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Misexpression of the White-Phase-Specific Gene *WH11* in the Opaque Phase of *Candida albicans* Affects Switching and Virulence†

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Candida albicans **WO-1 switches between a white- and an opaque-colony-forming phenotype. The gene *WH11* is expressed differentially in the white phase. The *WH11* open reading frame was inserted downstream of the promoter of the opaque-phase-specific gene *OP4* in the transforming vector pCWOP16, and resulting transformants were demonstrated to misexpress *WH11* in the opaque phase. Misexpression had no effect on the ability to switch from the white to the opaque or the opaque to the white phase, and it had no effect on the genesis of the unique opaque-phase cellular phenotype, even though the Wh11 protein was distributed throughout the cytoplasm in a manner similar to that observed for the endogenous gene product in the white phase. Misexpression did, however, increase the frequency of the opaque-to-white transition 330-fold and markedly increased the virulence of cells in the opaque phase in a mouse tail injection model.**

Most strains of the opportunistic pathogen *Candida albicans* switch between two or more general phenotypes characterized by different colony morphologies and, in some cases, by different cellular phenotypes (24–26). In the case of *C. albicans* WO-1, cells switch spontaneously between a white and an opaque phase at frequencies of approximately 10^{-3} (3, 5, 20, 23, 27). In the white phase, cells generate hemispherical white colonies and exhibit a round budding shape with subcellular architecture common to the dominant forms of most strains of *C. albicans* (24). In the opaque phase, cells generate gray or opaque colonies, are twice as big as white-phase cells, are bean shaped and asymmetric rather than round, bud in an abnormal fashion, contain pimples in the cell wall, and are dominated by a large vesicle-filled vacuole (1–3, 23). Opaque-phase cells also differ from white-phase cells in their sensitivity to leukocytes and oxidants (10), the environmental constraints on the budhypha transition (1), the secretion of aspartyl proteinase (17, 36), accessibility to dyes (3), and virulence in a mouse model (15, 19). White- and opaque-phase cells also differ in the expression of a number of phase-specific genes. While opaque-phase cells selectively express the genes *OP4*, *PEP1* (*SAP1*), and *SAP3* (8, 17, 36), white-phase cells selectively express the gene *WH11* (31). *WH11* encodes a polypeptide of 66 amino acids (31) which is homologous to the low-molecular-weight heat shock protein Hsp12 (33, 35). *WH11* is transcribed exclusively in the round budding form of the white phase; it is not expressed in either the elongate hyphal form of the white phase or the elongate budding form of the opaque phase (31, 32). *WH11* expression is regulated by two major transcription activation sequences (28, 29) and a newly identified minor transcription activation sequence upstream of the *WH11* open reading frame (ORF) (32). The Wh11 protein is found only in white budding-phase cells and is absent in opaque budding-phase cells or white-phase cells extending a hypha (21). In addition, by indirect immunofluorescent staining, a Wh11-like antigen has been demonstrated to be present in a number of species closely related to *C. albicans* that grow in a round budding growth form but to be absent in related species growing in an elongate budding growth form (21). The correlation between growth in a round budding form and the expression of *WH11* suggests that Wh11 may have a regulatory or structural role in the genesis of a spherical cell morphology.

To explore further the function of *WH11* in the phenotypic transition between the white and opaque phenotypes, we constructed a misexpression plasmid in which the *WH11* ORF was fused downstream to the *OP4* promoter and used the plasmid to transform the *ade2* strain Red 3/6 by integration at the *ADE2* locus. The phenotypic characteristics, switching frequencies, and virulence of several transformants, which misexpressed the white-phase-specific gene *WH11* in the opaque phase, were then characterized.

MATERIALS AND METHODS

Maintenance of stock cultures. *C. albicans* WO-1, the *ade2* derivative strain Red 3/6, and transformed derivatives of Red 3/6 were maintained on agar storage slants containing the nutrient composition of Lee's medium (11) supplemented with 70 μg of arginine per ml and 0.1 μM $ZnSO_4$ (4) and, in the case of Red 3/6, with 0.6 mM adenine. For experimental purposes, cells were removed from a slant, clonally plated on agar containing supplemented Lee's medium, and incubated at 25°C for 4 to 5 days. Cells from individual white or opaque colonies were in turn inoculated into liquid supplemented Lee's medium. In all cases in which white- or opaque-phase cells were used directly for experiments, an aliquot was plated to assess the phenotypic homogeneity of the population.

Construction of the misexpression plasmid pCWOP16. The primer pair WPP (5′-AACTGCAGATGTCCCACTTAGGTA-3′)-XN (5′-CATGCCATGGTGA GACGCGACAGAC-3′) was used to amplify the *WH11* cassette containing the *WH11* ORF and the *WH11* transcription termination sequence (32). A *Pst*I site was placed immediately upstream of the ATG codon, and an *Nco*I site was placed immediately downstream of the untranslated region of *WH11*. The primer pair TS1 (5′-CTAATTTAGCTGTGACCCCCCT-3′)-TS2 (5′-TCCTGCAGTG TAAATTATTTTTATTTTGTATGTG-3′) was used to amplify 850 bp upstream of the ORF of *OP4* (17) which contained the *OP4* promoter (29). TS2 was designed with a *Pst*I site at the 5′ end, which is the 3′ end of the *OP4* promoter (29). The TS1 primer contained no restriction sites. The parental plasmid pCRW3 (Fig. 1B) contained the functional *C. albicans ADE2* gene (Fig. 1A) for selection in the *ade2* strain Red 3/6, the *Renilla reniformis* luciferase (*RLUC*) ORF fused to the *WH11* transcription termination sequence, and a multiple cloning site positioned immediately upstream of the *RLUC* ORF (30). The *OP4* promoter fragment was directionally inserted at the multiple cloning site of pCRW3, creating plasmid pCROP31. The *RLUC* ORF and *WH11* transcription termination sequence were removed by *Pst*I/*Nco*I digestion and replaced by the *WH11* cassette containing the *WH11* ORF and termination sequence, creating

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†This work is dedicated to Arne M. Kvaal (1944–1997).

