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Murine Model of Dextran Sulfate Sodium-induced Colitis Reveals *Candida glabrata* Virulence and Contribution of β -Mannosyltransferases*

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Background: β -1,2-Linked mannosides (β -Mans) are adhesins present in the cell wall of the pathogenic yeast *Candida glabrata*.

Results: β -Mans are associated with intensive gut colonization by *C. glabrata*, which exacerbates intestinal inflammation and induces mouse mortality.

Conclusion: β -Mans affect the virulence of *C. glabrata* in the host.

Significance: This study confirms and extends our knowledge about β -Mans as therapeutic targets for combating life-threatening *Candida* infections.

Candida glabrata, like *Candida albicans*, is an opportunistic yeast pathogen that has adapted to colonize all segments of the human gastrointestinal tract and vagina. The *C. albicans* cell wall expresses β -1,2-linked mannosides (β -Mans), promoting its adherence to host cells and tissues. Because β -Mans are also present in *C. glabrata*, their role in *C. glabrata* colonization and virulence was investigated in a murine model of dextran sulfate sodium (DSS)-induced colitis. Five clustered genes of *C. glabrata* encoding β -mannosyltransferases, BMT2–BMT6, were deleted simultaneously. β -Man expression was studied by Western blotting, flow cytometry, and NMR analysis. Mortality, clinical, histologic, and colonization scores were determined in mice receiving DSS and different *C. glabrata* strains. The results show that *C. glabrata bmt2–6* strains had a significant reduction in β -1,2-Man expression and a disappearance of β -1,2-mannobiose in the acid-stable domain. A single gavage of *C. glabrata* wild-type strain in mice with DSS-induced colitis caused a loss of body weight, colonic inflammation, and mortality. Mice receiving *C. glabrata bmt2–6* mutant strains had normal body weight and reduced colonic inflammation. Lower numbers of colonies of *C. glabrata bmt2–6* were recovered from stools and different parts of the gastrointestinal tract. Histopathologic examination revealed that the wild-type strain had a

greater ability to colonize tissue and cause tissue damage. These results showed that *C. glabrata* has a high pathogenic potential in DSS-induced colitis, where β -Mans contribute to colonization and virulence.

Like *Candida albicans*, *Candida glabrata* is an endosaprophytic yeast that can cause severe infections in humans (1), including bloodstream and mucosal infections (2–4). Systemic *C. glabrata* infections are associated with higher mortality than *C. albicans* infections (5). In addition, *C. glabrata* is resistant to some antifungal drugs, particularly azoles, making clinical management difficult (6, 7). Experimental research on *C. glabrata* has been hampered by difficulties in establishing an animal model mimicking the pathogenic potential of this species in humans (8, 9).

Another common feature shared by *C. glabrata* and *C. albicans* is the ability to synthesize β -mannosides (β -Mans)³ (10, 11). Several studies in *C. albicans* have shown that β -Mans display a wide range of specific characteristics related to pathogenesis. β -Mans modulate cell wall hydrophobicity (12) and act as specific adhesins through interaction with galectin-3 (13, 14). This interaction promotes *C. albicans* adhesion and gut colonization and stimulates host cells to produce cytokines (15). Finally, anti- β -Man antibodies are protective in experimental models of *C. albicans* infection (15). No physiological role has been established so far for β -Mans in *C. glabrata*.

Recent research has resulted in the discovery of nine *C. albicans* BMT genes encoding β -mannosyltransferases in *C. albicans* (16). These enzymes transfer β -1,2-linked mannose units

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³ The abbreviations used are: β -Man, β -mannosides; PPM, phosphopeptidomannan; DSS, dextran sulfate sodium; Gal-3, galectin-3; ConA, concanavalin A; GNL, *G. nivalis* lectin.

β -Mans Impact on *C. glabrata* Virulence

TABLE 1

C. glabrata strains used in the study

Strain	Description	Parent strain	Genotype
<i>C. glabrata</i> BG2	Wild type		MATa
<i>C. glabrata</i> 183	<i>bmt2-6Δ1</i>	<i>C. glabrata</i> BG2	<i>bmt 2-6Δ::URA3</i>
<i>C. glabrata</i> 184	<i>bmt2-6Δ2</i>	<i>C. glabrata</i> BG2	<i>bmt 2-6Δ::URA3</i>

to β -1,2 linkage-containing side chains or attach β -1,2-linked mannose units to α -linked manno oligosaccharides (17). The mechanism of β -mannosylation of carrier molecules is complex, with various β -mannosyltransferases involved at specific stages of phosphopeptidomannan (PPM) or phospholipomannan β -mannosylation. Furthermore, the majority of cell wall mannoproteins may be β -mannosylated by processes that differ from those involved for PPM (18–19). Our knowledge of *C. glabrata* β -mannosylation is restricted to studies concerning PPM (17). However, due to its haploidy, *C. glabrata* provides an easier model than *C. albicans* for studies into the function of *BMT* genes and the biological effects resulting from their deletion. BLAST analysis has revealed seven putative genes encoding β -mannosyltransferases. Among them, *BMT2*–*BMT6*, associated in a cluster on chromosome B, provide an easy deletion model to analyze their effect on virulence.

In this study, *C. glabrata* was investigated in a murine model initially developed to study *C. albicans* intestinal colonization and inflammation. The model consisted of the induction of colitis using dextran sulfate sodium (DSS) (20, 21). The DSS-induced colitis model is used to experimentally mimic chronic inflammatory bowel diseases because it induces intestinal inflammation, has low mortality rates, and is highly reproducible (22). Low doses of DSS were used in order to establish *C. glabrata* colonization in mice, followed by the administration of drinking water to assess the effects of *C. glabrata* colonization on colonic epithelial regeneration. The objectives of the study were to investigate (i) the effect of DSS-induced inflammation on *C. glabrata* colonization; (ii) the effect of *C. glabrata* colonization on inflammation and whether *C. glabrata* colonization impairs colonic epithelium regeneration; and (iii) the role of β -Mans in this model using *C. glabrata* strains deficient in five genes encoding β -mannosyltransferases.

EXPERIMENTAL PROCEDURES

Yeast Strains—The *C. glabrata* strains used are shown in Table 1.

Disruption of *C. glabrata* β -Mannosyltransferase Genes—The disruption cassette was constructed by PCR fusion using a strategy similar to that described by Kuwayama *et al.* (23). For the *BMT2*–*BMT6* cluster, upstream and downstream gene fragments and the *URA3* marker were amplified using an HFPCR kit (Clontech, Palo Alto, CA). The primers used for these amplifications were as follows: *BMTX-3'5* (TACAACGTCGTGACTGGGCCA CGGTCATCTGGTACACATC), *BMTX-5'3* (GTCATAGCTGTTTCCTGTCCCT-TGGGTTAGTTGAAGGAG), and the reverse complements of MKRfBMTX (GATGTGTACCAGATGACCGTGGCC-AGTCACGACGTTGTA) and MKRrBMTX (CTCCTTCAAC-TAACCAAGGGACAGGAAACAGCTATGAC), respectively.

TABLE 2

^1H - ^{13}C anomer chemical shifts and $^1J_{\text{H/C}}$ coupling constant of phosphopeptidomannan isolated from *C. glabrata* wild-type strain

These values are given using acetone as the internal standard (2.225 for ^1H and 31.55 ppm for ^{13}C).

