O-Mannosylation in Candida albicans Enables Development of Interkingdom Biofilm Communities

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ABSTRACT Candida albicans is a fungus that colonizes oral cavity surfaces, the gut, and the genital tract. Streptococcus gordonii is a ubiquitous oral bacterium that has been shown to form biofilm communities with C. albicans. Formation of dual-species S. gordonii-C. albicans biofilm communities involves interaction of the S. gordonii SspB protein with the Als3 protein on the hyphal filament surface of C. albicans. Mannoproteins comprise a major component of the C. albicans cell wall, and in this study we sought to determine if mannosylation in cell wall biogenesis of C. albicans was necessary for hyphal adhesin functions associated with interkingdom biofilm development. A C. albicans mnt1Δ mnt2Δ mutant, with deleted α-1,2-mannosyltransferase genes and thus defective in O-mannosylation, was abrogated in biofilm formation under various growth conditions and produced hyphal filaments that were not recognized by S. gordonii. Cell wall proteomes of hypha-forming mnt1Δ mnt2Δ mutant cells showed growth medium-dependent alterations, compared to findings for the wild type, in a range of protein components, including Als1, Als3, Rbt1, Scw1, and Sap9. Hyphal filaments formed by mnt1Δ mnt2Δ mutant cells, unlike wild-type hyphae, did not interact with C. albicans Als3 or Hwp1 partner cell wall proteins or with S. gordonii SspB partner adhesin, suggesting defective functionality of adhesins on the mnt1Δ mnt2Δ mutant. These observations imply that early stage O-mannosylation is critical for activation of hyphal adhesin functions required for biofilm formation, recognition by bacteria such as S. gordonii, and microbial community development.

IMPORTANCE In the human mouth, microorganisms form communities known as biofilms that adhere to the surfaces present. Candida albicans is a fungus that is often found within these biofilms. We have focused on the mechanisms by which C. albicans becomes incorporated into communities containing bacteria, such as Streptococcus. We find that impairment of early stage addition of mannose sugars to C. albicans hyphal filament proteins deleteriously affects their subsequent performance in mediating formation of polymicrobial biofilms. Our analyses provide new understanding of the way that microbial communities develop, and of potential means to control C. albicans infections.

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Candida albicans is an opportunistic fungal pathogen found in the microbiota of the gut, oral cavity, and genital tract (1). In healthy individuals, C. albicans growth is kept in check by a combination of the resident microbiota and both the innate and acquired immune systems. Overgrowth of C. albicans, associated with administration of broad-spectrum antibiotics or immune dysfunction, may lead to superficial infections, such as oropharyngeal candidiasis (thrush) and vulvovaginal candidiasis (vaginal candidiasis). Systemic infections have become increasingly prevalent because more individuals are immunocompromised. C. albicans biofilm infections are common in patients with urinary or intravascular catheters or artificial joints or voice boxes (2). Indeed, C. albicans is associated with >90% of human oral fungal diseases and is becoming an increasingly serious problem in hospital infections (3).

C. albicans forms biofilms on a range of surfaces in vivo (4–6) and is well adapted to conditions in the oral cavity. Biofilm formation occurs in three or four phases, starting first with the deposition of yeast-form cells onto the substratum and then the formation of hyphae (7). The latter are critical for biofilm formation because mutants that do not form hyphae under biofilm-promoting conditions are unable to form robust biofilms (8). Biofilm formation is under genetic regulation by the transcription factors Bcr1 (9, 10), Efg1 (11), and Ace2 (12), among others. In particular, Bcr1 is known to control expression of cell surface proteins, such as Als3 and Hwp1 (13), that are involved in adhesion of hyphal filaments to each other (14) in the building of biofilms. A number of techniques may be utilized to grow C. albicans biofilms in vitro on surfaces of acrylic, rubber disks, catheter strips, or glass coverslips (15). Static biofilm models provide good in vitro data but do not take into account conditions in vivo, where C. albicans biofilms growing on medical implants, such as catheters, prosthetic joints and heart valves, would be exposed to flow forces and replenishment of nutrients from body fluids. In addi-
tion, biofilms formed under flow have been shown to develop architectures different from those of static biofilms grown under the same conditions (16).

The cell surface of *C. albicans* is the major point of contact between fungus and host, along with its constituent microbiota, and provides adhesive and immunomodulatory functions (17). The outer cell wall layer is comprised principally of mannanproteins which are embedded in a polysaccharide skeleton provided by β-(1,3)- and β-(1,6)-linked glucan chains and covalently linked chitin (18). The protein and carbohydrate components of the outer wall layers have both been implicated in adhesion to host surfaces (19) and in biofilm formation (20). It is predicted that ~115 proteins encoded by the *C. albicans* genome are glycosyl-phosphatidylinositol (GPI)-modified proteins (21). Among these are adhesins, such as Hwp1, Rbt1, Eap1, and the Als family of proteins (22), some of which have established roles in biofilm formation (13, 23, 24) and in interactions with host tissues (25). Glycosylation of these and other cell wall proteins is thought to be important for their functions (26). The N-linked glycans carry a conserved core structure and highly branched outer mannos chains (27). The O-linked glycans are, conversely, linear oligosaccharides of one to five α-1,2-linked mannos residues attached to serine or threonine residues (28). The N- and O-glycosylations are completed in the Golgi apparatus, mediated by a set of multifunctional mannosyltransferases (29). Mnt1 and Mnt2 are partially redundant α-1,2-mannosyltransferases that catalyze the addition of the second and third mannos residues in an O-linked mannos pentamer (30). The Mnt1 enzyme may also add the fourth and fifth mannos residues (31). *C. albicans* mutants deficient in the Mnt1 and Mnt2 proteins are modified in cell wall structure (30, 32), diminished in adherence to human buccal epithelial cells (28), and attenuated in virulence (33). In addition, mnt1Δ, mnt2Δ, and mnt1Δ mnt2Δ mutants form hyphae that are hypersensitive to killing by *Pseudomonas aeruginosa* (34).

In polymicrobial infections (35), *C. albicans* has been found in close association with *P. aeruginosa* (36), *Staphylococcus aureus* (37), and oral streptococci in denture- and mucosa-related diseases (38). The interactions of oral streptococci, such as *Streptococcus gordonii*, with *C. albicans* are proposed to facilitate oral carriage and persistence of *C. albicans* in mixed-species biofilms on natural or prosthetic surfaces (39, 40). The cell surface protein SspB expressed on *S. gordonii* is responsible at least in part for mediating adherence to *C. albicans* hyphal filaments (41, 42). *S. gordonii* appears to promote hyphal filament formation by *C. albicans* (42, 43), in contrast to *P. aeruginosa* and *Salmonella enterica* serovar Typhimurium, which kill hyphae (44, 45). The aims of this work were to determine if *C. albicans* cell wall O-mannosylation was necessary for interactions of hyphae with *S. gordonii* and for formation of dual-species biofilms.

