

# Filament Ring Formation in the Dimorphic Yeast *Candida albicans*

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**ABSTRACT** Stationary phase cultures of *Candida albicans* inoculated into fresh medium at 37°C synchronously form buds at pH 4.5 and mycelia at pH 6.5. During bud formation, a filament ring forms just under the plasma membrane at the mother cell-bud junction at roughly the time of evagination. A filament ring also forms in mycelium-forming cells, but it appears later than in a budding cell and it is positioned along the elongating mycelium, on the average 2  $\mu\text{m}$  from the mother cell-mycelium junction. Sections of filament rings in early and late budding cells and in mycelia appear similar. Each contains approximately 11 to 12 filaments equidistant from one another and closely associated with the plasma membrane. In both budding and mycelium-forming cells, the filament ring disappears when the primary septum grows inward. The close temporal and spatial association of the filament ring and the subsequent chitin-containing septum suggests a role for the filament ring in septum formation. In addition, a close temporal correlation is demonstrated between filament ring formation and the time at which cells become committed to bud formation at pH 4.5 and mycelium formation at pH 6.5. The temporal and spatial differences in filament ring formation between the two growth forms also suggest a simple model for the positioning of the filament ring.

In the budding yeast *Saccharomyces cerevisiae*, septation invariably occurs at the junction between mother cell and bud (1, 2). In this process, a chitin-containing ring grows inward, pinching off the plasma membrane between mother cell and bud to form the primary septum (3). This is followed by the deposition of secondary septal layers on both sides of the primary septum (3), and subsequent cell separation (4). Although the components involved in the regulation of chitin synthetase activity have been investigated in some detail (3, 5), it is still unclear why the chitin ring and subsequent septum form exactly at the mother cell-bud junction even though the enzyme chitin synthetase appears to be distributed throughout the plasma membrane (6). A clue to septum localization may lie in the ordered ring of 10-nm filaments which appears just under the plasma membrane at the junction of mother cell and evagination and which disappears sometime during the formation of the primary septum (7). Although the chemical nature of this ring is unknown, its location at the junction suggests that it may be related to septum formation, and the absence of organized filaments in particular cell division cycle mutants correlates with defective cytokinesis (8). However, several possible functions of the filament ring have not been completely ruled out, including positioning of the evagination, maintenance of an open channel at the mother cell-bud junc-

tion, involvement in nuclear migration and division, and constriction at the junction.

The yeast *Candida albicans* affords us with a very useful system for investigating the information and function of the filament ring. This dimorphic yeast is capable of growing either in a budding form, in a fashion similar to *Saccharomyces* (9), or in an elongate, mycelial form (10), depending upon environmental conditions and the growth history of the cells (11, 12, 13). In both growth forms, a chitin-containing septum is formed between the mother cell and daughter compartment (14, 15, 16), but the timing and position of septum formation differ between the two growth forms (14). During synchronous bud formation, the septum begins to form at approximately the same time as initial evagination and is invariably positioned at the junction of mother cell and bud (14), just as in *Saccharomyces* (1, 2, 3). In contrast, during synchronous mycelium formation, the septum begins to form roughly 20 to 30 min after evagination and is positioned along the mycelium, on the average, 2  $\mu\text{m}$  from the mother cell-mycelial junction (14). During the establishment of either growth form, the onset of septum formation correlates closely with the time at which a shift to the alternate pH does not affect the phenotype prescribed by the original pH, the point of phenotypic commitment (17). These temporal events are diagrammed in Fig. 1.

