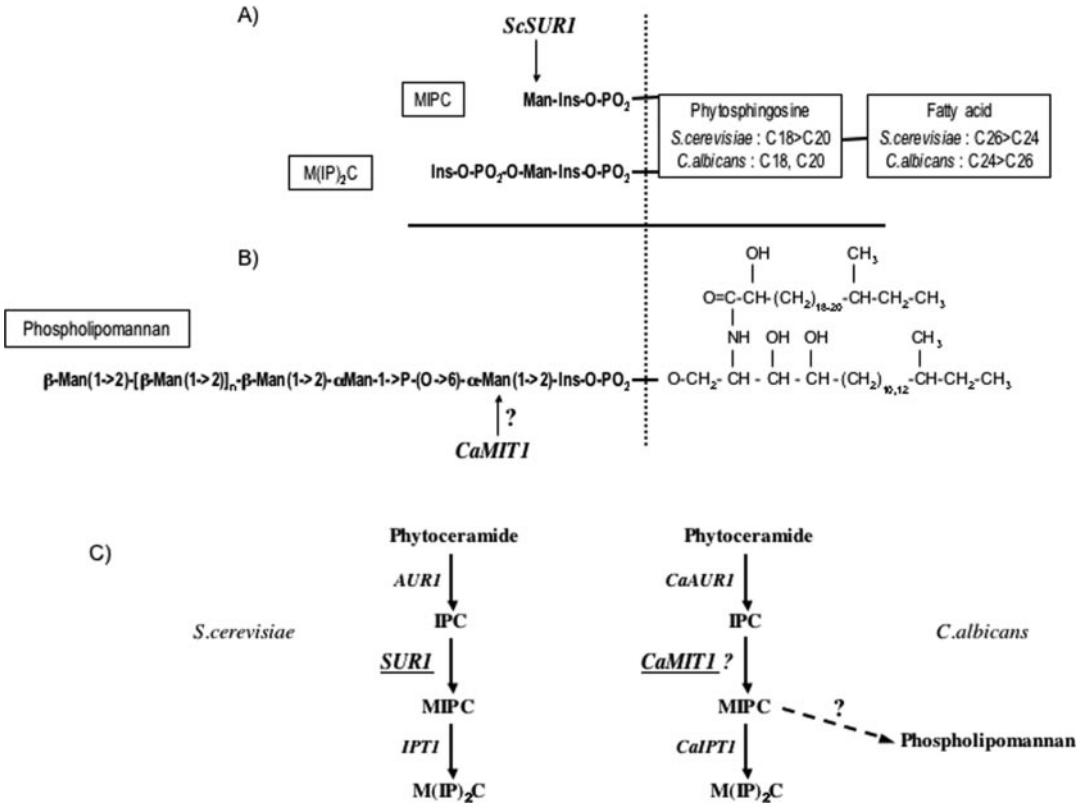


FIG. 1. **Comparison of *S. cerevisiae* and *C. albicans* sphingolipids.** Schematic structure of fungal MIPCs with a focus on the main divergence observed in the composition of the ceramide moiety in *S. cerevisiae* and *C. albicans* strains (*A*) and phospholipomannan of *C. albicans* (*B*). *C*, hypothesis for the biosynthetic pathway of PLM in *C. albicans* that shares common steps with the *S. cerevisiae* fungal sphingolipid pathway.

6.8, 2% SDS, 5% glycerol, 1% β-mercaptoethanol, and bromphenol blue). Lysates were collected and clarified by centrifugation for 10 min at 12,000 × *g* at 4 °C. Extracted proteins were separated by 10% SDS-PAGE. Membranes were probed with rabbit anti-ERK1/2 phospho-specific antibodies (New England Biolabs, Beverly, MA) diluted 1:1000 in 50 mM Tris-HCl, 150 mM NaCl, 0.01% Tween 20, pH 7.5 (TNT) 5% bovine serum albumin overnight at 4 °C. After washing several times, the membranes were incubated for 1 h at 20 °C with a 1:2000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Southern Biotechnology Laboratories) in TNT-5% bovine serum albumin. After washing, the membrane was incubated with ECL detection reagents (SuperSignal chemiluminescent substrate; Pierce) and exposed to hyperfilm ECL.

## RESULTS

*Identification of C. albicans MIT1 as the Potent Homologue of S. cerevisiae SUR1 Responsible for Mannosylation of the IPC Core, and Construction of mit1Δ and Revertant Strains*—Complete elucidation of the *C. albicans* PLM structure (10) strongly suggested that it was a member of the MIPC family, from which it differs by the presence of a second phosphate group and extensive mannosylation (Fig. 1, *A* and *B*). Among the genes involved in the *S. cerevisiae* MIPC biosynthetic pathway, *SUR1* is responsible for mannosylation of the IPC core (18), and its inactivation in *S. cerevisiae* results in viable mutants (18). It was considered that biosynthesis of PLM in *C. albicans* uses the MIPC biosynthetic pathway described in *S. cerevisiae* and that inactivation of the *C. albicans* homologue of *SUR1* could lead to *C. albicans* viable mutants defective in PLM biosynthesis (Fig. 1*C*). A *SUR1* homologue was identified in the *C. albicans* genome whose open reading frame is referred to as IPF2067 in the *C. albicans* CandidaDB data base (genolist.pasteur.fr/CandidaDB/). It encodes a protein sharing 59% similarity in its amino acid sequence with *S. cerevisiae* Sur1p. As another gene has previously been named *SUR1* in *C. albicans*, the current gene was designated *MIT1*, according to its putative function of encoding GDP-mannose:inositol-phospho-ceramide mannose transferase. Sur1p and Mit1p sequences contain four putative transmembrane domains and display the conserved "glycosyltransferase sugar-binding region containing the same DXD motif" domain (pfam Gly_transf_sug; PF04488) specific to some classes of glycosyltransferases (33). The main differences lie in protein size (544 amino acids for Mit1p *versus* 382 for Sur1p) and in the presence of two asparagine- and serine-rich domains in the Mit1p C-terminal region that are not present in Sur1p (Fig. 2).

Both alleles of the *MIT1* gene were disrupted using the URA-blaster technique (Fig. 3*A*) described by Fonzi *et al.* (22) and generated a set of single- and double-disrupted strains (Table I). A complemented strain was also constructed by reintroduction of a wild copy of the *MIT1* gene into the *mit-*Δ::*hisG*/*mit-*Δ::*hisG* strain. Each strain was tested by Southern blotting to confirm correct integration of the cassette (Fig. 3*B*). All studies were performed on strains having one copy of the *URA3* gene.

