Cell Cycle Dynamics and Quorum Sensing in *Candida albicans*
Chlamydospores Are Distinct from Budding and Hyphal Growth

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The opportunistic fungal pathogen *Candida albicans* grows in different environments depending on environmental conditions. *C. albicans* typically forms buds similar to *Saccharomyces cerevisiae* and other yeasts in standard growth medium but under different conditions can be induced to form elongated pseudohyphae, filamentous chains of cells termed hyphae, or large rounded cells termed chlamydospores (8, 24, 50, 60). A major underlying factor for *C. albicans* virulence is the ability to switch between budding and hyphal states, which differ in their ability to produce virulence factors, grow invasively, and evade the immune system (8, 24). Morphological transitions are also thought to be important for biofilm formation (36). In addition, *C. albicans* cells that are homozygous at the mating loci can undergo further morphological variation by an epigenetic switch between white and opaque phases, which produce rounded or elongated buds, respectively (46). *C. albicans* only mates efficiently when in the opaque phase (46, 57).

*C. albicans* can also be induced to undergo a complex morphological transition to form chlamydospores (24, 50), which are generally defined as thick-walled asexual spores that are derived from a hyphal cell. The role of chlamydospores in the *C. albicans* life cycle is not understood, as they are rarely observed at sites of infection, and there is no evidence that they confer long-term viability (33). However, a potential role is suggested by the fact that the only *Candida* species that characteristically form chlamydospores, *C. albicans* and *C. dubliniensis*, are two of the most prevalent human fungal pathogens. In fact, chlamydospore formation is an important diagnostic tool for distinguishing between different *Candida* species since the majority of *C. albicans* clinical isolates retain the ability to form chlamydospores (2, 3). These observations raise the possibility that this conserved process contributes an advantage for growth as either a commensal or a pathogen in human hosts.

Early studies of chlamydospores in *C. albicans* were limited to descriptions of their growth, development, and ultrastructural features (1, 33, 47, 54). The empirically determined conditions that favor chlamydospore production were found to include growth in the dark, microaerophilic conditions, room temperature, and nutrient-poor media containing complex carbohydrates. These conditions first trigger cells to grow in long filamentous chains; elongated suspensor cells then develop by branching off of the filaments, and then large rounded chlamydospores (8 to 12 μm in diameter) form at the end of the suspensor cells. In some cases, a chlamydospore can also form at the terminus of a filament. The filamentous growth that occurs during chlamydospore induction resembles hyphal filaments seen under other conditions, but to avoid confusion in this study, we will use the term hyphae to describe only the type of filamentous growth similar to that observed at 37°C under conditions that mimic infection. The mature chlamydospores contain a nucleus and other organelles but are distinguished by the presence of large lipid droplets (47), high RNA content (64), and a thick outer layer of cell wall that is contiguous with the suspensor cell (33, 47).
Another distinct type of septin organization was observed in chlamydospore production. Farnesol is a known inhibitor of the later stages of this process. This indicates an important role for Efg1 in chlamydospore-inducing conditions yet fail to produce chlamydospores (58). For example, in S. cerevisiae, the Efg1 transcription factor is important for inducing hyphal and chlamydospore morphogenesis and invasive growth in C. albicans (12, 14, 51, 62). Interestingly, comparison of septin localization with the nuclear division cycle during chlamydospore development in this study revealed that chlamydospore morphogenesis is distinct from budding and hyphal morphogenesis.

Recent studies taking advantage of new gene deletion methods have shown that chlamydospore development is influenced by some of the same genes that control hyphal development (49). However, these processes appear to be distinct, as not all genes that affect hyphal growth are needed for chlamydospore development, and some genes have differential effects. For example, the Efg1 transcription factor is important for inducing hyphal growth at 37°C but is not needed for the filamentous growth observed when cells are grown on chlamydospore-inducing medium or embedded within an agar matrix (7, 20, 58). Interestingly, efg1Δ mutants are hyperfilamentous under chlamydospore-inducing conditions yet fail to produce chlamydospores (58). This indicates an important role for Efg1 in the later stages of this process.

