JOURNAL OF BACTERIOLOGY, Dec. 1987, p. 5579–5588
0021-9193/87/125579-10$02.00/0

Vol. 169, No. 12

Unique Phenotype of Opaque Cells in the White-Opaque Transition of *Candida albicans*

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Received 24 July 1987/Accepted 22 September 1987

Select strains of *Candida albicans* switch reversibly and at extremely high frequency between a white and an opaque colony-forming phenotype, which has been referred to as the white-opaque transition. Cells in the white phase exhibit a cellular phenotype indistinguishable from that of most standard strains of *C. albicans*, but cells in the opaque phase exhibit an unusually large, elongate cellular shape. In comparing the white and opaque cellular phenotypes, the following findings are demonstrated. (i) The surface of the cell wall of maturing opaque cells when viewed by scanning electron microscopy exhibits a unique pimpled, or punctate, pattern not observed in white cells or standard strains of *C. albicans*. (ii) The dynamics of actin localization which accompanies opaque-cell growth first follows the pattern of budding cells during early opaque-bud growth and then the pattern of hypha-forming cells during late opaque-bud growth. (iii) A hypha-specific cell surface antigen is also expressed on the surface of opaque budding cells. (iv) An opaque-specific surface antigen is distributed in a punctate pattern.

Most strains of *Candida albicans* are capable of switching at a high frequency between a number of phenotypes distinguishable by colony form on agar (11, 12, 22; D. R. Soll, *in* D. E. Berg and M. M. Howe, ed., *Mobile DNA*, in press; D. R. Soll, B. Slutsky, S. Mackenzie, C. Langtimm, and M. Staebell, *in* I. Mackenzie and C. Squier, ed., *Diseases of the Oral Mucosa*, in press). There are at least three distinguishable switching systems which are strain specific and give rise to multiple colony phenotypes (22), but only one system, the white-opaque transition, has been demonstrated to affect the basic cellular form of the budding cell (12). In the white-opaque transition, cells switch reversibly at frequencies of roughly 10^{-2} to 10^{-3} between two major colony-forming phenotypes. Cells in the white phase generate a smooth white colony which is indistinguishable from the common colony form of other *C. albicans* strains, and cells in the opaque phase generate a larger, flatter grey colony which is uncharacteristic of standard strains of *C. albicans* (12). It has been demonstrated that although white and opaque budding cells contain roughly the same quantity of DNA, they differ in mass, volume, shape, budding pattern, constraints on the bud-to-hypha transition, generation time, and sensitivities to both low and high temperature (12; B. Slutsky, Ph.D. thesis, University of Iowa, Iowa City, 1986; Soll et al., in press). White cells in the budding form are round and bud with a pattern similar to that of common laboratory strains of *C. albicans* (4, 8, 14, 19). When challenged to form hyphae under the regime of pH-regulated dimorphism (5, 14, 17), they form hyphae in a fashion indistinguishable from that of common laboratory strains (17, 24). In marked contrast, opaque cells are at least two times as large as white cells, are elongate or bean shaped, exhibit a somewhat different budding pattern, and are unresponsive to standard methods for inducing hypha formation (12). The elongate shape of opaque budding cells suggests that they may represent an intermediate phenotype between the bud and hypha forms and that a switch from the white to opaque cellular phenotype may result from the partial expression of hypha-specific traits involved in daughter cell elongation (17). We have therefore examined opaque cells for several cellular characteristics which were previously demonstrated to differ between bud- and hypha-forming cells. The results presented in this report demonstrate the following points. (i) The surface of the cell wall of maturing opaque cells when viewed under scanning electron microscopy exhibits a unique pimpled pattern not observed in either white budding or white hypha-forming cells. (ii) The dynamics of actin localization (2) accompanying opaque cell growth follows the white budding cell pattern early in bud growth and the white hypha pattern later in bud growth. (iii) A hypha-specific cell surface antigen is expressed in opaque cells. (iv) An opaque-specific antigen is distributed in a punctate pattern in association with the cell surface of opaque cells. These results suggest that the genesis of the opaque phenotype involves not only temporally orchestrated modulation of bud- and opaque-specific processes, but also the expression of opaque-specific characteristics.

MATERIALS AND METHODS

Growth and maintenance of stock cultures. Stock cultures of strain WO-1 (12) were maintained on agar slants or plates according to methods previously described in detail (4, 18, 24). To initiate growth in suspension culture, cells from a clonal colony freshly generated on agar were suspended in 25 ml of defined medium (9) supplemented with 70 μg of arginine per ml and 0.1 μM zinc sulfate in a 125-ml plastic Erlenmeyer flask (1, 3, 16, 20). The culture flask was rotated at 200 rpm (New Brunswick Gyrotory water bath shaker model G76) at 25°C. Cells were diluted into fresh medium and grown for two more rounds of growth and then used in mid-log phase for experiment purposes.

Colony plating for analyses of switching. Cells were diluted into double-distilled water at a concentration of roughly 500 to 1,000 CFU/ml, and 0.1 ml was spread on 2% Bacto-Agar (Difco Laboratories, Detroit, Mich.) containing the nutrient components of the defined medium of Lee et al. (9) supplemented with 70 μg of arginine per ml and 0.1 μM zinc sulfate (3). Plates were then incubated at 24°C for at least 6 days before being scored for colony phenotype. Each plate contained between 50 and 100 colonies. The visualization of

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sectoring was facilitated by the use of agar containing phloxine B (5 μg/ml), a technique developed by J. Hicks, Department of Molecular Biology, Scripps Clinics, La Jolla, Calif.

Scanning electron microscopy. Cells were harvested by filtration onto membrane filters (pore size, 0.45 μm; Millipore Corp.) and washed with 75 ml of double-distilled H_2O. Cells were suspended in 5 ml of 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). After incubation overnight at 4°C, cells were postfixed in 1% OsO_4 in phosphate buffer for 50 min. Cells were then washed three times in phosphate buffer and treated with 6% thiocarbohydrazide at room temperature followed by fixation in 1% OsO_4 to enhance detail. After a rinse in distilled water, the cells were gradually dehydrated in ethanol. Cells were then placed in perforated Beem capsules and treated in an Emscope CPD 750 critical point dryer. After drying, samples were mounted on aluminum stubs and sputter coated with gold palladium in the Emscope SC500 sputter coater and viewed with an Hitachi 5-570 scanning electron microscope.

Induction of hypha formation for cytological analysis. To induce synchronous hypha formation, white budding cells in the mid-log phase of growth at 25°C were starved for 20 min according to the methods of Soll and Herman (21) and then diluted into fresh medium at 37°C (pH 6.8) (5, 17) and rotated at 200 rpm.