**RESULTS**

Mannosyltransferase Mnt1 is necessary for *C. albicans* to bind streptococci. Previous work has demonstrated that *S. gordonii* cells adhered to hyphae formed by *C. albicans* SC5314 (43). In the present study, *C. albicans* CAI4+Clp10 has been designated wild type, this being a Ura3– reintegrant generated from *C. albicans* CAI4 to control for any effects of ura3 gene deletion and transposition in mutagenesis (46). The strain behaved identically to SC5314, from which it was initially derived (see Table S1 in the supplemental material). Hyphal filament formation by the wild-type strain was induced in planktonic phase under our standard conditions with glucose (YPT [1× Difco yeast nitrogen base, 20 mM NaH2PO4–H2PO4 buffer, pH 7.0, and 0.1% Bacto-tryptone]-Glc). Hyphae were shown to avidly bind *S. gordonii* DL1 cells along their lengths (Fig. 1A). A homozygous mnt1Δ mutant, deficient in production of α-1,2-mannosyltransferase (30), which adds the second mannos residue to growing O-linked glycan chains (30), showed reduced binding of *S. gordonii*.
by wild-type or och1 UB1939 S. gordonii binding to hyphae (Fig. 1G). The mutant strain phorylation of S. gordonii forming and calculated from image capture software measurements, as described in Materials and Methods (*, corresponding biofilm biomass values for panel A images (*, 

In the homozygous double mutant strain UB1933 not noticeably impaired in streptococcal cell attachment (Fig. 1C). (Fig. 1B). Hyphae formed by a homozygous impaired mutants (47, 48) for the ability to recognize S. gordonii (Fig. 1D). Reintroduction of MNT1 into the double mutant resulted in restoration of S. gordonii attachment (Fig. 1E), while reintroduction of MNT2 did not (Fig. 1F). These results suggested that expression of MNT1 was essential in order to provide hyphal cell wall receptors for adherence of S. gordonii to C. albicans.

We also tested mnt4Δ and och1Δ N-linked glycosylation-impaired mutants (47, 48) for the ability to recognize S. gordonii. Deficiency in Mnn4, which normally regulates mannosyl phosphorylation of N- and O-linked glycans, had no effect on S. gordonii binding to hyphae (Fig. 1G). The mutant strain UB1939 och1Δ (48), deficient in production of α-1,6-mannosyltransferase, which initiates N-glycan outer chain branch addition, did not support adherence of S. gordonii (Fig. 1H). However, this mutant had a highly pleiotropic phenotype, including being impaired in growth rate and hypha production. Under the conditions of these experiments, Saccharomyces cerevisiae cells did not bind S. gordonii (Fig. 1I).

**Effect of growth medium on development of biofilms.** To investigate in more detail the role of O-mannosylation in biofilm-forming and S. gordonii-binding properties of C. albicans, we compared phenotypes of the wild type and the mnt1Δ mnt2Δ double mutant under different growth conditions. In biofilm studies with YPT-Glc medium, a high proportion of deposited C. albicans wild-type cells had formed hyphal filaments at 2 h (Fig. 2A). After a 3-h incubation, an intensified network of hyphae was apparent, with a concomitant increase in biofilm biomass (Fig. 2B). Although at 2 h, fewer mnt1Δ mnt2Δ mutant cells were associated with the substratum (Fig. 2A, column B), a proportion of cells similar to that for the wild type had formed hyphae. However, after 2 h, the mutant cells appeared to become arrested in biofilm formation (Fig. 2A, column B), and no further significant increase in biomass had occurred at 3 h (Fig. 2B). When N-acetylated-glucosamine (GlcNAc), a known inducer of hypha formation (49), was substituted for glucose, the previous inability of the mnt1Δ mnt2Δ mutant to further develop a biofilm and increased biomass over the 2- to 3-h period was reversed (Fig. 2A, columns C and D, and 2B). The numbers of cells forming hyphae at 3 h in YPT-GlcNAc were similar (65% ± 6.0%) for the wild type and double mutant (Fig. 2A). Fewer S. gordonii cells bound to wild-type hyphae in YPT-GlcNAc than in YPT-Glc (Fig. 2C). This we attributed to a deficiency in metabolism of GlcNAc by S. gordonii. We also utilized 10% saliva containing 0.01% glucose (saliva-Glc) as a growth medium, and in this medium, the C. albicans wild type showed patterns of biofilm formation and biomass production similar to those with YPT-Glc (Fig. 2A, column E, and 2B). With saliva-Glc medium, the mnt1Δ mnt2Δ mutant formed only sparse biofilms (Fig. 2A, column F) and showed no increase in biomass after 2 h (Fig. 2B).

Although the presence of GlcNAc rescued the mnt1Δ mnt2Δ mutant from deficiency in biofilm production in YPT-Glc medium, the ability of these double mutant hyphae to bind S. gordonii was not restored to wild-type levels (Fig. 2C). Confocal scan-
Effect of *mnt1Δ* and *mnt2Δ* mutations on formation of monospecies or dual-species biofilms. In monospecies biofilms, *C. albicans* wild-type cells formed a confluent mat of blastospores and hyphae after 6 h at 37°C in YPT-Glc or YPT-GlcNAc growth conditions (Fig. 4). When saliva-Glc was utilized to grow *C. albicans* biofilms, aggregates or clusters of cells were visible and consequently overall coverage of the substratum was lower (Fig. 4). Saliva-Glc medium did not support such extensive hyphal filament and biofilm formation. The *mnt1Δ mnt2Δ* mutant cells were unable to form biofilms in YPT-Glc or saliva-Glc medium (Fig. 4). However, the deleterious effects of the *mnt1Δ* and *mnt2Δ* mutations on hypha formation and biofilm formation in YPT-Glc and saliva-Glc media were not evident in YPT-GlcNAc medium (Fig. 4). Although the *mnt1Δ mnt2Δ* mutant biofilms still showed reduced coverage compared to findings for the wild type (Fig. 4), there was extensive hyphal filament formation (more detail can be seen in Video S1 and Video S2 in the supplemental material).

*C. albicans* wild-type cells formed dual-species biofilms with *S. gordonii* under the three different growth conditions, with more abundant growth and hypha formation in YPT-GlcNAc (Fig. 4). In YPT-Glc medium, *S. gordonii* cells formed an evenly distributed biofilm over the substratum surface between the deposited *C. albicans* cells, and hyphae appeared integrated within the streptococcal cell community (Fig. 4). More extensive *C. albicans* hypha formation in YPT-GlcNAc medium was accomplished by hyphae appearing to emanate from the *S. gordonii* community. In saliva-Glc medium, both *C. albicans* and *S. gordonii* formed patches of growth (Fig. 4) associated with saliva-mediated aggregation of the microorganisms. In dual-species biofilm experiments, the *mnt1Δ mnt2Δ* mutant was unable to form biofilms in YPT-Glc or saliva-Glc in the presence of *S. gordonii* (Fig. 4). In YPT-GlcNAc medium, a dual-species biofilm with *S. gordonii* was formed (Fig. 4), similar in architecture to the monospecies biofilm. Taken collectively with the results shown in Fig. 2, it is evident that the *mnt1Δ mnt2Δ* deletions result in the following: (i) reduced levels of adhesion to a salivary pellicle substratum, (ii) a depressed rate of hyphal filament extension, and (iii) a curtailed ability (in YPT-Glc or saliva-Glc medium) to form a robust biofilm.
Architecture of monospecies or dual-species biofilms. We then investigated in finer detail the architecture of *C. albicans* and *S. gordonii* in mono- or dual-species biofilms. In YPT-Glc biofilms, *C. albicans* was present as a mixture of blastospores and filaments integrated within and on top of a dense layer of streptococci (see Fig. S1A in the supplemental material). A vertical section (see Fig. S1B) showed *C. albicans* attached to regions of the pellicle substratum and to the streptococcal layer. In the presence of YPT-GlcNAc, *C. albicans* formed a more robust and filamentous biofilm (see Fig. S1, center panels) either alone (see panel E) or with *S. gordonii* (see panel D). The *S. gordonii* component of the dual-species biofilm was much less compact (panel C), and *S. gordonii* cells were more clearly interdigitated with the extending hyphal filaments. In saliva-Glc medium, a highly integrated dual-species biofilm was produced (see Fig. S1), with streptococci and hyphal filaments extending up to 115 μm from the substratum. Both of the microbial components grew more luxuriantly under these conditions, which was especially apparent when comparing *C. albicans* dual- and monospecies biofilms (see Fig. S1D and E).