To investigate further the relationship between hyphal and chlamydospore morphogenesis, as part of this study we examined the effects of the quorum-sensing factor farnesol on chlamydospore production. Farnesol is a known inhibitor of hyphal growth in C. albicans (30) but was found in the present study to increase chlamydospore production. We also examined the septin proteins, since this family of membrane-associated GTPases contributes to morphogenesis and also displays distinct patterns of localization that serve as useful spatial landmarks for the progression of morphogenesis (15, 22, 25).

For example, in S. cerevisiae and C. albicans, septin rings mark sites of incipient bud emergence, while later in the cell cycle they form a hourglass-shaped structure that spans the mother-bud junction (17, 34, 61, 65). Septins facilitate bud morphogenesis by acting as a boundary domain and as a scaffold to recruit proteins involved in bud site selection, cell cycle control, and septum formation (15, 21, 41). In contrast, septins form a more diffuse band at the base of cells induced to form highly polarized mating projections in S. cerevisiae (19, 34, 42). A similar diffuse array of septins was also observed in C. albicans at the base of newly formed hyphae (germ tubes) and also as a cap across the growing hyphal tip (61, 65). Gene deletion studies indicate that the septins play a role in proper hyphal morphogenesis and invasive growth in C. albicans (65, 66). Another distinct type of septin organization was observed in meiotic spores of S. cerevisiae, which contain long filamentous septin structures associated with the prosome membrane (12, 14, 51, 62).

### TABLE 1. C. albicans strains used in this study

| Strain       | Genotype                                      | Source or reference |
|--------------|-----------------------------------------------|---------------------|
| DAY185       | ura3Δ::imm434/ura3Δ::imm434 HIS1::hisG ARG4 URA3/arg4::hisG | 10                  |
| CA4          | ura3Δ::imm434/ura3Δ::imm434 his1::hisG ARG4 URA3/arg4::hisG | 16                  |
| YAW60        | CA4, except pADH-CDC10-GFP integrated         |                     |
| YAW-CDC10GFP | CA4, except CDC10-GFP integrated              |                     |
| BWP17        | ura3Δ::imm434/ura3Δ::imm434 his1::hisG ARG4 URA3/arg4::hisG |                     |
| YAW7 (cdc10Δ)| cdc10Δ::ARG4/cdc10Δ::HIS1 ura3::imm434/ura3::imm434 his1::hisG/his1::hisG arg4::hisG:ARG4:URA3 | 66                  |
| YAW11 (cdc11Δ)| cdc11Δ::ARG4/cdc11Δ::HIS1 ura3::imm434/ura3::imm434 his1::hisG/his1::hisG ARG4:URA3 | 66                  |
| YAW16 (spr3Δ)| spr3Δ::ARG4/spr3Δ::HIS1 ura3::imm434/ura3::imm434 his1::hisG/his1::hisG ARG4:URA3 | 66                  |
| YAW19 (spr28Δ)| spr28Δ::ARG4/spr28Δ::HIS1 ura3::imm434/ura3::imm434 his1::hisG/his1::hisG ARG4:URA3 | 66                  |
| YSM45 (bni1Δ)| bni1Δ::ARG4/bni1Δ::HIS1 ura3::imm434/ura3::imm434 his1::hisG/his1::hisG ARG4:URA3 | This study          |
| YSM47 (cla4Δ)| cla4Δ/cla4Δ, except pRS424-ARG4-URA3 integrated | This study (a)      |
| YSM56        | CA4, except pCDC12-GFP integrated             | This study (b)      |

(a) Source for cla4Δ/cla4Δ is reference 38.

### MATERIALS AND METHODS

#### Strains and media.

The yeast strains used in this study are described in Table 1. Cells were propagated on YPD (yeast extract, peptone, and dextrose) medium plus 80 mg/liter uridine (55). Chlamydospore growth was induced on medium containing 1.25% white cornmeal, 1% agar, and 1% Tween 80 (polyethylene- sorbitan monoooleate; Sigma catalog no. P-1754). To induce chlamydospore production, cells were grown overnight in YPD medium plus 80 mg/liter uridine, and diluted 1:250 in sterile water; 3 μl of this suspension was spotted onto cornmeal-Tween agar. Coverslips were placed on top of the spots and plates were kept at 23°C in the dark. To examine the effects of farnesol (3,7,11,trimethyl-2,6,10-dodecatrien-1-ol; Sigma catalog no. F-8627), it was dissolved in methanol to make a 1 M stock and then added to cornmeal-Tween agar at a final concentration of 10 mM. In studies testing the effect of farnesol on spore production, methanol was added to control plates at a final concentration of 1%. Consistent results were best obtained when cornmeal-Tween plates were inoculated within 1 to 2 days after pouring.

