Candida albicans Hwp1, Hwp2, and Rbt1 are related cell wall proteins expressed during the programs of sexual differentiation and filamentous growth. In this study, we compare strains lacking either single factors or a combination of these genes, and we demonstrate distinct but overlapping roles in mating and biofilm formation.

The fungus Candida albicans is often a harmless commensal in humans yet has the capacity to cause life-threatening infections, particularly in the immunocompromised host (27). The transition between commensal and pathogenic states is associated with morphological changes, among which the yeast-hypha switch is paramount. During this transition, cells switch from growing as budding yeast cells to growing as filamentous hyphae. Importantly, many genes associated with this transition are essential for virulence, including both cell wall proteins and secreted enzymes (7, 18, 33, 35).

Originally classified as an obligately asexual organism, a sexual (or parasexual) program has recently been uncovered in C. albicans (2, 11, 20, 31). The mating cycle is unique in that it is regulated by phenotypic switching; a and α cells only undergo efficient mating if they switch from the common “white” phase to the alternative “opaque” phase (19, 21). Furthermore, transcriptional profiling analyses have revealed an unexpected overlap between genes induced during mating (of opaque cells) and those induced during filamentation (in white cells) (4, 36). One hypothesis for this overlap is that genes originally involved in mating were co-opted during evolution for adherence and invasion of the host (4). It is therefore likely that studying the role of these genes in mating will also provide insight into their functions during pathogenesis.

Hwp1, Hwp2, and Rbt1 are three hypha-specific cell wall proteins that are also upregulated during mating of opaque cells (4, 5, 14, 32). Hwp1 is a well-characterized adhesin required for covalent attachment to host epithelial cells and virulence (32), as well as biofilm formation (5). Interestingly, expression and localization of Hwp1 during mating has been reported as being mating type specific; opaque α cells, were shown to express Hwp1 on their cell surface (8). This is reminiscent of agglutinin-type activity in Saccharomyces cerevisiae, where mating-type-specific proteins promote cell-cell adhesion between a and α cells (16). In this study, we examined the role of Hwp1 and the related proteins Hwp2 and Rbt1 in the mating program of C. albicans and extended this analysis to in vitro models of biofilm formation. Our results indicate the importance of these proteins in both mating and biofilm formation and, in particular, their nonredundant roles in these processes.

Hwp1 is expressed in both a and α cells of SC5314 during mating. Previous studies indicated that Hwp1 is expressed on the surface of conjugation tubes produced by opaque a cells, but not α cells, during mating (8). However, these experiments were performed using nonisogenic a and α clinical isolates of C. albicans. Furthermore, transcriptional profiling studies using derivatives of SC5314, the standard laboratory strain of C. albicans, suggested that the HWP1 gene was expressed in both cell types during mating (34). To establish the pattern of Hwp1 expression and localization in a and α strains of SC5314, Hwp1-green fluorescent protein (GFP)-Hwp1 fusion constructs were introduced into both cell types. In all cases, isogenic a and α strains were obtained by growth of C. albicans strains on sorbose medium, which selects for loss of one copy of chromosome 5 (containing the MTL locus), followed by reduplication of the remaining copy of chromosome 5 during growth on yeast extract-peptone-dextrose (12). The strains used in this study are listed in Table 1, and a list of the oligonucleotides is provided in Table 2. As shown in Fig. 1, Hwp1 protein was observed on the cell surface of both a and α cells undergoing mating and was also detectable in both halves of the conjugation bridge in zygotes (Fig. 1). Thus, in the SC5314 background, isogenic a and α cells express Hwp1 during mating, and the protein localizes to the cell surface of both of these cell types.