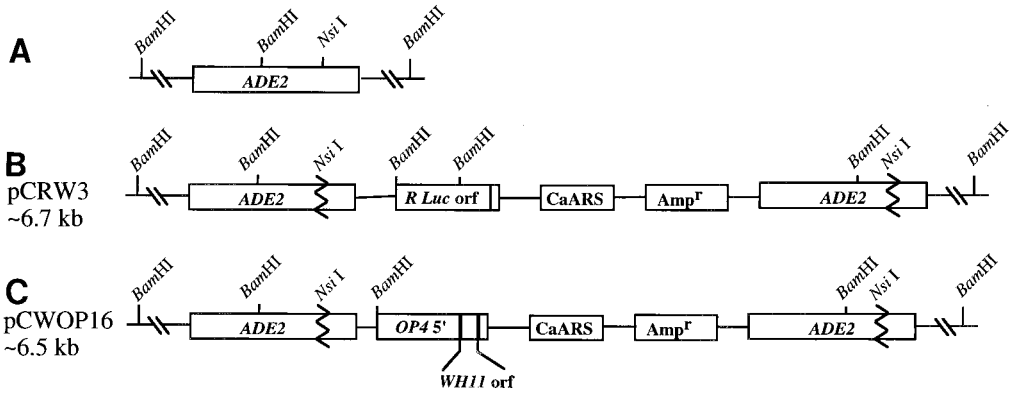

FIG. 1. Genesis of the transforming plasmid pCWOP16, in which the *WH11* ORF is under the regulation of the *OP4* promoter. (A) The endogenous *ADE2* gene with relevant restriction sites. (B) Plasmid pCRW3, which was used to construct pCWOP16. (C) The final transforming plasmid pCWOP16. *ADE2*, the *ADE2* gene of *C. albicans*; *R Luc* orf, the *RLUC* ORF; CaARS, a *C. albicans* autonomously replicating sequence; Amp^r, ampicillin resistance gene; *OP4* 5′, the 5′-upstream region of the gene *OP4*; *WH11* orf, the ORF of *WH11*.

plasmid pCWOP16 (Fig. 1C). The junction between the *WH11* ORF and the *OP4* promoter was sequenced to confirm the in-frame fusion.

Transformation of spheroplasts. Red 3/6 was used previously in transformation studies with pCRW3 and has been demonstrated to behave like the parent strain WO-1 in the switching process (30). For transformation, four to five independent white colonies were pooled, inoculated into 25 ml of YPD medium (22), and grown to late log phase at 25°C. From this initial culture, 2.5×10^7 cells were inoculated into 25 ml of minimal medium (22) and grown to a density of 1×10^8 to 2×10^8 cells/ml. Spheroplasts were generated by methods previously described (30, 32). Briefly, cells were pretreated with a solution containing 1 M sorbitol, 25 mM EDTA, and 50 mM dithiothreitol for 20 min at 37°C, followed by incubation for 60 to 90 min in a solution containing 1 M sorbitol, 0.1 M sodium citrate (pH 5.8), 10 mM EDTA, and Zymolyase 100T (0.4 mg/ml [wt/vol]; Seikagaku America, Rockville, Md.). Spheroplast formation was monitored microscopically. When 80% of cells had formed spheroplasts, they were washed twice with 1 M sorbitol and once with CaST (1 M sorbitol, 0.01 M $CaCl_2$, 0.01 M Tris-HCl [pH 7.5]) and then resuspended in CaST at a density of 10^9 per ml. Transformation was carried out for 20 min at 25°C in a final volume of 200 μl containing 2×10^8 spheroplasts, 10 μg of *Nsi*I-linearized pCWOP16, and 100 μg of denatured calf thymus DNA in 200 μl of CaST. This was followed by incubation in 1 ml of a solution containing 20% (wt/vol) polyethylene glycol 4000, 10 mM $CaCl_2$ and 10 mM Tris-HCl (pH 7.5) for 20 min. Spheroplast suspensions were recovered by incubation in a solution containing 1 M sorbitol, 0.35% (wt/vol) yeast extract, 0.7% (wt/vol) Bacto Peptone, and 6.5 mM $CaCl_2$ for 40 min at 25°C. The appropriate dilutions of spheroplasts were mixed with 5 ml of 1% top agar containing 1 M sorbitol and spread on minimal medium agar containing 2% (wt/vol) dextrose, 0.7% (wt/vol) Bacto yeast nitrogen base without amino acids, and 1 M sorbitol. Plates were incubated for 3 to 5 days at 25°C before transformant colonies were visible.

Southern and Northern blot analysis. To verify transformants by Southern blot hybridization, total DNA was extracted by methods previously described (17, 30) from cells grown for 4 days on agar plates containing supplemented Lee's medium. Five micrograms of DNA was then digested with *Bam*HI, and the resulting fragments were separated on a 0.8% (wt/vol) agarose gel containing 0.55% (wt/vol) Tris-HCl (pH 7.5), 0.25% (wt/vol) boric acid, and 0.0125 M EDTA. The DNA was transferred to Hybond-N nylon membrane (Amersham International, Buckinghamshire, England) and probed with the *C. albicans ADE2* gene. The *ADE2* probe was radiolabeled by using the random priming method and [α-^{32}P]dCTP (NEN, Boston, Mass.). For Northern blot hybridization, total cellular RNA was extracted by using the guanidine thiocyanate method (6) and a Bead Beater (Biospec Products, Bartlesville, Okla.). RNA was separated in an agarose formaldehyde gel containing 0.2% 3-(4-morpholino)propanesulfonic acid (Fisher Scientific, Fair Lawn, N.J.), 0.1 M sodium acetate, and 0.01 M EDTA (pH 7.0). RNA was transferred to Hybond-N nylon membrane and probed with the *C. albicans EF1α* gene (34) and the *WH11* ORF (31). Prehybridization and hybridization procedures were performed by the method of Church and Gilbert (7) for both Northern and Southern blots. Autoradiography was performed by exposing membranes at −70°C, using intensifying screens and Kodak XAR film (Eastman Kodak Co., Rochester, N.Y.).

Scanning electron microscopy. Cells grown to mid-log phase in liquid medium were pelleted by centrifugation, washed once in double-distilled H_2O, and fixed in 2.5% (wt/vol) glutaraldehyde. Cells were attached to poly-L-lysine-coated coverslips, washed in sodium cacodylate buffer, and postfixed in 4% (wt/vol) OsO_4 in phosphate buffer. Cells were then washed in double-distilled water, gradually dehydrated in ethanol, and finally dehydrated in hexamethyldisilazane. Cells were sputter coated with gold palladium in an Emscope SC500 (Emscope Laboratories Ltd., Ashford, England) and viewed with a Hitachi S-4000 scanning electron microscope (Hitachi Corp., San Diego, Calif.).

