-1,2-Mannosyltransferases 1 and 3 Participate in Yeast and Hyphae O- and N-Linked Mannosylation and Alter Candida albicans Fitness During Infection

Courjol, Flavie; Jouault, Thierry; Mille, Céline; Hall, Rebecca; Maes, Emmanuel; Sendid, Boualem; Mallet, Jean Maurice; Guerardel, Yann; Gow, Neil A R; Poulain, Daniel; Fradin, Chantal; Hall, Rebecca

DOI: 10.1093/ofid/ofv116

License:
Creative Commons: Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):
Courjol, F, Jouault, T, Mille, C, Hall, R, Maes, E, Sendid, B, Mallet, JM, Guerardel, Y, Gow, NAR, Poulain, D, Fradin, C & Hall, R 2015, "-1,2-Mannosyltransferases 1 and 3 Participate in Yeast and Hyphae O- and N-Linked Mannosylation and Alter Candida albicans Fitness During Infection", Open Forum Infectious Diseases, vol. 2, no. 3, pp. 1-11. https://doi.org/10.1093/ofid/ofv116

Link to publication on Research at Birmingham portal

General rights
Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

• Users may freely distribute the URL that is used to identify this publication.
• Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
• Users may use extracts from the document in line with the concept of ‘fair dealing’ under the Copyright, Designs and Patents Act 1988 (??)
• Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy
While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

Download date: 15. Jul. 2021
β-1,2-Mannosyltransferases 1 and 3 Participate in Yeast and Hyphae O- and N-Linked Mannosylation and Alter Candida albicans Fitness During Infection

Flavie Courjol,1,2 Thierry Jouault,1,2 Céline Mille,1,2 Rebecca Hall,3 Emmanuel Maes,4,5 Boualem Sendid,1,2,6 Jean Maurice Mallet,7 Yann Guerardel,4,5 Neil A. R. Gow,3 Daniel Poulain,1,2,6 and Chantal Fradin1,2

1Université de Lille, and 2Institut National de la Santé et de la Recherche Médicale, Lille Inflammation Research International Center–Unité Mixte de Recherche 995, France; 3Aberdeen Fungal Group, School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, United Kingdom; 4Université de Lille, Unité de Glycobiologie Structurale et Fonctionnelle, and 5Centre National de la Recherche Scientifique, Unité Mixte de Recherche 8576, Villeneuve d’Ascq, 6Centre Hospitalier Régional Universitaire de Lille, Centre Biologie et Pathologie, and 7Laboratoire des Biomolécules Unité Mixte de Recherche 7203, Université Pierre et Marie Curie, Ecole Normale Supérieure, Paris, France

β-1,2-mannosylation of Candida albicans glycoconjugates has been investigated through the identification of enzymes involved in the addition of β-1,2-oligomannosides (β-Mans) to phosphopeptidomannan and phospholipomannan. β-1,2-oligomannosides are supposed to have virulence properties that they confer to these glycoconjugates. In a previous study, we showed that cell wall mannoproteins (CWMPs) harbor β-Mans in their O-mannosides; therefore, we analyzed their biosynthesis and impact on virulence. In this study, we demonstrate that O-mannans are heterogeneous and that α-mannosylated O-mannosides, which are biosynthesized by Mnt1 and Mnt2 α-1,2-mannosyltransferases, can be modified with β-Mans but only at the nonreducing end of α-1,2-mannotriose. β-1,2-mannosylation of this O-mannotriose depends on growth conditions, and it involves 2 β-1,2-mannosyltransferases, Bmt1 and Bmt3. These Bmts are essential for β-1,2-mannosylation of CWMPs and expression of β-Mans on germ tubes. A bmt1Δ mutant and a mutant expressing no β-Mans unexpectedly disseminated more in BALB/c mice, whereas they had neither attenuated nor enhanced virulence in C57BL/6 mice. In galectin (Gal)3 knockout mice, the reference strain was more virulent than in C57BL/6 mice, suggesting that the β-Mans innate receptor Gal3 is involved in C. albicans fitness during infection.

Keywords. β-1,2-oligomannosides; Candida albicans; fungal virulence; mannosyltransferase; O-mannosylation.

Candida albicans is a successful pathogen in immunosuppressed patients, leading to frequent nosocomial infections with high mortality rate [1]. The fungal cell wall and its components are the natural and dynamic interface with the host, forming a moving target in terms of host recognition mechanisms [2]. Cell wall glycans are immunologically active components, which are present either as polysaccharides or glycoconjugates. Amongst its glycan diversity, C. albicans expresses β-1,2-oligomannosides (β-Mans), which are unusual as only evidenced in few nonmammalian eukaryotes and prokaryotes [3]. β-Mans are present in the N-glycan of the cell wall outer layer phosphopeptidomannan (PPM) [4]. Within this molecule, they are linked to a phosphomannose and at the nonreducing end of α-1,2-oligomannosides (Figure 1A). β-Mans are also associated to a cell wall glycolipid named phospholipomannan (PLM) (Figure 1B) [5]. β-Mans
biosynthesis involves β-1,2-mannosyltransferases (Bmts), which have distinct substrate specificities and catalyze specific steps of β-1,2-mannosylation [3].