Hwp1, Hwp2, and Rbt1 affect mating efficiency in C. albicans. Hwp1 is part of a family of related cell surface factors that includes Hwp2 and Rbt1 (5, 6). The genes encoding these factors are clustered together in the genome, suggestive of a
common ancestry (Fig. 2A). All three genes are induced in opaque cells in response to mating pheromones (4, 36), and quantitative PCR revealed a greater-than-100-fold increase in mRNA levels. Deletion strains lacking HWP1, HWP2, or RBT1 were constructed in a and α derivatives of SC5314, along with hwp2/hwp2 rbt1/rbt1 and hwp1/hwp2 hwp2/hwp2 rbt1/rbt1 strains. Quantitative mating assays were performed on the mutants by crossing strains with different auxotrophic markers (1, 3) and revealed that each of the mutant strains exhibited a significant decrease in mating efficiency (Fig. 2B). Since agglutinin function in S. cerevisiae mating is important only for cell-cell adhesion in liquid medium (17, 29), the frequency of

| Name | Sequencea |
|------|-----------|
| Hwp1-GFP oligo 1 | CGGTCAAAAATAACCGCGCATTTTCAATTTC |
| Hwp1-GFP oligo 2 | GTAACCATACCTTTAAGTGTAAC |
| Hwp1 oligo 1 | GCTCATCACTTAGAACGAAGAACAG |
| Hwp1 oligo 3 | GTACGCGCCGATCTTCTAGCAGGAAGAATTTC |
| Hwp1 oligo 4 | GTACGCGCCGATCTTCTAGCAGGAAGAATTTC |
| Hwp1 oligo 6 | CAAGGAAATTCGGAAGATTTCGAG |
| Rbt1 oligo 1 | GGCAAGACATTTTTTC |
| Rbt1 oligo 3 | CAGGGCGCGCTAGCAGGGCGAGATTGCAATTC |
| Rbt1 oligo 4 | GTACGCGCCGATCTTCTAGCAGGAAGAATTTC |
| Rbt1 oligo 6 | GAATTGACATCAAGAGAC |
| Hwp2 oligo 1 | ATCCTCGAGTTTCGAGAG |
| Hwp2 oligo 3 | CGGCGCGCGCGAGCAGATCTTACAGTCAG |
| Hwp2 oligo 4 | GTACGCGCCGATCTTCTAGCAGGAAGAATTTC |
| Hwp2 oligo 6 | CTAAAGGCGAGAAGATAG |
| Hwp1 (-500) | GGCGCGGCGCGGGCGAATCTGGAATTTCGAG |
| Hwp1 (0) rev | GGCGCGGCGCGGGCGAATCTGGAATTTCGAG |
| Hwp1 (+1900) | GGCGCGGCGCGGGGCGATTTGCGATCTTCA |
| Hwp1 (+2300) rev | GGCGCGGCGCGGGGCGATTTGCGATCTTCA |

* All strains are derivatives of SC5314 and, except for RBY1040, -1042, -1045, and -1046, are URA3/ura3 IRO1/iro1::ura3/ura3.

**TABLE 2. Oligonucleotides used in this study**
**C. albicans** mating was analyzed in both liquid and solid media. In comparing single gene deletions, loss of *HWP2* resulted in the largest decrease in mating efficiency, with only 4% of cells mating in liquid media and 8% on solid media, while 50% of the mating efficiency (orders of magnitude) would be expected in liquid media. The three genes of interest were shown as filled boxes, and intervening genes were open boxes. (B) A quantitative mating assay was used to determine mating efficiency in both liquid and solid media, as previously described (30). Mutant strains were derived from RBY1132 (a/a) or RBY 1134 (a/α) and are listed in Table 1. A PCR fusion technique was used for gene deletion (26) with the oligonucleotides listed in Table 2. For each gene knockout, one flank of the gene was amplified with oligos 1 and 3 and the opposite flank was amplified with oligos 4 and 6. The resulting PCR products were then used to generate a targeting cassette containing the *HIS1*, *LEU2*, or *ARG4* marker, as described previously (26). Following transformation, correct integration of each construct was confirmed by PCR across the DNA junctions at the site of integration. For the double deletion of *RBT1/HWP2*, a single construct was used to target both genes, based on their adjacent position in the genome. PCRs of the 5' flank of *RBT1* (oligos 1 and 3) and the 3' flank of *HWP2* (oligos 4 and 6) were combined in the targeting cassette and used to remove both genes simultaneously. For the triple mutant lacking *HWP1*, *HWP2* and *RBT1*, *HWP1* was deleted in the *RBT1/HWP2* mutant background by using the *SAT1* flippur construct (28). In this case, the 5' and 3' flanks of *HWP1* were PCR amplified using *HWP1* (+500) for/*HWP1* (-500) rev and *HWP1* (+1900) for/*HWP1* (+2300) rev, respectively, and cloned into the pSFS2A plasmid (28). The resulting construct was digested with Apal and SacI and used to target *HWP1*. The standard practice for analyzing gene disruptions in *C. albicans* involves either comparing multiple independent disruptions or complementation by reintroducing a wild-type copy of the gene of interest. Due to the analysis of strains with multiple gene deletions, we chose the former approach, and multiple strains were therefore analyzed in this study (Table 2). Each of the mutant strains showed a statistically significant decrease (*P* < 0.05) in the number of mating products formed in both liquid and solid mating assays compared to the wild-type strain. Results are means ± standard errors of the mean of 2 to 12 experiments with each strain, and statistical analyses were performed using two-sample f tests. All *P* values are two tailed and are based on comparisons with the wild type.