Measurement of phenotypic switching. Cells from white or opaque colonies were grown in liquid supplemented Lee's medium as described above. Cells from mid-log-phase cultures were serially diluted to 500 cells per ml. Then 200 μl of the final cell suspension, containing 100 cells, was spread on each of 15 agar plates (11-cm diameter) containing supplemented Lee's medium with phloxine B (5 μg per ml) which preferentially stains opaque-phase cells (3). Colony phenotypes were assessed after 7 days of incubation at 25°C.

Indirect immunofluorescent staining of Wh11. Cells were washed once in PD solution (136 mM NaCl, 2 mM KCl, 10 mM $Na_2HPO_4 \cdot 7H_2O$, 1 mM KH_2PO_4, 5% [vol/vol] dimethyl sulfoxide) at room temperature. Cells were resuspended in 500 μl of PD solution, mixed with 500 μl of 8% paraformaldehyde, and incubated first for 30 min at 37°C and then for 90 min at 25°C. Fixed cells were washed twice in 1 ml of PD, resuspended in 250 μl of PD plus 10 μl of Zymolase 100T, and incubated for 15 min in the case of opaque-phase cells or 90 min in the case of white-phase cells. Cell wall digestion was monitored microscopically. When 80% of the cells had become spheroplasts, they were washed twice in 1 ml of PD, resuspended in 400 μl of PD, and dispersed on 22- by 22-mm glass coverslips coated with Cell-tak cell and tissue adhesive (Collaborative Biomedical Products, Bedford, Mass.) according to the manufacturer's specifications. After 60 min, the coverslips were washed with PD, and 1.5 ml of PDT (PD, 0.2% [vol/vol] Triton X-100) was added for 10 min to permeabilize the cells. The coverslips were washed twice in DBP (PD containing 1.0% [wt/vol] bovine serum albumin) and once in a solution containing 1.2 M sorbitol and 100 mM Tris-HCl (pH 8.3). To stain for Wh11, cells were incubated in blocking solution containing 25% (vol/vol) goat serum, 1% (wt/vol) bovine serum albumin 10% (wt/vol) Carnation milk powder, and 5% (vol/vol) dimethyl sulfoxide in phosphate-buffered (PBS) for 60 min, then incubated in a 1:500 dilution of rabbit anti-rWh11 antiserum (21) for 90 min, and finally washed five times in PD. Coverslips were then incubated in a 1:300 dilution of fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G antiserum (ICN Pharmaceuticals Inc., Costa Mesa, Calif.) in DBP for 45 min in a dark chamber. To stain for DNA, the same cells were washed six times with DBP and then incubated in 0.02 μg of 4′,6-diamino-2-phenylindole (DAPI) per ml for 5 min. After staining for either Wh11 or DNA, coverslips were placed cell side down on 25 μl of Vectashield (Vector Laboratories, Burlingame, Calif.). Cells were photographed by using epiluminescent fluorescence in a Zeiss ICM405 inverted microscope. For visualizing cells stained with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G antiserum, a blue I 450-nm excitation filter was used; for DAPI-stained cells, a UV 365-nm excitation filter was used. The same preparations were examined with a Bio-Rad 600 MRE confocal microscope.

Assessment of virulence in a mouse model. Cells from either white- or opaque-phase colonies were grown in liquid supplemented Lee's medium to late log phase (2.5×10^7 spheres/ml). Cells were washed twice in sterile 1× PBS and resuspended at a cell density of 4×10^6 per ml. Cell phenotype was verified microscopically. Seven to ten-week-old female ND-4 mice (Sprague-Dawley, Madison, Wis.) weighing 21 to 26 g were injected in the lateral tail veins with 0.25 ml of the prepared cell suspension; for each strain tested, 30 mice were injected with white-phase cells and 30 mice were injected with opaque-phase cells. At days 1, 2, 4, 8, and 12 postinjection, six mice were sacrificed, and the right kidney was removed and weighed. The kidney was then homogenized in 10 ml of sterile 1× PBS. The suspension of cells from each kidney was serially diluted 100-, 500-, and 1,000-fold with PBS, and cells from the original suspension and the three serial dilutions were distributed on plates containing modified Lee's medium plus phloxine B. After 5 to 7 days of incubation at 25°C, colonies on each plate were

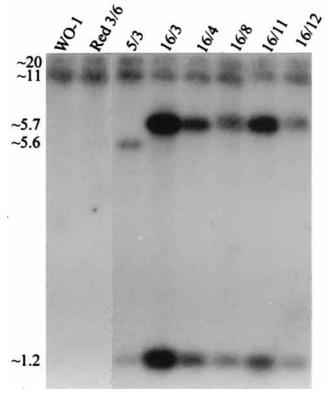

FIG. 2. Southern blot analysis of transformant DNA digested with *Bam*HI and hybridized with *ADE2*. WO-1 and Red 3/6 exhibit the two expected bands at ~20 and ~11 kb for the endogenous *ADE2* gene. Strain 5/3 transformed with pCRW3 exhibits the expected bands for the endogenous gene and expected bands for the plasmid gene at ~5.6 and ~1.2 kb. Strains 16/3, 16/4, 16/8, 16/11, and 16/12 transformed with pCWOP16 exhibit the expected bands for the endogenous gene and the expected bands for the plasmid gene at ~5.7 and ~1.2 kb for the plasmid gene. Approximate sizes in kilobases are presented to the left.

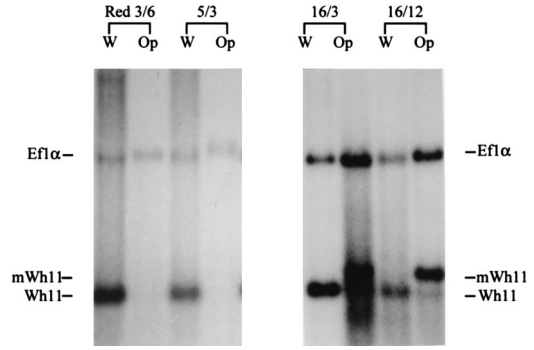

FIG. 3. Northern blot analysis of RNA from white (W) and opaque (Op) cell cultures of parent strain Red 3/6, control strain 5/3, and misexpression strains 16/3 and 16/12 probed with *EF1*α and *WH11*. Parental strain Red 3/6 and control strain 5/3 expressed the *WH11* transcript exclusively in the white phase. Strains 16/3 and 16/2 expressed the *WH11* message in the white phase and the *mWH11* transcript in the opaque phase. 16/3 and 16/12 also expressed low levels of the *WH11* message in the opaque phase due to high levels of white-phase cell contamination.

counted and scored for phenotype (white colonies were white to light pink, and opaque colonies were stained red); values were converted to CFU per gram of kidney and averaged for the four plates of each serial series and then for the six mice.