#### Construction of bni1Δ homozygous deletion mutant and BNI1-GFP strain.

The C. albicans BNI1 gene was identified in the C. albicans genome database (http://www.candidagenome.org/) based on sequence similarity with the S. cerevisiae BNI1 gene. To create a homozygous deletion strain, the open reading frame of one allele was replaced by homologous recombination with a PCR-generated fragment containing ARG4, and the other allele was replaced in a similar manner with a fragment containing HIS1 in strain BWP17, as described previously (68). The deletions removed the entire open reading frame and stop codon. PCR mixtures contained approximately 10 ng of plasmid template (pGEM-HIS1 or pRS-ARG4/Spel [68]), primer 5′ BNI1KO (ATTTACTACGTTTTTTTTTTTTTTGTTTCATCATCATCATTCCATTACCATCATCATTACCATGTAAGAGATGGGCGA), and primer 3′ BNI1KO (AAAAATCATTACCAAAAAAAGAAAACACCTCC TTATATTATACGTGTTCTCTGTTTTCCTCCACGTACGAGTTC). To verify that the BNI1 gene was deleted, genomic DNA was extracted from Arg1 His1 transformants (27) and used as a template for PCR with primers that lie outside the BNI1 open reading frame (5′ BNI1detect, CCTGATCACATGCTGATACAGCGT, and 3′ BNI1detect, CTTGATCACATGCTGATACAGCGT) to detect the deletion alleles. As a control to make sure that all copies of BNI1 were deleted, a separate PCR was carried out using a primer internal to the BNI1 open reading frame (5′ BNI1internal, CTTGATCACATGCTGATACAGCGC, and 3′ BNI1detect primer).

A BNI1-GFP strain was constructed by fusing the coding region of a codon-
optimized version of a gene encoding the green fluorescent protein (GFP) in frame with the 3’ end of the BNI4 open reading frame. PCR amplification was used to add ~60 bp of homology corresponding to the 3’ end of the BNI4 open reading frame on each side of a module containing GFP and the URA3 selectable marker (18) using primers F2-BNI4-GFP (AAGAATTGATAGTTTAAA AGTGAAATGGAAATCTAGTTGAATCAAAAATTTTATACACATTTTT TTGCTGTTTGTTCTAAAGTTGAAGAAATATTATTTTGCTTTGTTTTGC) and R1-BNI4-GFP (TCT ATTTCTTACCACTCAACAAAAAAAGAAAAACACCTCCTTATAT TATACCTTGTCTTTGTTCTAGAAAGGACCATTTTGTAGG). The module was then transformed into strain BWP17, and proper homologous integration was verified by PCR and also by microscopic analysis of the BNI4-GFP.

Time lapse microscopy. Developing chlamydospores and colony morphologies on cornmeal-Tween plates were analyzed by direct microscopic observation through a 20× lens objective. Samples were photographed every hour starting at 30 h after the plates were seeded until mature chlamydospores were produced. Images were captured with an Olympus BH2 microscope using a Zeiss AxioCam run by Openlab 3.0.8 software from Improvision, and then the composite image was constructed using Photoshop (Adobe).

Cell staining and fluorescence microscopy. For all cell stains, cells were scraped off of cornmeal-Tween with a sterile toothpick and processed for staining. DNA staining was performed on formaldehyde-fixed cells by using 5 μg/ml DAPI (4’,6-diamidino-2-phenyindole) dilactate in Prolong Antifade mounting medium (Molecular Probes, Eugene, OR) and viewed under UV. Chitin staining was performed using 0.2 μg/ml calcofluor white for 5 to 10 min and viewed without fixation under UV. For double stains using calcofluor white and DAPI, cells were fixed with formaldehyde before staining. Lipid particles were stained using 10 μg/ml Nile Red for 5 to 10 min and viewed without fixation by fluorescence microscopy using the same filter sets used to detect rhodamine. For analysis of septin-GFP localization, strains expressing CDC12-GFP under control of the native promoter (65) or CDC10-GFP under control of the ADH promoter (65) were scraped off of cornmeal-Tween plates and examined by fluorescence microscopy.