β-Mans are involved in the interplay between C. albicans and its host, for example, mediating adhesion of C. albicans to macrophages and epithelial cells [6, 7], inducing cytokine production [8], and generating protective antibodies against vaginal and disseminated candidiasis [9, 10]. However, very little is known about their relative and respective functions inside the wall. Biological activity of β-Mans depends on their carrier molecule. PPM and PLM phosphomannosides both display β-Mans, but they have distinct immune-modulatory properties [11–13].

In a previous study, we revealed the presence of β-Mans epitopes on cell wall mannoproteins (CWMPs) O-mannan. O-mannosylation is an essential glycosylation process for protein modification, but it is also important for C. albicans virulence [14]. Therefore, we determined which Bmts are responsible for addition of β-Mans on O-mannosides and generated the appropriate mutant to analyze (1) the contribution of N- and O-mannans to the surface expression of β-Mans and (2) the impact of these β-Mans on Candida virulence. We additionally investigated the global role of β-Mans on Candida virulence by generating a mutant expressing no β-Mans.

**METHODS**

**Fungal Strains and Growth Conditions**

All strains used are listed in Table 1. Yeast cells were grown in YPD broth (1% yeast extract, 2% bactopeptone, 2% dextrose [Difco]) and grown at 28°C or 37°C. Hyphae were obtained after inoculation of RPMI 1640 medium (Invitrogen) with yeast cells and incubation for 3 hours at 37°C. For animal experiments, cells were grown in YPD at 28°C for 16 hours.

**Lectin and Monoclonal Antibody**

Biotinylated concanavalin A ([ConA] Sigma-Aldrich) detects terminal α-D-mannosyl residues. Monoclonal antibodies (mAbs) 5B2 (rat-mouse immunoglobulin [Ig] M) is specific for β-Mans with a mannobiose as minimal epitope [15]. The mAb 16B1 is a mouse IgG specific for hyphal wall protein 1 (Hwp1) [16].

**Whole-Cell Protein Extraction and Western Blotting**

Total extracts were obtained and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described [16, 17]. Membranes were probed with mAb 5B2 and then an alkaline phosphatase-conjugated anti-rat IgM diluted both 1:2000. Enzyme activity was detected with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate substrate (Promega). For lectin staining, membranes were incubated with biotinylated ConA and then horseradish peroxidase-labeled streptavidin (AbCam) diluted both 1:1000. Peroxidase activity was detected with diamidobenzidine (Sigma-Aldrich).

**Whole-Cell Hydrolysis and Fluorophore-Assisted-Carbohydrate-Electrophoresis Analysis**

Cells were boiled for 1 hour in 10 mM HCl. After cooling and neutralization, the supernatants containing phosphomannosides were harvested by centrifugation. Remaining cells were washed and then incubated for 16 hours at room temperature in 100 mM NaOH. After neutralization, O-mannosides were harvested by centrifugation. Both supernatants were filtered through a 0.22 µm polyvinylidene fluoride filter (Millipore).

Hydrolases were tagged with 0.15 M 8-amino-naphthalene-1,3,6-trisulfonate ([ANTS] Sigma-Aldrich) as previously described [18]. Electrophoresis of ANTS-labeled oligomannosides
was performed on 25%–30% acrylamide gels. Purified oligomannosides from PPM were tagged with ANTS and used as carbohydrate standards. Gels were acquired with the Gel Doc 2000 apparatus (Bio-Rad).

Generation of *Candida albicans* Cells Expressing 6xHis Hwp1

Primers HWP1-1/HWP1-2 (Table 2) were designed to amplify the promoter and the HWP1 open reading frame (orf19.1321; [http://www.candidagenome.org/](http://www.candidagenome.org/)) missing its last 75 nucleotides. The amplified fragment was cloned in pYES2.1-TOPO (Invitrogen) to fuse the gene to DNA sequence coding for 6xHis. A fragment of pYES2.1-TOPO-6xHIS-HWP1 was amplified using the primers HWP1-1/V5-His (Table 2) and cloned in pCR2.1-TOPO. The sequence coding for 6xHis-Hwp1 was ed using the primers HWP1-1/V5-His (Table2) and cloned in pYES2.1-TOPO. The sequence coding for 6xHis-Hwp1 was analyzed by Western blotting. PD-10 Desalting Columns (GE Healthcare Life Sciences), following the manufacturer

Candida β-Mannosylation and Virulence ● OFID ● 3
For PNGase F treatment, proteins samples were suspended in sodium phosphate 0.5 M, pH7.5 and incubated with peptide-N-glycosidase F following the manufacturer’s recommendation (Sigma-Aldrich).