Staining of F-actin with rhodamine-conjugated phalloidin. Detailed methods for formaldehyde fixation, Glusulase treatment, and staining with tetramethylrhodaminylphalloidin were previously reported (2). Stained cells were viewed and photographed with a Zeiss ICM 405 inverted microscope equipped for rhodamine epifluorescence microscopy. Photographs were taken with Kodak Plus-X pan film. Rhodamine-conjugated phalloidin was a generous gift from T. Wieland, Max-Planck Institute for Medical Research, Heidelberg, Federal Republic of Germany.

Genesis of antiserum, immunoabsorption, and indirect immunofluorescent staining. Opaque cells from a mid-log-phase culture grown in suspension at 25°C were heat killed (68°C, 1 h), washed in distilled water, suspended at a concentration of 2×10^8 spheres per ml in sterile saline, and mixed with Freund complete adjuvant (final concentration, 20%). A 0.5-ml sample of this mixture was injected subdermally into a New Zealand albino white rabbit. Injections were repeated after 20-, 10-, and 8-day intervals. A 1-to-10 dilution of the original antiserum was absorbed with 1×10^{10} cells per 1.5 ml of serum (type of cell designated in the text). Absorptions were carried out by gentle agitation at 37°C for 30 min or by end-over-end mixing at 4°C overnight. After absorption treatment, cells were pelleted in a Beckman Microfuge and the serum was carefully removed. For immunostaining, 2×10^8 cells in the mid-log phase of growth were harvested by filtration onto a Millipore filter (pore size, 0.45 μm), washed with 75 ml of double-distilled H_2O and suspended in phosphate buffer solution (PBS) (2). The cells were heat fixed at 68°C for 1 h, suspended in PBS plus 1 mg of sodium azide per ml, and stored at 4°C. Fixed cells at 6.7×10^6 spheres per ml were incubated with 20 μl of antiserum diluted in PBS plus bovine serum albumin (BSA) (10 mg of BSA per ml, 1 mg of NaN_3 per ml) for 1 h at 37°C and then washed three times in PBS. Washed cells were incubated for 1 h at 37°C with 20 μl of fluorescein-conjugated, affinity-purified goat anti-rabbit immunoglobulin G (heavy- and light-chain specific) (Organon Teknika, Malvern, Pa.) diluted 1:25 in PBS plus BSA. Treated cells were washed three times in PBS and suspended in 3 drops of Gelvatol (a gift from Monsanto Polymers and Petrochemicals Co., St. Louis, Mo.) containing 100 mg of 1,4-diazabicyclo[2.2.2]octane per ml (Aldrich Chemical Co., Inc., Milwaukee, Wis.). Cells were placed on glass slides and viewed with a Zeiss ICM 405 inverted microscope equipped for epifluorescence microscopy. Photographs were made on Kodak Plus-X pan film and developed in Diafine according to the instructions of the manufacturer. Preimmune and unabsorbed serum were also tested in an enzyme-linked immunosorbent assay at a dilution of 1:500. Peroxidase-conjugated, affinity-purified goat anti-rabbit immunoglobulin (heavy- and light-chain specific) was used as the second antibody at a dilution of 1:1,000. The substrate was orthophenylenediamine dihydrochloride (40 mg/100 ml) with 40 μl of 30% hydrogen peroxide. Plates were read at 492 nm in a Titertek Multiskan (Flow Laboratories, McLean, Va.).

RESULTS

High-frequency switching of colony phenotype in the white-opaque transition. The original strain of *Candida* which exhibited the white-opaque transition was isolated from the blood and lungs of an immunosuppressed patient with a systemic infection (12; Slutsky, Ph.D. thesis; Soll et al., in press). This initial strain was labeled WO-1 and represented the first of a series of isolates from vaginal, oral, and systemic infections exhibiting a white-opaque type transition (12, 22; Soll et al., in press). In the white-opaque transition, cells switch back and forth between a white (W) phenotype characterized by a very white, hemispherical colony and an opaque (Op) phenotype characterized by a wider, flatter grey colony when plated on standard agar (Fig. 1A and B). When cells from a white colony are clonally plated, the majority of colonies are white, a minority are opaque (Fig. 1A), and a minority are white with opaque sectors. When cells from an opaque colony are clonally plated, the majority of colonies are opaque, a minority are white (Fig. 1B), and a minority are opaque with white sectors. Sectoring is best exemplified by plating cells on agar containing phloxine B. White colonies or sectors appear creamy pink, and opaque colonies or sectors appear bright red (Fig. 1C through F). To demonstrate the complete and continued reversibility of switching between the two phenotypes and to demonstrate the unusually high frequency of switching in both directions, cells from one white and one opaque colony were clonally plated, and the frequency of opaque and white colonies was measured after 7 days at 25°C. With sectored colonies, only the major colony phenotype was scored. In turn, the cells from a colony of opposite phenotype were clonally plated, and the frequency of opposite phenotypes was measured. The results of this experiment are outlined in Fig. 2A. For the initial white colony, the plating sequence was W → Op → W → Op → W → Op, and for the initial opaque colony, the plating sequence was Op → W → Op → W → Op → W. It is clear that the average frequencies neither increased nor decreased with continued plating. When the frequencies of opaque colonies emanating from white colonies (Fig. 2A) were averaged, a mean frequency of 3.3×10^{-3} (standard deviation [SD], $\pm 2.5 \times 10^{-3}$) was obtained, and when the frequencies of white colonies emanating from opaque colonies (Fig. 2A) were averaged, a mean frequency of 2.3×10^{-2} (SD, $\pm 2.6 \times 10^{-2}$) was obtained (Fig. 2B). It should be noted that for sectored colonies (for an example, see Fig. 1C and D), only the major colony phenotype was scored. If one were to take into consideration that the multiplication rate of white cells is twice that of opaque cells (12), then the