RESULTS

Farnesol promotes chlamydospore production. Chlamydospore morphogenesis was induced by plating cells under a coverslip on cornmeal-Tween agar in the dark at room temperature (23°C) (Fig. 1). During preliminary optimization studies, chlamydospore production was observed to be more abundant when cells were seeded onto plates at higher densities. This suggested the possibility that chlamydospore morphogenesis may be regulated by the quorum-sensing factor farnesol. Farnesol produced by C. albicans accumulates in the culture medium in a cell density-dependent fashion and is able to suppress the ability of cells to switch from budding to hyphal growth at 37°C (30). In contrast, the addition of farnesol to cornmeal-Tween agar resulted in increased numbers of chlamydospores relative to mock-treated controls after 3 days (Fig. 1). Quantitation of this effect showed that farnesol did not affect the filamentous growth phase, but it instead increased the number of chlamydospores per filament. Although there was variability between independent experiments carried out on different days, farnesol treatment always resulted in more chlamydospores relative to control cells, indicating that it positively regulates chlamydospore production (Fig. 1B).

It was surprising that farnesol did not affect the filamentous growth of cells under chlamydospore-inducing conditions (Fig. 1), since it suppresses hyphal growth in C. albicans incubated at 37°C with various inducers, such as serum (30). To better understand the role of farnesol in regulating filamentous growth under different inducing conditions, cells were seeded onto cornmeal-Tween medium with or without farnesol and incubated at 23 or 37°C for 4 to 5 days (Fig. 2). At 23°C, filamentous growth and chlamydospore production were observed in both the presence and absence of farnesol as expected (Fig. 2A and B). At 37°C in the absence of farnesol, highly filamentous cells formed, but chlamydospore production was extremely rare (Fig. 2C). These filamentous cells resembled serum-induced hyphae at 37°C in that they were thinner (diameter, 1.81 ± 0.17 μm; n = 50) than filaments formed at 23°C (diameter, 2.94 ± 0.18 μm; n = 50). In addition, inclusion of farnesol in the cornmeal-Tween agar prevented filamentous growth at 37°C (Fig. 2D). These results indicate that chlamydospore production is subject to different regulation than hyphal growth and that chlamydospore-producing filaments are distinct from true hyphae. For clarity in this report, we will use the term hyphae to describe only the type of filamentous growth observed at 37°C to distinguish it from the filamentous growth that occurs under chlamydospore-inducing conditions.

Time course analysis of chlamydospore morphogenesis. An initial examination of the morphology of developing suspensor

FIG. 1. Farnesol promotes chlamydospore production in C. albicans. (A) Wild-type cells (DAY185) were seeded onto cornmeal-Tween agar with or without 10 mM farnesol and then photographed through a 20× microscope lens at the indicated times. (B) Chlamydospore production in the absence (squares) or presence (circles) of farnesol was quantified as the number of spores produced per filament over a 3-day time course. Representative data from three independent experiments are shown.

FIG. 2. Chlamydospore morphogenesis in the presence of serum. (A) Wild-type cells (DAY185) were incubated at 37°C with various inducers, such as serum (30). To better understand the role of farnesol in regulating filamentous growth under different inducing conditions, cells were seeded onto cornmeal-Tween medium with or without farnesol and incubated at 23 or 37°C for 4 to 5 days (Fig. 2). At 23°C, filamentous growth and chlamydospore production were observed in both the presence and absence of farnesol as expected (Fig. 2A and B). At 37°C in the absence of farnesol, highly filamentous cells formed, but chlamydospore production was extremely rare (Fig. 2C). These filamentous cells resembled serum-induced hyphae at 37°C in that they were thinner (diameter, 1.81 ± 0.17 μm; n = 50) than filaments formed at 23°C (diameter, 2.94 ± 0.18 μm; n = 50). In addition, inclusion of farnesol in the cornmeal-Tween agar prevented filamentous growth at 37°C (Fig. 2D). These results indicate that chlamydospore production is subject to different regulation than hyphal growth and that chlamydospore-producing filaments are distinct from true hyphae. For clarity in this report, we will use the term hyphae to describe only the type of filamentous growth observed at 37°C to distinguish it from the filamentous growth that occurs under chlamydospore-inducing conditions.
cells and chlamydospores raised the question as to whether chlamydospores separate from suspensor cells through cell fission or by budding. Therefore, time course analysis was performed in order to better understand how the filamentous cells give rise to the suspensor cells and chlamydospores (Fig. 3). Cells were seeded onto cornmeal-Tween agar containing 10 mM farnesol and photographed at hourly intervals from 30 h after seeding until mature chlamydospores were observed in the culture. Filaments emerged from colonies of budding cells and grew in a highly polarized fashion until a slight swelling of the tip formed the suspensor cell at 38 h after seeding (Fig. 3, arrowhead). At 41 h, a chlamydospore was observed to form at the tip of the suspensor cell (Fig. 3, arrow). The chlamydospore continued to enlarge until it reached its maximum size at approximately 46 h. Septum formation subsequently separated chlamydospores from suspensor cells, although cell separation did not take place. Previous electron microscopy studies revealed that at this stage the chlamydospores develop a thick cell wall, the outer layer of which remains contiguous with the suspensor cell (33, 47). Thus, chlamydospores form through a budding process but are distinguished from buds by the larger diameter of the septum at the suspensor-chlamydospore junction relative to the septum produced during budding (see below) and by lack of cell separation.