Indirect Immunofluorescence Assays
Hyphae were fixed with formalin on reaction wells of microscope slides (Thermo Scientific). Wells were blocked with phosphate-buffered saline containing 5% bovine serum albumin and incubated for 1 hour at 37°C with mAb 5B2 or biotinylated ConA diluted 1:500. Wells were washed and incubated with the corresponding RPE-conjugated anti-Ig or fluorescein isothiocyanate-conjugated streptavidin diluted 1:100, respectively.

Slides were examined under a Leica fluorescence microscope. Images were captured and analyzed with a Leica DC camera and its software.

Gene Disruption
All mutants were generated in the CAI-4 background using the mini ura-blaster method as previously described [19] using primers BMT1 knockout Fwd/BMT1 knockout Rev (Table 2). Selection URA3 marker was recycled with 5-fluoroorotic acid. Disruption of both alleles for each gene was checked by Southern blot with probes obtained with primers probe Fwd/probe Rev (Table 2). A BMT1-reconstituted strain was constructed by cloning the BMT1 region (−652 to +870) into CIp10 [20] and transforming Ura–bmt1Δ strain with the resulting linearized CIp10–BMT1. Linearized CIp10 was transformed into CAI-4 and the different mutants. Single integration of these plasmids at the RPS1 locus in the same allele was confirmed by Southern blot analysis with probes obtained with primers probe Fwd/probe Rev (Table 2).

Animal Experiments
All experiments, approved by the national ethics committee (Reference no. 00374.03), were conducted following the French Guide for the Care and Use of Laboratory Animals and the Guidelines of the European Union.

For survival assays, cells were suspended in sterile physiological saline. For each C. albicans strain, 5 female BALB/c mice (8–10 weeks old) were given intravenous injections of

| Primers | Sequences |
|---------|-----------|
| BMT1 knockout Fwd | CTAAAAAAGTAAAGGAAATTTATGTTTCACATGTATCAATGATGAAAAGTTTTCCAGCTACGAGCAGTT |
| BMT1 knockout Rev | TCTTTTTCATTGGTACAGGAAAATATTCTTGTTGACGCAAAAAATTAAATAGGAATTTCAATGATGAAAAGTTTTCCCAGTCACGACGTT |
| BMT2 knockout Fwd | CCTTATAAGGGTAAAATAGGGAATATTCTTGTTGACGCAAAAAATTAAATAGGAATTTCAATGATGAAAAGTTTTCCCAGTCACGACGTT |
| BMT2 knockout Rev | TAAACCCATAATCATTCAATTCTAATAATTCTTGTTGACGCAAAAAATTAAATAGGAATTTCAATGATGAAAAGTTTTCCCAGTCACGACGTT |
| BMT5 knockout Fwd | GCAGTACCGATTGTGTTTTTGATGACATCATTTGTGTTTCTATGTTTTGTTGATATGAAAAGTTTTCCCAGTCACGACGTT |
| BMT5 knockout Rev | CTGTGTTTTTCAATATTATGCAATGATGAAAAGTTTTCCCAGTCACGACGTT |
| probe BMT1 Fwd | ATACAATCTATTTATCATAAATATATCAGATAATATGCAATGATGAAAAGTTTTCCCAGTCACGACGTT |
| probe BMT1 Rev | ATACTGGGATAGGGGCGATT |
| probe BMT2 Fwd | GAGAAATGTCGCTGTGTGA |
| probe BMT2 Rev | TGTTTTTTCCAGGGATATGT |
| probe BMT5 Fwd | GACCTGACCGATATTGACAT |
| probe BMT5 Rev | ATGGGCACACAAATACCAT |
| probe URA3 Fwd | GCCTACACAGATGACACAAGCAT |
| probe URA3 Rev | GCATTCCACACCAGCATCTTATAC |
| probe HWP1 Fwd | CAATTTGAGCTCCCTACTCC |
| probe HWP1 Rev | GTCAATTGAGCCGAGGAC |
| probe RPS1 Fwd | ATGGCTGTCGGTAAAAACAAG |
| probe RPS1 Rev | AAAGCCCAATAATGAAACCAAAG |
| probe ACT1 Fwd | ACCGGAGCTCCATGAATCCA |
| probe ACT1 Rev | GGATGGACCAGATTTGTCGT |
| HWP1-1 | CTCAATTGAGCCGAGGAC |
| HWP1-2 | TTCAAAATGAGAAATAGGAC |
| V5-HIS | CTCAATTGAGCCGAGGAC |

Abbreviations: Fwd, forward; Rev, reverse.