Biofilm formation under flow. Under flow conditions, which might more closely mimic those in the human oral cavity, there were some remarkable changes in morphology and composition of single- or dual-species biofilms. *C. albicans* monospecies biofilms that formed in YPT-Glc under flow contained more hyphae than when grown under nonflow conditions, and hyphae extended further from the surface (107 μm) (Fig. 5B) than under nonflow (73–80 μm) (see Fig. S1D and E in the supplemental material). *C. albicans* wild-type biofilms formed in YPT-GlcNAc produced hyphal filaments that protruded far out into the environment (to 239 μm), giving a brush-like appearance (Fig. 5B; see also Video S3). In saliva-Glc medium, extensive hypha formation was also observed, but the hyphae tended to lie flatter across the surface of the biofilm (Fig. 5B). Conversely, the *mnt1Δ mnt2Δ* mutant was unable to form robust biofilms under flow conditions, even in YPT-GlcNAc (Fig. 5C and D; see also Video S4).

In dual-species biofilms, patterns of hypha formation by *C. albicans* wild-type cells were observed that were similar to those of monospecies biofilms (see Video S5 in the supplemental material). Hyphae formed by the *C. albicans* wild type in saliva-Glc were less protruding and lay flatter over the *S. gordonii* (Fig. 5B). The presence of *S. gordonii* did not promote incorporation of *mnt1Δ mnt2Δ* mutant cells into the flow biofilm except in YPT-GlcNAc medium (Fig. 5). In this medium, the presence of *S. gordonii* stimulated hypha formation by the *mnt1Δ mnt2Δ* mutant (Fig. 5C; see also Video S6), suggesting that metabolic signaling between these two strains was occurring.

These interactions under flow are shown in greater detail in Fig. S2 in the supplemental material, in which the dual fluorescence channels are separated. Hyphae produced by the *C. albicans* wild type in YPT-Glc medium grew within, and protruded through the *S. gordonii* biofilm (see Fig. S2B in the supplemental material). In YPT-GlcNAc, hyphal filaments extended into the environment by at least 250 μm and streptococcal cells were seen to be associated with the hyphae through the depth of the biofilm (see Fig. S2B and C and Video S5). In saliva-Glc medium, hyphal filaments tended to grow within and across the surface of the *S. gordonii* biofilm (see Fig. S2B, C, and D), the biofilms being more compact than the corresponding monospecies *C. albicans* flow biofilm (see Fig. S2E). The *mnt1Δ mnt2Δ* mutant formed a dual-species flow biofilm with *S. gordonii* in YPT-GlcNAc medium (see Fig. S2). However, the *S. gordonii* component of the biofilm appeared to be reduced compared to that for the wild type (see Video S6 for greater detail). Taken collectively, these results show that Mnt1/Mnt2 deficiency can severely restrict *C. albicans* biofilm development but that this restriction can be at least partly negated in the presence of GlcNAc. Importantly, *C. albicans* wild-type monospecies biofilms or dual-species biofilms with *S. gordonii* appeared to be more substantive and vigorous when formed under flow conditions. This suggests that suppressing factors under nonflow conditions or shear forces under flow may play a role in modulating biofilm development.

Expression of *C. albicans* Als3. We have shown that a major adhesin for *S. gordonii* on the hyphal cell surface is Als3 (42), a filament-specific glycophasphatidylinositol (GPI)-modified cell wall protein with multiple adhesin functions (50, 51). Therefore, a possible explanation for the inability of *mnt1Δ mnt2Δ* mutant

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**FIG 4** CSLM images of biofilms formed by *C. albicans* wild type or *mnt1Δ mnt2Δ* mutant strains in the absence (monospecies) or presence (dual species) of *S. gordonii* DL1. Monospecies or dual-species biofilms were prepared as described in Materials and Methods and were grown for 6 h at 37°C in the three different growth media indicated. The *mnt1Δ mnt2Δ* mutant was unable to form monospecies or dual-species biofilms in YPT-Glc or saliva-Glc but produced robust monospecies and dual-species biofilms in YPT-GlcNAc. Bar = 50 μm.
hyphae to bind S. gordonii was that expression of Als3 was affected. To test this, we reacted hyphal filaments formed by wild-type or mnt1Δ mnt2Δ mutant cells after a 3-h incubation in YPT-GlcNAc with a monoclonal antibody specific to an epitope within the N-terminal region of Als3 (52). Immunofluorescence microscopy showed that hyphal filaments of the wild type expressed Als3 evenly along their lengths (Fig. 6A and B). However, hyphal filaments formed by the mnt1Δ mnt2Δ mutant cells also reacted with the antibody along their lengths (Fig. 6C and D), with no evidence for any differences in distribution or intensity of antibody reactivity compared to findings for the wild type.

To investigate the functionality of Als3, hypha-forming cells of the wild type or mutant were incubated with fluorescently labeled cells of Lactococcus lactis expressing the S. gordonii adhesin SspB. This streptococcal cell wall-anchored protein has been shown to interact with Als3 (42). L. lactis SspB+ cells adhered to wild-type hyphae (Fig. 6E and F) but not to hyphae produced by the mnt1Δ mnt2Δ mutant (Fig. 6G and H). L. lactis MG1363 control cells did

FIG 5  CSLM images of mono- or dual-species biofilms formed under flow conditions by the C. albicans wild type or mnt1Δ mnt2Δ mutant in three different growth media as indicated. Biofilms were prepared as described in Materials and Methods and grown for 6 h at 37°C at a medium flow rate of 6 ml/h. In the left-side group of 12 panels, monospecies biofilms of C. albicans wild-type and mnt1Δ mnt2Δ mutant strains are shown; the right-side group of 12 panels shows dual-species biofilms with S. gordonii. The image at the top of each group of 6 is a representative xy stack of the biofilm assembled from the top down (A and C). The lower image in each column (B and D) is the corresponding xz image showing organization and thickness (depth) of the biofilm. Red, C. albicans; green, S. gordonii. The significant structural and architectural differences associated with the different growth conditions employed are described in the text. The values shown in μm are average thickness (depth) measurements calculated from across the sections of the biofilms shown. Bar = 100 μm.