RESULTS

Verification of pCWOP16 transformants. To verify that putative revertants which have regained adenine prototrophy contained pCWOP16 vector sequences, Southern blots of *Bam*HI-digested DNA were probed with *ADE2*. Strains WO-1 and the *ade2* derivative Red 3/6 contained two *ADE2*-containing *Bam*HI fragments with molecular sizes of ~20 and ~11 kb (Fig. 2), representing the expected fragments of the endogenous gene (Fig. 1A). The putative transformants 16/3, 16/4, 16/8, 16/11, and 16/12 contained these two fragments plus two additional *ADE2*-containing fragments at molecular sizes of 5.7 and 1.2 kb (Fig. 2). The latter two represented the expected DNA fragments of the *ADE2* gene for pCWOP16 inserted at the *ADE2* locus (Fig. 1C). If insertions occurred at sites other than the *ADE2* locus of Red 3/6, different-size bands would have been observed between the independent transformants. The absence of such band variability demonstrates that all analyzed transformants contained insertions of pCWOP16 at the *ADE2* locus. The similar intensities of the ~20- and ~11-kb bands, which represent the endogenous *ADE2* sequences, and the variable intensities of the 5.7- and 1.2-kb bands in the five transformants suggested variable copy number of the insertion sequence. Strain 16/3, with the highest copy number, was used in subsequent misexpression experiments. In addition, the *ade2* parental strain Red 3/6 was transformed with pCRW3 (Fig. 1B) in order to generate the *ADE2* derivative 5/3, which served as a control strain for the misexpression mutants. The Southern blot hybridization pattern of strain 5/3 contained the two endogenous *ADE2* bands plus bands at approximately 5.6 and 1.2 kb, the predicted sizes for *ADE2*-containing fragments in pCRW3 (Fig. 1B).

Mistranscription of the *WH11* transcript in the opaque phase. pCWOP16 contains the *WH11* ORF fused downstream from the *OP4* promoter. In constructing pCWOP16, the *WH11* transcription start site was replaced by the *OP4* transcription start site. This resulted in a misexpressed *WH11* transcript 20 nucleotides longer than the transcript of the endogenous gene, allowing discrimination between the transcripts of the misexpressed gene (*mWH11*) and the endogenous gene. Northern blots of RNA from white- and opaque-phase cells of the original strain WO-1, the *ade2* derivative strain Red 3/6, and two transformant strains, 16/3 and 16/12, were probed with *WH11* (Fig. 3). To compare loading between white-phase and opaque-phase RNAs for each tested strain, the blots were also probed with the constitutively expressed gene *EF1*α (34). For each strain, patterns of *EF1*α hybridization were similar for white- and opaque-phase cells (Fig. 3). For Red 3/6 and control strain 5/3, the endogenous *WH11* gene was transcribed only in the white phase, as expected (31). In the two putative misexpression strains 16/3 and 16/12, endogenous *WH11* was expressed in the white phase and at greatly reduced levels in the opaque phase (Fig. 3), which, as demonstrated below, is the result of increased white-phase cell contamination in opaque-phase cell populations of misexpression mutants. More importantly, *mWH11* was expressed differentially in opaque-phase cell populations of the two putative misexpression mutants, demonstrating that *mWH11* transcription was under the regulation of the *OP4* promoter. In addition, the band representing the *mWH11* transcript was more intense in strain 16/3 than in strain 16/12 (Fig. 3), which was predicted from the difference in plasmid copy number (Fig. 2).

Misexpression of *WH11* does not suppress the unique opaque-phase cellular phenotype. Opaque-phase cells exhibit a number of unique phenotypic traits (24). To test whether cells which misexpress *WH11* in the opaque phase form morphologically normal opaque-phase cells, white- and opaque-phase cells were compared between strain WO-1 and the misexpression mutant 16/3. In each case, cells from a white-phase colony and cells from an opaque-phase colony were independently grown in liquid nutrient medium and then examined by phase-contrast microscopy or by scanning electron microscopy. Figure 4 shows phase-contrast micrographs of white budding and opaque budding cells, respectively, of strain WO-1. While the majority of white-phase cells were round, the majority of opaque-phase cells were bean shaped, asymmetrical, and roughly twice the volume of white-phase cells, as previously reported (3, 23, 24). The same was true for white- and opaque-phase cells of the misexpression mutant strain 16/3. Examples of phase-contrast micrographs of round white-phase cells and elongate, bean-shaped opaque-phase cells of