Chlamydospores display an altered nuclear division cycle. To better understand cell cycle dynamics in developing chlamydospores, DAPI staining was used to investigate the spatial and temporal regulation of nuclear division during chlamydospore morphogenesis. Unexpectedly, the nucleus did not move into a position adjacent to the neck of the chlamydospore as it does during budding. Instead, nuclear division was found to occur inside of suspensor cells, producing a binucleate cell (Fig. 4, stages 1 and 2). Subsequently, one nucleus then migrated into the immature chlamydospore (Fig. 4, stages 3 and 4). Ultimately, septation occurred after nuclear migration into the chlamydospore was completed (Fig. 4, stages 5 and 6). Nuclear division across the suspensor-chlamydospore junction was never observed, as is the typical case at the necks of budding cells. This pattern of nuclear division also differs from that seen in hyphae, where the nucleus moves out of the mother cell and into the germ tube so that the nucleus divides...
across the future site of septation. Thus, these differences in nuclear division indicate that chlamydospores and suspensor cells undergo distinct cell cycle dynamics compared to buds or hyphae.

**Septin localization during chlamydospore morphogenesis.** Septin proteins have been used as spatial landmarks for monitoring cell cycle progression and morphogenesis in several species of fungi (15, 22, 25). To examine septin localization during chlamydospore development in *C. albicans*, cells producing the septin Cdc10p with a C-terminal GFP tag were seeded onto cornmeal-Tween agar and observed using fluorescence microscopy after 2 to 5 days of growth. Septin rings similar to those seen in budding cells were observed at the septation sites in filaments and at the junctions between filaments and suspensor cells and were initially detected at the suspensor-chlamydospore junction (Fig. 5A). These septin rings appear to be similar to those formed during budding, except that the diameters of the septin rings at the neck of chlamydospores (1.91 ± 0.22 μm; n = 76) were significantly larger than the diameters of septin rings observed in budding cells (1.37 ± 0.15 μm; n = 100). Immature chlamydospores showed a broad band of Cdc10-GFP at the neck that resolved into two rings in cells that showed an obvious septum (Fig. 5A). Interestingly, at later stages of chlamydospore formation, only the ring on the suspensor cell side remained obvious (Fig. 5A). The ring on the chlamydospore side became less obvious, and, instead, increased Cdc10-GFP fluorescence was detected around the periphery in a significant proportion of chlamydospores (46.7%; n = 45). In some cases it appeared that the septins formed filaments that ran from the ring toward the tip of the chlamydospore (Fig. 5A). Similar results were observed for a strain producing a different septin, Cdc12-GFP (data not shown).