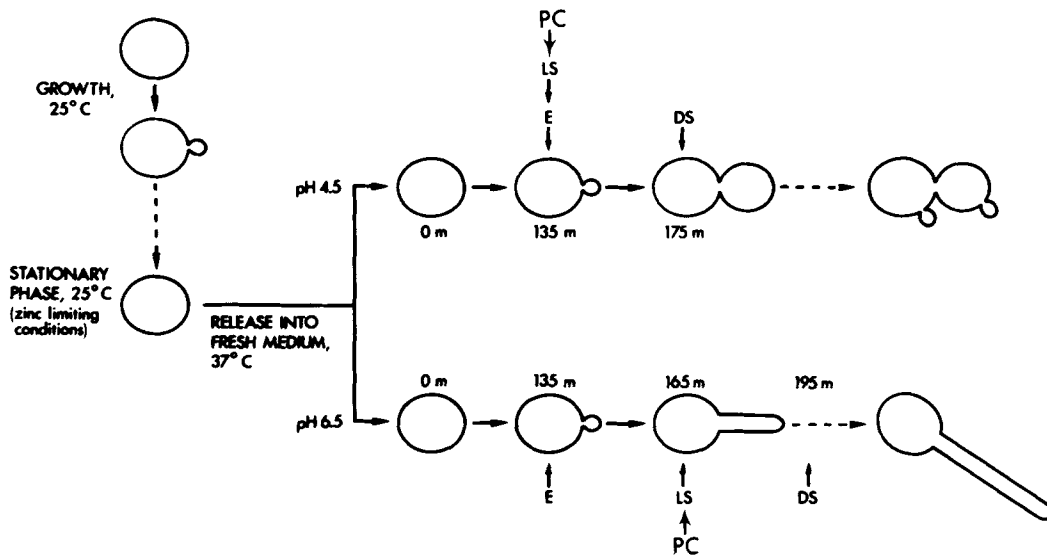


FIGURE 1 A scheme of the regulation of dimorphism in *Candida albicans* by pH. Cells were grown to stationary phase at 25°C in zinc-limiting medium (19). Under these conditions, cells accumulated as unbudded singlets. These cells were then released into fresh medium at 37°C and at either pH 4.5—to induce bud formation, or pH 6.5—to induce mycelium formation. *E*, average time of evagination; *LS*, light staining septal region (with the

fluorescent chitin stain Calcofluor); *DS*, brightly staining septal region (with Calcofluor); *PC*, average time of phenotypic commitment to the bud phenotype at pH 4.5 and to the mycelial phenotype at pH 6.5 (17).

In the present report, we have investigated filament ring formation during pH-regulated cell divergence in *Candida albicans* by electron microscopy of thin sections. We have found that a filament ring similar to the one in *Saccharomyces* is formed in both budding and mycelium-forming cells. By comparing the temporal and spatial characteristics of filament ring and septum formation between the two growth forms, we have assessed the relationship between the two structures. If the filament ring were to form at the mother-daughter cell junction in both bud- and mycelium-forming cells, then it would be highly unlikely that the ring is involved in septum formation since the septum forms in mycelia, on the average, 2  $\mu\text{m}$  distal to this point. However, if the filament ring were to form at the junction in budding cells, and, on the average, 2  $\mu\text{m}$  distal to the junction in mycelium-forming cells, then it would be likely that the filament ring plays a role in septation. The results presented in this report demonstrate that the latter is the case. In turn, these results suggest a simple model for the positioning of the filament ring and subsequent septum during outgrowth in the alternate growth forms.

## MATERIALS AND METHODS

**Growth of Stock and Experimental Cultures:** Stock cultures of *Candida albicans*, strain 3153A, were maintained according to methods previously reported (18, 19). For experimental purposes, cells were cloned from agar plates, grown to stationary phase in defined medium (11) limiting for zinc (19, 20, 21) at 25°C, and then tested for normal generation time (18), normal final stationary phase concentration (19), and normal outgrowth kinetics (13) before they were used for experimental purposes. Mass cultures were never maintained for more than 2 wk to ensure genotypic homogeneity.

**Regulation of Synchronous Bud or Mycelium Formation:** The methods for the regulation of phenotype by pH are described in detail elsewhere (13, 17, 21). In brief, to induce bud formation, stationary phase cells were diluted into fresh nutrient medium at 37°C, pH 4.5 and rotated at 200 rpm. To induce mycelium formation, we diluted stationary phase cells into fresh nutrient medium at 37°C, pH 6.5 and rotated them at 200 rpm (Fig. 1). The initial cell concentration in either culture was  $2 \times 10^6$  cells per ml. At time intervals, samples were removed and scored for the percent evagination and phenotype under  $\times 400$  magnification. One hundred cells were scored at each time point.

**Electron Microscopy:** The methods for fixation and embedding were similar to those described by Byers and Goetsch (22). Cells were pelleted, resuspended in 3% glutaraldehyde solution, and incubated at room temperature for 24 h. Cells were then washed twice in phosphate-citrate buffer at pH 5.8, and

then incubated in a 5% solution of glutusase for 4 h. Mycelium preparations were incubated for an additional 4 h in fresh glutusase solution. Cells were then washed in 0.2 M sodium cacodylate solution, pH 6.8, postfixed in 1% OsO<sub>4</sub> for 2 h at room temperature, rinsed with distilled water, and stained with 2% aqueous uranyl acetate for 60 min at room temperature. Cells were then dehydrated through a series of alcohol transfers and embedded in Spurr's resin. Thin sections were cut with a Porter-Blum MT2B ultramicrotome and examined in a Zeiss EM10A electron microscope.