Residues	^1H ppm	^{13}C ppm	$^1J_{\text{H/C}}$ Hz
M β M α -P (a)	5.56	95.5	173
M α 2M α 2M α 2 (b, c)	5.30	102.0	174
M β 2M α 2M α 2 (d)	5.18	101.4	172
M α 3M α 2 (e)	5.15	103.7	172
M α 6M α 6M α 6 (f)	5.10	99.6	172
M α 3M α 2M (g)	5.05	103.6	171
M α 2M α 2 (h)	5.05	103.6	171
M β 2M β 2M α 2 (i)	4.88	100.3	161
M β 2 M β 2M α 2 (j)	4.86	102.4	161
M β M α -P- (k)	4.84	100.1	162
M β 2M α 2 (l)	4.79	99.9	160
M α 6M α 6 (m)	4.92	100.9	171
M α 6M α 6 (n)	4.92	100.9	171

In addition, 5 ng of each of the three amplified fragments was used as a substrate for PCR fusion with the primers *BMTX-5'5* (GCAACAACGTTTAGACCAGTGC) and *BMTX-3'3* (CAGATCCACACGAGAGATACG) and the following program: 94 °C for 30 s, followed by 35 cycles of 94 °C for 15 s, and 68 °C for 4 min. The final PCR fragment represented the *bmt2-6Δ::URA3* cassette. The BG14 (*ura3*) strain was transformed with the deletion cassette, as described previously (24), and *Ura*⁺ transformants were then selected on –*URA* DO plates. Proper integration of the cassette was determined by PCR using a primer that annealed to a region outside the disruption cassette (*BMTXex*, GCGAGCTGAAGTATACCAGACC) and a primer that annealed to a sequence within the marker (*URA3F*). A second pair of primers was used to check for correct integration of the cassette (*BMTXex2*, TACTACCTGCACCAGACAC-CAG-*URA3R*). PCR amplifications of the *BMTXex*–*BMTex2* region were used to verify knock-out of the wild-type gene in each putative deletion strain. Southern blot analysis was used to confirm gene deletion. Two knock-out strains were produced independently for each gene or cluster and stored in 40% glycerol at –80 °C for further analysis.

Phosphopeptidomannan Extraction—PPM from cells grown in YPD medium was extracted as described previously (25). Briefly, cell pellets were suspended in 20 mM 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 SpeedVac concentrator.

NMR Experiments—Liquid NMR experiments were performed using a 9.4 T Avance Bruker[®] spectrometer, where ^1H and ^{13}C resonate at 400.33 and 100.67 MHz, respectively. Data were acquired with a Broad Band Inverse self-shielded z -gradient probe head. Spectra were recorded at 318 K in D_2O after two chemical exchanges with $^2\text{H}_2\text{O}$ (Euriso-top, Gif-sur-Yvette, France). Durations and power levels were optimized for each experiment. Spectra were recorded without sample spinning. The chemical shifts were expressed in ppm downfield from the signals of internal acetone: ^1H , 2.225 ppm; ^{13}C , 31.55 ppm.

Lectins and Monoclonal Antibodies—Lectins were used to study the expression of β -Man residues. These consisted of biotinylated concanavalin A (ConA) specific for terminal α -D-mannosyl and α -D-glucosyl residues and biotinylated

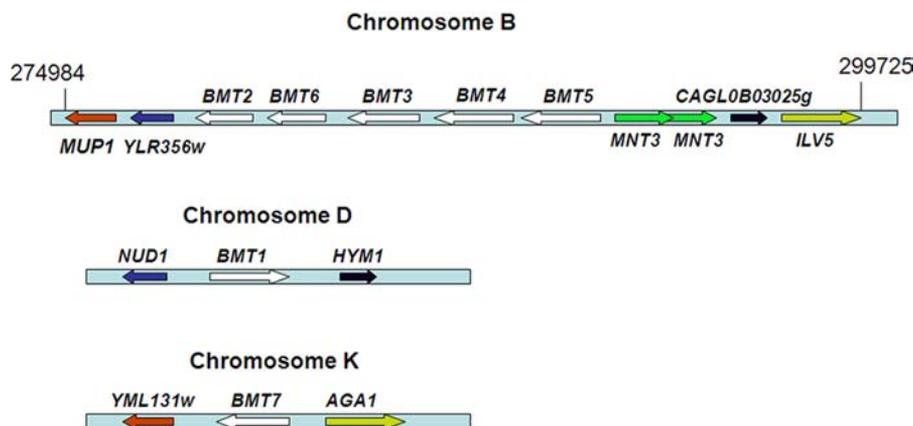


FIGURE 1. Disruption of *C. glabrata* β -mannosyltransferase genes.

Galanthus nivalis lectin (GNL) specific for terminal α -D-mannosyl, preferentially α -1,3 residues (19). Monoclonal antibodies (mAbs) were used to study the expression of β -Man residues. mAb 5B2 (26, 27) has a specificity analogous to serum factor 5, and its reactivity may overlap with factor 6 (28).

mAb 26G7 (provided by Prof. J. Ponton), obtained after immunization with a *C. glabrata* 150-kDa glycoprotein, binds to this species and to *C. albicans* serotype A strains (29, 30). Thus, it corresponds, at least in part, to factor 6, recognizing β -Man residues associated at the terminal non-reducing end of β -linked oligomannose chains (28).

Whole-cell Protein Extraction and Western Blot Analysis—Strains were grown for 16 h at 25 and 37 °C in Sabouraud broth. Proteins and glycoconjugates were recovered by alkaline extraction under reducing conditions (27). The protein content of each extract was estimated using a bicinchoninic acid protein assay (Pierce) and was adjusted to the same protein concentration (60 μ g) prior to analysis by SDS-PAGE (31) on a 10% acrylamide gel. Subsequently, the proteins were probed with either mAb 5B2 (1:2000 dilution) or 26G7 (1:1000 dilution), or biotinylated lectin ConA or GNL, both diluted 1:1000. Alkaline-labeled secondary antibodies and horseradish peroxidase-labeled streptavidin (1:2000 dilution) (Southern Biotech) were used to detect bound mAbs or lectins, respectively.

Flow Cytometry—*C. glabrata* was grown on Sabouraud agar at 24 °C for 48 h in conditions leading to optimal epitope expression for slide agglutination serotyping. *C. glabrata* cells (10⁶) were washed with phosphate-buffered saline (PBS), 2% fetal calf serum and incubated with either 26G7, 5B2, or fluorescein isothiocyanate-GNL, as described previously (32).

Animals—6–8-week-old female BALB/c mice were used. All mice were maintained by Charles River Laboratories. Two complete experimental series were performed independently. A total of 52 mice/experiment were divided into five control groups, including assessment of the effect of DSS alone, and three experimental groups, as shown in Table 2.

Ethics Statement—All mouse experiments were performed according to protocols approved by the Subcommittee on Research Animal Care of the Regional Hospital Center of Lille, France and in accordance with the European legal and institu-

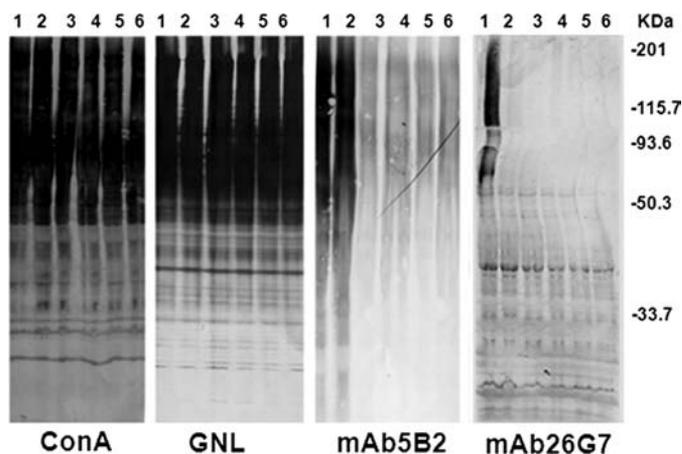


FIGURE 2. Mapping of α - and β -1,2-oligomannoside epitopes on mannoproteins present in wild-type and mutant strains of *C. glabrata*. Total cell extracts of wild-type (lanes 1 and 2), *bmt2*- Δ 1 (lanes 3 and 4), and *bmt2*- Δ 2 (lanes 5 and 6) strains grown in Sabouraud broth at 25 °C (lanes 1, 3, and 5) or 37 °C (lanes 2, 4, and 6) were separated by SDS-PAGE before blotting and staining with lectins specific for α -oligomannosides, ConA and GNL, and mAbs specific for β -1,2 oligomannosides, 5B2, and 26G7.

tional guidelines (86/609/CEE) for the care and use of laboratory animals.