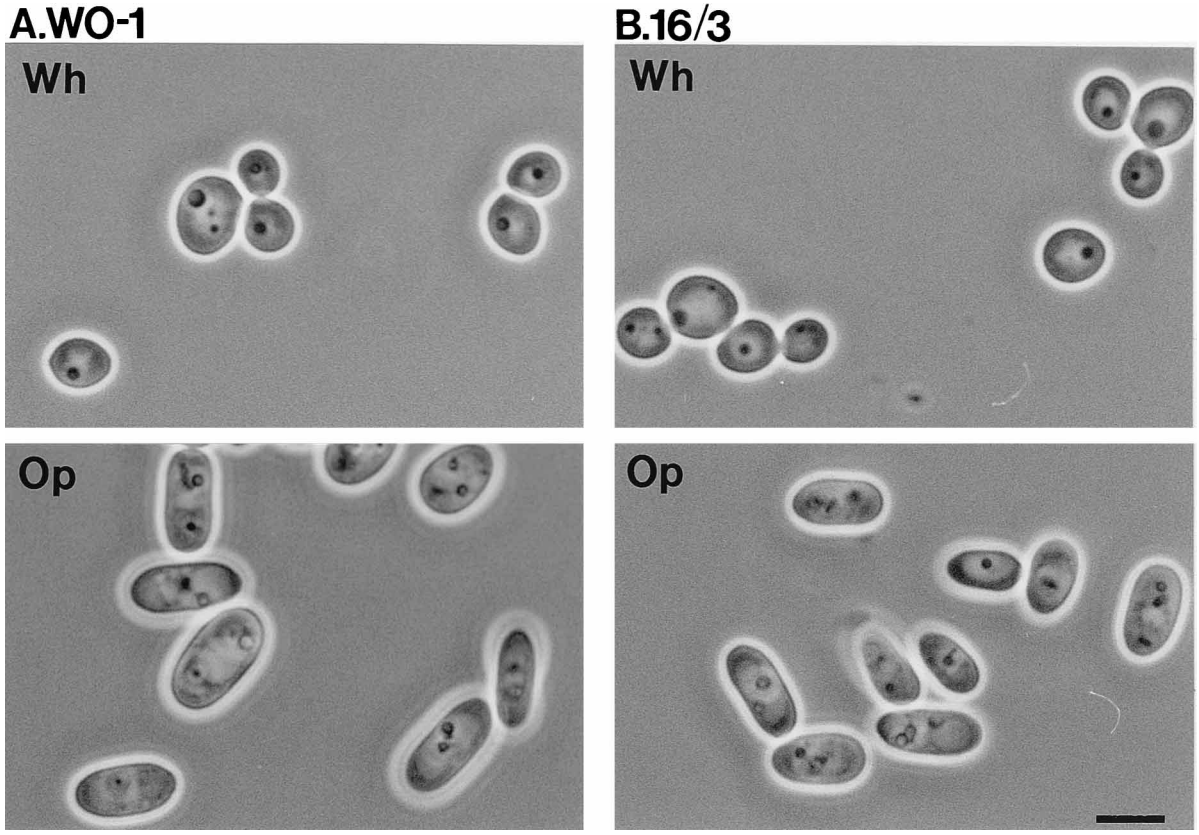

FIG. 4. Phase-contrast micrographs of white (Wh)- and opaque (Op)-phase cells of strains WO-1 (A) and 16/3 (B). Scale bar, 3 μm.

mutant 16/3 are presented in Fig. 4B. Similar results were obtained for control strain 5/3 and for the misexpression mutants 16/8 and 16/12 (data not shown).

Figures 5A and C show representative scanning electron micrographs of a representative white-phase cell and a representative opaque-phase cell of strain WO-1. While the wall of the mature white-phase cell was relatively smooth, the wall of the mature opaque-phase cell contained unique wall pimples, as previously described in detail (2, 3, 24). The same was true for white- and opaque-phase cells of the misexpression mutant 16/3 (Fig. 5B and D, respectively). These results demonstrate that misexpression of *WH11* in the opaque phase does not suppress the genesis of the unique opaque-phase cellular phenotype.

Misexpression of the Wh11 protein in the opaque phase. To test whether misexpression mutants express the Wh11 protein in the opaque phase, cells were stained with antiserum generated against recombinant Wh11 (21). In studies using this antiserum, it was previously demonstrated that in white-phase cells of *C. albicans* WO-1, Wh11 is distributed throughout the cytoplasm but is excluded from the nucleus, cytoplasmic vesicles, plasma membrane, and cell wall, and that in opaque-phase cells, it is not detectable. The Wh11 staining patterns of white-phase cells and the absence of staining of opaque-phase cells of strain Red 3/6 (Fig. 6B and F, respectively) were similar to those previously reported for the original parent strain WO-1 (21). The Wh11 staining pattern of white-phase cells of the misexpression mutant 16/3 (Fig. 6D) was also the same as those of WO-1 (21) and Red 3/6 (Fig. 6B). However, in contrast to strains WO-1 and Red 3/6 (Fig. 6F), opaque-phase cells of strain 16/3 stained for the Wh11 antigen (Fig. 6H), and the distribution of the antigen was similar to that observed in white-phase cells (Fig. 6B and D). Similar results were obtained for the misexpression strains 16/8 and 16/12 (data not shown).

Misexpression of *WH11* selectively increases the frequency of switching from opaque to white. It was immediately apparent after generating misexpression strains 16/3, 16/4, 16/8, 16/11, and 16/12 that the opaque-phase cell population of each was more heavily contaminated with white-phase cells than opaque-phase cell populations of strains WO-1, Red 3/6, and 5/3, suggesting that misexpression mutants switch more frequently from opaque to white. Cells from clonal white- and opaque-phase colonies of control strains 5/3 and the misexpression strains 16/3, 16/8, and 16/11 were individually grown in liquid medium and then plated at low density to determine the level of contamination of alternative cell phenotypes. These levels reflect the frequency of switching to the alternative phenotype. White-phase cell populations of control strain 5/3 contained opaque-phase cells at an average frequency of 1.3×10^{-3}, and opaque-phase cell populations contained white-phase cells at an average frequency of 6.0×10^{-4} (Table 1). These frequencies were close to those found in previously published studies in which switching was estimated by measuring white and opaque CFU in clonal populations (3, 23). White-phase populations of the *WH11* misexpression mutants 16/3, 16/8, and 16/11 contained opaque-phase cells at frequencies ranging between 1.0×10^{-3} and 3.0×10^{-3}, with a mean frequency for the pooled data of the three mutants of 1.7×10^{-3}, which is very close to the frequency of opaque-phase cells in white-phase cell populations of the control strain 5/3 (Table 1). However, the frequency of white-phase cells in