*Deletion of MIT1 Has No Effect on C. albicans Growth and Morphogenesis but Induces Increased Sensitivity to Some Agents Used to Detect Cell Wall Defects*—When the growth of each strain in YPD or Sabouraud's broth was monitored, no significant difference was observed among the wild-type, revertant, or null strains (data not shown). Similarly, no difference was observed when strains were incubated in media inducing either yeast-hyphal transition or chlamydospore formation. All strains were able to produce hyphae and chlamydospores (Fig. 4). Therefore, deletion of *MIT1* had no obvious microscopic effect on growth or morphogenesis.

When strains were incubated on medium containing increasing concentrations of calcium, calcofluor white, and SDS, the *mit1*Δ strain was more sensitive to calcium and SDS than the wild-type and revertant strains (Fig. 5, *A* and *C*) but was

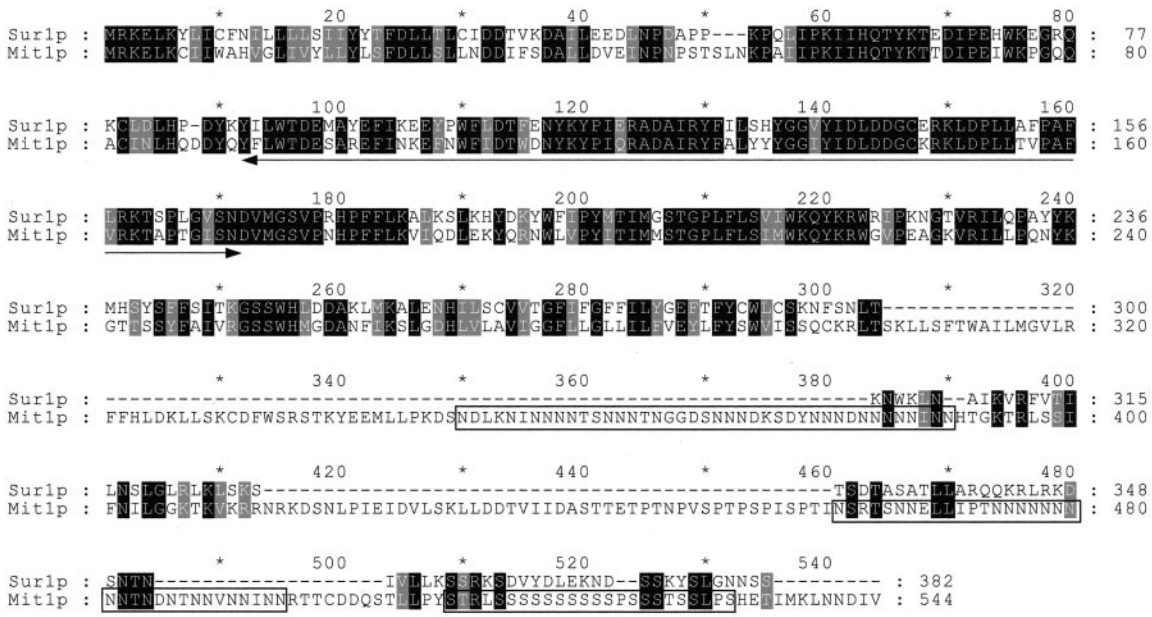


FIG. 2. **Conserved regions between Mit1p and Sur1p sequences.** The alignment was done with ClustalW 1.83 and edited with Genedoc. The *black arrow* underlines the glycosyltransferase sugar-binding domain. The aspargine- and serine-rich domains are *boxed*.

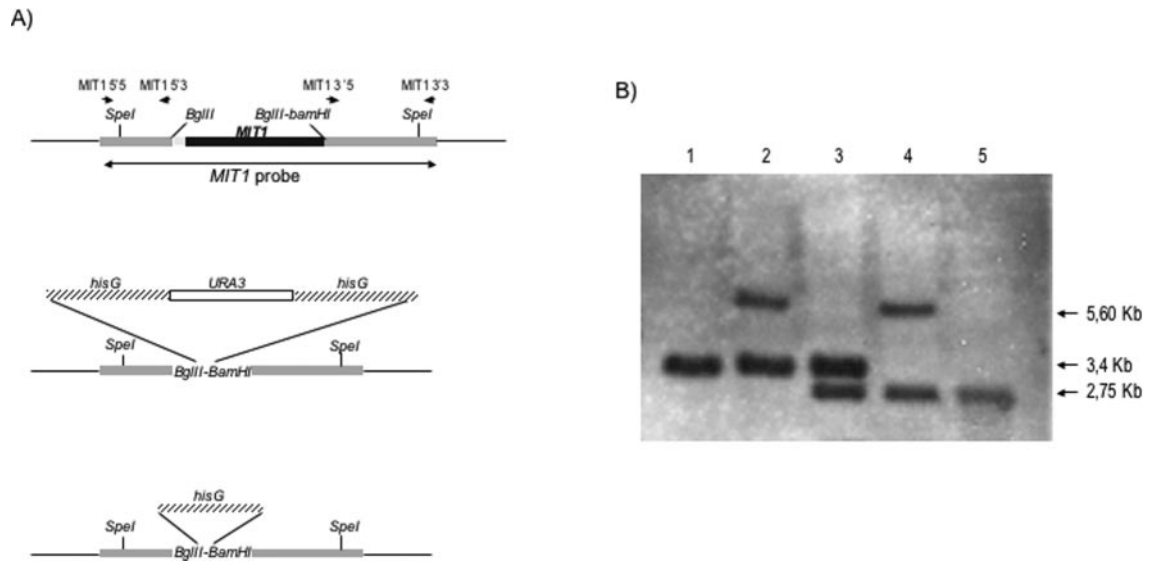


FIG. 3. *A*, disruption of the *CaMIT1* gene (see "Experimental Procedures" for details of construction). *B*, Southern blot used to verify the construction. *Lane 1*, CAI4 (parental strain); *lane 2*, C41.11 (*MIT1/mit1*-Δ::*hisG-URA3-hisG*); *lane 3*, C41.11A (*MIT1/mit1*-Δ::*hisG*); *lane 4*, S11.A14 (*mit1*-Δ::*hisG-URA3-hisG/mit1*-Δ::*hisG*), and *lane 5*, S11.14A (*mit1*-Δ::*hisG/mit1*-Δ::*hisG*). For each strain, 10 μg of SpeI-digested genomic DNA was electrophoretically separated, transferred to a nylon membrane, and analyzed with a *MIT1*-specific probe.