Cdc10-GFP localization was difficult to photograph, in part because the GFP fluorescence was always much weaker in cells grown under chlamydospore-inducing conditions. This was true for other GFP fusion proteins and was not specific to septin proteins. To better visualize septins inside of chlamydospores, we examined a strain overexpressing *CDC10-GFP* under control of the constitutive *ADH* promoter. Septin localization in an *ADH-CDC10-GFP* strain is easier to detect but still shows normal septin localization in buds, pseudohyphae, and hyphae (67). Under chlamydospore-inducing conditions, cells growing as buds or filaments showed typical septin ring formation. Immature chlamydospores showed either a wide band of Cdc10-GFP or a double septin ring at the junction with the suspensor cell (Fig. 5B). At later times the septin ring on the chlamydospore side of the junction appeared to extend into filamentous structures adjacent to the chlamydospore membrane. Interestingly, the mature chlamydospores commonly showed filaments of Cdc10-GFP that ran parallel to the apical-basal axis of the chlamydospore and appeared to form a complex filamentous system along the cell periphery (Fig. 5B and C). These filamentous septin structures were detected in 81.5% (n = 92) of chlamydospores examined. No corresponding structures were observed in filamentous cells or suspensor cells, suggesting that these septin filaments are unique to chlamydospore development. Furthermore, these septin filaments were not observed in immature chlamydospores, suggesting that these structures are produced during chlamydospore maturation at a time when the thickening of the chlamydospore cell wall occurs (33, 47).
Septins are required for proper chlamydospore morphogenesis. To test whether septins have a role in chlamydospore morphogenesis, five different septin mutant strains were seeded onto cornmeal-Tween agar and viewed after 4 to 5 days. Mutant phenotypes in chlamydospore morphology were observed in *cdc10*/*H9004* and *cdc11*/*H9004* strains but not in *sep7*/*H9004*, *spr3*/*H9004*, or *spr28*/*H9004* strains (Table 2). Since *cdc10*/*H9004* and *cdc11*/*H9004* strains produced similar phenotypes, detailed descriptions will only be presented for the *cdc11*/*H9004* strain.

Analysis of the early stages of chlamydospore induction revealed significant bending of the *cdc11Δ* cells growing in a filamentous manner that was not seen for the wild-type strain (Fig. 6). Quantification revealed that 46.5% (*n* = 99) of filaments produced by the *cdc11Δ* strain were bent more than 45° from the main axis of filament growth, while only 6.5% (*n* = 62) of wild-type filaments showed curvatures greater than 45°. In addition, curvature in wild-type filaments was gradual, while the *cdc11Δ* cells often showed sudden kinks and bending along their axis of growth (Fig. 6, arrows). These observations are reminiscent of the hyphal curvatures previously detected in *cdc10Δ* and *cdc11Δ* strains of *C. albicans* (67).

At higher magnifications, defects in cytokinesis between the suspensor cell and chlamydospore were observed in the *cdc11Δ* strain.

**TABLE 2. Analysis of chlamydospore morphology in septin mutant strains**

| Strain       | % Normal | % Abnormal (no. of chlamydospores) |
|--------------|----------|------------------------------------|
| DAY185 (wild type) | 100      | 0 (100)                            |
| YAW7 (*cdc10Δ*)   | 83       | 17 (100)                           |
| YAW11 (*cdc11Δ*)  | 67       | 33 (126)                           |
| YAW22 (*sep7Δ*)   | 100      | 0 (100)                            |
| YAW16 (*spr3Δ*)   | 100      | 0 (100)                            |
| YAW19 (*spr28Δ*)  | 100      | 0 (100)                            |
| YSM45 (*bni4Δ*)   | 100      | 0 (100)                            |
strains. The suspensor-chlamydospore junctions in the mutants were usually wider than wild type and often showed no evidence of a septum when stained with calcofluor white (Fig. 7A to D). When a septum was detected in mutant strains, it was abnormal compared to septa formed in wild-type cells (Fig. 7C and D, inset). In addition, ectopic chitin deposition was frequently observed throughout the cell wall of mutant chlamydospores (Fig. 7D).

The cdc11Δ septin mutant cells were frequently multinucleate (20%; n = 60), indicating a defect in nuclear positioning relative to the wild type (Fig. 7E to H). Chlamydospores produced by the cdc11Δ strain containing multiple nuclei were sometimes observed (Fig. 7H). In addition, suspensor cells containing up to three nuclei were detected (Fig. 7H, inset). These results are consistent with previously described defects in nuclear segregation in septin mutant strains of *S. cerevisiae* and *C. albicans* (37, 67).