FIG 6  Hypha-forming cells of the C. albicans wild type or mnt1Δ mnt2Δ mutant immunolabeled with monoclonal antibody to the hyphal cell wall protein Als3 or reacted with L. lactis cells expressing SspB. C. albicans cells were induced to form hyphae in YPT-GlcNAc medium suspension culture, as described in Materials and Methods, for 3 h at 37°C, harvested by centrifugation, and then incubated with anti-Als3 MAb 3-A5 (52). (A to D) Antibody binding was detected with FITC-conjugated goat anti-mouse F(ab)2 fragment-specific antibody, and wet mounts were observed microscopically by phase contrast and fluorescence (52). (E and F) Light and corresponding fluorescence microscopic images of FITC-labeled L. lactis cells expressing SspB binding to hyphae. (A, B, E, and F) C. albicans wild type. (C, D, G, and H) mnt1Δ mnt2Δ mutant. Bar = 20 μm.
not bind to hyphae, as previously shown (42). Thus, it was concluded that although Als3 was present on the surface of mnt1Δ mnt2Δ mutant hyphae, as detected by antibody binding, the protein was not expressed in a conformation that was recognized by the S. gordonii SpbP protein.

**C. albicans WT and mnt1Δ mnt2Δ cell wall proteomes.** To determine if there were significant differences in the expression of cell wall proteins (CWPs) by *C. albicans* wild-type and mutant strains under the different growth conditions, cell wall preparations (see Materials and Methods) were subjected to trypsin digestion and the resulting peptides were identified by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry. The experiments allowed us to reproducibly identify 23 CWPs (Table 1). Thirteen of these were produced by both wild type or mutant undergoing hyphal filament formation in three different media.
of mutant (53) for ability to form biofilms and to support adhesion, was considered. To test this, we examined a
pression might be necessary for activating receptors, e.g., Als3 for
protein consistently reduced in abundance from cell wall prepara-
the cell wall proteome data indicated that Sap9 was the only pro-
dium and conditions employed to induce hypha formation. Since
hyphal cell wall protein composition depending upon the me-
were not statistically significant within the data set. In summary,

of the proteins Als1, Als3, Phr1, Rbt1, Sap9, Utr2, and Scw1 for the
mnt1Δ mnt2Δ mutant compared with those for the wild type (Ta-
cept from
decreases in cell wall protein abundance between the wild type and
incorporation of glycan-modifying enzymes, e.g., glycosidases
and adhesins, into the cell wall when hyphal induction was per-
P
Crh11 proteins being more abundant than in YPT-GlcNAc, and a
Sap9 from the cell wall of
S. gordonii
that the hyphal cell wall Als3 receptor for
S. gordonii
expressing Eap1 (D and H) is shown. Magnification, ×20 or ×40, as indicated.

C. albicans
S. cerevisiae
C. albicans
S. cerevisiae
Saccharomyces cerevisiae
cells expressing Als3 on their surfaces (24) (Fig. 7B) were able to interact with
hypophase formed by the C. albicans
wild type but not with hyphophase formed by the mnt1Δ mnt2Δ mutant (Fig. 7F). Wild-type
S. cerevisiae
expressing empty vector pBC542 (54) did not interact with hypophase produced by either strain of C. albicans (Fig. 7A and E).
S. cerevisiae
cells expressing Hwp1 (Fig. 7C) also bound to hyphophase induced by the
S. cerevisiae
expressing Als3 (B and F), S. cerevisiae expressing Hwp1 (C and G), or S. cerevisiae expressing Eap1 (D and H) is shown. Magnification, ×20 or ×40, as indicated.

S. cerevisiae
BY4742 (pBC542)  
C. albicans
wild type
(pBC542-Als3*)  
(pBC542-Hwp1*)  
(pBC542-Eap1*)  
C. albicans
mnt1Δmnt2Δ

FIG 7 Interactions of C. albicans wild type or mnt1Δ mnt2Δ mutants with S. cerevisiae strains expressing C. albicans cell wall proteins. S. cerevisiae cultures were grown to mid-exponential phase in CSM medium, and the cells were FITC labeled and then mixed with C. albicans cells that had been induced to form hyphal filaments for 3 h at 37°C in YPT-GlcNAc medium (see Materials and Methods). After a 2-h incubation of cultures at 30°C with gentle agitation, samples (10 μl) were removed and placed on glass microscope slides, coverslips were applied, and the cells were visualized using a Leica microscope under phase contrast or fluorescence. The images show phase micrographs merged with respective green fluorescence images of S. cerevisiae cells. (A to D) C. albicans wild type; (E to H) C. albicans mnt1Δ mnt2Δ mutant. S. cerevisiae BY4742 empty vector pBC542 control (A and E), S. cerevisiae expressing Als3 (B and F), S. cerevisiae expressing Hwp1 (C and G), or S. cerevisiae expressing Eap1 (D and H) is shown. Magnification, ×20 or ×40, as indicated.
protein functions and hence interkingdom biofilm formation with S. gordonii.

**DISCUSSION**

A number of studies have demonstrated that mannosylation reactions in *C. albicans* are required for adherence, invasion of host tissues, and virulence (30, 33). The marked reduction in adherence of *mnt1Δ mnt2Δ* mutants to a number of substrata observed previously suggested that O-linked glycosylation might indeed be specifically required for adhesion (56). It has not been precisely clear why mannosylation reactions necessarily affect these properties. One possibility is that deficiency in mannosylation directly affects the synthesis, expression, or activities of cell wall proteins and adhesins crucial for invasion and virulence. Oligosaccharide chains are believed to confer stabilizing properties upon the extended regions of fungal extracellular glycoprotein adhesins (57). Thus, mannosylation could be key to formation of a fully functional *C. albicans* cell surface, especially since there is now good evidence that a number of CWP families, e.g., Als and Rbt, crucial to biofilm formation and virulence, carry extensive amino acid residue repeat blocks in their C-terminal regions that are heavily O-glycosylated (58). In addition to being attenuated in adherence and invasion, *C. albicans* glycosylation mutants induced lower levels of cytokine production by human peripheral blood monocytes (29). Disruption of the mannosylation processes seems therefore to have a range of damaging effects on the fungus and interactions with the host (28). Despite these observations, the potential for mannan synthesis to be an antifungal target has generally received less attention.

Previously, it was suggested that deletion of *MNT1* or *MNT2* in *C. albicans* might not result in marked changes in the tertiary structure of cell surface mannoproteins, because protein stabilization is thought to be achieved by addition of the first O-linked mannoside rather than subsequent residues (30). However, our results suggest that the subsequent (second and third) steps in mannosylation are important. Biofilm development and interactions of *C. albicans* with streptococci were dependent upon expression of *MNT1* and *MNT2*, encoding the enzymes adding the second and third mannoside residues to the growing mannan chain. Interestingly, the phenotypic effects of the *mnt1Δ mnt2Δ* double deletion on biofilm development were environmentally sensitive. When induced to form hyphal filaments by nitrogen starvation (YPT-Glc) or with salivary glycoproteins, the double mutant produced a mixture of pseudohyphae and true hyphae. However, when induced with GlcNAc, the double mutant formed hyphae similar to wild-type hyphae in structure and proteome.