TABLE I
*C. albicans strains used in this study*

| Strain | Parental strain | Genotype | References |
|---|---|---|---|
| CAF2–1 | SC5314 | *ura3*-Δ::*imm434/URA3* | (23) |
| CAI4 | CAF2–1 | *ura3*-Δ::*imm434/ura3*-Δ::*imm434* | (23) |
| C41.11 | CAI4 | *mit1*-Δ::*hisG-URA3-hisG/MIT1* | Thisstudy |
| C41.11A | C41.11 | *mit1*-Δ::*hisG/MIT1* | Thisstudy |
| S11.A14 | C41.11A | *mit1*-Δ::*hisG/mit1*-Δ::*hisG-URA3-hisG* | Thisstudy |
| S11.14A | S11.A14 | *mit1*-Δ::*hisG/mit1*-Δ::*hisG* | Thisstudy |
| AS1 | S11.14A | *mit1*-Δ::*hisG/mit1*-Δ::*hisG* + *MIT1-URA3* | Thisstudy |

equally resistant to calcofluor white (Fig. 5*B*). These results suggest a plasma membrane or cell wall structural defect in the mutant that could not be observed by microscopic analysis.

*MIT1 Is Involved in the PLM Biosynthetic Pathway*—The effect of *MIT1* deletion on expression of β-1,2 oligomannoside epitopes was analyzed on Western blots stained with an anti-β-1,2 oligomannoside monoclonal antibody (Fig. 6). Whole-cell extracts of strains grown at pH 6.0 (Fig. 6*A*) displayed β-1,2 oligomannoside epitopes on mannoproteins, but no PLM was apparent in the *mit1*Δ strain (*arrow*). PPM extracted from *mit1*Δ grown under the same conditions still displayed α-oligomannosides epitopes (data not shown) but revealed a strong decrease in β-1,2 oligomannoside epitope expression (Fig. 6*C*). When strains were grown at pH 2.0, a growth condition known

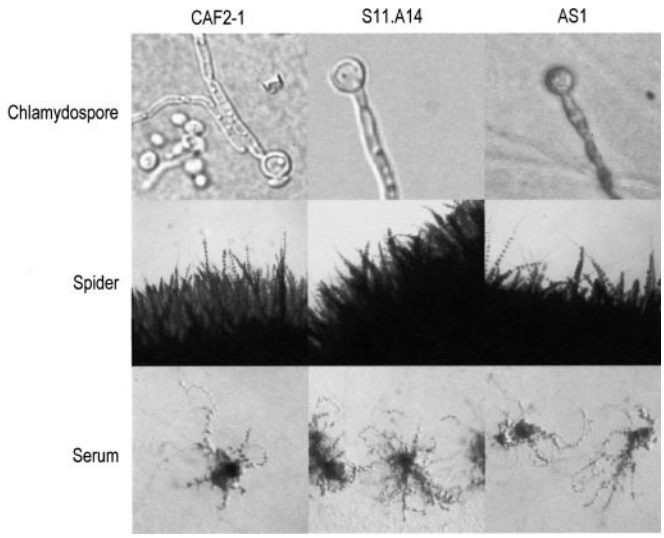


FIG. 4. ***MIT1* deletion does not affect cell morphogenesis.** Strains were grown on media to induce either chlamydospore or hyphae formation (Spider medium or medium containing serum) at 28 or 37 °C, respectively, for 2–5 days.

to reduce PPM β-mannosylation, all strains displayed a strong reduction in PPM β-1,2 oligomannoside expression compared with growth at pH 6.0 (Fig. 6*D*). This reduction in expression of β-1,2 oligomannoside epitopes was also observed on mannoproteins but was less pronounced in *mit1*Δ (Fig. 6*B*), suggesting that in the absence of PLM and under conditions where PPM is poorly mannosylated a compensatory mechanism promotes additional mannosylation of mannoproteins. These results strongly suggest that in *C. albicans* all processes of β-mannosylation are related and are under the control of a global regulatory mechanism.

*MIT1 Is Involved in the Conversion of IPC into MIPC*—Under the solvent and staining conditions used for TLC of sphingolipid extracts, PLM remained at the origin with other insoluble components, and two spots corresponding, respectively, to $M(IP)_2C$ and MIPC were observed (Fig. 7*A*). TLC of extracts from all strains showed that *mit1*Δ (*lane 2*) did not express MIPC or $M(IP)_2C$. These results were confirmed by mass spectrometry of sphingolipid extracts. Strain CAF2–1 displayed (M-H) molecular-related ions revealing the presence of IPC, MIPC, and $M(IP)_2C$ (Fig. 7*B*, *panel 1*). As observed for $M(IP)_2C$ from strain VW32 (10), each of these sphingolipids displayed a series of five peaks arising from variability in their ceramide moiety, which combines C18 or C20 phytosphingosines with C24-C26 mono- or dihydroxylated fatty acids. The major peak resulted from linkage of the OH C24 fatty acid with C20 phytosphingosine. The *mit1*Δ strain still displayed IPC peaks but, as expected from TLC analysis, was devoid of MIPC and $M(IP)_2C$ (Fig. 7*B*, *panel 2*), which were recovered in the complemented strain (*panel 3*). These results confirm that *MIT1* is needed for the conversion of IPC to MIPC.

*The mit1*Δ *Mutant Is Less Virulent in a Mouse Systemic Model of Infection*—To determine whether *MIT1* is involved in virulence, the mortality induced in a murine model of hematogenously disseminated candidiasis was determined (Fig. 8). All mice infected with the wild-type or revertant strains died within 12 days postinjection. At this time, 65% of mice infected with *mit1*Δ were still alive. Fifty percent were fully resistant to infection within 1 month postinjection. These differences in virulence were also obvious during the initial acute phase of infection (1–4 days) when 70% of mice infected with CAF2–1 died compared with 10% of those infected with *mit1*Δ.

*Deletion of MIT1 Reduces C. albicans Survival in Macrophages and Alters Macrophage Transduction Pathways*—A significant difference was observed in the number of colony-forming units of *mit1*Δ and wild-type strains recovered after coculture with J774 cells, demonstrating that the mutation resulted in increased sensitivity of yeasts to macrophage lysis (Fig. 9*A*).

Parallel analysis of macrophage transduction pathways concerned ERK1/2 phosphorylation (Fig. 9*B*), which has previously been shown to be an early triggering step in macrophage apoptosis (15). The results confirmed the down-regulation of ERK1/2 phosphorylation in *C. albicans* strain CAF2–1 (*lane 3*) demonstrated previously in *C. albicans* strain VW32 (or its PLM) compared with *S. cerevisiae* strain Su1 (*lane 2*). However, in contrast to CAF2–1, depletion of *MIT1* resulted in increased ERK1/2 phosphorylation (*lane 4*), leading to signals similar to those induced by *S. cerevisiae*.