Table 2. Candida albicans Primers Used in This Study

| Primers | Sequences |
|---------|-----------|
| BMT1 knockout Fwd | CTAAAAAAGTAAAGGAAATTTATGTTTCACATGTATCAATGATGAAAAGTTTTCCAGCTACGAGCAGTT |
| BMT1 knockout Rev | TCTTTTTCATTGGTACAGGAAAATATTCTTGTTGACGCAAAAAATTAAATAGGAATTTCAATGATGAAAAGTTTTCCCAGTCACGACGTT |
| BMT2 knockout Fwd | CCTTATAAGGGTAAAATAGGGAATATTCTTGTTGACGCAAAAAATTAAATAGGAATTTCAATGATGAAAAGTTTTCCCAGTCACGACGTT |
| BMT2 knockout Rev | TAAACCCATAATCATTCAATTCTAATAATTCTTGTTGACGCAAAAAATTAAATAGGAATTTCAATGATGAAAAGTTTTCCCAGTCACGACGTT |
| BMT5 knockout Fwd | GCAGTACCGATTGTGTTTTTGATGACATCATTTGTGTTTCTATGTTTTGTTGATATGAAAAGTTTTCCCAGTCACGACGTT |
| BMT5 knockout Rev | CTGTGTTTTTCAATATTATGCAATGATGAAAAGTTTTCCCAGTCACGACGTT |
| probe BMT1 Fwd | ATACAATCTATTTATCATAAATATATCAGATAATATGCAATGATGAAAAGTTTTCCCAGTCACGACGTT |
| probe BMT1 Rev | ATACTGGGATAGGGGCGATT |
| probe BMT2 Fwd | GAGAAATGTCGCTGTGTGA |
| probe BMT2 Rev | TGTTTTTTCCAGGGATATGT |
| probe BMT5 Fwd | GACCTGACCGATATTGACAT |
| probe BMT5 Rev | ATGGGCACACAAATACCAT |
| probe URA3 Fwd | GCCTACACAGATGACACAAGCAT |
| probe URA3 Rev | GCATTCCACACCAGCATCTTATAC |
| probe HWP1 Fwd | CAATTTGAGCTCCCTACTCC |
| probe HWP1 Rev | GTCAATTGAGCCGAGGAC |
| probe RPS1 Fwd | ATGGCTGTCGGTAAAAACAAG |
| probe RPS1 Rev | AAAGCCCAATAATGAAACCAAAG |
| probe ACT1 Fwd | ACCGGAGCTCCATGAATCCA |
| probe ACT1 Rev | GGATGGACCAGATTTGTCGT |
| HWP1-1 | CTCAATTGAGCCGAGGAC |
| HWP1-2 | TTCAAAATGAGAAATAGGAC |
| V5-HIS | CTCAATTGAGCCGAGGAC |

Abbreviations: Fwd, forward; Rev, reverse.
2.10^5 colony-forming units (cfu) into the lateral tail vein. Mice were monitored daily and humanely killed when they showed signs of distress.

For determination of organ fungal burdens, cells were suspended in sterile physiological saline. For each *C. albicans* strain, 5 female BALB/c mice (8–10 weeks old) or 4 C57BL/6 or gal3-/- female mice (8–10 weeks old) were given intraperitoneal injections of 5.10^7 cfu. Three days after injection, mice were sacrificed and organs (spleen, kidneys and liver) were removed aseptically, weighed, homogenized, and suspended in 5 mL sterile water. Homogenates were plated on Sabouraud chloramphenicol agar and incubated for 24 hours at 37°C. Colony-forming units were counted and reported to the organ’s weight.

The results shown are from 3 independent experiments. Survival data were analyzed by the Kaplan-Meier survival analysis. Fungal burdens were analyzed by the Kruskal-Wallis test followed by post hoc testing using the unpaired Mann-Whitney U test. *P* values <.05 were considered significant.

**RESULTS**

**β-1,2-mannosyltransferases 1 and 3 Are Involved in O-Mannosides β-1,2-Mannosylation**

O-mannosides released from whole yeast cells were analyzed by fluorophore-assisted-carbohydrate-electrophoresis (FACE), which separates oligosaccharides according to their degree of polymerization, to the type of saccharide and to their glycosidic linkage [18]. O-mannoside residues depended upon growth temperature (Figure 2A). The O-mannopentaose migrated as a doublet; the lower band was the main mannopentaose recovered at 28°C. This residue and the mannotetraose were the only O-mannosides resistant to α-mannosidase digestion, suggesting that they may contain terminal β-Mans. To confirm the presence of β-Mans in *C. albicans* O-mannan and to characterize their biosynthesis, we applied the strategy used previously to identify the 6 Bmts involved in the β-1,2-mannosylation of PPM and PLM [21, 22] (Figure 1A and B). O-mannosides from mutants lacking one of the 9 Bmts and their reference strain, grown at 28°C before their O-mannosides were released by β-elimination treatment. All the mutants and their reference strains are Ura+ with Clp10 integrated in the same RPS1 allele. Different carbohydrate standards were used to evaluate the monomer number in the oligomannoside chains. Abbreviations: M3, mannotriose; M4, mannotetraose; M5, mannopentaose; M6, mannohexaose; Mnt, α1,2-mannosyltransferase.
biosynthesized by a protein mannosyltransferase isoform [23] and by α1,2-mannosyltransferases (Mnt) 1 and/or 2 [14, 24] and formed a heteropolymer (Figure 1C). As expected, FACE analysis of O-mannosides from the double mnt1Δ/mnt2Δ mutant revealed that, unlike the reference strain, they contained neither a mannnotetraose nor a mannopentaose (Figure 2C). Additional bands detected in mnt1Δ/mnt2Δ mutant were certainly nonmannosyl products as previously reported [14]. Reactivity to mAb 5B2 was reduced on CWMPs of mnt1Δ/mnt2Δ mutant compared with the control strain, whereas α-Mans levels were comparable between the 2 strains (Figure 3A).