GlcNAc induces two sets of responses in *C. albicans*. One causes it to switch from budding to hyphal growth, activated by stimulation of adenyl cyclase and increased CAMP signaling (59). The second activates a pathway independently of cAMP to induce expression of genes necessary to catabolize GlcNAc (60), but this is not required for hypha induction (61). The hyphal induction signal from GlcNAc is transmitted through Ngt1 and then to the hyphal gene transcription regulator Efg1 (62). Conversely, the induction signals of low nitrogen or salivary (serous) proteins are received through Mep2 or Ras1, with activation of Cyr1 (adenyl cyclase). Possibly the latter hyphal induction pathway is more sensitive to the effects of *MNT1/MNT2* deletions, such as cell wall stress (63), than the Ngt1 induction pathway.

GlcNAc is not involved directly in O-mannosylation, but GlcNAc treatment induces chitin synthesis and could potentially be converted to other monosaccharide cell wall building blocks via its conversion to fructose-6-phosphate. Therefore, the effect of GlcNAc in effectively complementing the *mnt1Δ mnt2Δ* mutant phenotypes may be multifactorial. Despite GlcNAc enhancing biofilm and hypha formation by the *mnt1Δ mnt2Δ* mutant, the hyphae were still deficient in binding S. gordonii and in interacting with the *C. albicans* cell wall adhesins Als3 and Hwp1, expressed on the surface of *S. cerevisiae*. This provides biochemical and physiological demonstration that without the functions of *MNT1* and *MNT2*, the intermicrobial cell adhesins are not presented in a way that promotes dual-species community development. Nevertheless, there must be sufficient levels of interaction between *C. albicans mnt1Δ mnt2Δ* cells in GlcNAc to enable monospecies biofilm formation.

The ability of oral streptococci to interact with *C. albicans* may be relevant to longer-term carriage or persistence of *C. albicans* in the oral cavity (29). Evidence suggests that streptococci and *C. albicans* exhibit growth synergy, implying mutual benefit in cocolonization (43, 64). In this article, dual-species communities formed under flow or nonflow conditions, and *S. gordonii* cells could be seen within the biofilms closely associated with hyphal filaments (see Video S5 in the supplemental material). In biofilms formed under medium flow, hyphal filaments extended further into the environment (see Video S3) than they did under nonflow conditions, where the biofilms were structurally more compact (see Video S1). The stimulation of biofilm development under flow could result from better provision of nutrients, removal of inhibitory metabolic end products, shear-force-induced gene expression, or diffusion away from the biofilm of compounds, such as farnesol (65), that inhibit hypha formation.

To determine the effects of *mnt1Δ mnt2Δ* mutation on the production of hyphal cell wall proteins, we prepared cell walls from hypha-forming cells and subjected them to trypsin digestion and proteome analyses. In *C. albicans* cell walls, the more abundant covalently attached proteins are GPI modified, whereas the least abundant are attached via an alkali-labile linkage (66). Our experiments identified peptides from proteins present in the cell wall through covalent linkage. Proteins missing from the cell wall extracts would be either not expressed (transcription) or not properly incorporated (covalently linked) into the cell wall or lacking amenable trypsin cleavage sites. In the *mnt1Δ mnt2Δ* mutant grown in YPT-Glc, there were significant reductions in a wide range of CWPs that would be expected to have major effects on phenotypes such as adhesion, biofilm formation, and invasion of host cells (9, 50, 51). In the presence of GlcNAc, however, the *mnt1Δ mnt2Δ* mutant showed a cell wall proteome much more similar to that of the wild type. Peptides from the GPI-modified hypha-specific adhesin Hwp1 were not detected by liquid chromatography-tandem mass spectrometry (LC-MS/MS), as also reported by others (67). During hypha formation there is enrichment of carbohydrate-active or cell wall-remodeling enzymes Cht2, Crh11, Mpi65, Pga4, Phr1, Phr2, and Utr2 (49, 67). We have identified six of these proteins in our studies. Actual relative amounts will be dependent upon the methods employed in cell wall purification and MS analysis and most importantly the precise conditions utilized for growth and hypha formation.

A protein consistently deficient in the *mnt1Δ mnt2Δ* cell wall proteome was Sap9, one of a family of 10 aspartyl proteinases (Sap1 to 10) that are differentially expressed in *C. albicans* (68).
The main effects of SAP9 deletion are reduced chitinase activity and increased adhesion to epithelial cells (53). It has been suggested that Sap9 is able to trim cell surface proteins, on *C. albicans* or the host, to modulate receptor-ligand interactions (69–71). In a model for Als3-mediated interactions *in vitro*, soluble or membrane-bound Saps are hypothesized to partly digest proteins on the host cell surface, thus providing additional ligands for Als3 to interact with (71). However, it was concluded that this protease activity did not function in providing receptor availability for binding of *S. gordonii*.

Initial adherence in *C. albicans* biofilm formation is mediated at least in part by Als1 (25), and ALS1 expression is under control of the transcriptional regulator Bcr1. Other adhesin targets of Bcr1 are Als3 and Hwp1, which mediate cell-cell interactions in biofilms (14). The cell wall protein Eap1 is required for biofilm formation *in vitro* and *in vivo* (55) and may be needed for initial layer formation on specific substrata (24). It has been suggested that the Eap1 and Als proteins may play a role in environmental sensing as well as directly as adhesins (72). Genes encoding transglycosidases and 3 h biofilm development by the *mnt1* mutant appeared not to bind GlcNAc (Table 1), are coregulated with other hyphal or virulence genes, and may be needed for initial layer formation on specific substrata (24). It has been suggested that the Eap1 and Als proteins may play a role in environmental sensing as well as directly as adhesins (72). Genes encoding transglycosidases involved in maintenance of cell wall integrity, such as CRH11 (Table 1), are coregulated with other hyphal or virulence genes, while BGL2, PHR1, and XOG1 encode enzymes that are crucial for delivery of β-1,3-glucan to the biofilm matrix and for accumulation of matrix biomass (73). Levels of a number of these factors were altered in the *mnt1Δ mnt2Δ* mutant cell wall proteomes from cells forming hyphae in YPT-Glc and salivary-Glc medium, possibly disabling biofilm formation. The observation that between 2 h and 3 h biofilm development by the *mnt1Δ mnt2Δ* mutant effectively ceased is similar to that made for an *als3Δ* mutant (42). This might be consistent with the 80 to 90% reduction in Als3 in the cell wall proteome of the double mutant under YPT-Glc or salivary-Glc medium conditions. On the other hand, GlcNAc-induced hyphae formed by the *mnt1Δ mnt2Δ* mutant had a full complement of adhesins (Table 1). We conclude that in the presence of GlcNAc, the functional expression of adhesins on the *C. albicans* cell surface for binding *S. gordonii* but not for initial biofilm formation is affected by the block in mannosylation.

Evidence that adhesins expressed on the surface of GlcNAc-induced hyphae produced by the *mnt1Δ mnt2Δ* mutant were not properly functional was provided by the experiments utilizing *S. cerevisiae* strains expressing the adhesin Als3, Hwp1, or Eap1. Strains expressing these proteins bound avidly to *C. albicans* wild-type hyphae but feebly to hyphae produced by the *mnt1Δ mnt2Δ* mutant. This suggested that the *mnt1Δ mnt2Δ* mutant hyphae did not present a correct configuration of adhesins to interact with these CWP adhesins. Such effects would be extremely difficult to detect by current imaging techniques and are implicated by probing for functionality, such as ability to interact with known ligands. Of note is that some hyphae in all populations (wild type or mutant) appeared not to bind *S. gordonii* cells, thus indicating potential heterogeneity of hyphal adhesin expression or activation within hyphal filament populations.