**Visualization of Chitin-containing Septae:** The method used to visualize the septum was previously described in detail (14). In brief, cells were pelleted and resuspended in water. The fluorescent stain, Calcofluor, was added to a final concentration of 0.02 mg/ml. The cells were then washed in water, air-dried on slides, and stored dry for further use. Before viewing, a droplet of 0.6 M sucrose was placed over the preparation and a coverslip mounted on the droplet. Preparations were then examined under a Leitz fluorescence microscope, filter module H, with a hydrogen light source.

## RESULTS

### Regulation of Synchronous Bud and Mycelium Formation

*Candida albicans* grew exclusively in the budding form in zinc-limiting medium at 25°C, accumulating at stationary phase as singlets in G<sub>1</sub> of the nuclear division cycle (13, 18, 19). When stationary phase cells were diluted into fresh medium at 37°C, pH 4.5 (low pH), they evaginated synchronously after an average period of 135 min (Fig. 2), and then grew exclusively in the budding form (9, 13). Alternatively, when stationary phase cells were diluted into fresh medium at 37°C but at pH 6.5 (high pH), they evaginated synchronously after the same average period of 135 min (Fig. 2) but then grew exclusively in the mycelial form (10, 13). Thus, pH was used as the sole environmental determinant of phenotype in this model system of cell divergence (21), which is outlined in Fig. 1.

### Filament Ring during Synchronous Bud Formation

To assess the timing and position of filament ring formation during bud formation, we removed samples from low pH cultures at 140 min, just after the T<sub>50</sub> of evagination (Fig. 1), and at 170 min, when buds had achieved maximum volume (9, 13). These samples were prepared for electron microscopy according to the general methods of Byers and Goetsch (22),

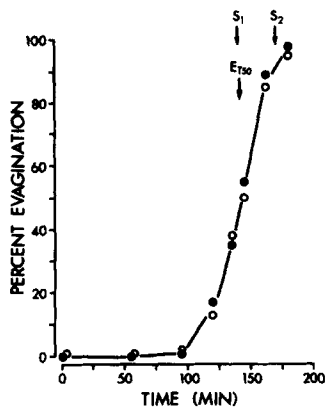


FIGURE 2 The kinetics of evagination in a budding (●) or mycelium-forming (○) population. As diagrammed in Fig. 1, stationary phase singlets were released into fresh medium at 37°C, pH 4.5, to induce bud formation, and into fresh medium at 37°C, pH 6.5, to induce mycelium formation. Time zero represents release from stationary phase.  $E_{50}$ , the time at which 50% of the population had formed evaginations (in this case, it was 135 to 140 min for both budding and mycelium-forming populations);  $S_1$  and  $S_2$ , the two times at which both populations were sampled for electron microscopy.

except that glucosylase treatment was increased to 4 h for budding cells and to 8 h for mycelium-forming cells to obtain sufficient penetration by the fixative, stain and embedding material for clear visualization of the plasma, mitochondrial, and vesicular membranes in the junctional region. Even with this extended digestion regime, the majority of mother cells still possessed a thick cell wall and usually did not stain with the detail of the junctional region and daughter cell cytoplasm. Electron micrographs were taken of cell sections which exhibited a mother cell of average diameter (4–5  $\mu\text{m}$ ), a complete transverse section through the junction between mother-cell and daughter compartment, and clearly distinguishable plasma and mitochondrial membranes in the neck and daughter compartment regions. These micrographs were in turn scored for the presence of a filament ring.

Evaginating cells of *Candida albicans* sampled at 140 min from low pH cultures (budding) contained a ring of filaments, equidistant from each other, and immediately under the plasma membrane of the junctional region joining mother cell and bud (Fig. 3A–C). This ring was similar in appearance to the ring observed in the junctional region of budding *Saccharomyces cerevisiae* (7). In most sections, the ring began in the proximal region of the junction and ended distally along the flared region of evagination (Fig. 3A–C). In most sections, the filament ring was observed below the opposing sections of the plasma membrane at the junction (Fig. 3A–C). A filament ring was observed in 92% of analyzed budding cell sections in a 140-min culture (Table I). The ring was located invariably at the mother cell-bud junction; similar filament condensations were never observed in the mother cell or bud in the nonjunctional regions. The average number of clearly defined filaments per ring section was  $12.1 \pm 2.3$  SD, but this may be a slight underestimate because of the difficulty in clearly identifying terminal filaments in ring sections. However, the low variability in the number of filaments per ring section (Table I) indicates that the ring contains a relatively set number of individual filament rings, if each filament cross-section represents an independent ring, or that the filament ring represents a coil with a constant number of turns.