**BMT1 Deletion Dramatically Affects Mannoproteins β-1,2-Mannosylation**

In contrast to PPM, β-Mans epitopes were not detected on CWMPs from the bmt1Δ mutant, whereas CWMPs from the other bmtΔ mutants were β-1,2-mannosylated like the reference strain (Figure 3B). Enhanced staining was obtained for the bmt1Δ mutant with ConA compared with the other strains (data not shown). This was likely to be due to the unmasking of terminal α-mannosyl. These data reveal that CWMPs and PPM are glycosylated in a different manner and that CWMPs β-1,2-mannosylation is only initiated by Bmt1.

The β-1,2-mannosylation of a highly O-glycosylated CWMP, the Hwp1 [25], was then studied. After replacement of the glycosylphosphatidylinositol signal sequence with a 6xHis tag, recombinant Hwp1 was purified from culture supernatants of CAI-4 and bmt1Δ strains by affinity chromatography (Figure 3B). CAI-4 6xHis-Hwp1 was assessed by Western blot analysis using mAb 16B1 (Figure 3C). Protein staining showed that 6xHis-Hwp1 copurified with another protein of 75 kDa (data not shown). Mass spectrometry analysis of this protein, which was performed by the SICaPS platform of IMAGIF (www.imagif.cnrs.fr), confirmed that it was not a digestion product of Hwp1 but was the hyphal cell wall protein Pra1 (pH Regulated Antigen 1) [26]. The Pra1 is secreted by hyphal cells [27], and thus it was not surprising to recover it from culture supernatants. Furthermore, Pra1 is a zinc-binding protein [28], with affinity for other divalent cations, which may explain why Pra1 copurified on the nickel column. Western blot analysis with mAb 5B2 showed that similar to Hwp1, Pra1 was β-1,2-mannosylated; however, in contrast to Hwp1 [16], Pra1 had β-Mans epitopes in both O- and N-glycans (Figure 3C). PNGase treatment of Pra1 resulted in a clear molecular weight shift of this protein (Figure 3C) and a reduction in mAb 5B2 specificity.

Deletion of BMT1 deprived 6xHis-Hwp1 and Pra1 of their β-Mans epitopes (Figure 3D). Similar results were obtained with other anti-β-Mans antibodies (data not shown). Staining with mAb 16B1 and ConA confirmed that CAI-4 and bmt1Δ expressed approximately equal amounts of proteins. Taken together, these results suggest that, as for yeast CWMPs, initiation of β-Mans expression on hyphal CWMPs is dependent on Bmt1.
β-1,2-mannosyltransferase 1 Is Responsible for Surface Expression of Hyphal β-1,2-oligomannosides

We showed previously that single BMT deletions have no effect on β-Mans surface expression [21]. Considering that β-Mans biosynthesis is initiated by at least 3 Bmts, it is not surprising that single deletion has no impact on surface expression of β-Mans. Phosphopeptidomannan differs between yeast and hyphal cells [29], with reduced amounts of β-Mans in PPM phosphomannosides of hyphal cells. Therefore, we assessed whether deletion of BMT1 would affect exposure of β-Mans on hyphae. Staining of hyphal cells from selected strains with mAb 5B2 confirmed that, compared with CAI-4, the bmt1Δ mutant expressed β-Mans on the mother cells but not on the lateral walls of germ tubes (Figure 4). Expression of β-Mans in germ tubes was recovered by the reintroduction of BMT1. In contrast, bmt2Δ and bmt5Δ mutants expressed β-Mans at the surface of both germ tubes and mother cells (data not shown). This immunofluorescence confirmed that, in contrast to mother cells, germ tubes do not express β-Mans from N-glycan phosphomannosides and PLM (Figure 1A and B) on their surface. The mnt1Δ/mnt2Δ mutant expressed, even if at a reduced level, β-Mans on germ tubes (Figure 4), certainly at the nonreducing ends of N-glycans (Figure 1A). These data show that the glycosylation motifs on the cell wall surface vary between germ tubes and mother cells. The β-Mans expressed on the germ tube surface were therefore synthesized by Bmt1 and Bmt3 and correspond to β-Mans from O-mannans and N-mannans at the nonreducing end of α-1,2-Mans (Figure 1A and C).