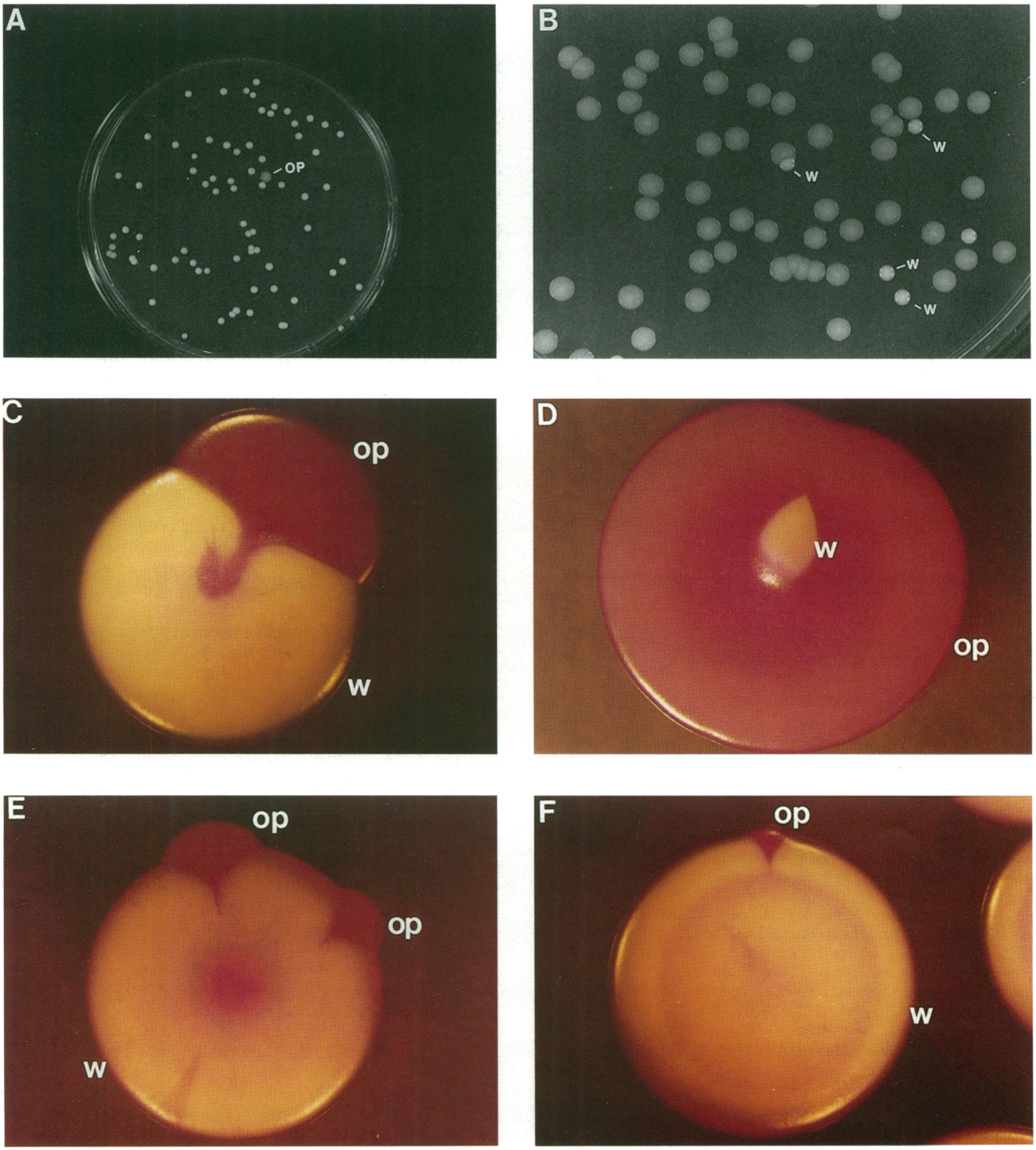

FIG. 1. The white-opaque transition. (A) Colonies generated by plating cells from a single white colony (note all colonies retain the small, white phenotype except one, which exhibits the wider, flatter grey opaque phenotype [OP]). (B) Colonies generated by plating cells from a single opaque colony (note that the majority of colonies retain the wide, flat, grey phenotype of opaque cells, but three colonies and one sector exhibit the small, white, hemispherical phenotype of white cells). (C) A phloxine B-stained white colony (w) with an opaque sector (op). (D) A phloxine B-stained opaque colony with a white sector. (E) A phloxine B-stained white colony with two opaque sectors. It should be noted that the pink core region of the white colony represents dead white cells which take up the dye, not opaque cells. (F) A white colony with a single opaque sector formed late in colony development.

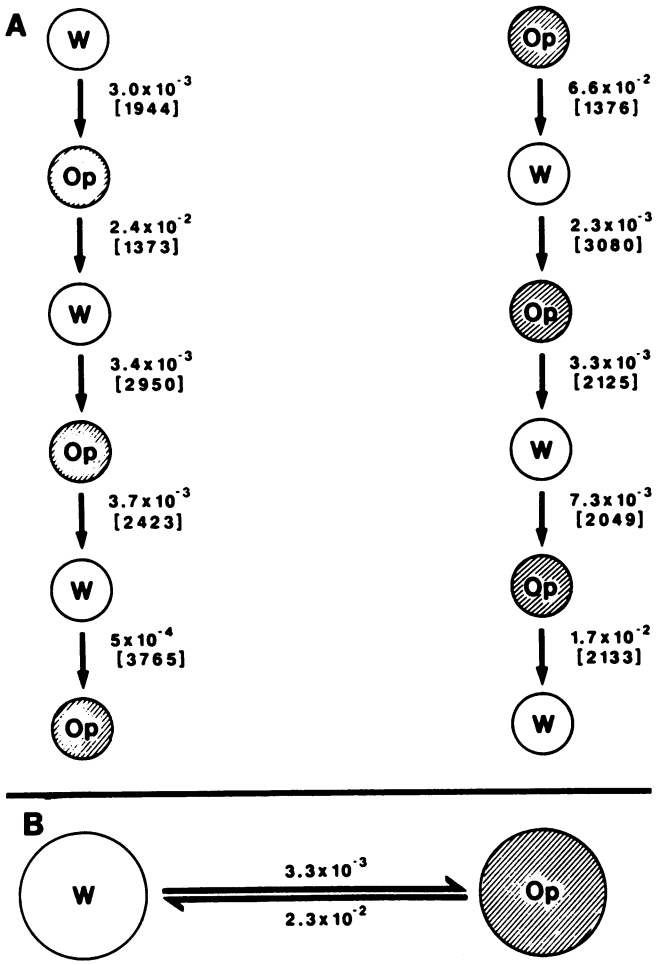

FIG. 2. Sequential switching in the white-opaque transition. Cells from a single white colony (left, top of panel A) and a single opaque colony (right, top of panel A) were first plated, and the frequency of opaque colonies, in the former case, and the frequency of white colonies, in the latter case, were counted. The frequency and total number of colonies analyzed are presented next to the arrow from W to Op, in the former case, and from Op to W, in the latter case. Cells from a single opaque colony from the initial plating of cells from the first white colony, and cells from a single white colony from the initial plating of cells from the first opaque colony were in turn plated, and the frequencies of the alternative phenotype were counted. In each case, the frequency of the alternative phenotype and the number of colonies assessed (in brackets) are presented below the original colony phenotype in each sequence. In panel B, the mean frequency of opaque cells in white colonies is presented above the top arrow and the mean frequency of white cells in opaque colonies is presented below the bottom arrow.

frequency of white CFU in opaque colonies would be doubled to 6.6×10^{-3} and the frequency of opaque CFU in white colonies would be halved to 1.2×10^{-2}. Again, these frequencies are fraught with problems of enrichment because of spatial differences in growth pattern on agar (12), but they do demonstrate that the rate of switching is high in both directions and that it continues indefinitely. Indeed, cell lines monitored through more than 50 switches back and forth between white and opaque colonies still exhibited the same frequencies of opaque CFU in white colonies and white CFU in opaque colonies. It should also be noted that in addition to the two major phenotypes, fuzzy and irregular phenotypes also appear in white and opaque populations but at frequencies below those observed for the transition between white and opaque colonies (Fig. 2B).