In view of the morphological and nuclear segregation defects observed in chlamydospores produced by cdc11Δ strains, other aspects of spore morphogenesis were examined. In par-
ticular, we examined the characteristic formation of a large, central lipid particle inside of the chlamydomospore (54). This process was unaffected in cdc11Δ strains, even when severe morphological defects were evident (Fig. 7I to L).

Chlamydomospore production in bni4Δ and cla4Δ strains. Septins interact with two general classes of proteins: those that regulate septin assembly and function and those whose function is regulated by virtue of their septin interactions. We therefore examined the effects on chlamydomospore development of deleting the genes encoding one member from each class.

In S. cerevisiae, localization of the enzymes involved in chitin ring synthesis during budding, Chs3p and Chs4p, depends on septin ring integrity (11). Chs3p and Chs4p are tethered to the septin ring by the septin-binding protein Bni4p. Deletion of BNI4 in S. cerevisiae results in mislocalized chitin deposition and abnormal morphology of bud scars (11). Interestingly, complete deletion of BNI4 in C. albicans did not cause any discernible effect during chlamydomospore development (Fig. 8 and data not shown). Budding and hyphal growth were also not dramatically affected. bni4Δ cells stained with calcofluor white showed relatively normal chitin ring deposition and septa. The most apparent phenotype was that about 53% (n = 873) of the cells showed abnormal protrusions at previous division sites similar to what was seen in S. cerevisiae bni4Δ strains (11). While this report was in preparation, a partial BNI4 deletion mutant was reported to cause a similar defect in bud morphogenesis and a defect in hyphal morphogenesis on certain solid media (52). Since CaBni4p contained only a low level of overall similarity with ScBni4p, we constructed a C. albicans strain producing a BNI4-yellow fluorescent protein fusion protein and confirmed that CaBni4p localized primarily to a ring at the mother cell side of the bud neck, similar to the S. cerevisiae ortholog (data not shown).

In S. cerevisiae, proper septin ring assembly requires the protein kinase Cla4p (23, 53, 63). Deletion of CLA4 in S. cerevisiae results in abnormal bud morphology, septin mislocalization, and ectopic septum production. In C. albicans, deletion of CLA4 caused abnormal bud morphogenesis, a defect in hyphal morphogenesis, and reduced virulence in a mouse model of systemic infection (38). Chlamydomospore production was significantly impaired in the cla4Δ strain, and chlamydomospores were only rarely detected. When chlamydomospores were produced, they formed at the end of truncated filaments (Fig. 8). In addition, morphology was abnormal in cla4Δ strains, with many suspensor cells showing a bulge in the center and a higher degree of branching filaments than were observed for the wild type. However, because Cla4p regulates many aspects of morphogenesis, the defects in chlamydomospore morphology seen in cla4Δ strains cannot be attributed solely to a failure in septin regulation.

**DISCUSSION**

Regulation of chlamydomospore morphogenesis. Chlamydomospore development was investigated to better understand how the different morphogenesis programs are controlled in C. albicans. The first stage of C. albicans chlamydomospore development, the transition from budding to a filamentous pattern of morphogenesis, was strongly influenced by temperature. Incubation of cells on chlamydomospore-inducing medium (corn-meal-Tween agar) at both 23°C and 37°C induced filamentous growth, but the filamentous growth observed at 23°C was thicker in diameter and not affected by the quorum-sensing factor farnesol. In contrast, farnesol inhibited filamentous growth at 37°C on cornmeal-Tween agar (Fig. 1 and 2), similar to the way it inhibits hyphal growth at 37°C (30). Interestingly, other studies have shown that the Efg1 transcription factor stimulates hyphal growth at 37°C but is not needed for the filamentous growth response when cells are embedded within an agar matrix or grown under chlamydomospore-inducing conditions at 23°C (20, 40, 58, 59). The temperature dependency of chlamydomospore development suggested a possible relationship to white-opaque switching, which is promoted at 23°C and accounts for the increased mating efficiency of C. albicans at 23°C (46, 57). However, we did not detect a significant difference in chlamydomospore formation by opaque cells, and we did not detect activation of opaque-specific gene expression during chlamydomospore development (data not shown).