Samples removed at 170 min from a low pH culture (budding) gave similar results (Fig. 3D–F). Filament rings were observed in 71% of analyzed sections (Table I). Filaments were again found invariably and exclusively at the mother cell-bud junction in close association with the plasma membrane. In cells in which an obvious primary septum was growing inward, no filament ring was observed (Fig. 4A). The sections of

filament rings of 170-min cells were similar in appearance to those of earlier cells and contained on the average  $10.8 (\pm 1.1$  SD) filaments per ring section, roughly the same number as in 140-min cells (Table I). Therefore, unlike the situation in *Saccharomyces* (7), the number of filaments per ring section did not appear to increase as buds matured.

### Filament Ring during Synchronous Mycelium Formation

Unlike budding cells, few of the mycelium-forming cells sampled at 140 min contained filament rings. The average length of mycelia at this time was 2  $\mu\text{m}$ , half the average diameter of mother cells (Fig. 5A and B). Only 15% of the evaginated cells at 140 min contained filament rings (Table I), and this minor fraction contained mycelia which were on the average 4  $\mu\text{m}$  long, twice the length of the average mycelium at this time. However, 65% of the cells sampled at 170 min contained filament rings (Fig. 5C, D, E, and F; Table I). These cells had an average length of roughly 5  $\mu\text{m}$  ( $\pm 2$  SD). Therefore, although evagination occurred at the same average time in bud and mycelium-forming cells (Fig. 2), the filament ring formed later in the latter population.

The basic structure of the ring in mycelium-forming cells was similar to that in budding cells (for instance, compare Figs. 3E and 5E). Filaments were again equally spaced immediately under the plasma membrane (Fig. 5C–F), and the average number of clearly defined filaments per ring section was  $12.1 \pm 2.4$  SD (Table I). Again, no similar filament structures were observed in mother cells. However, the position of the filament ring in mycelium-forming cells differed markedly from its position in budding cells. As noted previously, in budding cells the ring was invariably positioned at the junction of mother cell and bud (Table I). However, in mycelium-forming cells, the ring formed along the mycelium on the average 2  $\mu\text{m}$  from the junction of mother cell and daughter mycelium, with a range of 0 to 5  $\mu\text{m}$  (Fig. 6). In a few sections, a constriction was not apparent along the mycelium at the point of filament formation, but in the majority of sections a slight indentation was visible (e.g., Fig. 5C and D). In some cases in which cell wall had not been completely removed from the mycelium by digestion, the wall appeared thicker in the region adjacent to the ring. In mycelia in which the primary septum was growing inward, no filament ring could be distinguished (Fig. 4B), as was the case in budding *Candida* (Fig. 4A) and budding *Saccharomyces* (7).

### Relationship Between Filament Ring and Septum Formation

Using the fluorescent stain Calcofluor, we previously determined the timing and position of septum formation during synchronous bud and mycelium formation (14). We demonstrated that in budding cells a lightly staining septum (LS in Fig. 1) formed invariably at the mother cell-bud junction at approximately the same time as initial evagination. Roughly 30 to 40 min later, the septum abruptly converted to a brightly staining septum (DS in Fig. 1). However, in mycelium-forming populations, the light septum formed 30 min after evagination and abruptly converted to a brightly staining septum 30 min later. Since the clone used in the previous experiments (14) exhibited average evagination times of 145 min, and since the clones used in the present study exhibited average evagination times of 135 min, we repeated the staining experiments to