Unique cellular form of opaque colonies. Cells in the white phase grew exclusively in the budding form at 25°C in liquid nutrient medium. White budding cells were round (Fig. 3A through C) and formed round buds with the same evagination polarity (12) and constriction at the mother cell-bud junction (Fig. 3A through C) as the more carefully studied laboratory strain 3153A (8, 14, 15, 19). Mature white budding cells exhibited an average volume of 33 μm^3 (SD, ±19; n = 100) and an average roundness measurement (width divided by length) of 0.9 (SD, ±0.1; n = 100). White budding cells could be stimulated to form hyphae by either growing them to stationary phase at 25°C, then diluting them into fresh medium at 37°C (pH 6.8) (5, 14, 17), or growing them to mid-log phase at 25°C, starving them in buffer for 20 min, then diluting them into fresh medium at 37°C (pH 6.8) (14, 21). In both cases, the hyphae which were formed (Fig. 3D through F) were narrow, with no apparent constriction at the mother cell-daughter cell junction as was the case in bud formation, and, in general, were identical to hyphae formed under the same regimens by laboratory strain 3153A (10, 14, 15, 23). In contrast to budding white cells, budding opaque cells are elongate or bean shaped (Fig. 3G through I). Mature opaque cells exhibit an average volume of 114 μm^3 (SD, ±62; n = 100) and an average roundness measurement of 0.6 (SD, ±0.1; n = 100). Therefore, the average volume of opaque budding cells is roughly twice that of white budding cells, and the roundness measurement is roughly two-thirds that of white cells.

When the surface of mother and daughter cells of white budding (Fig. 3A through C) and white hypha-forming (Fig. 3D through F) cells were examined by scanning electron microscopy, they were found to be smooth whether examined early or late in daughter-cell development. In contrast to the smooth surface of mature white budding or hypha-forming cells, mature opaque cells exhibited an unusual punctate or pimpled surface pattern reminiscent of a cucumber (Fig. 3G through I). Early buds of opaque cells (Fig. 3G) and buds with lengths up to half the length of their mother cells (Fig. 3H) were smooth, but buds approaching final volume began to develop a pimpled surface (Fig. 3I). The opaque pimples appeared randomly spaced, with no obvious pattern of rings, columns, or coils. All of the cell surface of a mature cell was covered by pimples; no unpimpled regions were observed on 10 carefully scrutinized mature cells.

Dynamics of F-actin localization during opaque-cell growth shares characteristics of bud and hypha growth. The elongate shape of the opaque cell suggested to us that the opaque phenotype may represent a mixture or intermediate between the very round, white budding cell and the narrow, elongate white hypha. Since the spatial dynamics of F-actin localization differ dramatically between a growing bud and a growing hypha (2), we examined this parameter during expansion of the opaque cell, using rhodamine-conjugated phalloidin to visualize F-actin. It was previously demonstrated in strain 3153A (2) that at the time of evagination of both bud and hypha formation, F-actin granules clustered in the incipient evagination. As a bud expanded, these granules distributed themselves evenly throughout the cytoplasmic cortex and remained in this pattern throughout bud growth. In contrast, as a hypha elongated, the granules remained localized mainly in the hyphal apex. The same differences in actin localization were observed between bud and hypha forma-