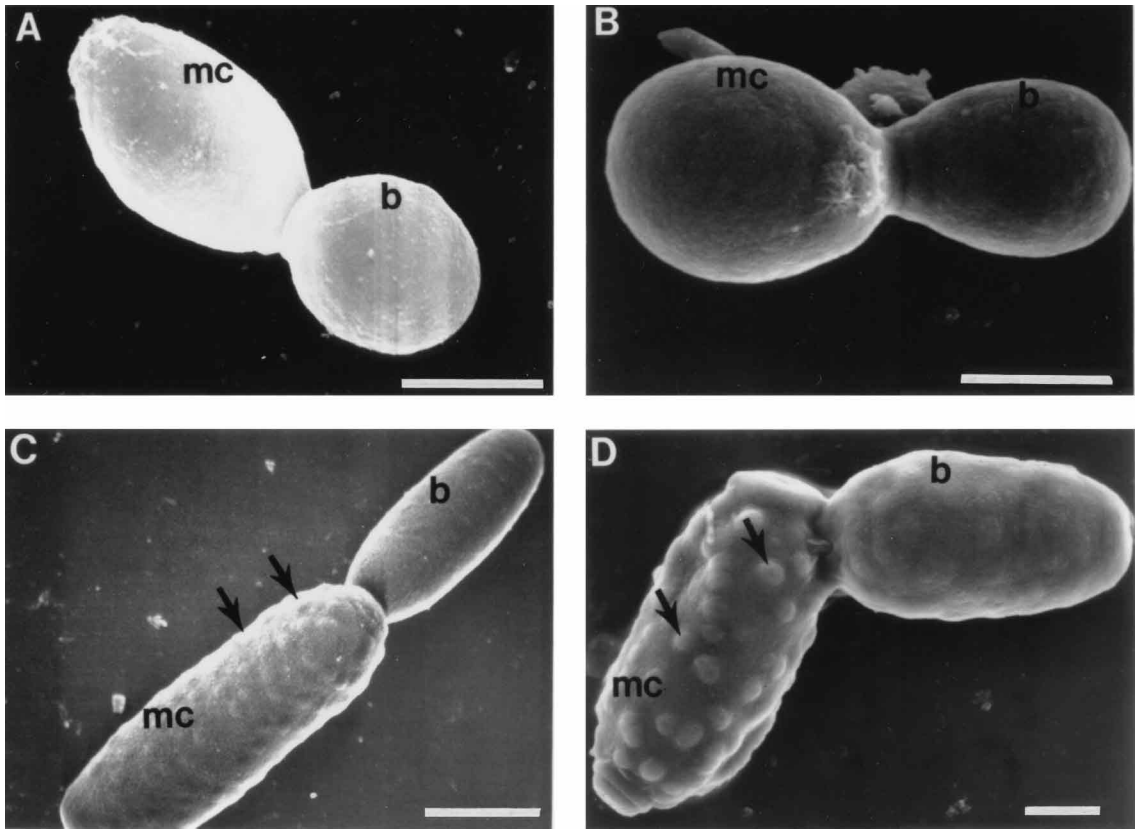

FIG. 5. Scanning electron micrographs of budding white- and opaque-phase cells of original strain WO-1 and misexpression strain 16/3. (A and B) Budding white- and opaque-phase cells, respectively, of strain WO-1. (C and D) Budding white- and opaque-phase cells, respectively, of strain 16/3. Arrows point to unique opaque-phase pimples. mc, mother cell; b, bud or daughter cell. Scale bars, 2 μm.

opaque-phase cell populations was 2.0×10^{-1}, which was 330 times higher than the frequency of white-phase cells in opaque-phase cell populations of control strain 5/3. These results demonstrate that although misexpression of *WH11* does not affect the transition from white to opaque, it dramatically increases the switching frequency from opaque to white.

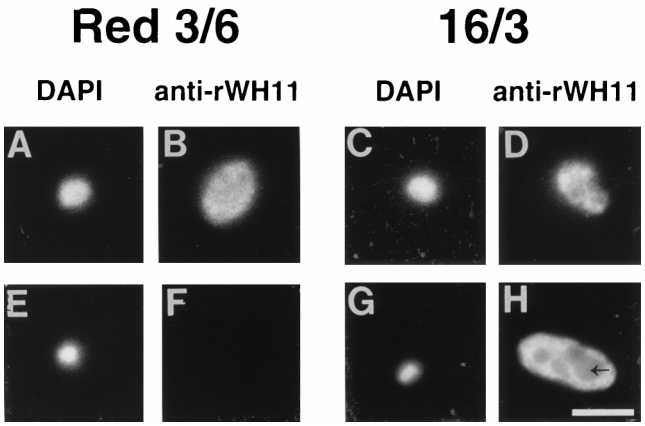

FIG. 6. Distribution of the Wh11 protein in the opaque phase of the misexpression mutant 16/3. Cells were double stained with DAPI, to visualize the location of the nucleus, and anti-Wh11 antibody, to visualize the distribution of Wh11. (A and B) White-phase cell of strain Red 3/6 stained with DAPI and anti-Wh11 antibody, respectively; and (C and D) white-phase cell of strain 16/3 stained with DAPI and anti-Wh11 antibody, respectively; (E and F) opaque-phase cell of strain Red 3/6 stained with DAPI and anti-Wh11 antibody, respectively; (G and H) opaque-phase cell of strain 16/3 stained with DAPI and anti-Wh11 antibody, respectively. The arrow in panel H points to vacuole. Scale bar, 3 μm.

The effects of misexpression on virulence in a tail injection mouse model. To assess the effects of misexpression on virulence, we compared the accumulation of yeast in the kidneys of mice injected with white- or opaque-phase cells of control and misexpression mutant strains. White-phase cells of the original strain WO-1 injected into the tail vein of mice colonized the kidney after 1 day at a total cell density of 1.3×10^5 CFU/g (Fig. 7A). The density increased to 2.5×10^6 CFU/g after 4 days and remained relatively stable at this density through day 12. The vast majority of cells colonizing the kidney through the 12 days following injection formed white colonies when plated

TABLE 1. Frequencies of opaque-phase cells in clonal white-phase cell populations and white-phase cells in clonal opaque-phase cell populations

Strain(s)	Clonal population	No. of colonies analyzed	Frequency of cells with alternative phenotype
5/3 (Red 3/6 transformed with pCRW3)	White	4,000	1.3×10^{-3}
	Opaque	4,000	6.0×10^{-4}
16/3, 16/8, and 16/11[a]	White	8,000	1.7×10^{-3}
	Opaque	8,000	2.0×10^{-1}

[a] Since the results were highly similar for the three independent misexpression mutants 16/3, 16/8, and 16/11, the data were pooled.