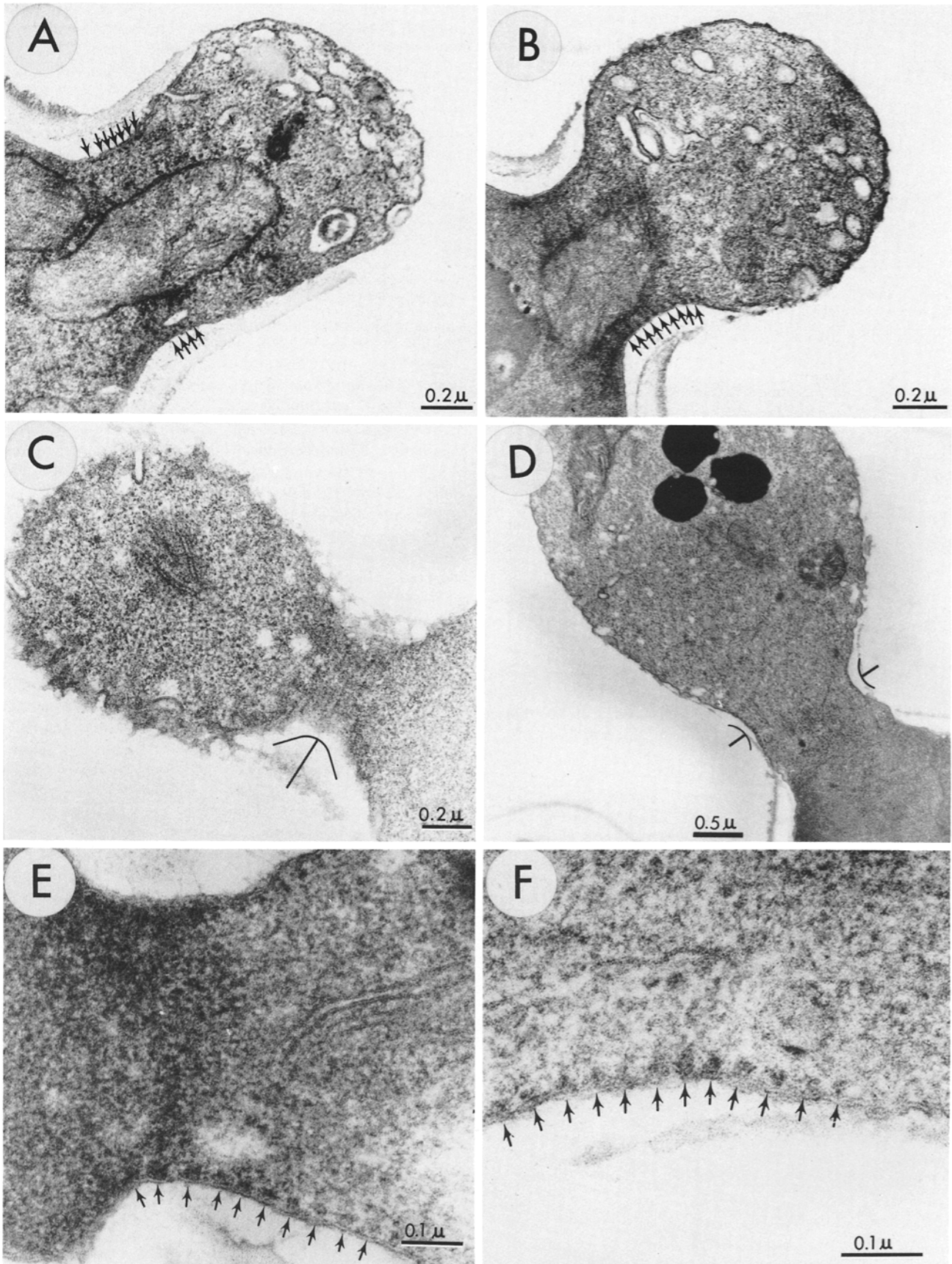


FIGURE 3 Electron micrographs of thin sections of a budding population of *Candida albicans*. Cell samples were removed from a low pH culture (see Fig. 2) at 140 min (A, B, and C) and 170 min (D, E, and F). The sharp arrows point to filament cross-sections. The rounded arrows in C and D point to the general ring area. In C, a tangential "shave" of the filament ring exposes the length of the coiling filament(s). Note the abundance of vesicles in the early budding profiles (A, B, and C), but the absence of vesicles in the area of the ring.

TABLE I  
Temporal and Spatial Parameters of Filament Ring Formation during Bud or Mycelium Formation

	$T_{50}$ evagination	% Cells with filament ring*	Av. No. of filaments/ring‡	% Cells with filament ring*	Av. No. of filaments/ring‡	Av. distance of filament ring from junction§	Av. distance of septum from junction¶
			140 min		170 min		
Budding cells	135 min	92% (23/25)	12.1 ± 2.3 (N = 15)	71% (10/14)	10.8 ± 1.1 (N = 10)	0 μm (N = 25)	0 μm
Mycelium-forming cells	135 min	15% (4/26)	—	65% (11/17)	12.1 ± 2.4 (N = 24)	1.8 μm ± 1.2 (N = 53)	2.0 μm

\* Cells were scored only when the section was through a mother cell of average diameter, the channel between mother and daughter cell was filled with cytoplasm, and a clear plasma membrane was distinguishable at the junction between mother and daughter compartment.

‡ Cells were scored only when the cytoplasm and plasma membrane on either side of the filament ring were in focus.

§ The distance was taken from the continued perimeter of the mother cell to the mid-point of the ring structure. See Fig. 6 for distribution.

¶ Data taken from Mitchell and Soll (14). Also, see Fig. 6 for distribution.