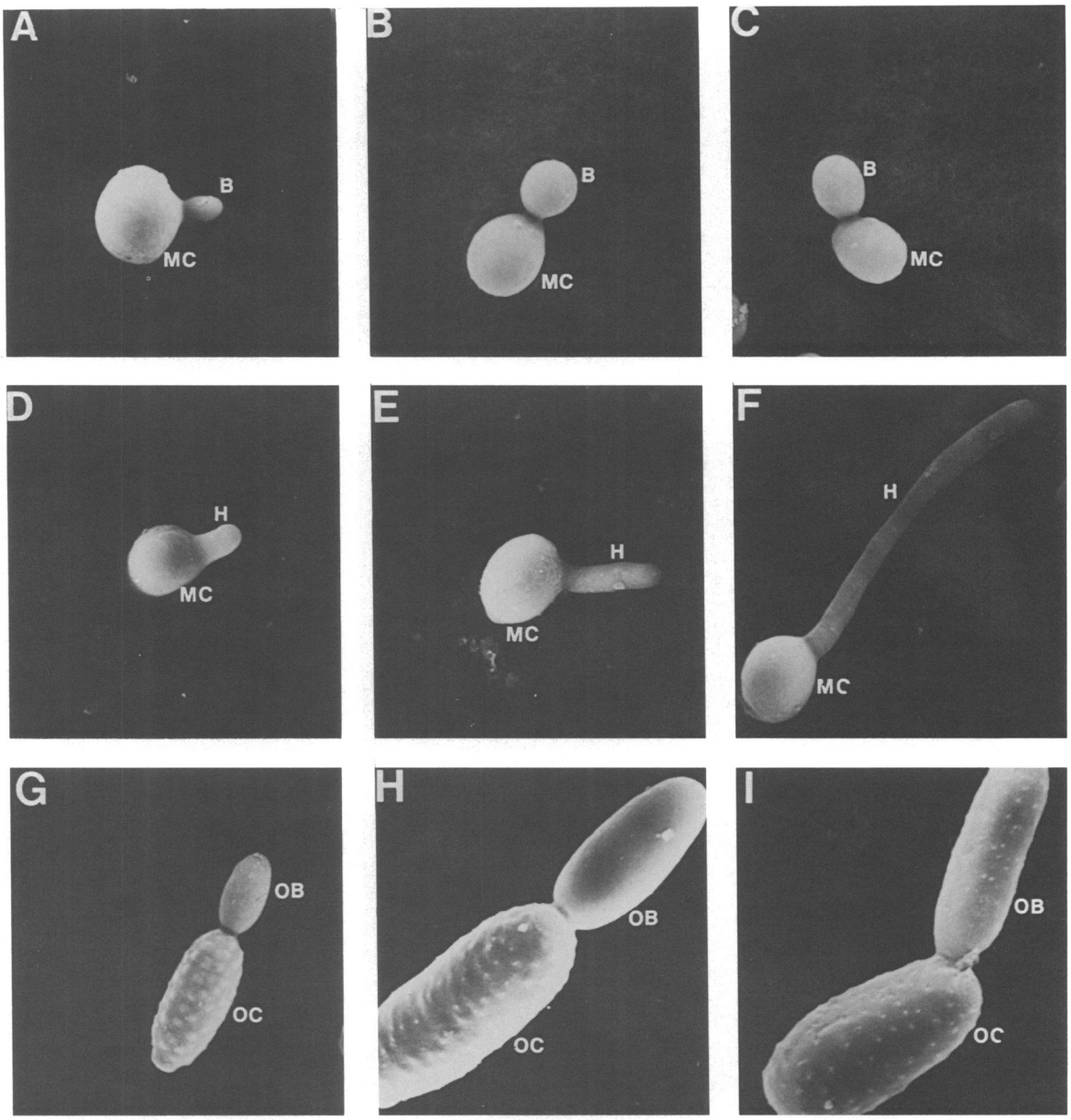

FIG. 3. Scanning electron micrographs of budding white cells (A through C), hypha-forming white cells (D through F) and budding opaque cells (G through I). (A) A budding cell with a small evagination (note the constriction at the mother cell (MC)-bud (B) junction). (B) A mother cell with medium-sized bud. (C) A mother cell with a slightly larger bud (note the constrictions in both panels B and C between mother cell and bud). (D) A mother cell with an incipient hypha (H) or germ tube (note the lack of a constriction between mother cell and daughter tube). (E) A mother cell with a hypha the length of which is equal to the diameter of the mother cell. (F) A mother cell with a long hypha. (G) An opaque mother cell (OC) with a young opaque bud (OB) (note the pimpled or punctate pattern on the mother cell surface and the lack of this pattern on the bud). (H) An opaque mother cell with punctate surface pattern, with a medium-sized opaque bud lacking the punctate pattern. (I) An opaque mother cell with a large opaque bud with punctate pattern.

tion in the white phase of strain WO-1 (Fig. 4 and Table 1). To examine F-actin localization during bud formation, white budding cells in the mid-log phase of growth at 25°C were stained with rhodamine-conjugated phalloidin. All cells with evaginations (Table 1) contained a cluster of F-actin granules filling the evaginations with F-actin fibers entering the mother cells (Fig. 1A). As the evaginations grew into mature buds, the F-actin granules remained dispersed in the cytoplasmic cortex (Fig. 1B and C) in the majority of cells analyzed (labeled "edge" in Table 1). To examine F-actin localization during hyphal growth in the white phase, white cells were grown to mid-log phase in the budding phenotype at 25°C, starved for 20 min (15, 21), and then released into fresh medium at 37°C (pH 6.8) to induce synchronous hypha formation. Again, all cells with evaginations (Table 1) contained F-actin granules clustered in the evagination (Fig. 4D) in a fashion similar to the evagination of a budding cell (Fig. 4A). When evaginations had expanded into daughter cells with lengths roughly one-fourth the diameter of their mother cells (small hyphae), over 50% exhibited apically clustered granules, and when daughter cells achieved a length roughly one-half the diameter of their mother cells (medium hyphae),

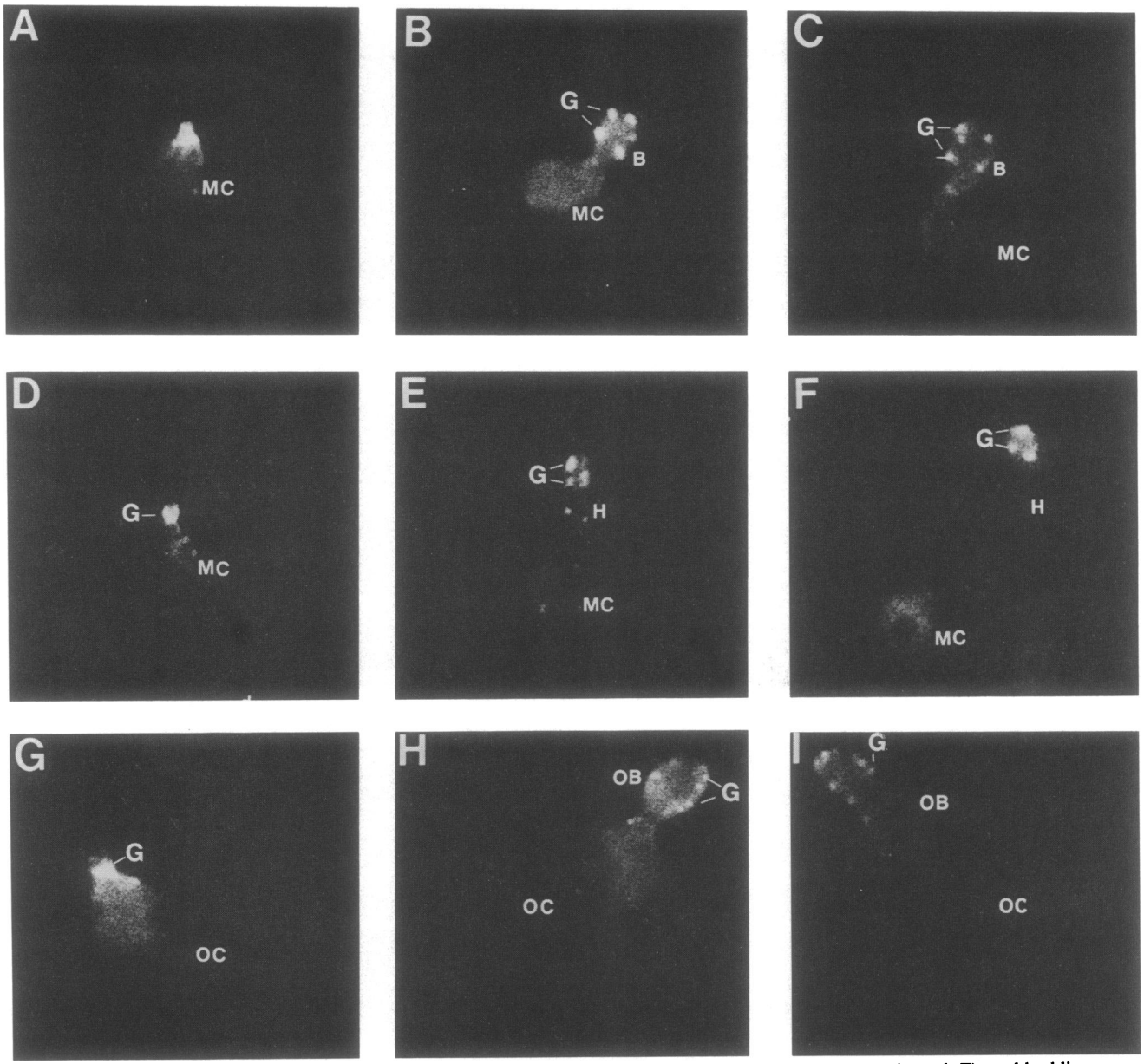

FIG. 4. Patterns of F-actin granules in budding white cells (A through C), hypha-forming white cells (D through F), and budding opaque cells (G through I). Fluorescent micrographs are presented of cells stained with rhodamine-conjugated phalloidin. (A) F-actin granules clustered at the site of evagination, with F-actin fibers entering the mother cell proper (MC). (B) Edge granules (G) in the cytoplasmic cortex of a medium-sized bud (B). (C) Edge granules in the cytoplasmic cortex of a slightly large bud. (D) Granule cluster in the evagination of a hypha-forming white cell. (E) Granules clustered in the apex of a hypha (H) roughly twice the length of the mother cell. (F) Granules clustered at the apex of a hypha roughly three times the length of the mother cell. (G) Granules clustered into the site of evagination of an opaque mother cell (OC). (H) Edge granules in the cytoplasmic cortex of an opaque bud. (I) Granules localized in the distal third of a large opaque bud (OB).

over 80% exhibited apically clustered granules (Table 1). When hyphae had achieved a length roughly equal to or greater than that of their mother cells (large hyphae), over 90% exhibited apically clustered granules (Table 1). Examples of apical clusters in growing hyphae are presented in Fig. 4E and F.