**FIG. 2.** Mating efficiency in strains lacking Hwp1, Hwp2, or Rbt1. (A) Schematic of the region on chromosome 4 containing *HWP1*, *HWP2*, and *RBT1* genes. (B) Loss of wild-type cells underwent mating under these conditions (Fig. 2B). Loss of *HWP1* or *RBT1* also reduced the overall mating frequency in both liquid and solid media (Fig. 2B), although double and triple mutants did not show a further reduction in mating. Significantly, the difference between mating efficiencies in liquid and solid media were small in all of the crosses, suggesting that these proteins do not exhibit the classical agglutinin function, with which a much larger reduction in mating efficiency (orders of magnitude) would be expected in liquid media.
Hwp1, Hwp2, and Rbt1 are required for efficient biofilm formation. We also examined the role of the three cell surface proteins in biofilm assays. Biofilm formation is dependent on adhesins to mediate both the attachment of cells to the substrate surface as well as the adherence of cells to one another (23, 25). Assays were performed using white-phase cells, as these undergo hypha formation, during which expression of HWP1, HWP2, and RBT1 is upregulated (5, 13), and this mode of growth is necessary for efficient biofilm formation (23). We observed decreased biofilm formation in each of the mutants, with hwp1/hwp1 strains showing a greater decrease than either hwp2/hwp2 or rbt1/rbt1 mutants (Fig. 3). This is also in agreement with recent studies that implicated Hwp1 as having an important role in C. albicans biofilm formation both in vitro and in vivo (24, 25). The triple mutant strain hwp1/hwp1 hwp2/ hwp2 rbt1/rbt1 showed the greatest defect in biofilm formation, suggesting that the three surface factor genes are related but non-overlapping, roles in biofilm formation (Fig. 3).

Concluding remarks. Our results demonstrate that the three cell surface proteins, Hwp1, Hwp2, and Rbt1, play important roles in both white and opaque phases of C. albicans biology. These factors are induced in the opaque phase by pheromones and enhance mating between a and α cells. However, they do not exhibit classic agglutinin functions, as the mating frequencies of mutants were comparable in both liquid and solid media. Hwp1 was also shown to be expressed on the surface of both a and α cells in isogenic derivatives of SC5314. This result contrasts with that reported in clinical isolates of C. albicans (8), and the fact that both mating types express this gene again distinguishes Hwp1 from the sexual agglutinins of S. cerevisiae (16). Perhaps C. albicans no longer requires classical agglutinins if conjugation occurs on the surface of the skin (15) or in a confined three-dimensional matrix (9). Hwp1, Hwp2, and Rbt1 also play a defined role in C. albicans for promoting biofilm formation by white-phase cells, as biofilm masses were diminished in each of the three mutant backgrounds. Hwp1 was the most important of the three, a result in keeping with recent studies (24, 25), yet strains lacking all three factors showed the greatest defect in biofilm formation. These results indicate that there is partial redundancy between these cell wall proteins and that loss of one factor can be compensated for, at least in part, by another. Our findings also suggest that further analysis of these factors in mating and adhesion will extend their prospective roles in biofilm formation and host pathogenesis.

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