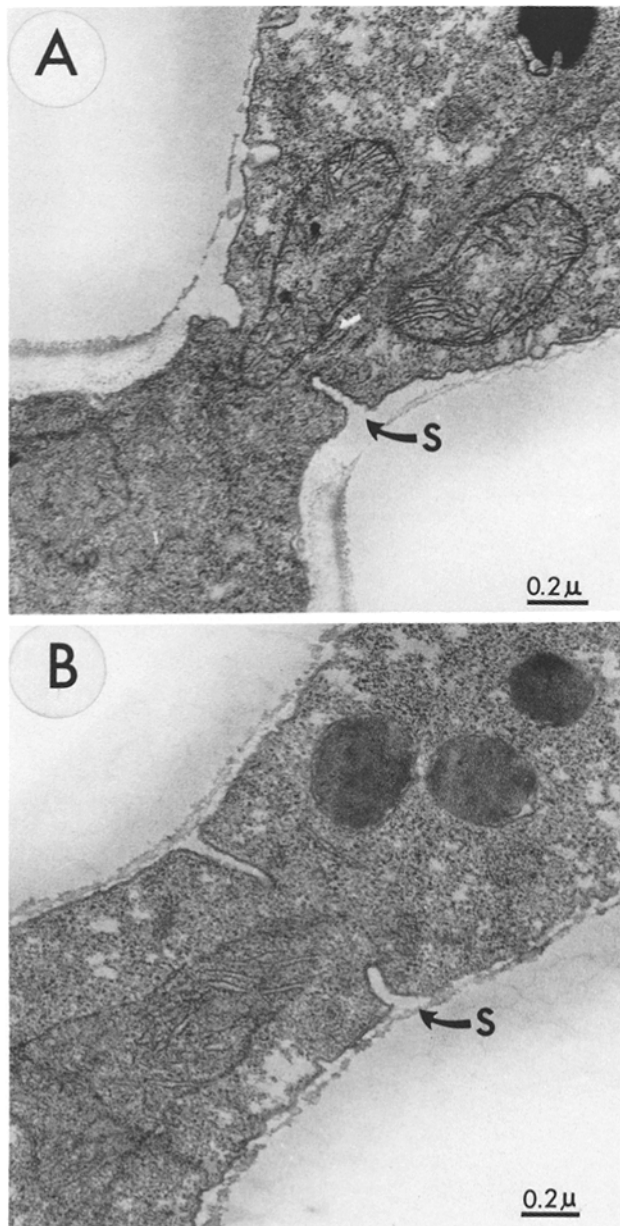


FIGURE 4 The absence of filament rings in the septal regions of budding (A) and mycelium forming (B) cells during primary septum formation. In both cases, cells were sampled at 170 min (see Fig. 2). S, centripetally growing primary septum.

assess the temporal relationship of septum formation and evagination. Again, the average time of light septum staining in budding populations corresponded with the average time of evagination, both occurring at 135 min, and the average time of light septum staining in mycelium-forming populations was approximately 165 min, 30 min after the average time of evagination (135 min). These results are summarized in Fig. 1.

Since septa were visualized by fluorescence microscopy of whole cells, and since filament rings were visualized by electron microscopy of thin sections of cells previously treated with glusulase, the septum and ring could not be critically analyzed in the same cell. This problem is not critical in our evaluation of septum and filament ring positions in budding populations since both are invariably formed at the junction of mother cell and bud. However, in mycelium-forming cells, the variability in position makes it difficult to conclude that the septum invariably forms in the same location as the filament ring in each cell. Nevertheless, the average distances of both septum and ring from the mother cell junction are similar (Table I), and the distributions of distances are similar (Fig. 6), indicating quite strongly that the septum forms at the location of the filament ring in mycelia.

## DISCUSSION

We have demonstrated that during bud formation in *Candida albicans* a filament ring is formed just below the plasma membrane at the junction between mother cell and bud which is similar in location and appearance to the filament ring of *Saccharomyces cerevisiae* (7). Its transient appearance in two budding species of yeast and its absence in cytokinesis defective mutants of *Saccharomyces* (8) indicate that it may play an essential role in the yeast cell cycle. In addition, we have found that a filament ring is formed during mycelium formation and that this ring is similar to the ring in budding cells in both general appearance and filament number. In both growth forms of *Candida*, approximately 11 to 12 filaments are observable in each ring section. This number remains stable with time, in contrast to *Saccharomyces* in which it has been reported that the number increases to more than 20 as the bud matures (7). However, just as in *Saccharomyces* (7), the filament ring in both growth forms of *Candida* disappears when the primary septum begins growing centripetally.