Experimental Design—Mice were inoculated on day 1 by oral gavage with 5×10^7 cells of different *C. glabrata* strains in 200 μ l of PBS. From day 1 to day 14, mice were given 1.5% DSS (21) in drinking water to induce intestinal inflammation. After 14 days of DSS administration, mice were given tap water only until sacrifice on day 17.

Assessment of Clinical and Histologic Parameters—The mortality rate of DSS-treated mice was determined daily, and a colon biopsy was taken post mortem for histologic analysis. Total body weight was measured daily. The data are expressed as mean percentage change from starting body weight. A daily clinical activity score ranging from 0 to 8 was calculated as described elsewhere (20, 33). Histologic scores were evaluated by two independent, blinded investigators on two colon sections per mouse at magnifications of $\times 10$ and $\times 100$, as described previously (21). In order to localize *C. glabrata* cells in the colon, the tissue sections were incubated with fluorescein isothiocyanate-GNL and counterstained with PBS containing 0.02% Evans blue (21).

β -Mans Impact on *C. glabrata* Virulence

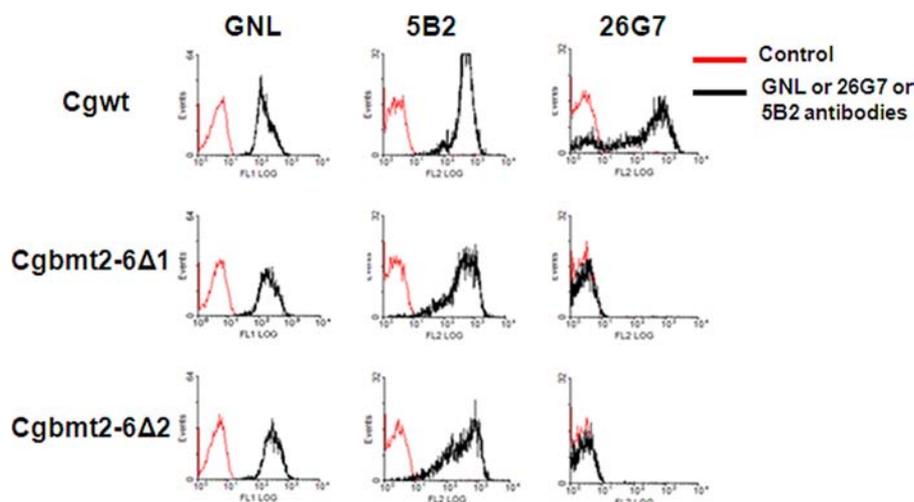


FIGURE 3. **Flow cytometry analysis of cell wall surface glycan expression in *C. glabrata*.** Cell wall surface glycan expression was analyzed in *C. glabrata* wild-type and mutant strains using mAbs 5B2 and 26G7 and GNL immunofluorescent staining. Red peaks (controls) are isotype mAb background fluorescence for each specific mAb or immunofluorescent stain investigated.

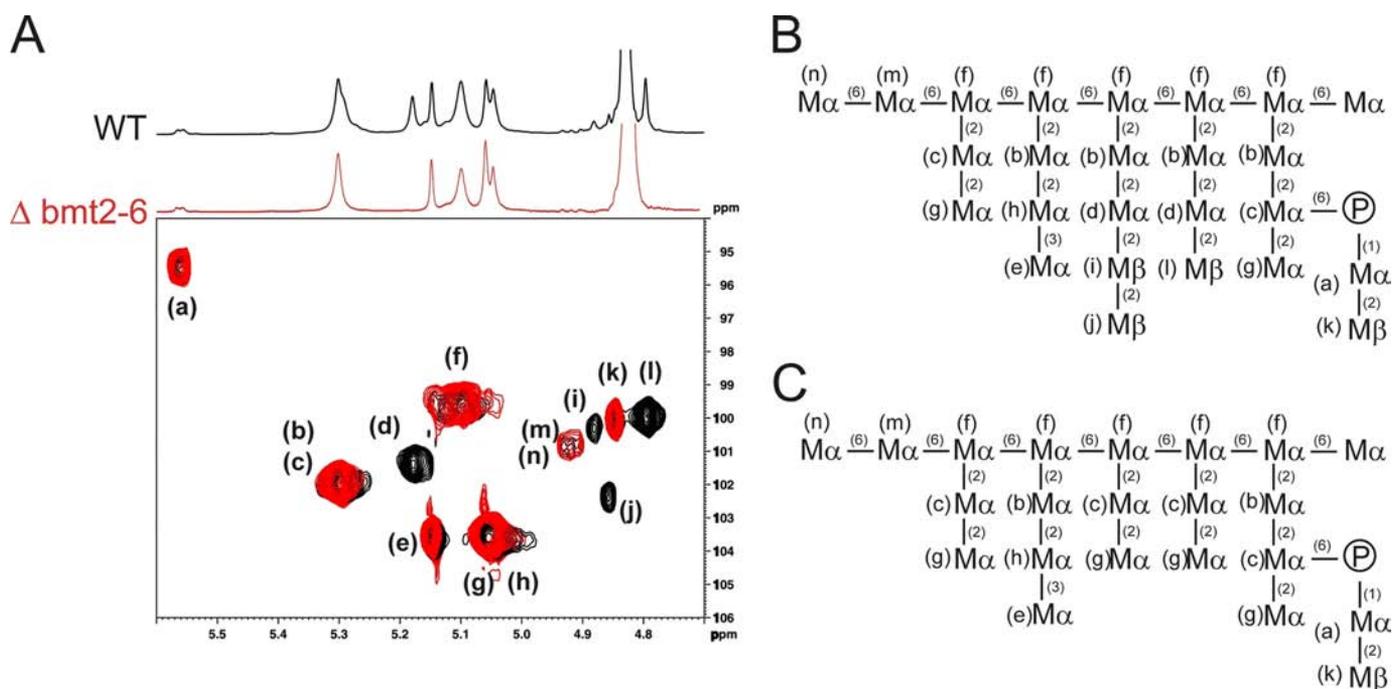


FIGURE 4. **Structural analysis of phosphopeptidomannan isolated from wild-type and *bmt2-6*-deficient strains.** A, anomer region of ^1H and ^1H - ^{13}C HSQC NMR spectra of wild-type (in black) and $\Delta bmt2-6$ strains of *C. glabrata* (in red). Shown are structural models of mannan moieties of PPMs isolated from wild-type (B) and $\Delta bmt2-6$ strains (C) of *C. glabrata*.

Analysis of Colonization—The presence of yeasts in the intestinal tract was determined by performing plate counts of feces (~ 0.1 g/sample) collected daily from each animal. Serial dilutions of the fecal samples were performed. The results were noted as colony-forming units (cfu) per μg of feces (20).