BMT1 Deletion Affects Candida albicans Virulence

To investigate the possible involvement of β-Mans to O-mannosides virulence [14, 30], we analyzed the survival of the bmt1Δ mutant in a murine model of invasive candidosis [31]. BALB/c mice were chosen because they have been widely used to identify factors that contribute to C. albicans virulence. Mice received a tail vein injection of either bmt1Δ mutant or its reference strain for mortality studies. Deletion of BMT1 reduced the survival of BALB/c mice, suggesting that the bmt1Δ mutant displays hypervirulence (Figure 5A). Similar to β-Mans epitope biosynthesis, wild-type virulence was partially recovered by complementing the mutant with a single copy of BMT1. To analyze the global role of β-Mans on virulence, we generated a strain devoid of β-Mans. We deleted BMT2 and BMT5, coding for Bmts that initiate the synthesis of β-Mans on PPM phosphomannosides and PLM (Figure 1A and B) in the bmt1Δ mutant background. We confirmed by Western blot analysis (data not shown) and immunofluorescence (Figure 4) that the bmt1Δ bmt2Δ bmt5Δ mutant had no β-Man in its cell wall. This mutant was as virulent as the bmt1Δ mutant (Figure 5A). To rule out faster elimination of the reference strain by blood cells or serum components, we used a second murine model of invasive candidosis. Intraperitoneal injection of C. albicans has been used in different studies as a systemic infection model to measure tissue dissemination of the yeast [32–34]. Comparable results to the survival assay were obtained when spleen, kidneys, and liver fungal burdens were determined following BALB/c mice intraperitoneal infection (Figure 5B). Organs, particularly spleen and liver, contained more fungal cells after mice challenge with bmt1Δ mutant than reference strain (Figure 5B). All independent experiments gave similar results, and even with the strong heterogeneity of the mutant fungal burdens between mice, the differences between the bmt1Δ mutant and the reference strain were significant in the liver. Restoration of the native BMT1 gene into bmt1Δ mutant attenuated significantly its dissemination. The differences between the bmt1Δ bmt2Δ bmt5Δ mutant and the wild-type strain were significant in the different organs analyzed.

The lectin, galectin-3 (Gal3), has been described as a β-Mans receptor [35], and it is proposed to induce killing of C. albicans [36]. Spleen, kidneys, and liver fungal burdens in Gal3−/− mice
and C57BL/6 control mice were therefore analyzed following intraperitoneal infection with the $bmt1\Delta$ and $bmt1\Delta \ bmt2\Delta \ bmt5\Delta$ mutants and the control strain. The 2 mutants had the tendency to disseminate less than the wild-type strain in liver of C57BL/6 mice, but the difference was not significant (Figure 5C). However, dissemination in the spleen and the kidneys was similar for the 3 strains. It is interesting to note that spleen from Gal3$^{-/-}$ mice infected with CAI-4 contained significantly higher fungal burdens than those from C57BL/6 mice ($P < .05$) (Figure 5C and D). In contrast, no significant difference was observed with the 2 $bmts\Delta$ mutants. Gal3$^{-/-}$ mice displayed lower fungal burdens in the spleen when infected with the $bmt1\Delta/\ bmt2\Delta/\ bmt5\Delta$ mutant (Figure 5D).

DISCUSSION

Surface mannoproteins have specific functions related to C. albicans virulence including adhesion to host tissues, biofilm formation, and triggering of the immune system [37]. Some of these virulence attributes are conferred through proteins $N$- and/or $O$-glycans [14, 38]. Emphasis has then been placed on the role of the cell wall glycans in the interplay between C. albicans and its host. The commensal/pathogen transition is critically dependent on the biological activities of specific oligomannosides, including $\beta$-Mans that are recognized by the soluble lectin Gal3 [35]. These epitopes have immunomodulatory properties [8, 11] and seem to be related to C. albicans infections [10, 39]. Structural studies performed by Shibata et al [4] have described $\beta$-Mans in PPM $N$-glycans, whereas Trinel et al [5] characterized $\beta$-Mans in the PLM glycolipid, a member of the mannose-inositol-phosphoceramide family.