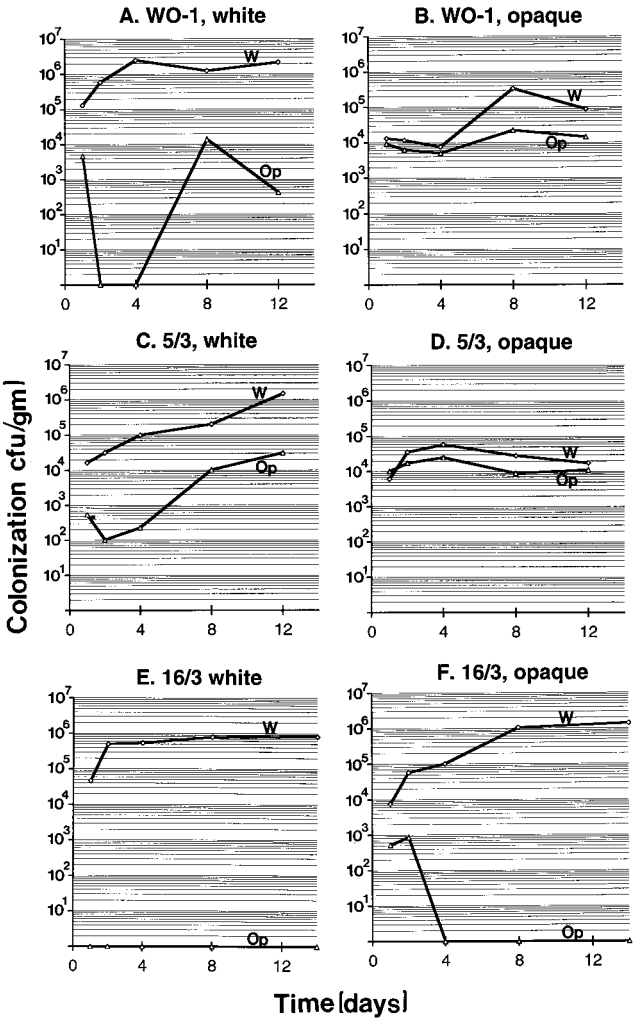

FIG. 7. Density of colonization and proportion of white (W) and opaque (Op) phenotypes of colonizing cells in the kidneys of mice injected with white- or opaque-phase cells of the indicated strains. Time is in days postinjection. Colonization is expressed as CFU per gram of kidney.

on agar (Fig. 7A), demonstrating that the majority of injected white-phase cells maintained their original white-phase phenotype. Opaque-phase cells injected into the tail vein of mice colonized the kidney after 1 day at a total cell density of approximately 1.5×10^4 CFU/g (Fig. 7B), roughly an order of magnitude lower than the total cell density 1 day after white-phase cell injection (Fig. 7A). Colonization peaked at 8 days, at a density of 3.3×10^5 CFU/g (Fig. 7B), again roughly an order of magnitude lower than that measured 8 days after injection of white-phase cells (Fig. 7A). Although the original opaque-phase cell population contained over 99% opaque-phase cells at the time of injection, approximately two-thirds of cells colonizing the kidney 1 day after injection were in the white phase and one-third were in the opaque phase, suggesting that mass conversion from the opaque to the white phenotype (5, 20, 23, 31) and/or selection of white-phase cells occurred after injection. By 8 days, at the peak of colonization, white-phase cells accounted for 94% of the colonizing population and opaque cells accounted for 6%. Similar results were obtained with control strain 5/3. Again, mice injected with white-phase cells were colonized primarily with white-phase cells (Fig. 7C), mice injected with opaque-phase cells were colonized by a majority of white-phase cells after 1 day (Fig. 7D), and the level of colonization after white-phase cell injection was at least 1 order of magnitude greater than that after opaque-phase cell injection (compare Fig. 7C and D).

White-phase cells of the misexpression strain 16/3 injected into the tail veins of mice colonized the kidney at levels (Fig. 7E) similar to those observed for white-phase cells of the parental strain WO-1 (Fig. 7A) and the control strain 5/3 (Fig. 7C). However, while low levels of opaque-phase cells were always observed in the kidneys of mice injected with white-phase cell populations of strain WO-1 (Fig. 7A) and strain 5/3 (Fig. 7C), no opaque-phase cells were ever retrieved from the kidneys of mice injected with white-phase cells of strain 16/3 (Fig. 7E). More dramatic differences were observed in the level of colonization and the phenotypic makeup of colonizing cells after injection with opaque-phase cells of strain 16/3. When opaque-phase cells of strain 16/3 were injected, the level of colonization of the kidney after 8 days was 1.1×10^6 CFU/g (Fig. 7F), which was approximately 10-fold higher than that observed in mice injected with opaque-phase cells of either the original parental strain WO-1 (Fig. 7B) or the control strain 5/3 (Fig. 7D). In addition, no opaque-phase cells were observed in the kidneys of mice injected with opaque-phase cells of strain 16/3 at 4, 8, and 12 days postinjection (Fig. 7F), even though the original opaque phase-cell populations contained approximately 80% opaque-phase cells at the time of injection. This contrasts markedly with colonizing cells from mice injected with opaque-phase cells of strain WO-1, which contained 42% opaque-phase cells after 1 day and 15% after 12 days (Fig. 7D), and with opaque-phase cells of strain 5/3, which contained 63% opaque-phase cells after 1 day and 35% opaque-phase cells after 12 days (Fig. 7B).

DISCUSSION

WH11 is expressed exclusively in the white budding phase of *C. albicans* WO-1 as well as other strains of *C. albicans* which undergo the white-opaque transition (12, 31). *WH11* is not expressed in either the opaque phase of the white-opaque transition or the hyphal phase of the bud-hypha transition of strain WO-1. *WH11* is also differentially expressed in the budding phase but not the hyphal phase of other *C. albicans* strains (31). In both the white-opaque phase transition and the bud-hypha transition of strain WO-1, the same regulatory mechanism appears to control *WH11* expression (32). In the white budding phase, one or more transcription activation factors which interact with the transcription activation sequences of the *WH11* promoter are activated or expressed, while in the hypha or opaque phases, the inactivation or absence of one or more of these factors results in the absence of *WH11* expression (29, 32). It was initially noted that *WH11* expression correlated with a round cell phenotype and that the absence of expression (i.e., in the hyphal or opaque phase) correlated with an elongate cell phenotype (31), and an analysis of WH11 expression in related species supported this suggestion (21).

Although Wh11 is homologous to the glucose-lipid-regulated protein Glp1 of *Saccharomyces cerevisiae* (33), which has also been identified as the low-molecular-weight heat shock protein Hsp12 (18), *WH11* expression in *C. albicans* is neither influenced by the glucose and lipid content of the medium nor induced and expressed exclusively after heat shock (31). Although the exact function of Hsp12 has not been demonstrated, there are indications that other low-molecular-weight heat shock proteins function as chaperones (9, 13, 14), a possible role now being investigated for Wh11.

Misexpression of *WH11* in the opaque phase did not inhibit the white-to-opaque transition. It also did not affect the unique phenotypic characteristics exhibited by opaque-phase cells, including pimples on the cell wall, the large vacuole, the elongate asymmetric shape, or increased size. Wh11 misexpressed in opaque-phase cells was distributed throughout the cytoplasm but was excluded from the cell wall, plasma membrane, and vacuole, which is the same distribution previously described for *WH11* encoded by the endogenous gene and expressed in the white phase (21). Most importantly, misexpression of Wh11 did not suppress the elongate shape characteristic of opaque-phase cells, suggesting that even if Wh11 proves to be involved in generating the round shape of budding cells, this function is not epistatic to genes involved in the mechanism generating the elongate morphology of opaque-phase cells.