Presence of *C. glabrata* Colonization in Gastrointestinal Tract—To check for *C. glabrata* colonization, the animals were sacrificed, and the gastrointestinal tract was removed and separated into the stomach, ileum, and colon. The tissues were cut longitudinally. After removal of intestinal contents, the tissues were washed several times in PBS to minimize surface contamination from organisms present in the lumen. Serial dilutions of homogenates were performed. The results were noted as *C. glabrata* cfu/mg of tissue. Fluorescence

microscopy was performed to assess surface oligomannose expression on yeast colonies isolated from the stomach. Yeast suspensions deposited on slides were incubated with mAb 26G7 and with fluorescein isothiocyanate-GNL, as described previously (20).

Statistical Analysis—Data are expressed as the mean \pm S.E. for eight mice in each group. The results shown are from two independent experiments. All comparisons were analyzed using the Mann-Whitney *U* test. Statistical analyses were performed using StatView 4.5 (SAS Institute Inc., Meylan, France). Survival data were analyzed with the log rank test using SigmaPlot 10 (Systat Software, Cleveland, OH). Differences were considered significant when the *p* value was < 0.05 .

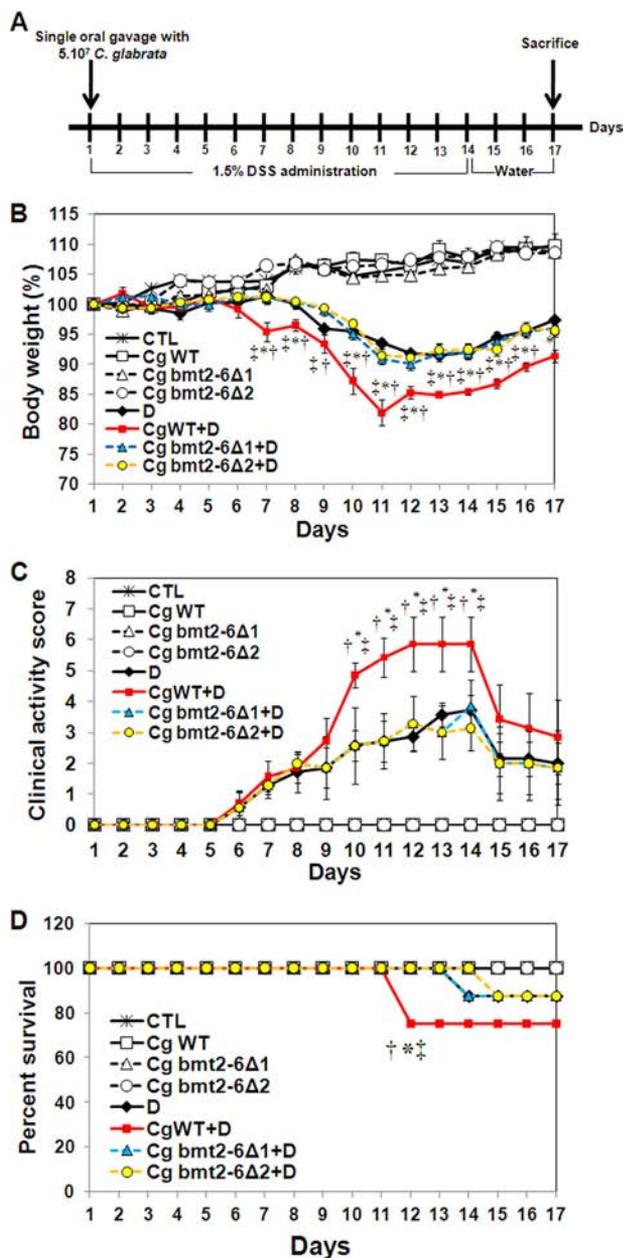


FIGURE 5. A, experimental design. Low doses of DSS were administered for 2 weeks, followed by the administration of drinking water for 3 days. The *C. glabrata* inoculum of 5×10^7 was the most adapted inoculum, as revealed by consistent intestinal colonization in a preliminary murine trial in which mice were challenged with a range of *C. glabrata* doses ranging from 10^7 to 10^8 cells/mouse. B, difference in body weight loss in mice colonized with *C. glabrata* during and after induction of colitis by DSS. Each data set represents the mean value for each body weight ($n = 10$ mice/control group and $n = 16$ mice/experimental group) and represents two independent experiments. *, $p < 0.05$ for CgWT+D mice versus D mice; †, $p < 0.05$ for CgWT+D mice versus Cg bmt2-6Δ1+D mice; ‡, $p < 0.05$ for CgWT+D mice versus Cg bmt2-6Δ2+D mice. C, clinical analysis of DSS-induced colitis in mice. Clinical score was determined by assessing weight loss, change in stool consistency, and presence of gross bleeding. The clinical score ranged from 0 to 8. *, $p < 0.05$ for CgWT+D mice versus Cg bmt2-6Δ1+D mice; †, $p < 0.05$ for CgWT+D mice versus Cg bmt2-6Δ2+D mice. D, percentage survival of mice colonized with *C. glabrata* during and after induction of colitis by DSS. Results are displayed as percentage survival from the time of *C. glabrata* challenge and DSS treatment ($n = 16$ mice/group from duplicate experiments). The survival data were statistically different by the log rank test. *, $p < 0.05$ for CgWT+D mice versus D mice; †, $p < 0.05$ for CgWT+D mice versus Cg bmt2-6Δ1+D mice; ‡, $p < 0.05$ for CgWT+D mice versus Cg bmt2-6Δ2+D mice. Error bars, \pm S.E.

TABLE 3
Groups of mice used in the experiments

Group	Inoculum and DSS treatment	n^a	Survival %
CTL	Water	10	100
CgWT	<i>C. glabrata</i> WT	10	100
Cg bmt2-6Δ1	<i>C. glabrata</i> bmt2-6Δ1	10	100
Cg bmt2-6Δ2	<i>C. glabrata</i> bmt2-6Δ2	10	100
D	DSS	16	87.5
CgWT+D	<i>C. glabrata</i> WT + DSS	16	75
Cg bmt2-6Δ1+D	<i>C. glabrata</i> bmt2-6Δ1 + DSS	16	87.5
Cg bmt2-6Δ2+D	<i>C. glabrata</i> bmt2-6Δ2 + DSS	16	87.5

^a Number of mice in two independent experiments.

RESULTS

Reduction of β-1,2-Oligomannoside Expression in C. glabrata bmt2-6Δ Mutants—BLAST analysis of *C. glabrata* revealed the presence of seven genes encoding a protein similar to *C. albicans* β-mannosyltransferase. Five of these genes (*BMT2*–*BMT6*) made up a gene cluster on chromosome B; the other two were on chromosomes D and K, respectively (Fig. 1). Two identical *C. glabrata* bmt2-6Δ strains were constructed independently by inactivating the five-gene cluster. In all our experiments, the two independently constructed *C. glabrata* bmt2-6Δ mutants displayed the same phenotype. In preliminary *in vitro* testing, they did not show any morphologic change or growth defects at either 25 or 37 °C; nor did they show increased susceptibility to osmotic imbalance when compared with the parental strain (data not shown).