A current model for the broad and strong adhesive activity of Als3 suggests that Als3 molecules are able to cluster on the cell surface and bind to each other through rapidly dissociable hydrophobic interactions (74). A long glycosylated C-terminal region stalk elevates the N-terminal region and allows flexibility needed for the adhesins to form amyloids. The Als3 ligand binding cleft is located within the N-terminal region (71). We suggest that the initial SspB-Als3 interaction unfolds or extends the Als3 protein such that further interactions are substantiated, possibly between amyloid-like regions of the proteins. The Als3 protein contains at least one amyloid sequence (32) and SspB carries three potential amyloid sequences within the central region and C-terminal regions of the polypeptide (VVYTYT, IWFAF, and TTSFVLN), as predicted by the TANGO computer algorithm (75), that could have amyloidogenic activity.

Amyloid interactions between adhesins may provide cohesive strength to *C. albicans* Als proteins. Formation of Als5 adhesin nanodomains on the cell surface was observed in response to mechanical stimuli, which probably caused the T region (Thr-rich N-terminally located sheet) to partially unfold and expose the amyloid-forming sequences (76). The formation of adhesin clusters could thus explain why Als proteins mediate strong adherence (51). These various *in vitro* observations taken collectively with our results in this article provide the basis for a model (see Fig. S3 in the supplemental material) through which we envisage O-mannosylation to play a critical role in development of CWP adhesin functions. In this model, O-mannosylation catalyzed by the enzymes Mnt1 and Mnt2 initially acts by stabilizing adhesin structure and then allowing for subsequent unfolding and extension. The apical N-terminal domains of Als3 protrude from the hyphal cell wall surface and are recognized by *S. gordonii* cells. Binding causes force-induced unfolding of Als3, which would be enhanced in biofilms under flow conditions (as shown in Results). The adhesive (pulling) forces then elicit clustering of Als3 protein molecules to form nanodomains over the cell surface, thus promoting multiple force interactions of Als3 proteins with bacteria. This would be consistent with observations that streptococci can be seen on some hyphae to show localized attachment and accumulations, suggesting that they are interacting with clusters of adhesins. In Fig. S3, Sap9 aspartyl proteinase is shown as modulating the activities of these nanodomains by proteolytically disrupting the intermolecular forces. This would disaggregate hyphal filaments, leading to increased biofilm permeability and more flexibility for recognition of additional binding partner molecules. Dispersal of intermicrobial cell contacts might also be regulated, at least in part, via Sap9-catalyzed hydrolysis of intramolecular peptide bonds of proteins engaged in intermolecular adhesion events. To date, the evidence for adhesin activation has been derived from *biophysical* *in vitro* analyses (76), but our data provide direct experimental evidence at the physiological level for the hypothesis that Als-mediated adhesion largely depends upon conformational modifications of existing adhesins and that this is dependent upon early-stage O-mannosylation reactions. These reactions are clearly worthy of further consideration as potential targets for control of *C. albicans* colonization and virulence.

**MATERIALS AND METHODS**

**Microbial growth conditions.** The microbial strains utilized in this study are listed in Table S1 in the supplemental material. *C. albicans* strains, all derived from CAH4 (77), were cultivated on Sabouraud dextrose agar (Lab M) aerobically at 37°C. *S. gordonii* strains were cultivated anaerobically at 37°C on BHYN agar (per liter: 37 g brain heart infusion broth, 5 g yeast extract, 5 g Neopeptone, and 15 g agar), which was supplemented with erythromycin (5 μg/ml) for *S. gordonii* expressing green fluorescent protein (GFP). *Lactococcus lactis* strains were grown anaerobically at 30°C on M17-glucose agar (Oxoid) containing 5 μg erythromycin/ml where appropriate. *Saccharomyces cerevisiae* strains (24, 78) were cultivated aerobically at 30°C on CSM medium (per liter: 6.7 g Difco yeast nitrogen base, 0.77 g complete supplement mixture [CSM] drop-out [CSM-Ura] | For-
medium, Hunstanton, United Kingdom], 20 g glucose, and 30 g agar). Suspension cultures of C. albicans were grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) in conical flasks at 37°C with shaking (200 rpm). S. gordonii strains were grown in BHY medium (brain heart infusion, containing 5 g/liter yeast extract), stationary, at 37°C. YPT medium (1× Difco yeast nitrogen base, 20 mM NaH₂PO₄·H₂PO₄ buffer, pH 7.0, and 0.1% Bacto-tryptone) supplemented with 0.4% glucose (YPT-Glc) or 0.4% N-acetyl-D-glucosamine (YPT-GlCNac) was utilized to support growth of S. gordonii and C. albicans in planktonic cultures or biofilms.

**Generation of S. gordonii expressing GFP.** Plasmid pAR4 (rpsT-gfp-mut3b*pAR2), kindly provided by Patrick Piggot (Temple University biofilms, 79] by a modified alkaline-lysis technique (41). The plasmid was purified made competent for transformation as previously described (80), and transformants containing pAR4 were selected on BHY Agar containing 5 µg erythromycin/ml (see Table S1 in the supplemental material).

**Preparation of microbial cells.** C. albicans strains were grown for 16 h in YPD. Cells were harvested by centrifugation (5,000 × g for 5 min), washed twice in YPT, and suspended at an optical density at 600 nm (OD₆₀₀) of 1.0 (approximately 1 × 10⁷ cells/ml). S. gordonii or L. lactis cells were grown for 16 h in 10 ml YPT-Glc, harvested by centrifugation (5,000 × g for 7 min), and washed twice with YPT (no glucose). Bacteria were suspended in 0.05 M Na₂CO₃-0.1 M NaCl containing 1.5 mM fluorescein isothiocyanate (FITC) and incubated in the dark at 20°C for 30 min with gentle agitation. The bacterial cells were then washed three times with YPT and suspended at an OD₆₀₀ of 0.5 (2 × 10⁶ cells/ml) in YPT.

**C. albicans interactions with S. gordonii in planktonic phase.** Portions (0.2 ml, 2 × 10⁶ cells) of C. albicans cell suspension in YPT were added to glass tubes containing warm YPT-Glc, YPT-GlCNac, or saliva-Glc medium (1.8 ml). The cultures were incubated for 37°C for 3 h with shaking at 220 rpm; S. gordonii (or in some experiments L. lactis) cell suspension (1 ml, 2 × 10⁶ cells) labeled with FITC or expressing GFP was then added, and incubation was continued at 37°C for 1 h. Samples of suspension (10 µl) were applied to glass microscope slides and visualized by light or fluorescence microscopy (Leica DMLB) or with a Leica SP5-AOBS confocal laser scanning microscope attached to a Leica DM 16000 inverted epifluorescence microscope. The Volocity software program was utilized to determine density of fluorescence associated with multiple hyphal filaments over at least 10 randomly selected fields of view (expressed as density units per 50 hyphae).