### Filament Ring and Septum Formation

Our observation that the filament ring forms, on the average, 2 μm from the junction of mother cell and daughter tube in

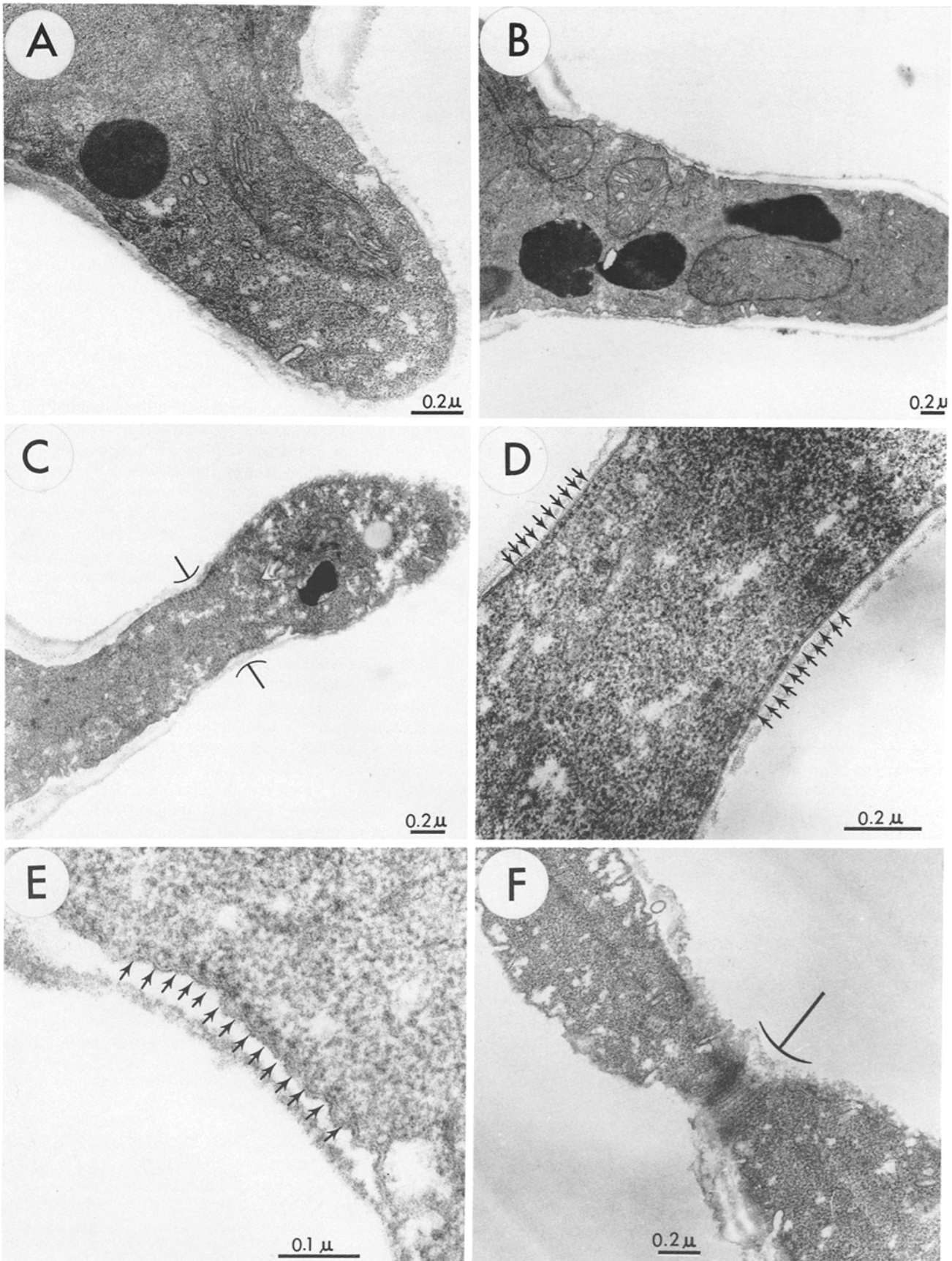


FIGURE 5 Electron micrographs of thin sections of a mycelium-forming population of *Candida albicans*. Cell samples were removed from a high pH culture (see Fig. 2) at 140 min (A, B) and 170 min (C-F). The sharp arrows point to filament cross-sections. The rounded arrows in C and F point to the general ring areas. In F, a tangential "shave" of the filament ring is presented. Note the lack of a filament ring in early mycelia (A and B) and the lack of vesicles in the ring regions of C to F.