Western blots were then performed on whole cell extracts from parental and mutant strains to examine β-1,2-oligomannoside expression using mAb 5B2, which reacts with two β-1,2-mannose residues as a minimal epitope, and mAb 26G7, which recognizes at least two β-1,2- and two α-1,2-linked mannose residues. As shown in Fig. 2, mAb 5B2 recognized a wide range of mannoproteins in the wild-type strain (Fig. 2, lanes 1 and 2), whereas only weak signals were obtained on mannoproteins larger than 90 kDa in the bmt2-6 mutant strains (Fig. 2, lanes 3–6). This confirms a reduction of β-Man epitope expression in the mutants. The results were similar at both 37 and 25 °C. This reduction of β-Man epitope expression was confirmed when immunoblots were revealed with mAb 26G7. However, as reported previously, the corresponding epitope (antigen 6) is not expressed *in vitro* at 37 °C (34). At 25 °C, this mAb recognized very large mannoproteins (molecular mass >115 kDa) in the wild-type extract. In the mutant strains, reactivity was almost undetectable when mAb 26G7 was used. In contrast, expression of α-Man residues, analyzed using ConA or GNL, was not affected by the *BMT* deletions (Fig. 2). Similar data were obtained with cells grown in YPD broth at 37 °C (data not shown).

Because it was intended to investigate *C. glabrata* colonization involving cell wall surface structures, the analysis of oligomannose expression was complemented by flow cytometry (Fig. 3). Surface expression of terminal α-1,3 mannosides was not affected by the gene deletions. By using this technique, the expression of β-1,2-mannosides assessed with mAb 5B2 was comparable with the *C. glabrata* wild-type strain, although some alteration occurred in the distribution of mAb 5B2-la-

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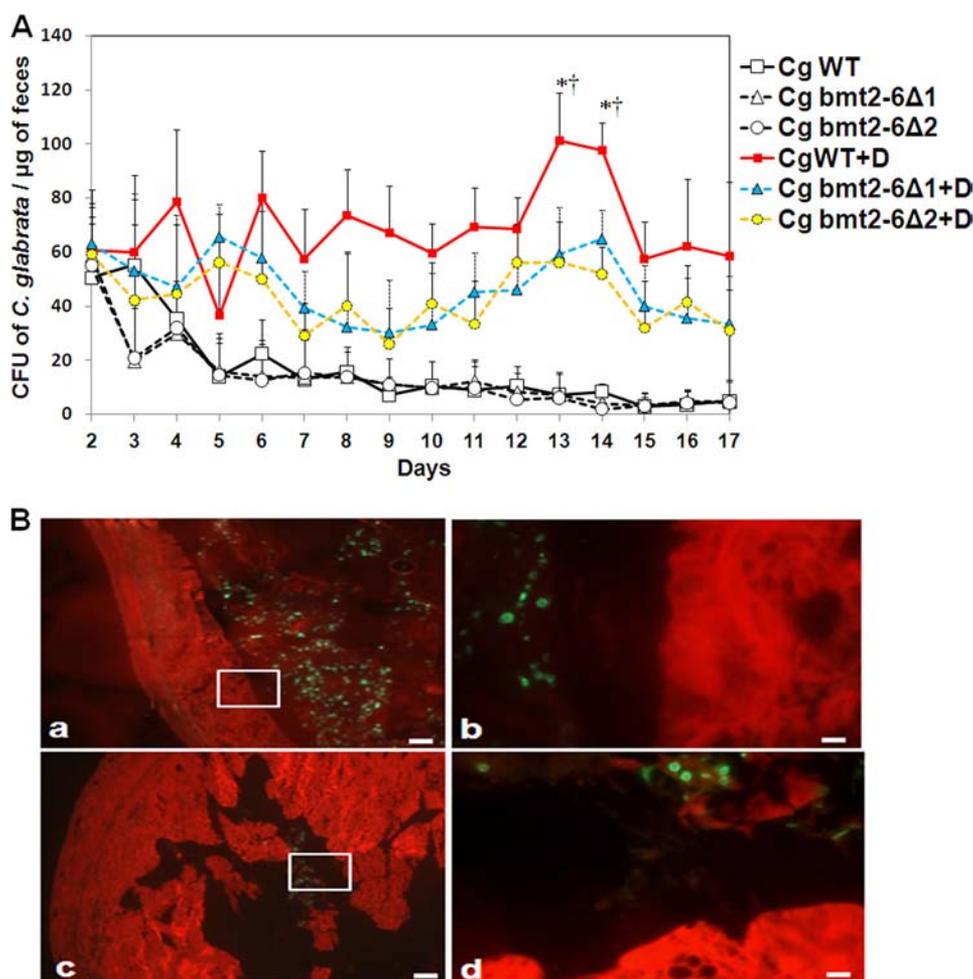


FIGURE 6. *A*, number of *C. glabrata* cfu recovered from stools. Each data set represents the mean value of counts for 16 mice per group from duplicate experiments. *, $p < 0.05$ for CgWT+D mice versus Cg bmt2-6 Δ 1+D mice; †, $p < 0.05$ for CgWT+D mice versus Cg bmt2-6 Δ 2+D mice. *B*, immunostaining of *C. glabrata* within the colon lumen. Representative sections of colon from a CgWT+D mouse (*a*) and Cg bmt2-6 Δ 2+D mouse (*c*). The boxed regions in *b* and *d* are shown at a higher magnification than in *a* and *c*, respectively. *C. glabrata* mutant and wild-type strains can be observed in the colon lumen. The scale bars represent 50 μ m (*a* and *c*) and 5 μ m (*b* and *d*). Error bars, \pm S.E.

beled yeasts. Expression of β -1,2-mannosides, assessed with mAb 26G7, on the *C. glabrata* wild-type strain revealed a broad range of reactivity. Deletion of *BMT2-6* resulted in the complete disappearance of this population (Fig. 3). Finally, the presence and location of β -1,2-mannosides on PPM as a major cell wall surface molecule were assessed on each strain by liquid ^1H and $^1\text{H}/^{13}\text{C}$ HSQC NMR analyses. Individual anomer signals were attributed based on previous assignments of PPM isolated from *C. glabrata*, *C. albicans*, and *Candida stellatoidea* (Table 2) (35–37). Experiments on PPM purified from the wild-type strain revealed the presence of four β -Man residue anomer signals (*i*, *j*, *k*, and *l*) ($^1J_{\text{H1/C1}}$ 160–162 Hz) with different locations within the polymannosylated chain (Fig. 4). According to their $^{13}\text{C1}$ chemical shift around 100 ppm, signals *i*, *k*, and *l* were attributed to *Man*(β 1,2)*Man*(α 1-) motifs, whereas signal *j* was attributed to *Man*(β 1,2)*Man*(β 1-) motifs ($^{13}\text{C1}$ at 102.4 ppm). Observation of a single α -Man-P $^1\text{H}/^{13}\text{C}$ signal at δ 5.56/95.5 and β -Man signal *k* established that the phosphomannan moiety of PPM isolated from the wild-type strain of *C. glabrata* was constituted by a single oligosaccharide, *Man*(β 1,2)*Man*(α 1-)P.

Altogether, NMR analysis established that PPM isolated from the wild-type strain was β -mannosylated on both acid-

stable and acid-labile domains (Fig. 4*B*). In comparison, $^1\text{H}/^{13}\text{C}$ HSQC NMR spectra of PPM isolated from *BMT2-6* deleted strains showed the absence of *d*, *i*, *l*, *j* anomer signals, which established the disappearance of β -Man residues associated with the acid-stable domain (Fig. 4). Conversely, observation of *k* and a signals demonstrated that the acid-labile domain was not modified compared with the wild-type strain (Fig. 4*C*). Altogether, NMR analyses established that the deletion of *bmt2-6* induced a loss of β -mannosylation exclusively on the acid-labile domain, whereas the β -Man residues on phosphomannan domains were untouched. Taken together, structural analysis of β -Mans by NMR is consistent with that assessed by Western blotting and flow cytometry because both 5B2 and 26G7 mAbs did not recognize *Man*(β 1,2)*Man*(α 1-) on the phosphodiesterified acid-labile domain.