To examine F-actin localization during bud growth in the opaque phase, opaque cells were grown to mid-log phase at 25°C, starved for 20 min, and then released into fresh medium at 37°C (pH 4.5). As in the case of budding and hypha-forming white cells, all opaque cells with evaginations (Table 1) contained F-actin granules clustered in the evagination (Fig. 4G). When evaginations had expanded into daughter cells with lengths roughly one-fourth the lengths of their mother cells (small buds), 75% exhibited edge granules (Table 1), a pattern similar to that of the majority of small buds but dissimilar to that of the majority of small hyphae. An example of a small opaque bud with edge granules is presented in Fig. 4H. Of the small opaque buds, 4% exhibited apical granule localization (Table 1). When opaque buds achieved a length roughly half the diameter of their mother cells (medium buds), roughly 40% exhibited the edge granule

TABLE 1. Proportion of cells exhibiting different patterns of F-actin granule localization[a]

Daughter-cell type and major F-actin pattern[b]	% of cells		
	White bud	White hypha	Opaque bud
Evagination (20/4/19)			
Clustered	100	100	89
Edge	0	0	11
Small (25/15/24)			
Edge	84	34	75
Apical cluster	4	53	4
General	12	13	21
Medium (17/17/13)			
Edge	94	18	38
Apical cluster	0	82	62
General	0	0	0
Large (8/33/25)			
Edge	88	6	48
Apical cluster	0	94	52
General	0	0	0

[a] Only small granule localization was assessed; large-neck granules and F-actin fibers were not included in the analysis of pattern (2).

[b] Clustered, Clustered granules in the evagination proper; edge, granules distributed evenly in the cytoplasmic cortex; apical cluster, granules clustered in the daughter-cell apex; general, granules distributed throughout the cytoplasm. Numbers in parentheses are numbers of white budding cells, white hypha-forming cells, and opaque budding cells examined, respectively.

pattern and roughly 60% exhibited apical granule clustering (Table 1). When opaque buds achieved a length close to that of their mother cells (large buds), roughly half still exhibited apical clustering (Table 1). An example of apical clustering of F-actin granules in a large bud is presented in Fig. 4I. The remaining half still exhibited the edge granule pattern (Table 1). It is important that in large white buds, 88% exhibited the edge granule pattern, and in large white hyphae, 94% exhibited the apical granule cluster pattern. It therefore appears that early in opaque bud expansion, the edge pattern of the white budding cell predominates, but as the opaque daughter cell expands, the pattern changes to that of the apical cluster pattern of the hypha.

Opaque cells express a hypha-specific and an opaque-specific antigen. The temporal dynamics of F-actin localization suggest that in the formation of an opaque bud, hyphal characteristics are modulated. Since the differences in synthesis of major polypeptides between bud and hypha are minimal, even when examined by two-dimensional polyacrylamide gel electrophoresis (6, 17) and since differentially expressed genes have not yet been cloned for Northern (RNA) blot analyses, we used the hypha-specific wall antigen (for examples, see references 13 and 26) as a means to test whether opaque cells expressed hypha-related genes. Antiserum was first raised against opaque budding cells by injecting heat-killed cells into rabbits (see Materials and Methods). The original antiserum stained white budding cells (Fig. 5A) and white hypha-forming cells (Fig. 5B) as well as opaque budding cells (Fig. 5C). Although preimmune serum also stained the three cell types, a 1:500 dilution resulted in an enzyme-linked immunosorbent assay value 45 times lower than that of a 1:500 dilution of original immune serum. The original antiserum was first absorbed with heat-killed white budding cells. This absorbed antiserum did not stain white mother cells and buds (Fig. 5D), and it did not stain mother cells in the process of forming hyphae (Fig. 5E). It did, however, intensely stain 98% of the hyphae forming on white mother cells under the regimen of pH-regulated dimorphism (Table 2). Staining in these preparations was absolutely hypha-specific, beginning exactly at the junction of mother cell and daughter hypha (Fig. 5E). To demonstrate this specificity, white cells with small buds in the mid-log phase of growth at 25°C were starved for 20 min in buffered salts solution and then diluted into fresh nutrient medium at 37°C (pH 6.8). This regimen causes small buds to reinitiate growth in the hyphal form, first forming a shmoo shape which then tapers into an elongate hypha with growth (21). In this case, the mother cell and original bud did not stain, but the hypha emanating from the original bud stained intensely, beginning at the junction of bud and hypha (Fig. 5F). This antiserum, which had been absorbed with budding cells, also stained 96% of opaque cells (Table 2). Small opaque buds stained intensely (Fig. 5G), but opaque mother cells and large buds exhibited a punctate staining pattern at the cell periphery (Fig. 5G and H) which was distinguishable from the more even staining pattern with hyphae (Fig. 5E and F). These results demonstrate that opaque budding cells possess antigens in common with white hyphae which are not present on white buds.

To test whether the original antiserum contained antibodies against antigens specific to opaque cells, the original antiserum was absorbed with white cells forming hyphae. This treatment should have removed antibodies against budding cell antigens on the mother cells as well as hypha-specific antigens. This absorbed antiserum did not stain white mother cells or white buds and only stained 30% of white hyphae (Table 2). However, it continued to stain 89% of opaque mother cells and opaque buds (Fig. 5I; Table 2). Again, staining was punctate at the surface of the opaque cell.

As a control, the original antiserum was absorbed with opaque budding cells. This antiserum lost the capacity to stain white mother cells, white buds, white hyphae, and opaque mother cells and buds (Table 2). Together, the staining experiments suggest that opaque cells contain one or more antigens common to white buds, white hyphae and opaque budding cells, one or more antigens present on hyphae but missing on white cells, and one or more opaque-specific antigens.