Misexpression of *WH11*, therefore, does not affect the ability to switch from the white to opaque phase or the frequency of that transition, and it does not affect the capacity to switch from the opaque to white phase. It does, however, dramatically affect the frequency of the transition from the opaque to the white phase. Earlier measures of the number of opaque CFU in clonal white-phase cell populations and the number of white CFU in clonal opaque-phase cell populations at 25°C resulted in estimates of switching frequencies of approximately 5×10^{-4} to 10^{-3} for both white-phase cells and opaque-phase cells (3, 23). Estimation of the rate of switching by using the Luria-Delbruck fluctuation formula (20) resulted in lower rates, and estimations using long-term microscopic analysis of switching in single-cell lineages resulted in a higher rate for white to opaque and a lower rate for opaque to white (5, 27). Here, we found that the frequency of opaque CFU in white-phase cell populations of the control strain 5/3 at 25°C was 1.3×10^{-3} and the frequency of white CFU in opaque-phase cell populations was 6×10^{-4}, which are very close to the frequencies estimated for the original strain WO-1. The frequency of opaque CFU in clonal white-phase cell populations of the misexpression mutants at 25°C averaged 1.7×10^{-3}, which is very close to that measured for control strain 5/3, but the frequency of white CFU in clonal opaque-phase cell populations was 2.0×10^{-1}, which is 330 times higher than that of the control strain 5/3. It should be noted that this selective increase in the frequency of the transition from opaque to white phase was observed in three independent misexpression transformants, which excludes the possibility that the effect was due to a parallel mutation, reorganization, or unpredicted insertion. This conclusion was supported by Southern analysis of the independent transformants.

The increase in the rate of switching from opaque to white suggests that misexpressing the Wh11 protein in the opaque phase destabilizes the opaque phase. How this could be accomplished is not immediately obvious since nothing is known of the molecular nature of the actual switch event (24–26). If Wh11 were simply involved in the genesis of shape, one would not expect it to influence the switching frequency, since the latter is a reflection of a basic regulatory event, presumably at a gene locus, which changes the transcription pattern of a number of phase-specific genes at least in part through the synthesis or activation of *trans*-acting factors (25, 26, 29, 32). A gene downstream of the switch event would, presumably, have no effect on the switch event unless, of course, the signal for switching originates in the cytoplasm or at the plasma membrane. In such a case, a molecule distributed throughout the cytoplasm which plays a role in cell morphology or in the stabilization of phenotype could, conceivably, influence the frequency of switching.

Although we have examined the effects of *WH11* misexpression in the opaque phase, we have not performed a similar analysis in the hyphal growth phase, in which *WH11* is also silent. The *OP4* promoter, which was used in pCWOP16 to misexpress *WH11*, is not activated in the hyphal phase as it is in the opaque phase (16). It is possible that *WH11* misexpression in the hyphal phase will have a pronounced effect on phenotype. Misexpression may suppress or destabilize the hyphal phenotype, driving cells which begin to make hyphae back to the round budding cell phenotype, just as it drives opaque-phase cells back to the white phenotype. This would reinforce suggestions that the opaque phase and hyphal phase, although distinct, share regulatory mechanisms (24–26, 32). Experiments are now in progress in which a promoter of a hypha-specific gene will be substituted for the *OP4* promoter in pCWOP16.

In previous experiments in which the time of death was used as a measure of virulence in a mouse tail injection model, it was demonstrated that the average time of death is dramatically delayed in mice injected with opaque-phase cells compared to those injected with white-phase cells, and an analysis of the phenotypes of cells from autopsied tissue of animals injected with populations containing over 99% opaque-phase cells contained at the time of death predominately white-phase cells (17, 19). These latter results suggested that the delay in the death of animals injected with opaque-phase cells was due to the time necessary either for the colonization of the kidney by the minority of white-phase cells or for temperature-induced mass conversion from the opaque to the white phase. Since an increase from 25 to 37°C results in mass conversion from the opaque to white phenotype in vitro (5, 20, 23, 27, 31), the latter mechanism seems more likely. Here, we monitored kidney colonization as a function of time after tail vein injection as a measure of virulence and again found that white-phase cells of parental strain WO-1 and control strain 5/3 were more virulent than opaque-phase cells. The former colonized the kidney within 1 day after injection at higher concentrations than opaque-phase cells and maintained that advantage over 12 days. In addition, the kidneys of animals injected with opaque-phase cells of parental or control strains contained a majority of white CFU only 2 days postinjection, suggesting that the injected opaque-phase cell population had undergone mass conversion to the white phase and/or white-phase cells contaminating the injected opaque phase cell population had been selected for. In addition, colonization of the kidneys of animals injected with opaque-phase cells of strain 5/3 was roughly an order of magnitude lower than that of animals injected with white-phase cells throughout the entire 12-day postinjection period. When mice were injected with white-phase cells of the misexpression strain 16/3, the levels of total colonization were similar to those of mice injected with white-phase cells of the parental strain WO-1 and control strain 5/3, but there was absolutely no indication of contaminating opaque-phase cells. In addition, when mice were injected with opaque-phase cell populations of strain 16/3, the levels of colonization increased over 12 days more rapidly than that of mice injected with opaque-phase cells of either the parental or control strains, and there was no detectable level of opaque-phase cells after 4 days; i.e., all cells retrieved from the kidney were white. These results suggest that misexpression of Wh11 in the opaque phase leads to increased virulence of cells in the opaque phase as a result of the increased frequency of conversion to the white phase in vivo. The in vitro demonstration of an increased frequency of switching from the opaque to the white phase was predictive of this result.

Misexpression of the white-phase-specific gene *WH11* in the opaque phase, therefore, appears to destabilize the opaque

phase, driving cells back to the white phase, suggesting that *WH11* normally plays a role in the maintenance of the white budding phase. Misexpression of *WH11* also increases the virulence of opaque-phase cells by increasing the rate at which they revert to the more virulent white-phase phenotype. *WH11*, therefore, encodes the first identified gene product of *C. albicans* which affects the switching process and in turn virulence.

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