Clinical Effects of Oral C. glabrata in Mice Receiving DSS—Different combinations of 1.5% DSS exposure and administration of *C. glabrata* strains were investigated for their clinical effects (Fig. 5*A*). In the absence of DSS, no significant differences in body weight or clinical activity scores were observed between control animals (not inoculated) and those that received *C. glabrata* wild-type or mutant strains (Fig. 5, *B* and *C*).

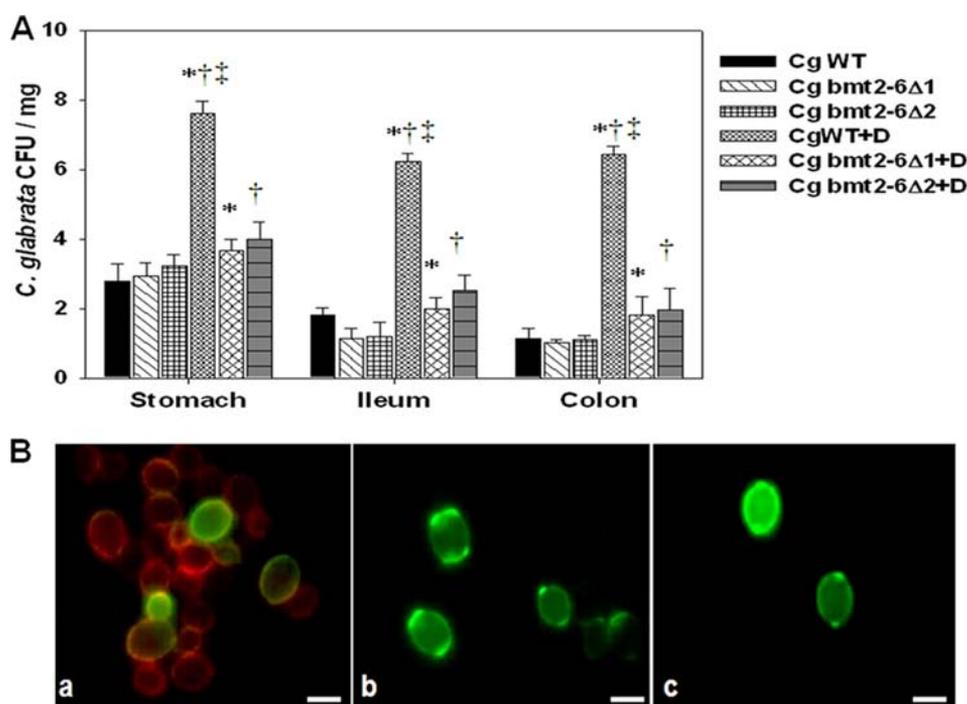


FIGURE 7. *A*, number of *C. glabrata* cfu recovered from different gut compartments. Each data set represents the mean value of counts for 16 mice/group from duplicate experiments. In mice receiving DSS, numbers of *C. glabrata* wild-type cfu from the stomach, ileum, and colon were significantly higher than those of *C. glabrata* bmt2-6Δ1 and *C. glabrata* bmt2-6Δ2. * and †, $p < 0.05$. A significantly higher number of *C. glabrata* wild type cfu were recovered from the stomach compared with those recovered from the ileum and colon in mice receiving DSS. ‡, $p < 0.05$. *B*, microscopic examination of β -1,2 expression in *C. glabrata* wild-type and bmt2-6 mutant strains recovered from the stomach. *C. glabrata* wild-type (*a*), bmt2-6Δ1 (*b*), and bmt2-6Δ2 (*c*) cfu recovered from the stomach were stained with anti-*C. glabrata* mAb 26g7 (red) and GNL (green). Scale bars, 5 μ m. Error bars, \pm S.E.

When mice were treated with DSS, the induced colitis caused a statistically significant loss of body weight and clinical signs of inflammation starting on day 7. DSS-treated mice that received *C. glabrata* wild-type strain lost significantly more body weight (around 20% at day 11) and exhibited more clinical signs of inflammation starting from day 10 than mice that did not receive any yeasts. The two independently constructed *C. glabrata* bmt2-6Δ mutants caused fewer clinical changes than the wild-type strain in DSS-treated mice. The symptoms disappeared after the removal of DSS from the drinking water, and both body weight and clinical activity score gradually returned to normal levels (Fig. 5, *B* and *C*).

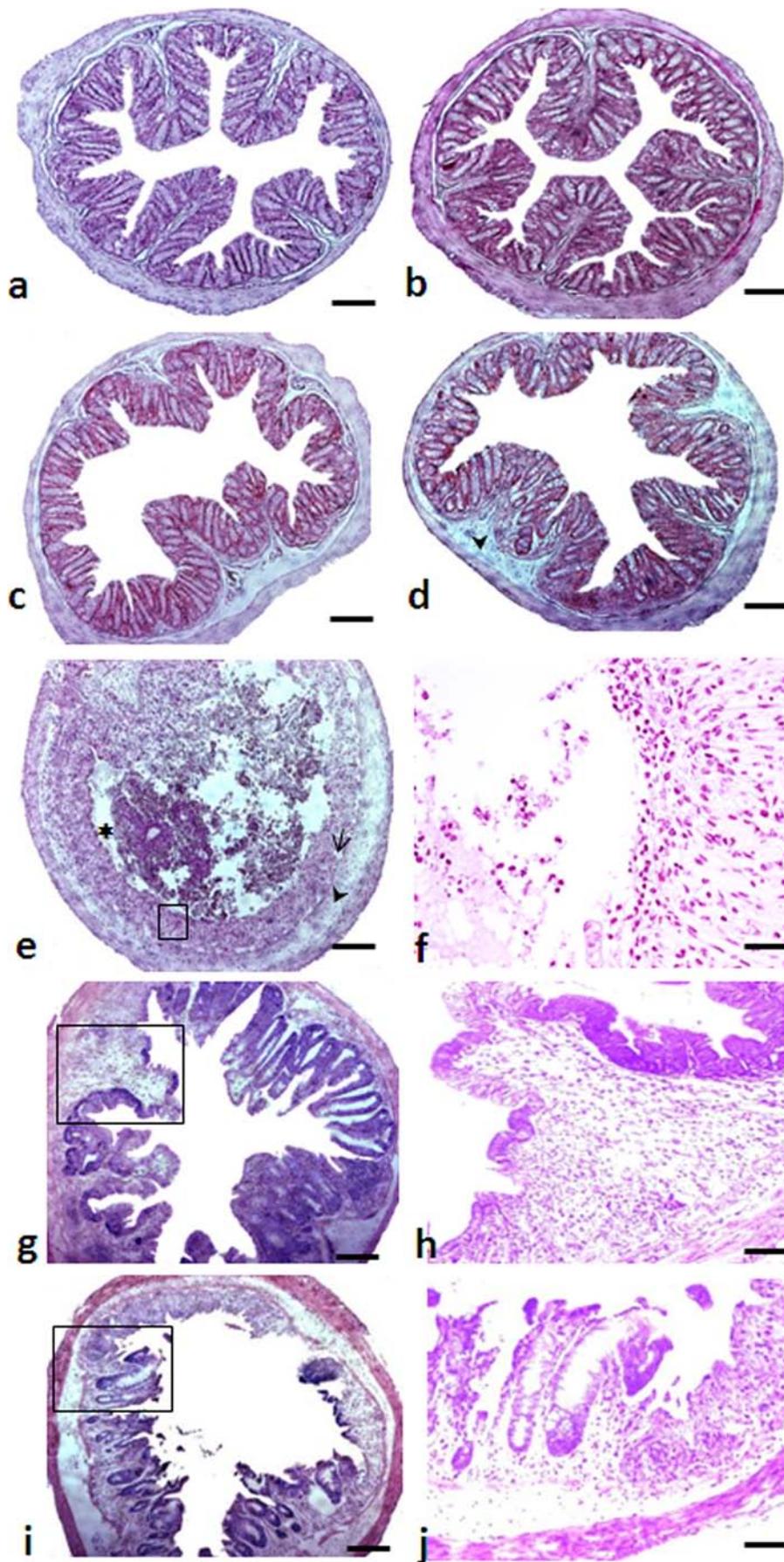
As shown in Fig. 5*D*, no animal died in the absence of DSS treatment, whether they received *C. glabrata* or not. At day 14, 87.5% of DSS-treated mice survived when they did not receive *C. glabrata* (Table 3). When the animals received *C. glabrata* wild-type cells, the survival rate of DSS-treated mice decreased to 75% at day 12. In contrast, the presence of *C. glabrata* bmt2-6Δ mutants in the intestinal tract had no influence on survival rates of DSS-treated mice (87.5% at day 14) (Fig. 5*D*).