**Planktonic-phase interactions of S. cerevisiae with C. albicans.** S. cerevisiae BY4742 strains expressing C. albicans cell wall proteins (see Table S1 in the supplemental material) were inoculated into warm CSM (10 ml) and incubated for 16 h at 30°C with shaking at 220 rpm. Portions (5 ml) were centrifuged (5,000 × g, 5 min), washed twice with YPT, and fluorescently labeled with FITC as described above. The labeled cells were washed thoroughly with carbonate buffer and twice with YPT before being suspended in YPT-Glc at an OD₆₀₀ of 1.0 (1 × 10⁷ cells/ml). A 1-ml suspension of each strain was transferred to a glass tube containing 1 ml C. albicans cells preinduced to form hyphae for 3 h in YPT-GlCNac and incubated at 30°C for 2 h with shaking at 220 rpm. Portions (10 µl) of the cell suspensions were visualized by transmitted light or fluorescence microscopy.

**Monospecies and dual-species biofilms of C. albicans and S. gordonii.** Sterile 19-mm glass coverslips were incubated with 10% filter-sterilized saliva at 4°C for 16 h. Individual coverslips were then transferred to 12-well tissue culture plates containing 1.9 ml YPT-Glc, YPT-GlCNac, or saliva-Glc, and 0.1 ml C. albicans cell suspension (1 × 10⁶ cells) was added. The plates were incubated at 37°C for up to 6 h with gentle motion (50 rpm) in a humid environment. Coverslips were removed at intervals, rinsed gently with phosphate-buffered saline (PBS), dried, and stained with 0.5% crystal violet solution for light microscopy or estimation of biomass after releasing the stain with 10% acetic acid and measuring the OD₅₉₅ (43).

For visualization of S. gordonii interactions with C. albicans hyphae in early biofilms, coverslips were incubated with C. albicans as described above for 3 h, removed, and placed in wells containing fresh YPT-Glc medium (0.5 ml). Suspensions (0.5 ml) of FITC-labeled S. gordonii were added, and the cultures were incubated for a further 1 h. For comparative monospecies biofilms of C. albicans, culture medium alone (0.5 ml) was added. Coverslips were then removed, gently rinsed once with PBS, inverted onto clean glass microscope slides, and examined by transmitted light or fluorescence microscopy as described above.

**Preparation of biofilms for CSLM.** Plastic culture dishes (35-mm diameter; Mat Tek) with 14-mm No. 1.0 coverslip base glass windows were incubated with 2 ml 10% saliva at 4°C for 16 h. The saliva was aspirated, and 1.8-ml growth medium (YPT-Glc, YPT-GlCNac, or saliva-Glc) was added to each dish, followed by 0.2 ml C. albicans cell suspension (2 × 10⁶ cells). Dishes were incubated in a humid environment at 37°C for 1 h with gentle motion at 50 rpm. All subsequent incubation steps were carried out under these growth conditions. The culture suspensions were aspirated and replaced with the appropriate medium, and the dishes were incubated for 2 h. For dual-species biofilms, the C. albicans suspension was gently aspirated, and 0.2 ml S. gordonii UB2549 (expressing GFPmut3b*) suspension in appropriate medium was added. The dishes were incubated for 30 min, the S. gordonii culture suspension was aspirated, and 2 ml appropriate growth medium was added for further incubation at 37°C for 4 h. The cell suspensions were then aspirated, the dishes were washed gently with sterile deionized water, and C. albicans cells were stained with calcofluor white (0.2 µg/ml) just prior to visualizing by confocal scanning laser microscopy (CSLM) with a Leica SP5-AOBS confocal microscope attached to a Leica DM 16000 inverted epifluorescence microscope. Velocity software was utilized to prepare three-dimensional (3D) images and to calculate biofilm heights (in µm). For monospecies biofilms, exactly the same protocols were applied, minus bacteria or C. albicans, substituted with sterile growth medium.

**Flow-cell biofilms.** Flow cell units, consisting of two parallel chambers sealed with a glass coverslip, were prepared as described by Palmer (81). The growth medium input line was connected, medium was drawn through the cells with a syringe, and the cells were injected with 0.5 ml 10% human saliva and incubated at 4°C for 16 h to coat the inside surfaces of the chambers with salivary glycoproteins. The effluent line to a peristaltic pump was then connected, and appropriate growth medium was drawn through the flow cell for 15 min at a flow rate of 6 ml/h. C. albicans cell suspension in growth medium (0.2 ml) was injected into the flow cell chamber and incubated statically at 37°C for 1 h. The growth medium (containing 1 µg/ml calcofluor white) was then drawn through the flow cell chambers at a rate of 6 ml/h for up to 16 h. For dual-species biofilms with S. gordonii, the medium flow was stopped after 2 h, and S. gordonii UB2549 (expressing GFPmut3b*) cell suspension (0.2 ml) was injected into the chamber and incubated without medium flow at 37°C for 30 min. Flow of growth medium was then recommenced and continued for 4 h at a rate of 6 ml/h. Biofilms were visualized by CSLM.

**Immunolabeling of C. albicans hyphae.** Suspension cultures of C. albicans were grown in YPD medium for 16 h at 37°C with shaking (200 rpm). Cells were harvested by centrifugation, washed with YPT medium, suspended at an OD₆₀₀ of 0.5 in YPT-GlCNac medium, and incubated with shaking for 3 h at 37°C. Cells were harvested by centrifugation,
washed, blocked with goat serum, and then reacted with MAb 3-5A monoclonal antibody to the protein Als3, as previously described (52). Antibody binding was detected with FITC-labeled goat anti-mouse IgG F(ab') fragment-specific antibody, and wet mounts were visualized by light microscopy using an Olympus BX50 microscope.

**C. albicans cell wall purification.** Cell wall protein samples were prepared by a modification of the method previously described (82). C. albicans cells (200-ml cultures) were grown at 37°C for 6 h with shaking to an OD₆₀₀ of 0.7 to 0.9. The cells were harvested by centrifugation (5,000 x g, 5 min), washed with 10 mM Tris-HCl, pH 7.5, at 4°C, suspended in 0.2 ml Tris-HCl buffer, and mixed with 0.5 g cold silica beads (Biocsep). Protease inhibitor cocktail (Sigma) and 1 mM phenylmethylsulfonyl fluoride (PMSF) were added, and the cells were disrupted by shaking using a FastPrep cell breakage machine (FastPrep-24 bead beater; MP Biomics). The contents of the tubes were washed out into cold 50-ml tubes with 1 M NaCl, the beads were allowed to settle, and the supernatant was removed (repeated twice). Cell wall suspensions were centrifuged (3,000 x g, 5 min, 4°C), and the pellets were washed 5 times with 1 M NaCl and once with double-distilled water (ddH₂O). The crude cell walls were then twice extracted with SDS-mercaptoethanol buffer (50 mM Tris, 2% SDS, 0.3 M β-mercaptoethanol, and 1 mM EDTA [pH 8.0]) at 100°C for 10 min to remove noncovalently bound proteins. Cell walls were then washed thoroughly by alternate suspension in ddH₂O and centrifugation (5 times) and freeze-dried.