## DISCUSSION

A large number of concordant studies have shown that sequences of β-1,2 oligomannosides synthesized by *C. albicans* contribute specifically to *Candida*-host relationships and may be among the *C. albicans* virulence attributes. They act as adhesins for human enterocytes (34) and can prevent colonization of the mouse gut by *C. albicans* (5). They also bind to macrophages (3, 35), stimulating these cells to produce mediators or effectors of the immune response (4, 36). They elicit the production of specific antibodies that are protective in rodent models of systemic or vaginal candidiasis (37, 38). All these conclusions about the biological effects of β-1,2 oligomannosides have been drawn using oligomannosides derived from PPM or its synthetic analogues. Additional evidence to prove that these residues are involved in *C. albicans* virulence could be obtained by producing mutant strains depleted in β-1,2 oligomannosides.

Although β-1,2 mannosyltransferase activity has been described in *C. albicans* (39), the corresponding gene has not yet been identified. As an alternative approach, this study aimed at depleting β-1,2 oligomannosides from a *C. albicans* molecule that expresses them electively. This molecule was *C. albicans* PLM, whose structure has recently been described (10). Like the β-1,2 oligomannosides of its polysaccharide moiety, PLM induces TNF-α secretion from cells of macrophage lineage (40), which requires toll-like receptor-2 (13). PLM has also been shown to induce apoptosis of macrophages (15) that have ingested yeasts via upstream modulation of the ERK pathway (19). Through the construction of this mutant, which did not express β-1,2 oligomannosides on PLM, interrelated questions about *C. albicans* virulence and MIPC and β-mannosylation pathways were addressed.

Western blots of whole-cell extracts of the *mit1*Δ mutant showed that PLM could no longer be detected with an anti-β-1,2 oligomannoside antibody. This demonstrated that *MIT1* is required upstream for the presence of β-1,2 oligomannoside epitopes in PLM. TLC revealed that the absence of PLM reactivity on Western blots could arise from a lack of MIPC. Mass spectrometry confirmed these results and showed that the biosynthetic pathway was interrupted upstream between IPC and MIPC. Despite slight differences between *S. cerevisiae* and *C. albicans* phytosphingosine and fatty acid chains already observed with another *C. albicans* strain, these results confirm that the *C. albicans* gene *MIT1*, like its homologue *SUR1* in *S. cerevisiae* (18), is responsible for the addition of mannose to inositol of IPCs. As expected, inactivation of *MIT1* prevented further addition of inositol phosphate leading to the synthesis of $M(IP)_2C$. This inactivation also prevented the addition of mannose phosphate, the first step in the extensive mannosylation of sphingolipids deduced from complete elucidation of the

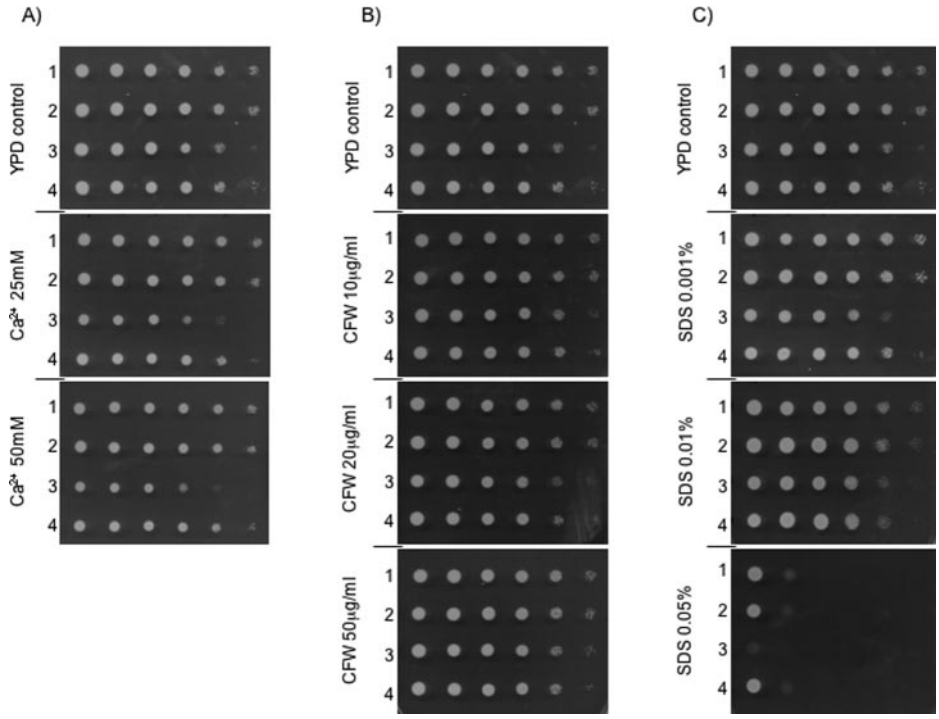


FIG. 5. ***MIT1* deletion affects $Ca^{2+}$ and SDS sensitivity of *C. albicans* cells.** Serial 10-fold dilutions of strains were inoculated on YPD agar medium containing different concentrations of either $CaCl_2$ (*A*), calcofluor white (*B*), or sodium dodecyl sulfate (*C*). Results were observed after 24 h of incubation at 37 °C. *Lanes 1*, CAF2–1 (*MIT1/MIT1*); *lanes 2*, C41.11 (*MIT1/mit1*-Δ::*hisG-URA3-hisG*); *lanes 3*, S11.A14 (*mit1*-Δ::*hisG-URA3-hisG/mit1*-Δ::*hisG*), and *lanes 4*, AS1 (*mit1*-Δ::*hisG/mit1*-Δ::*hisG* + *MIT1-URA3*).