We have later identified the 6 Bmts responsible for their biosynthesis [21, 22]. We have shown that $\beta$-Mans epitopes are widely distributed among the different CWMPs [16]. We also demonstrated that, in contrast to PPM, $O$-mannosylation is part of the C. albicans $\beta$-1,2-mannosylation process. We report here the presence of $\beta$-Mans and their biosynthetic process in C. albicans $O$-mannan. Two mutants, $bmt1\Delta$ and $bmt3\Delta$, had truncated $O$-mannosides with an accumulation of $O$-mannotriose and $O$-mannotetraose, respectively. Alpha-mannosidase treatment of $O$-mannosides from wild-type strain digests all mannosides containing less than 4 mannoside residues, showing that a heteropolymer rather than a homopolymer made up $\alpha$-mannosidase-resistant $O$-mannans. The Bmt1 is known to add $\beta$-mannose on $\alpha$-1,2-Mans in N-mannan, initiating then a heteropolymer composed of $\alpha$-1,2-Man with terminal $\beta$-Mans (Figure 1A) [21]. This Bmt1 substrate would also be available on $O$-mannosides after

Figure 5. Virulence assays are shown. (A) Cumulative mortality of mice given injections of $4 \times 10^5$ cells from $bmt1\Delta$, $bmt1\Delta/\ BMT1$, and $bmt1\Delta \ bmt2\Delta \ bmt5\Delta$ mutants and their reference strain, CAI-4. Difference between CAI-4 and $bmt1\Delta$ or $bmt1\Delta \ bmt2\Delta \ bmt5\Delta$ mutants were significant ($P < .05$). Fungal burdens of different Candida albicans strains in the spleen, kidney, and liver of BALB/c (B), C57BL/6 (C), and Gal3$^{-/-}$ (D) mice, 3 days postintraperitoneal infection. Mice received intraperitoneal injection of $5 \times 10^7$ CAI-4, $bmt1\Delta$, and $bmt1\Delta \ bmt2\Delta \ bmt5\Delta$ cells. The results shown are from 3 independent experiments. *, $P < .05$; **, $P < .01$. All the mutants and their reference strains are Ura$^+$ with Clp10 integrated in the same $RPS1$ allele.
Mnt1 and possibly Mnt2 activities (Figure 3D) [14, 24]. The mutant that lacks these 2 enzymes was deprived of β-mannosylated O-mannopentaoase and expressed less β-Mans on CWMPs than the reference strain. As for β-Mans at the nonreducing end of PPM α-1,2-Mans [21], Bmt3 adds the second β-mannose.

We highlighted heterogeneity of O-mannosidases released from yeast cells, and we demonstrated variability in the β-Mans content determined by growth temperature. The β-Mans expression is complex because yeasts grown at 37°C express less β-Mans on N-mannans than yeasts grown at 28°C [40, 41]. C. albicans cells display 2 O-mannopentaoses (Man₅) mainly composed of an α-mannosidase-sensitive Man₃ at 37°C and an α-mannosidase-resistant Man₅ at 28°C. These data suggest that β-Mans from both N- and O-mannans undergo the same temperature-dependent regulation.

β-1,2-oligomannosidases at the nonreducing end of α-1,2-Mans are major CWMPs β-Mans in both yeasts and hyphae. Yeast CWMPs from bmt1Δ mutant did not harbor β-Mans epitopes, and 2 purified hyphal proteins, Hwp1 and Pra1, were no more β-1,2-mannosylated when expressed in this mutant. Lack of Bmt1 activity has been reported to have no impact on yeast surface expression of β-Mans [21], certainly due to expression of alternative sources of β-Mans such as those present on PLM and N-mannans phosphomannosidases. We were surprised to find that surface expression of β-Mans on germ tubes was initiated only by Bmt1. β-1,2-mannosylation of Hwp1 and Pra1 was not affected in the bmt2Δ mutant (data not shown), but it was diminished in the mnt1Δ/mnt2Δ mutant, suggesting that hyphae-specific N-mannans contain β-Mans only at the nonreducing end of α-1,2-Mans. Supporting this hint, others have observed presence of non-β-1,2-mannosylated phosphomannose in hyphae [42].

Surface cell wall molecules are important because they can directly interact with the host. However, β-Mans biosynthesized by Bmt1 apparently have no detrimental impact on the host, because mutants lacking Bmt1 were more or equally virulent than control strains in 2 different genetic background mice, BALB/c and C57/BL6 mice, respectively. Wild-type strain clearly disseminated in the mnt1Δ/mnt2Δ mutant, suggesting that hyphae-specific N-mannans contain β-Mans only at the nonreducing end of α-1,2-Mans. Supporting this hint, others have observed presence of non-β-1,2-mannosylated phosphomannose in hyphae [42].

Surface cell wall molecules are important because they can directly interact with the host. However, β-Mans biosynthesized by Bmt1 apparently have no detrimental impact on the host, because mutants lacking Bmt1 were more or equally virulent than control strains in 2 different genetic background mice, BALB/c and C57/BL6 mice, respectively. Wild-type strain clearly disseminated in the mnt1Δ/mnt2Δ mutant, suggesting that hyphae-specific N-mannans contain β-Mans only at the nonreducing end of α-1,2-Mans. Supporting this hint, others have observed presence of non-β-1,2-mannosylated phosphomannose in hyphae [42].