**Preparation of cell wall peptides.** Freeze-dried cell wall sample (1 to 2 mg) was mixed with 0.5 M ammonium bicarbonate in water containing 3 mM diithiothreitol and heated at 60°C for 20 min. Iodoacetamide (30 µl of 55 mM stock solution) was then added, and the suspension was incubated at 25°C for 10 min in the dark. Trypsin (30 µl of 20 µg/ml stock) was added, and the suspension was incubated at 37°C for 14 h and centrifuged at 14,000 x g for 10 min. The supernatant was freeze-dried and extracted with 10% formic acid, and peptides were purified using ZipTip mC18 pipette tips (Millipore) and dissolved in 0.1% formic acid.

**Proteomic analysis.** Samples (3 µl) were injected into an LC-MS system which comprised an UltiMate 3000 LC instrument (Dionex Ltd., United Kingdom) fitted with a PepSwift monolithic poly(styrene-co-divinylbenzene) (PS-DVB) column (200-µm inside diameter [i.d.] by 5 cm; Dionex) coupled to an LC/MS (electrospray ionization (ESI) source and controlled by HyStar software (version 4.0; Bruker Daltonik GmbH, Bremen, Germany) fitted with a low-flow nebulizer in the electrospray ionization (ESI) source and controlled by HyStar software (version 4.0; Bruker Daltonik). Peptides were separated at a flow rate of 2 µl/min using a linear gradient of 0 to 40% acetonitrile-water-formic acid (80:20:0.04) (solvent B) in water-acetonitrile-formic acid (97:3:0.05) (solvent A) over 40 min, followed by a 1-min column wash in 90% solvent B and a 12-min equilibration step in solvent A. MS/MS data (scan range, 120 to 2,200; averages = 2) were acquired in positive data-dependent AutoMS(n) mode using the expectoreControl software program (version 6.2; Bruker Daltonik). Up to three precursor ions were selected from the MS scan (range, m/z 100 to 2,100; averages = 3) in each AutoMS(n) cycle. Precursors were actively excluded after being selected twice within a 1-min window, and singly charged ions were also excluded. Peptide peaks were detected (maximum of 9,999 abundance above an intensity threshold of 50,000) and deconvoluted automatically using DataAnalysis software (version 3.4; Bruker Daltonik). Mass lists in the form of Mascot Generic Format (*.mgf) files were created automatically and used as inputs to Mascot MS/MS ion searches via a local Mascot server (version 2.2; Matrix Science, London, United Kingdom) with a database built from the <CALBL_prot.txt> file (date stamp, 5 February 2002; 6,166 sequences) downloaded from CandidaDB (ftp://ftp.pasteur.fr/pub/GenomeDB/CandidaDB/FlatFiles). Search parameters used were the following: enzyme = trypsin; fixed modifications = carbamidomethyl (C); variable modifications = oxidation (M); mass values = monoisotopic; peptide mass tolerance = 1.5 Da; fragment mass tolerance = 0.5 Da; max missed cleavages = 1; instrument type = ESI-TRAP. Search results were displayed by Mascot after selection of the following parameters: standard scoring; require bold red; ion score or expect cutoff = 0.05. An open-source web application (emPAI Calc) was utilized for estimation of protein abundance (83, 84).

**SUPPLEMENTAL MATERIAL**


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**REFERENCES**

for normal biofilm formation and development in Candida albicans.

factor Ace2 regulates metabolism and is required for filamentation in hy-
EC.00155-06.

Filler SG, Mitchell AP. 2006. Critical role of Bcr1-dependent adhesins in
http://dx.doi.org/10.1371/journal.ppat.0020063.

14. Nobile CJ, Schneider HA, Nett JE, Sheppard DC, Filler SG, Andes DR,
2008.09.017.


model of Candida albicans biofilms formed under conditions of flow:
development, architecture, and drug resistance. Mycopathologia 168:

17. Gow NA, Hube B. 2012. Importance of the Candida albicans cell wall
http://dx.doi.org/10.1016/j.mib.2012.04.005.

albicans morphogenesis and host defence: discriminating invasion from
nmicro2711.

2002. The Golgi GDPase of the fungal pathogen Candida albicans affects
morphogenesis, glycosylation, and cell wall properties. Eukaryot. Cell 1:

20. Peltecro-Lucasahuanga H, Goyard S, d’Enfer C, Pirl SK, Ernst JF.
2006. Protein O-mannosyltransferases isoforms regulate biofilm for-
http://dx.doi.org/10.1128/AAC.00606-06.

NA, Gaillardin C, Munro CA, Richard ML. 2008. Functional analysis of
Candida albicans GPI-anchored proteins: roles in cell wall integrity and
dx.doi.org/10.1016/j.fgb.2008.08.003.

22. Hoyer LL, Green CB, Oh SH, Zhao X. 2008. Discovering the secrets of
the Candida albicans agglutinin-like sequence (ALS) gene family—a sticky
35317.


sion of Candida albicans cell wall-associated adhesins in Saccharomyces
cerevisiae reveals differential specificities in adherence and biofilm for-
tion and in binding oral Candida albican. Infection and in binding oral
dx.doi.org/10.1128/AAC.00606-06.

25. Kickert WT, Green CB, Oh SH, Zhao X. 2008. Discovering the secrets of
the Candida albicans agglutinin-like sequence (ALS) gene family—a sticky
35317.


27. Nobbs AH, Vickerman MM, Jenkinson HF. 2010. Heterologous expres-
sion of Candida albicans cell wall-associated adhesins in Saccharomyces
C. albicans MNT1 and Biofilm Communities

A, Wallet F, Wallaert B, Dei-Cas E, Sime-Ngando T, Chabé M, Vis-
cogliosi E. 2012. The airway microbiota in cystic fibrosis: a complex fungal
and bacterial community involved in the regulation of host mediators.

bloodstream infections involving Candida species: analysis of patients and
dx.doi.org/10.1016/j.diagmicrobio.2007.07.001.

Biofilm microbial communities of denture stomatitis. Oral Microbiol. Im-

omi.12012.

32. Diaz PI, Xu Z, Sobue T, Thompson A, Biyikoglu B, Ricker A, Ikonow-
eco L, Dongari-Bagtzoglou A. 2012. Synergistic interaction between
Candida albicans and commensal oral streptococci in a novel in vitro
ALO8966-11.

33. Holmes AR, Gilbert C, Wells JM, Jenkinson HF. 1998. Binding prop-
eties of Streptococcus gordonii Spa and SspB (antigen I/I family) polype-
ptides expressed on the cell surface of Lactococcus lactis MG1363. Infect.

34. Silverman RJ, Nobbs AH, Vickerman MM, Barbour ME, Jenkinson HF.
2010. Interaction of Candida albicans cell wall Al3 protein with Strepto-
coccus gordonii SspB adhesin promotes development of mixed-species
AL08685-10.

35. Bamafov CV, d’Mello A, Nobbs AH, Dutton LC, Vickerman MM,
Jenkinson HF. 2009. Streptococcus gordonii modulates Candida albicans
biofilm formation through intergeneric communication. Infect. Immun.

sensing molecule influences Candida albicans morphology. Mol. Micro-

Salmomella enterica serovar Typhimurium is mediated by sopB effectors,
C. albicans MNT1 and Biofilm Communities


61. Nasci R, Jenkinson HF. 1998. Altered adherence properties of a Streptococcus gordonii hppa (oligoepitope permease) mutant result from tran-