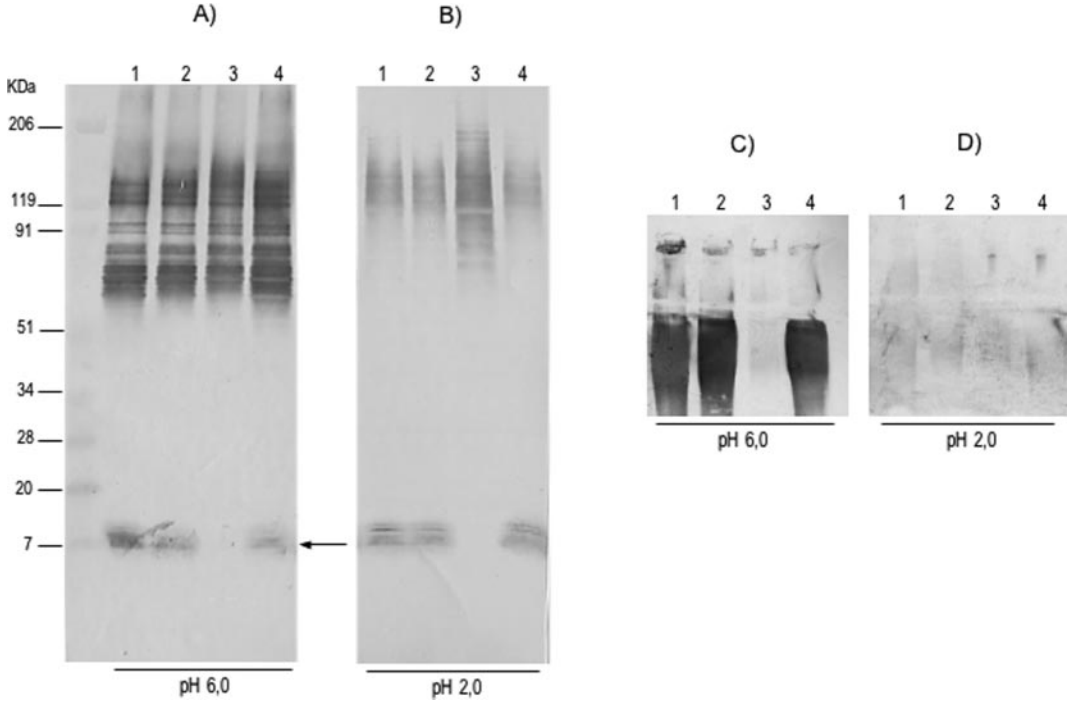


FIG. 6. ***MIT1* deletion affects the synthesis of phospholipomannan and β-1,2 mannosylation of *C. albicans* glycoconjugates.** Western blots of whole-cell extracts (*A* and *B*) or phosphopeptidomannan (*C* and *D*) from strains grown at pH 6.0 (*A* and *C*) or pH 2.0 (*B* and *D*) stained with anti β-1,2 oligomannoside monoclonal antibody, 5B2. *Lanes 1*, CAF2–1 (*MIT1/MIT1*); *lanes 2*, C41.11 (*MIT1/mit1*-Δ::*hisG-URA3-hisG*); *lanes 3*, S11.A14 (*mit1*-Δ::*hisG-URA3-hisG/mit1*-Δ::*hisG*), and *lanes 4*, AS1 (*mit1*-Δ::*hisG/mit1*-Δ::*hisG* + *MIT1-URA3*).

PLM structure. The present results showing the absence of PLM synthesis following disruption of *MIT1* confirm that PLM is derived from the MIPC pathway.

In *C. albicans*, $M(IP)_2C$ and PLM appear as two end products of the sphingolipid pathway and only diverge after the MIPC step. *MIT1* therefore encodes a protein that is responsible for mannosylation of IPC, the last biosynthesis step common to these two components that have highly different targets in the cell, mainly in the plasma membrane or cell wall, according to their hydrophobic/hydrophilic properties. These assertions are in agreement with mass spectrometry analysis that revealed the same heterogeneity of their ceramide moiety (10) and a similar effect of growth temperature on ceramide moiety composition, mainly a shift of the major phytosphingosine from C18 to C20 when the temperature increases from 28 to 37 °C (10).

*mit1*Δ is the first *C. albicans* strain totally affected in the β-mannosylation of a glycoconjugate. The effects of this deletion on phenotype and, more particularly, on virulence were also analyzed. Morphogenesis was not affected by the deletion, including the yeast-to-hyphal transition considered to be a