Effect of DSS-induced Colitis on *C. glabrata* Colonization in Mice—In the control groups (no DSS), few cfu were recovered from stools irrespective of the *C. glabrata* strain (Fig. 6*A*). In contrast, in DSS-treated mice, large numbers of *C. glabrata* cfu were observed. However, the number of *C. glabrata* bmt2-6Δ cfu was lower than wild-type strain cfu from day 7, when acute inflammation started, and was highly significant on day 14, at the peak of inflammation.

When *C. glabrata* cells were localized in the colon using GNL fluorescence staining on day 17, the relative quantities of these cells correlated with the wild-type and mutant strain cfu isolated from stools (Fig. 6*B*). No direct contact could be observed between yeast cells and epithelial cells, which were separated by a thick mucus layer (Fig. 5*B*).

Recovery of *C. glabrata* Wild-type Strain and bmt2-6Δ Mutants from Stomach, Ileum, and Colon of DSS-treated Mice—To assess *C. glabrata* colonization efficiency in the gut in more detail, the yeast content of three sequential sections of the gut (stomach, ileum, and colon) was determined by culture. In the control groups (without DSS), the stomach was the most colonized part of the gut, but no difference was observed between *C. glabrata* wild-type and mutant strains (Fig. 7*A*). In DSS-treated mice, more cfu were recovered from the stomach, ileum, and colon compared with untreated mice, irrespective of the strain. Once again, the stomach was the most colonized section. bmt2-6Δ strains were less effective at colonizing the three sections of the gut than the wild-type strain in DSS-treated mice (Fig. 7*A*).

Fluorescence microscopy was performed to analyze surface oligomannose expression on yeast colonies isolated from different gut sections. A representative example, double-stained with GNL and mAb 26G7, is shown in Fig. 7*B*; this concerns colonies isolated from the stomach and grown at 37 °C. As expected, *C. glabrata* bmt2-6 mutants were not stained by mAb 26G7 at this temperature and were only stained by GNL. Surprisingly, the wild-type strain isolated from tissues was stained with mAb 26G7. Because yeast cells prepared



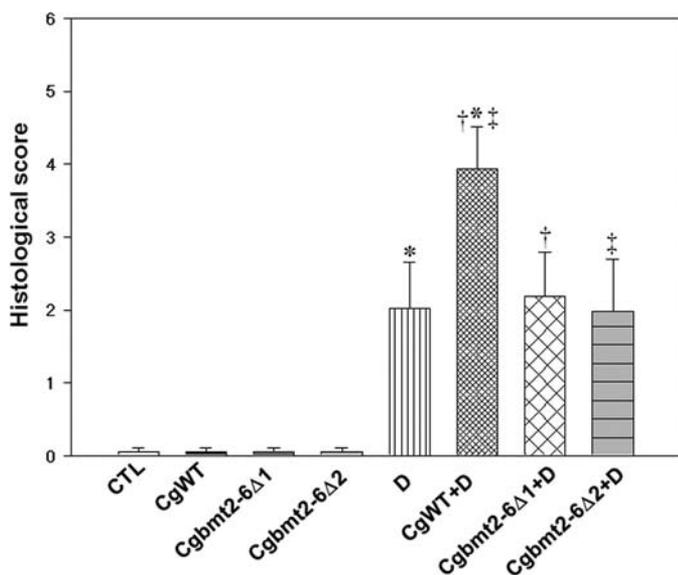


FIGURE 9. **Histologic score.** Histologic scoring was performed by two independent, blinded examiners. Data are expressed as mean \pm S.E. ($n = 10$ mice/control group (CTL) and $n = 16$ mice/experimental group) and represent two independent experiments. The histologic score ranged from 0 to 6. All colon biopsies were included in this scoring. Pairs of symbols (*, †, and ‡) represent significant ($p < 0.05$) differences between the corresponding groups. Error bars, \pm S.E.

from the original wild-type inoculum maintained *in vitro* were unstained (data not shown), this suggests that *in vivo* conditions (host environmental pressure) for 17 days could influence β -mannosyltransferase activity or surface expression of molecules containing β -Mans.

Colon Histopathology—In addition to macroscopic examination at sacrifice, the microscopic changes caused by *C. glabrata* colonization and DSS treatment of the colon were also investigated. Blind histologic injury scoring was carried out on the colon from all surviving animals and those that died during the experiments. Histologic examination of colon sections from control mice (without DSS) receiving water (CTL) or *C. glabrata* wild-type or *C. glabrata bmt2-6 Δ* mutant strains showed no signs of inflammation. Colonic inflammation induced by DSS was mostly associated with mucosal cell loss, crypt damage, mucosal ulceration, and accompanying submucosal edema (Fig. 8). The histologic score (assessing the extent of infiltration of inflammatory cells and degree of tissue damage, ranging from 0 to 6) showed significantly greater inflammation and crypt damage by the *C. glabrata* wild-type strain in DSS-treated mice compared with mice treated with DSS alone (no yeast) (Fig. 9). In addition, *C. glabrata* wild-type colonization impaired colonic epithelium regeneration in surviving animals (75%) when DSS injury had been stopped 3 days previously. In contrast, the two *C. glabrata bmt2-6 Δ* mutants did not affect the histologic score or impairment of colonic epithelium regeneration; histologic signs of colon inflammation

observed in mice receiving *C. glabrata* mutants plus DSS were similar to those in mice treated with DSS alone (Fig. 9).

DISCUSSION

Like *C. albicans*, *C. glabrata* has adapted to colonize all segments of the human gastrointestinal tract and vagina (8, 38), suggesting that these two species share some physiologic traits. A common feature between *C. albicans* and *C. glabrata* is the presence of β -Mans at their cell wall surface (11, 17). These glycan residues, corresponding to antigenic factors 5 and 6 (28), have been extensively studied for their role in *C. albicans* virulence but have never been investigated in *C. glabrata* (15, 39).