DISCUSSION

The white-opaque transition has been observed in a number of *Candida* strains isolated from healthy and infected mouths (Soll et al., in press), acute vaginal infections (22; D. R. Soll, M. Staebell, C. J. Langtimm, M. Phaller, J. Hicks, and T. V. G. Rao, submitted for publication), and systemic infections (12; D. R. Soll, personal observations). This switching system is strain specific since it has never been observed to occur in the common laboratory strain 3153A or in other strains with the 3153A-type switching system (11; Soll et al., in press). Strain WO-1, a systemic isolate from the blood and lungs of a bone marrow transplant patient (12), switches back and forth between the white and opaque phase at relatively high frequencies and retains the same high frequency of switching even after 50 transitions. White clonal colonies contain, on the average, 3.3×10^{-3} opaque cells and opaque colonies contain, on the average, 2.3×10^{-2} white cells after 7 days at 24°C on agar containing an amino acid-rich defined medium (3, 9), indicating that the frequencies of switching are indeed high. Exact switching frequencies are difficult to assess because of differences in generation time, growth patterns on agar, final densities at

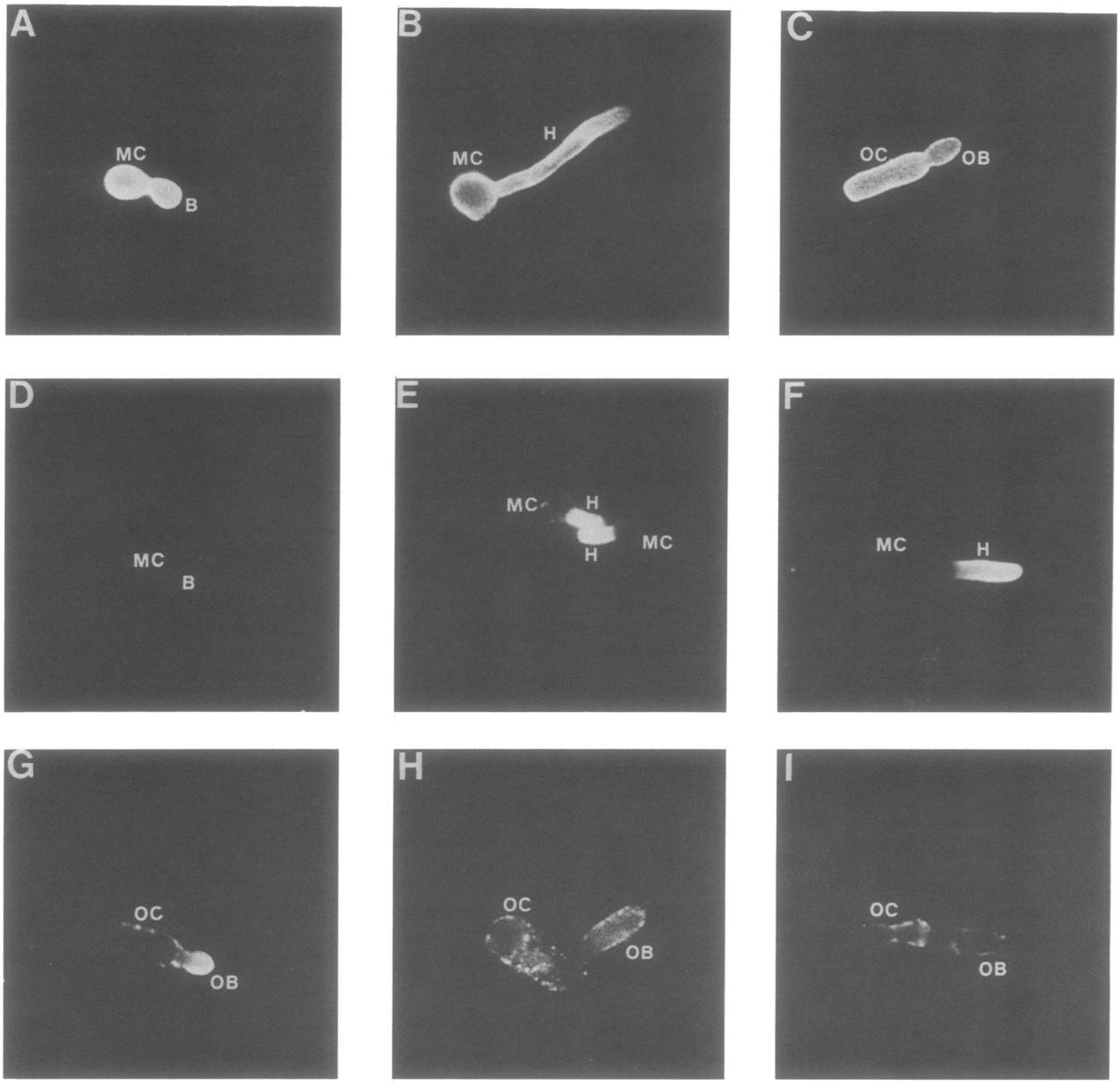

FIG. 5. Indirect immunofluorescent staining with unabsorbed and absorbed antiserum developed against opaque cells. (A) Budding white cell (MC) with medium-sized bud (B) immunostained with unabsorbed antiserum. (B) Hypha-forming white cell with long hypha (H) immunostained with unabsorbed antiserum. (C) Opaque cell (OC) with small bud (OB) immunostained with unabsorbed antiserum. Note that the cell surfaces in panels A, B, and C are uniformly stained. (D) Budding white cell with medium-sized bud immunostained with antiserum absorbed with budding white cells (note the lack of any staining). (E) Hypha-forming white cell immunostained with antiserum absorbed with budding white cells (note the lack of staining of mother cells and the intense staining of hyphae). (F) Hypha-forming cell with a small bud immunostained with antiserum absorbed with budding white cells (note the lack of staining of mother cell and bud and the intense staining of the hypha emanating from the small bud). (G) Opaque cell with small bud immunostained with antiserum absorbed with budding cells. (H) Opaque cell with medium bud immunostained with antiserum absorbed with budding white cells. (I) Opaque cell with large bud immunostained with antiserum absorbed with hypha-forming cells.

stationary phase, and the differential influence of different environmental conditions on the frequency of switching (12; Slutsky, Ph.D. thesis; E. J. Wilson, J. M. Anderson, and D. R. Soll, manuscript in preparation). Strain WO-1 in the white budding form types as *C. albicans* by its sugar assimilation pattern (12; Soll, in press) and contains roughly 15 copies of each of two midrepeat sequences, JH3 and JH7, which are specific to the species *C. albicans* (22; Soll et al., submitted; Hicks et al., in preparation). It lacks a midrepeat sequence, JH13-8, specific to *Candida tropicalis* (Soll et al., submitted). Cells in the opaque phase differ from cells in the white phase in the assimilation of four sugars (Soll, in press) but still exhibit the same level of nuclear DNA (12), the same banding pattern on Southern blots hybridized with JH3 (22; Soll et al., in press; Hicks and Soll, in preparation), and no hybridization with JH13-8 (22).