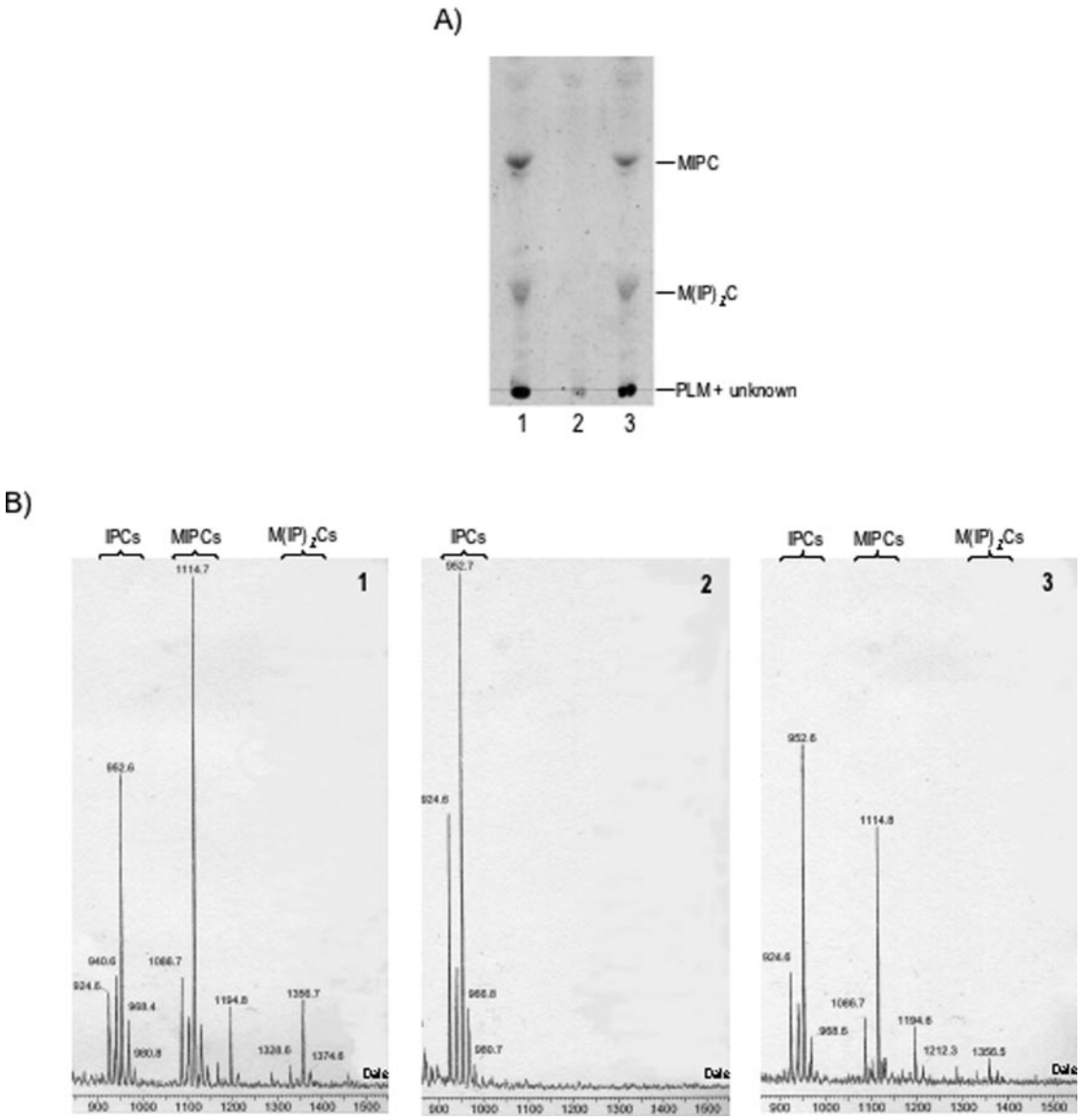


FIG. 7. ***MIT1* deletion inhibits the synthesis of MIPC and $M(IP)_2C$.** Cell pellets were obtained from strains grown on Sabouraud dextrose agar for 16 h at 37 °C. Sphingolipids were then extracted by incubation of the cell pellets in ethanol/water/diethylether/pyridine/ammonia (15/9/5/1/0.018) for 1 h at 60 °C. *A*, thin layer chromatography performed on silica gel plates using a chloroform/methanol/ammonia 4.2 N (9/7/2) solvent system and revealed with orcinol. *Lane 1*, CAF2–1 (*MIT1/MIT1*); *lane 2*, S11.A14 (*mit1*-Δ::*hisG-URA3-hisG/mit1*-Δ::*hisG*); *lane 3*, AS1 (*mit1*-Δ::*hisG/mit1*-Δ::*hisG* + *MIT1-URA3*). *B*, electrospray mass spectrum showing (M-H) molecular-related ions. Each labeled sphingolipid displays a series of peaks that arise from the heterogeneity of the ceramide moiety.

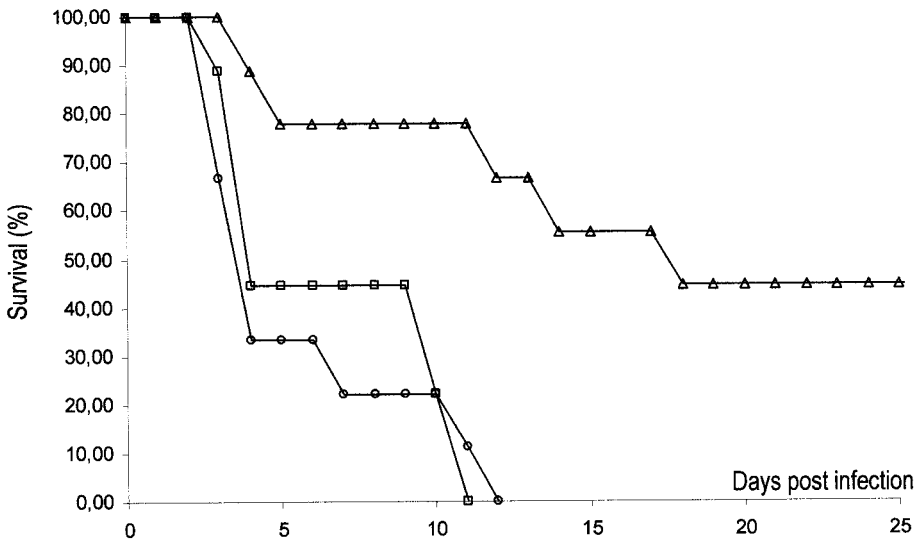


FIG. 8. **Virulence assay.** Cumulative mortality of mice injected with $2 \times 10^5$ yeasts. CAF2–1 (*MIT1/MIT1*) (*circles*). S11.A14 (*mit1*-Δ::*hisG-URA3-hisG/mit1*-Δ::*hisG*) (*triangles*). AS1 (*mit1*-Δ::*hisG/mit1*-Δ::*hisG* + *MIT1-URA3*) (*squares*).

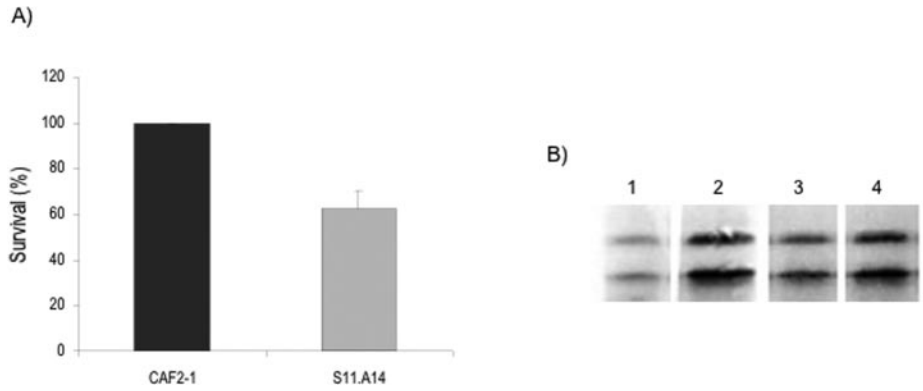


FIG. 9. **Effect of *MIT1* deletion on yeast-macrophage interactions.** *A*, reduction in number of colony-forming units recovered after co-culture of strain S11.A14 (*mit1*-Δ::*hisG-URA3-hisG*/*mit1*-Δ::*hisG*) (*gray bar*) and strain CAF2–1 (*MIT1*/*MIT1*) (*black bar*) with macrophages. *B*, Western blot analysis of J774 ERK1/2 phosphorylation (*lane 1*, control; *lane 2*, Su1; *lane 3*, CAF2–1 (*MIT1*/*MIT1*); *lane 4*, S11.A14 (*mit1*-Δ::*hisG-URA3hisG*/*mit1*-Δ::*hisG*)).

pathogenic trait of *C. albicans*. However, as observed for *sur1*Δ in *S. cerevisiae* (18), the mutant was more sensitive to calcium. This result, suggesting a plasma membrane or cell wall defect, was confirmed by increased sensitivity of the strain to SDS. Such an increased sensitivity is commonly observed in *C. albicans* mutants, including genes that are not directly related to the synthesis of cell wall components, because of the complexity of the cellular machinery leading to fungal cell wall biogenesis. However, the absence of sensitivity to calcofluor suggests that an increase in chitin content, a common compensation mechanism triggered by cell wall defects (41), is not triggered in response to the *mit1*Δ cell wall defect. Transcriptional analysis would be interesting to determine whether this results in differences in the set of genes that are co-regulated in a large number of mutants with cell wall defects (42).