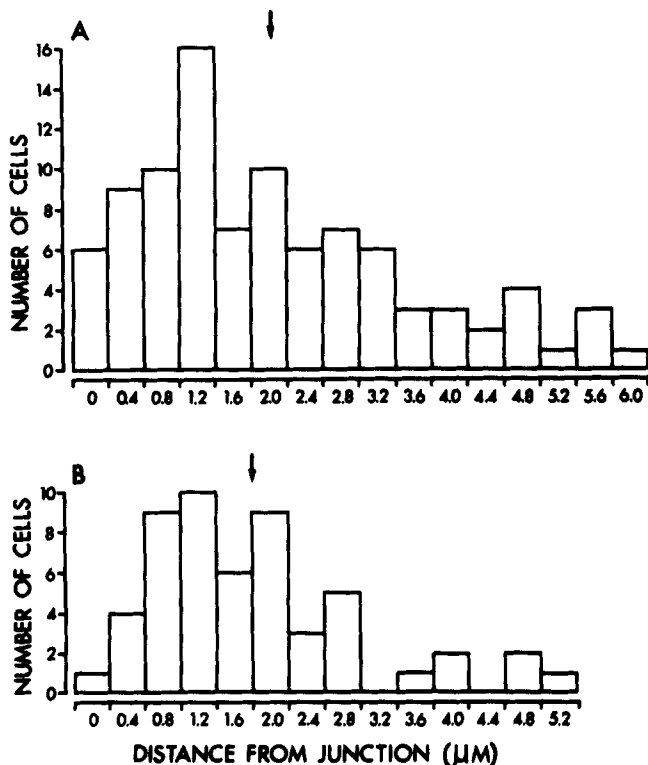


FIGURE 6 The distributions of the distance between the mother-daughter cell junction and the septum (A) or filament ring (B) in mycelium-forming populations. Arrow points to mean distance.

mycelium-forming cells suggests that the filament ring is not involved in the positioning of evagination, or in the maintenance of an open channel between mother cell and immature bud. Since the ring disappears during septum formation, it is also unlikely that the ring is involved in constriction, as in the case of the contractile ring in cleaving embryos (23) and dividing mammalian cells (24). Rather, we have found a very close temporal and spatial correlation between the formation of the filament ring and the formation of the chitin-containing septum. In cell preparations in which cell wall is still visible along the mycelium after glucuronase treatment, the wall is disproportionately thicker and the tube is sometimes slightly constricted in the region of the filament ring, indicating the beginning of chitin ring formation (2, 3). In addition, the distributions of filament ring and septal distances from the mother cell-mycelial junction are similar, indicating that each cell forms a filament ring at the future position of the septum.

The correlation between filament ring and septum location suggests that the ring may be involved in the localized deposition of septal polysaccharides. Byers and Goetsch (7) observed that the ring filaments were in physical contact with the plasma membrane. Since the enzyme chitin synthetase appears to be distributed throughout the plasma membrane (6), it is worth considering the possibility that the filament ring structure may be involved in the localized activation of chitin synthetase (25, 26), a possibility which has not previously been considered in past discussions of the regulation of septum formation in *Saccharomyces* (3, 26).

### A Simple Model for Positioning the Filament Ring

If the filament ring dictates the position of the septum, what in turn dictates the position of the filament ring? Rather than

hypothesize the involvement of another localized structure, a simple temporal model will account for the results presented in this report. If synthesis of filament subunits occurs prior to evagination in budding cells, and if organization of subunits into filaments occurs only in the growth zone, then one would expect the ring to be formed immediately after evagination begins in budding cells and to be localized in the junctional zone as subsequent growth ensues. If the synthesis of filament subunits occurs after evagination in mycelium-forming cells, then the organization of subunits into filaments would follow soon after synthesis, at the tip of the already elongating mycelia. If this model is correct, then one would expect to find incomplete filament rings in the tip of mycelia caught in the act of filament ring formation and only complete filament rings along the length of mycelia. This prediction is now being tested.

### Filament Ring, Septation, and Phenotypic Commitment

In *Candida*, the time at which cells become committed to alternate phenotypes can be precisely defined by initiating outgrowth at one pH, then shifting to the alternate pH at various times (17). The average time at which a shift to the alternate pH does not result in the alternate phenotype, but rather results in the phenotype prescribed by the original pH, is considered the average time of phenotypic commitment (17). In *Candida*, the average time of light septal staining correlates closely with the average time of phenotypic commitment in the alternate growth forms (14, 17; see diagram in Fig. 1). Both occur at the time of evagination in budding cells and 30 min after evagination in mycelium-forming cells. Because of this unusually close temporal correlation, we previously suggested that septum formation may be involved in the commitment event (14). However, if the filament ring dictates the timing and location of septum formation, it is more likely that the formation of the filament ring rather than the septum is basic to commitment. Since the filament ring appears to be similar in structure in the two growth forms, the determination of distal phenotype may be dependent upon the time and location of filament ring formation rather than upon qualitative differences in the ring structure, a possibility now under intensive investigation.

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