Perhaps the most dramatic aspect of the white-opaque transition is the difference in cellular form between the two

phases (12). White budding cells exhibit the same round shape and budding pattern as budding cells of most strains of *C. albicans* (8, 14) or, for that matter, haploid strains of *Saccharomyces cerevisiae* (7, 14). In marked contrast, opaque cells exhibit roughly twice the volume and mass of white budding cells and are elongate or bean shaped rather than round (12). In many cases, one end of an opaque cell is broader than the other, a lack of symmetry rarely observed in white cells. Although the volume and mass of an opaque cell are at least twice those of a white cell, the DNA contents in the mid-log phase of growth are comparable (12). This difference in the mass-to-DNA ratio may explain why the average generation time of an opaque cell is close to twice that of a white budding cell at 25°C in suspension culture (12).

By visualizing the surface of the opaque budding cells with scanning electron microscopy, we have found to our surprise that mature cells and large buds exhibit a pimpled pattern. Neither the white budding or hypha-forming cell exhibits this unique pattern, nor do budding and hypha-forming cells from other strains (5). It is not yet clear whether these pimples represent thickenings of the cell wall, bulges in the cytoplasm, or specialized structures. Transmission electron microscopy studies are now under way to distinguish among these possibilities.

The elongate shape of opaque cells suggested to us that the switch to the opaque phenotype may involve the partial expression of hyphal traits involved in elongation (17). One trait which, as we previously demonstrated, differed markedly between bud and hyphal growth was the distribution of F-actin granules in the growing daughter cell, and it had been suggested (2, 17) that this difference was in turn related to the differences in the dynamics of cell wall expansion (25). Actin granules were found to be distributed throughout the cytoplasmic cortex of a growing bud but clustered in the apex of an elongating hypha. This finding correlated with the significant level of uniform wall expansion in an expanding bud versus the nearly complete expansion of wall at the hyphal apex (25). The dynamics of F-actin granule distribution during opaque-cell growth appears to represent a combination of the bud and hyphal patterns. Early during opaque-daughter-cell expansion, the distribution of F-actin granules more closely resembles the pattern observed in an expanding white bud; however, when the length of the opaque daughter cell reaches roughly half the length of the mother cell, the pattern changes to that observed in elongating white hyphae. Budding white cells, in contrast, rarely exhibit the apical cluster pattern of hyphae.

If opaque budding cells use hyphal as well as bud mechanisms to generate the opaque cell phenotype, then one would expect opaque cells to express hyphal genes. Unfortunately, of the 375 polypeptides which can be reproducibly visualized by two-dimensional polyacrylamide gel electrophoresis, only one appears to be bud specific and one appears to be hypha specific (6, 15), and there have been no published reports of cloned hypha-specific genes or cDNAs to hypha-specific mRNAs which could be used in Northern blot hybridization experiments to analyze hyphal gene regulation in opaque cells. However, there have been a number of reports of hypha-specific antigens on the cell surface which are easily identified by antisera (13, 26, 27). We therefore examined whether opaque budding cells contained surface antigens expressed in white hyphae but not in white budding cells. Our strategy was to generate a polyclonal antiserum to opaque cells and then selectively absorb from it antibodies specific to the budding cell surface. The result of this treatment was an antiserum which stained white hyphae and opaque cell surfaces by indirect immunofluorescence but which did not stain white budding cell surfaces. Since this antiserum was generated against a heat-killed opaque-cell preparation devoid of hyphae, it seems reasonable to conclude that opaque cells express a hypha-specific antigen. By absorbing the original antiserum with hypha-forming cells, we were surprised to find that the antiserum continued to stain opaque cells even though it had lost the capacity to stain either budding cells or hyphae. This result suggests that the genesis of an opaque cell does not simply involve the modulation of bud- and hypha-specific genes or a combination of bud and hypha-specific characteristics but may also involve the expression of opaque-specific genes. This suggestion is supported by the finding that the antiserum, after absorption with white budding cells, stains a 13,500-dalton antigen on Western blots (immunoblots) which is observed in opaque cells only; neither white budding cells nor white hypha-forming cells exhibit this antigen (J. M. Anderson and D. R. Soll, manuscript in preparation; Soll, in press). Finally, since cells exhibiting a white-opaque transition have been isolated from a number of patients with either systemic or acute vaginal infections (12, 22; Soll et al., in press), it is worth speculating on the possible role of this high-frequency switching system in pathogenesis. First, it has recently been demonstrated that white and opaque cells exhibit dramatic differences in hydrophobicity and adhesion to buccal epithelial cells (M. J. Kennedy, A. L. Rogers, L. A. Hanselman, D. R. Soll, and R. J. Yancey, Jr., submitted for publication; Soll, in press). In addition, conditions were recently discovered which stimulated hypha formation in opaque-cell cultures, including adhesion to polylysine-coated glass surfaces, adhesion to fibronectin-coated glass surfaces, and reduced aeration (J. Anderson, B. Schnars, and L. Cundiff, manuscript in preparation). Finally, it has been demonstrated that opaque cells secrete acid protease at a dramatically elevated level when compared with white cells (T. Ray, C. D. Payne, and D. R. Soll, manuscript in preparation). This combination of characteristics suggests that the reversible transition to the specialized opaque phenotype may play a transient role in adherence and penetration. The reason for suggesting that the role is transient emanates from the observation that the white phenotype will enrich in an opaque population at body temperature because of differential temperature sensitivity (12; Slutsky, Ph.D. thesis). Finally, it is hard to believe that such a carefully orchestrated high frequency and reversible-phase transition and such a specialized cell as the opaque phenotype evolved in a pathogen like *C. albicans* for any purpose other than patho-

TABLE 2. Proportion of cells stained with anti-opaque-cell serum preabsorbed with hyphae or opaque budding cells[a]

Cell type for absorption	% of cells staining[b]					
	White hypha-forming cells			Budding opaque cells		
	Heavy	Medium	No stain	Heavy	Medium	No stain
White bud	90	8	2	75	21	4
White hyphae	5	25	70	34	55	11
Opaque bud	0	13	87	0	0	100

[a] Rabbit antiserum raised against heat-killed opaque budding cells was absorbed with either budding white cells (one time), hypha-forming white cells (three times), or opaque budding cells (three times). Serum dilutions were 1:36, 1:36, and 1:20, respectively.

[b] Most opaque cells stained with a punctate pattern; only the hyphal portion of hypha-forming cells stained.

genesis. It should be noted that the antiserum absorbed with hypha-forming cells stained opaque cells in a punctate pattern. It is not clear if this punctate pattern is related to the pimpling on the cell surface observed by scanning electron microscopy. Experiments to test this interesting possibility are now in progress.

ACKNOWLEDGMENTS

We are indebted to Kimberly Drollinger for help in assembling the manuscript, Mark Lohman for help with the photography, and James Hicks of Scripps Clinics, La Jolla, Calif., for the phloxine B method of staining opaque cells and T. V. G. Rao for the phloxine B pictures.

This project was supported in part by Public Health Service grant AI23922 from the National Institutes of Health, a grant from the Iowa High Tech Council, and funds from the Cecil J. Rusley Memorial Fund.

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