Our investigation was based on the deletion of five genes encoding β -mannosyltransferases on chromosome B. The protocol involved two different sets of experiments to create the two mutant strains. These two independently constructed mutant strains were phenotypically similar when compared with their parental strain in terms of morphology, growth, and stability to osmotic imbalance (data not shown). In contrast to the parental strain, the two mutant strains gave identical results in both immunochemical and virulence analyses. A marked reduction in mAb 5B2 epitope and complete disappearance of mAb 26G7 epitope (corresponding to factors 5 and 6, respectively) were observed by flow cytometry. In Western blots, these phenotypes were present on a wide array of mannoproteins, including PPM. Structural analysis by NMR of PPM purified from both strains established that the loss of antibody reactivity with PPM was the result of the complete disappearance of β -Man residues associated with the acid-stable domain. Conversely, deletion of *bmt2-6* did not have any effect on the phosphomannan domain, which established that none of the five encoded β -mannosyltransferases are involved in the synthesis of the acid-labile domain. Such exquisite substrate specificity is reminiscent of the enzymes previously described in *C. albicans* that individually transfer β -Man residues at different positions in PPM and phospholipomannan (16). The presence of additional *C. glabrata* genes *BMT1* and *CgBMT7* probably explains the presence of residual Man(β 1,2)Man(α 1)-P in the *bmt2-6 Δ* mutants. Furthermore, similar to *C. albicans* (19), Western blot profiles strongly suggested the involvement of β -mannosyltransferases in the β -mannosylation of *C. glabrata* mannoproteins in addition to PPM.

To assess virulence changes in *C. glabrata* related to gene deletions and reduced β -Man expression, we adapted a model initially developed to study *C. albicans* intestinal colonization and inflammation (20, 21). β -Mans have also been shown to act as adhesins for *C. albicans* at the intestinal level (21). This model consists of inducing colitis in mice using DSS (33), which induces mucosal injury and inflammation. A direct toxic effect on epithelial cells is followed by recruitment and activation of

FIGURE 8. **Histologic characteristics of colon sections from mice.** Sections (4 μ m thick) stained with hematoxylin/eosin. *a*, *b*, and *c* correspond to control colon sections from CTL, CgWT, and Cg *bmt2-6 Δ* mice, respectively. *d*, colon section from a DSS-treated mouse. *e*, inflamed colon from a *C. glabrata* wild-type+DSS-treated mouse. *f*, colon from a *C. glabrata bmt2-6 Δ* DSS-treated mouse. Compared with colon sections from control animals (*a*–*c*), the colon sections from DSS-treated mice showed an inflammatory cell infiltrate in colonic wall structures (arrow in *d*). The colon sections from mice with DSS-induced colitis and inoculated with *C. glabrata* wild type by oral gavage revealed severe inflammation with large numbers of infiltrating cells (arrowhead in *e*), tissue destruction with almost complete loss of crypts, loss of epithelial integrity (asterisk in *e*), and edema (arrow in *e*). Scale bars, 250 μ m (*a*–*e*, *g*, and *i*), 10 μ m (*f*), and 50 μ m (*h* and *j*).

β -Mans Impact on *C. glabrata* Virulence

inflammatory cells and up-regulation of inflammatory mediators, leading to severe colitis (40). In this study, we used low doses of DSS in drinking water for 2 weeks, followed by the administration of drinking water alone for 3 days. Two weeks of DSS administration was programmed to induce a colonic inflammation to promote *C. glabrata* colonization without the generation of intensive colonic inflammation, which would have led to high and rapid mice mortality. The 3 days without DSS administration allowed epithelial restoration to start, to initiate a regain in body weight that was associated with persistent but decreasing *C. glabrata* colonization.

In this model, the *C. glabrata* wild type seemed to be virulent, as revealed by the high clinical scores, significant weight loss, and mortality. This correlated with the establishment of colonization, seen as an increase in number of cfu in stools and the presence of large quantities of yeasts in the colon lumen. DSS-induced inflammation was a prerequisite for intense *C. glabrata* colonization, which in turn increased the severity of inflammation, leading to intense bleeding and diarrhea, colonic epithelial damage, and inflammatory cell infiltration (21, 33).

When the *bmt2-6* mutants were administered under the same conditions, mortality did not differ from that in mice receiving DSS alone. All clinical parameters were reduced, as was the number of cfu isolated from stools or organs at autopsy. Thus, this model clearly demonstrates that β -Mans contribute to *C. glabrata* virulence. Regarding the mechanism, the primary explanation probably relates to the reduction of colonization. A role for β -Mans as an adhesin for *C. albicans* has been documented on buccal epithelial cells (41, 42), spleen, lymph node, and macrophage cell lines (15), vaginal cells (43), and intestinal cells, either *in vivo* with the infant mouse model (39) or *in vitro* using intestinal cell lines (44). The receptor for β -Mans has been identified as galectin-3 (Gal-3) (13). It has been shown *in vitro* that the direct binding of Gal-3 to β -Mans induces the death of *Candida* species specifically expressing these residues, including *C. glabrata* (14). In a previous study using the DSS model with *C. albicans*, we showed that Gal-3 knock-out mice had less *C. albicans* colonization than wild-type animals (21). In Gal-3 knock-out mice, stimulation of the expression of mucosal TNF α mRNA by *C. albicans* was also reduced together with TLR2 (21). This is consistent with the observation from our group that TLR2 associates with Gal-3 for TNF α induction in the presence of *C. albicans* (45). Finally, this interaction between Gal-3 and β -Mans might also play a role in the antiapoptotic activity induced by Gal-3 through activation of inflammatory cytokine production (46, 47).

The primary phenomenon leading to pathogenic differences between wild-type and *bmt2-6* mutant strains is probably linked to colonization. It is difficult to extrapolate from a mechanistic point of view whether the resultant effect of β -Mans or other pathogenic determinants prompted by the intensity of colonization have an effect on inflammation. Paradoxically, β -glucan was recently shown to interact with β -Mans and to exhibit anti-inflammatory effects (48). In a model of vasculitis induced by *C. albicans* cell wall extracts, growth conditions favoring the expression of β -Mans decreased the proinflammatory potential (49). Thus, although mannan has long been recognized as a potent modulator of the host response, the reper-

toire of oligomannoses that are recognized differently by the immune system is complex (50, 51) and varies depending on the growth conditions. Furthermore, oligomannoses may be expressed on a wide range of mannoproteins. The *C. glabrata* cell wall contains numerous mannoproteins, including the virulence factor EPA, which binds to host glycans and displays a versatile expression (52). Most of the *C. glabrata* mannoproteins are extensively *O*-glycosylated (52). The Western blot mannoprotein profiles observed in this study for the *C. glabrata* wild type support the hypothesis that some *C. glabrata* mannoproteins could be β -mannosylated through their *O*-glycans (19). Current studies on *C. albicans* show that the process of β -Man biogenesis and the association with different carrier molecules are extremely complex (19). Basic structural, immunochemical, and genetic studies that are run in parallel on *C. glabrata* will help to provide important clues regarding β -Man regulation processes and their relation to virulence.

Our future plans include experiments involving complete deletion of a *BMT* gene family. This deletion can be accomplished in *C. glabrata* but may not be technically accessible in *C. albicans* (16). It would be interesting to observe the impact of this deletion on both yeast cell biology and virulence. This raises the subsequent question of which phenotypes are associated with individual or joint deletions of *BMT* genes located on chromosomes D and K.

In summary, our results suggest that β -Mans may play a role in the pathogenicity of *C. glabrata*. Decreased expression resulting from disruption of the genes involved in their synthesis led to a decrease in colonization, clinical, and histologic scores and mortality. We also showed that DSS-induced colitis is a reliable model for studying *C. glabrata*-host interactions. This model provides a new opportunity to investigate the phenotypic impact of *C. glabrata* genetic alterations on colonization and virulence in the intestinal tract and the assessment of new therapeutic strategies.

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