Despite the unaffected growth rate *in vitro*, the *mit1*Δ strain was less virulent in a murine model of systemic candidiasis. Fifty percent of mice survived inoculation by *mit1*Δ compared with 0% with CAF2–1. This clear difference in pathogenic potential during the chronic phase was also obvious during the acute early phase of infection when PLM has previously been shown to be responsible for acute mortality correlating with high levels of TNF-α (43). Among the effects of *MIT1* deletion is the absence of $M(IP)_2C$ that could play a role in reduction of virulence. However, among the biological activities specific to *C. albicans* PLM that can be deleterious to the host is its ability to promote yeast survival after endocytosis through modulation of the MEK-ERK pathway (19). The current study shows that *mit1*Δ was more sensitive to macrophage lysis and was not able to modulate phosphorylation of mitogen-activated protein kinase of the MEK-ERK-p90 pathway. This relation between ERK1/2 phosphorylation and an efficient cell response toward *C. albicans* has been confirmed in other models (44). Like PD98059 (45), the addition of PLM interferes with specific activation of this pathway. One explanation for PLM-induced yeast survival is the induction of mitochondrial apoptosis (15) initiated by p90 dephosphorylation (46). Interestingly, among the molecules that can trigger such an apoptotic effect are yeast phytosphingosines (47), which are lipid moieties of *C. albicans* PLM (10). It is possible that the *mit1*Δ cell wall defects may render this mutant more sensitive to phagocytosis. However, it is clear that its inability to produce PLM and, therefore, to alter the ERK1/2 phosphorylation pathway probably contributes to the reinforcement of macrophage candicidal activity.

This study also revealed that disruption of *MIT1* had a overall effect on β-mannosylation of *C. albicans* mannoproteins. This was first demonstrated for PPM extracted from strains grown at pH 6.0. To assess the extent of these modifications, the cells were grown under less favorable conditions for the synthesis of β-oligomannoside epitopes. This consisted of growth at pH 2.0 where β-1,2 oligomannosides are known to be associated with PLM, but not with PPM (9, 16). Under these conditions, none of the strains appeared to contain β-1,2 oligomannoside epitopes in PPM, but *mit1*Δ compensated for the absence of β-1,2 oligomannosides in PLM by an increase in β-mannosylation of mannoproteins. This observation raises three important interrelated issues about the regulation and function of β-1,2 oligomannosides and their carrier molecules in *C. albicans* and its host. The first is the need for chemical evidence of the presence of β-1,2 oligomannosides in mannoproteins. At the moment, the presence of β-1,2 oligomannosides has only been chemically proven in PPM and PLM (6, 8). It is obvious from a large number of studies where blots were probed with anti-β-1,2 oligomannoside monoclonal antibodies that, besides PPM and PLM, and depending on the growth conditions, β-1,2 oligomannoside epitopes are also expressed on a wide range of cell wall proteins where they generally co-localize with α-oligomannoside epitopes (7–9, 26). In parallel, several *C. albicans* cell wall proteins have been shown to play an important role in pathogenesis or cell wall organization/remodeling. Most of these proteins display glycosylation sites but the chemical nature of the mannose oligomers attached is unknown, contrasting with recent studies indicating that cell wall protein post-translational modifications caused by variable glycosylation can control transcription (48). The second issue is the observation of β-1,2 oligomannoside epitope overexpression on mannoproteins at pH 2.0 in the absence of PLM. This strongly suggests overall regulation of β-1,2 oligomannoside expression at the cell level. The third issue is the impact of modulation of expression on the host response because the signal induced depends on the β-1,2 oligomannoside carrier molecule and co-receptors involved (49). Using this mutant and others, glycomic analysis of cell wall molecules, with a special focus on β-1,2 oligomannosides, is in progress. This will complement current proteomic and genomic analyses designed to study *C. albicans* virulence attributes and their regulation under conditions encountered in the host.

*Acknowledgments*—We thank Prof. Robert L. Lester (College of Medicine, Lexington, KY) for providing yeast sphingolipids and expert advice for the TLC analyses. We thank Bernadette Coddeville for assistance in electrospray ionization-mass spectrometry analyses. We thank Val Hopwood for help in editing the manuscript.

REFERENCES

1. Nitz, M., Ling, C. C., Otter, A., Cutler, J. E., and Bundle, D. R. (2002) *J. Biol. Chem.* **277,** 3440–3446
2. Han, Y., Kanbe, T., Cherniak, R., and Cutler, J. E. (1997) *Infect. Immun.* **65,** 4100–4107
3. Fradin, C., Poulain, D., and Jouault, T. (2000) *Infect. Immun.* **68,** 4391–4398
4. Jouault, T., Lepage, G., Bernigaud, A., Trinel, P. A., Fradin, C., Wieruszeski, J. M., Strecker, G., and Poulain, D. (1995) *Infect. Immun.* **63,** 2378–2381
5. Dromer, F., Chevalier, R., Sendid, B., Improvisi, L., Jouault, T., Robert, R., Mallet, J. M., and Poulain, D. (2002) *Antimicrob. Agents Chemother.* **46,** 3869–3876
6. Kobayashi, H., Shibata, N., and Suzuki, S. (1992) *Infect. Immun.* **60,** 2106–2109
7. Cantelli, C., Trinel, P. A., Bernigaud, A., Jouault, T., Polonelli, L., and Poulain, D. (1995) *Microbiology* **141,** Pt. 10, 2693–2697