Our data show that β-Mans expression is under the control of regulatory mechanisms that favor exposure of α-1,2-Mans and their associated virulence and facilitate the yeast to counteract Galectin-3 may be secreted by neutrophils to aid opsonization and phagocytosis of yeast cells [49]. However, incubation of bmt1Δ and bmt1Δ/bmt2Δ/bmt5Δ mutants and their reference strain with human neutrophils did not confirm this hypothesis, and these mutants were found to be more susceptible to these phagocytes (data not shown). Comparing dissemination in C57BL/6 and Gal3−/− mice, we showed that the wild-type strain was more virulent in the knockout mice, whereas the mutants had similar (bmt1Δ mutant) or reduced (bmt1Δ/bmt2Δ/bmt5Δ mutant) virulence. These data indicate that Galectin-3 potentiates the elimination of C. albicans; however, in Gal3−/− deficient cells, β-Mans expressing strains of C. albicans are more virulent.

CONCLUSIONS

Our data show that β-Mans expression is under the control of regulatory mechanisms that favor exposure of α-1,2-Mans and their associated virulence and facilitate the yeast to counteract Gal3-mediated host defenses. Regulation of Gal3 expression by the host and regulation of β-Mans expression by C. albicans may be crucial events controlling the balance between saprophytic and parasitic status of the yeast.

Acknowledgments

We gratefully acknowledge Professor A. P. Mitchell (Carnegie Mellon University, Pittsburgh, PA), Professor A. J. P. Brown (Department of Molecular and Cell Biology, Aberdeen, UK), and Professor R. Robert (GEIHP, Université d’Angers, France), for providing pDDB57 and CIp10 plasmids and mAb 16R1. We are indebted to Annick Masset for excellent technical assistance. This work has benefited from the facilities and expertise of the SIGAPS platform of IMAGIF (Centre de Recherche de Gif-ww.imagif.cnrs.fr). We thank the animal facility Département Hospitalo-Universitaire de Recherche Expérimentale from IMPRT-IFR114 for the maintenance of mice.

Financial support. This work was supported by the Agence Nationale de la Recherche (grant ANR-09-MIE-031-01); the European project AllFun from the 7th Framework Programme-Health (grant 260338), and the Collège Doctoral Lille Nord de France (Ouverture Internationale des Etudes et de la Formation Doctorale en Région Nord-Pas de Calais). N. A. R. G. was also supported by the Wellcome Trust (grants 080088, 086827, 075470, and 099215).
41. Goto K, Okawa Y. Activity and stability of alpha- and beta-mannosyltransferases in Candida albicans cells cultured at high temperature and at low pH. Biol Pharm Bull 2008; 31:1333–6.
42. Shibata N, Fukasawa S, Kobayashi H, et al. Structural analysis of phospho-α-mannan-protein complexes isolated from yeast and mold form cells of Candida albicans NIH A-207 serotype A strain. Carbohydr Res 1989; 187:239–53.
43. Schofield DA, Westwater C, Balish E. Divergent chemokine, cytokine and beta-defensin responses to gastric candidiasis in immunocompetent C57BL/6 and BALB/c mice. J Med Microbiol 2005; 54:87–92.
44. Shaub JF, Datta K, Rhee P. Niche-specific requirement for hyphal wall protein 1 in virulence of Candida albicans. PLoS One 2013; 8:e80842.
45. Weinstein Y, Ran S, Segal S. Sex-associated differences in the regulation of immune responses controlled by the MHC of the mouse. J Immunol 1984; 132:656–61.
46. Hall RA, Bates S, Lenardon MD, et al. The Mnt2 mannosyltransferase family modulates mannoprotein fibril length, immune recognition and virulence of Candida albicans. PLoS Pathog 2013; 9:e1003276.
47. Shinohara H, Nagi-Miura N, Ishibashi K, et al. Beta-mannosyl linkages negatively regulate anaphylaxis and vasculitis in mice, induced by CAWS, fungal PAMPs composed of mannanprotein-beta-glucan complex secreted by Candida albicans. Biol Pharm Bull 2006; 29:1854–61.
48. Linden JR, De Paepa ME, Laforce-Nesbitt SS, Bliss JM. Galectin-3 plays an important role in protection against disseminated candidiasis. Med Mycol 2013; 51:641–51.
49. Linden JR, Kunkel D, Laforce-Nesbitt SS, Bliss JM. The role of galectin-3 in phagocytosis of Candida albicans and Candida parapsilosis by human neutrophils. Cell Microbiol 2013; 15:1127–42.
50. Gillum AM, Tsay EY, Kirsch DR. Isolation of the Candida albicans gene for orotidine-5′-phosphate decarboxylase by complementation of Saccharomyces cerevisiae ura3 and Escherichia coli pyrF mutations. Mol Gen Genet 1984; 198:179–82.
51. Wilson RB, Davis D, Mitchell AP. Rapid hypothesis testing with Candida albicans through gene disruption with short homology regions. J Bacteriol 1999; 181:1868–74.
52. Fonzi WA, Irwin MT. Isogenic strain construction and gene mapping in Candida albicans. Genetics 1993; 134:717–28.