8. Trinel, P. A., Borg-von-Zepelin, M., Lepage, G., Jouault, T., Mackenzie, D., and Poulain, D. (1993) *Infect. Immun.* **61,** 4398–4405
9. Trinel, P. A., Cantelli, C., Bernigaud, A., Jouault, T., and Poulain, D. (1996) *Microbiology* **142,** Pt. 8, 2263–2270
10. Trinel, P. A., Maes, E., Zanetta, J. P., Delplace, F., Coddeville, B., Jouault, T., Strecker, G., and Poulain, D. (2002) *J. Biol. Chem.* **277,** 37260–37271
11. Poulain, D., Slomianny, C., Jouault, T., Gomez, J. M., and Trinel, P. A. (2002) *Infect. Immun.* **70,** 4323–4328
12. Jouault, T., Fradin, C., Trinel, P. A., Bernigaud, A., and Poulain, D. (1998) *J. Infect. Dis.* **178,** 792–802
13. Jouault, T., Ibata-Ombetta, S., Takeuchi, O., Trinel, P. A., Sacchetti, P., Lefebvre, P., Akira, S., and Poulain, D. (2003) *J. Infect. Dis.* **188,** 165–172
14. Lorenz, M. C., and Fink, G. R. (2001) *Nature* **412,** 83–86
15. Ibata-Ombetta, S., Idziorek, T., Trinel, P. A., Poulain, D., and Jouault, T. (2003) *J. Biol. Chem.* **278,** 13086–13093
16. Kobayashi, H., Giummelly, P., Takahashi, S., Ishida, M., Sato, J., Takaku, M., Nishidate, Y., Shibata, N., Okawa, Y., and Suzuki, S. (1991) *Biochem. Biophys. Res. Commun.* **175,** 1003–1009
17. Trinel, P. A., Jouault, T., Cutler, J. E., and Poulain, D. (2002) *Infect. Immun.* **70,** 5274–5278
18. Beeler, T. J., Fu, D., Rivera, J., Monaghan, E., Gable, K., and Dunn, T. M. (1997) *Mol. Gen. Genet.* **255,** 570–579
19. Ibata-Ombetta, S., Jouault, T., Trinel, P. A., and Poulain, D. (2001) *J. Leukoc. Biol.* **70,** 149–154
20. Rustchenko, E. P., Howard, D. H., and Sherman, F. (1994) *J. Bacteriol.* **176,** 3231–3241
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual* (Ford, N., Nolan, C., and Ferguson, M., eds) Cold Spring Harbor Laboratory Press, Plainview, NY
22. Fonzi, W. A., and Irwin, M. Y. (1993) *Genetics* **134,** 717–728
23. Sanglard, D., Ischer, F., Monod, M., and Bille, J. (1996) *Antimicrob. Agents Chemother.* **40,** 2300–2305
24. Cannon, R. D., Jenkinson, H. F., and Shepherd, M. G. (1992) *Mol. Gen. Genet.* **235,** 453–457
25. Kobayashi, H., Takahashi, S., Shibata, N., Miyauchi, M., Ishida, M., Sato, J., Maeda, K., and Suzuki, S. (1994) *Infect. Immun.* **62,** 968–973
26. Trinel, P. A., Faille, C., Jacquinot, P. M., Cailliez, J. C., and Poulain, D. (1992) *Infect. Immun.* **60,** 3845–3851
27. Laemmli, U. K. (1970) *Nature* **227,** 680–685
28. Sendid, B., Jouault, T., Coudriau, R., Camus, D., Odds, F., Tabouret, M., and Poulain, D. (2004) *J. Clin. Microbiol.* **42,** 164–171
29. Kocourek, J., and Ballou, C. E. (1969) *J. Bacteriol.* **100,** 1175–1181
30. Faille, C., Wieruszeski, J. M., Lepage, G., Michalski, J. C., Poulain, D., and Strecker, G. (1991) *Biochem. Biophys. Res. Commun.* **181,** 1251–1258
31. Dubois, M., Gilles, K., Hamilton, J. K., Rebers, P. A., and Smith, F. (1951) *Nature* **168,** 167
32. Hanson, B. A., and Lester, R. L. (1980) *J. Lipid Res.* **21,** 309–315
33. Wiggins, C. A., and Munro, S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 7945–7950
34. Dalle, F., Jouault, T., Trinel, P. A., Esnault, J., Mallet, J. M., d'Athis, P., Poulain, D., and Bonnin, A. (2003) *Infect. Immun.* **71,** 7061–7068
35. Fradin, C., Jouault, T., Mallet, A., Mallet, J. M., Camus, D., Sinay, P., and Poulain, D. (1996) *J. Leukoc. Biol.* **60,** 81–87
36. Jouault, T., Fradin, C., Trinel, P. A., and Poulain, D. (2000) *Infect. Immun.* **68,** 965–968
37. Han, Y., Riesselman, M. H., and Cutler, J. E. (2000) *Infect. Immun.* **68,** 1649–1654
38. Han, Y., Morrison, R. P., and Cutler, J. E. (1998) *Infect. Immun.* **66,** 5771–5776
39. Suzuki, A., Takata, Y., Oshie, A., Tezuka, A., Shibata, N., Kobayashi, H., Okawa, Y., and Suzuki, S. (1995) *FEBS Lett.* **373,** 275–279
40. Jouault, T., Bernigaud, A., Lepage, G., Trinel, P. A., and Poulain, D. (1994) *Immunology* **83,** 268–273
41. Kapteyn, J. C., Hoyer, L. L., Hecht, J. E., Muller, W. H., Andel, A., Verkleij, A. J., Makarow, M., Van Den Ende, H., and Klis, F. M. (2000) *Mol. Microbiol.* **35,** 601–611
42. Lagorce, A., Hauser, N. C., Labourdette, D., Rodriguez, C., Martin-Yken, H., Arroyo, J., Hoheisel, J. D., and Francois, J. (2003) *J. Biol. Chem.* **278,** 20345–20357
43. Trinel, P. A., Jouault, T., and Poulain, D. (1997) in *Host-Fungus Interplay* (Vanden Bossche, H., Stevens, D. A., and Odds, F. C., eds) pp. 37–45, The National Foundation for Infectious Diseases, Bethesda, MD
44. Zhong, B., Jiang, K., Gilvary, D. L., Epling-Burnette, P. K., Ritchey, C., Liu, J., Jackson, R. J., Hong-Geller, E., and Wei, S. (2003) *Blood* **101,** 3240–3248
45. Lunghi, P., Tabilio, A., Dall'Aglio, P. P., Ridolo, E., Carlo-Stella, C., Pelicci, P. G., and Bonati, A. (2003) *Leukemia* **17,** 1783–1793
46. Kang, C. D., Yoo, S. D., Hwang, B. W., Kim, K. W., Kim, D. W., Kim, C. M., Kim, S. H., and Chung, B. S. (2000) *Leuk. Res.* **24,** 527–534
47. Park, M. T., Choi, J. A., Kim, M. J., Um, H. D., Bae, S., Kang, C. M., Cho, C. K., Kang, S., Chung, H. Y., Lee, Y. S., and Lee, S. J. (2003) *J. Biol. Chem.* **278,** 50624–50634
48. Choi, W., Yoo, Y. J., Kim, M., Shin, D., and Jeon, H. B. (2003) *Yeast* **20,** 1053–1060
49. Poulain, D., and Jouault, T. (2004) *Curr. Opin. Microbiol.,* **7,** 342–349