The next stage of development, in which suspensor cells form off the filament and then produce a chlamydomospore, was enhanced in the presence of farnesol (Fig. 1 and 2). Farnesol increased the number of chlamydomospores per filament but not the number of filaments, suggesting that the effects of farnesol on chlamydomospore production occur later in development. Consistent with this, several mutants, including efg1Δ, hog1Δ, and isi2Δ, that fail to produce chlamydomospores were reported to be hyperfilamentous under chlamydomospore-inducing conditions (4, 49, 58). Thus, induction of the filamentous growth response is regulated differentially from chlamydomospore development.

The effects of farnesol also indicate that quorum sensing can have a positive role in stimulating morphological transitions, as well as the previously observed inhibitory effects on hyphal growth. The mechanism by which farnesol promotes chlamydomospore development is unknown, but several lines of evidence suggest that oxidative stress is involved. First, cells lacking the Hog1p mitogen-activated protein kinase are defective in responding to reactive oxygen species and are also unable to form chlamydomospores (4). Interestingly, farnesol may act through Hog1p, since it was recently reported that Chk1p, a two-component system histidine kinase that lies upstream of Hog1p, is required to observe the effects of farnesol on hyphal growth (35) and that farnesol stimulates phosphorylation of Hog1p (56). Farnesol may act directly on Chk1p, or it could act indirectly by stimulating reactive oxygen species. In S. cerevisiae, the addition of exogenous farnesol inhibits the mitochondrial electron transport chain and results in the production of reactive oxygen species (43, 44). Consistent with a role for mitochondrial function in creating reactive oxygen species, suv3 mutants, which are defective in mitochondrial biogenesis, are also defective in chlamydomospore production (49).

The effects of farnesol and reactive oxygen species on chlamydomospore development have several implications for the regulation of C. albicans morphogenesis in human hosts. For one, neutrophils attack C. albicans by releasing reactive oxygen species, suggesting that C. albicans may respond in part by inducing thick-walled chlamydomospores that are more resistant. It may also be significant that cyclophosphamide, a therapeutically used immunosuppressive agent, induces reactive oxygen species (13) and stimulates chlamydomospore production (9).
addition, treatment of \textit{C. albicans} with azole drugs that inhibit ergosterol synthesis increased farnesol production (31). Thus, therapeutic intervention may alter \textit{Candida} morphogenesis in ways that could facilitate colonization or infection of patients. \textit{Candida} morphogenesis may also be altered in mixed infections with \textit{Pseudomonas aeruginosa}, which produces a bacterial quorum-sensing factor that is structurally similar to farnesol and also has the ability to inhibit hyphal formation (29). Since \textit{P. aeruginosa} kills hyphal cells by attaching and forming a biofilm, but does not kill budding cells, the response of \textit{C. albicans} cells to the \textit{P. aeruginosa} quorum factor could play a role in their escape from the killing action of \textit{P. aeruginosa} in mixed infections (28). Thus, although chlamydospore formation is not observed efficiently at 37°C in vitro, it could occur in specialized host niches similar to what is thought to occur for \textit{C. albicans} mating, which is also not efficient at 37°C in vitro yet is readily detected in infected mice (32, 45).

\textbf{FIG. 8.} Phenotypes produced by \textit{bni4Δ} and \textit{cla4Δ} strains. (Wild type (DAY185) (A to D), \textit{bni4Δ} (YSM45) (E to H), and \textit{cla4Δ} (YSM47) (I to L) strains were analyzed. (A, E and I) Colony morphology of strains grown on cornmeal-Tween agar and photographed through a 20× lens after 4 to 5 days. (B, F, and J) Differential interference contrast images. (C, G, and K) Calcofluor white staining of chlamydospores. (D, H, and L) Calcofluor white staining of budding cells grown to mid-logarithmic phase in YPD medium plus uridine. Bar, 10 μm (B to D, F to H, and J to L). Inset in panel I shows an enlarged view of short filaments produced by the \textit{cla4Δ} mutant (indicated by arrows).

\textit{C. albicans} cells might have an additional role in their escape from the killing action of \textit{P. aeruginosa} in mixed infections (28). Thus, although chlamydospore formation is not observed efficiently at 37°C in vitro, it could occur in specialized host niches similar to what is thought to occur for \textit{C. albicans} mating, which is also not efficient at 37°C in vitro yet is readily detected in infected mice (32, 45).

\textbf{Nuclear division during chlamydospore development.} A novel pattern of nuclear division was observed during chlamydospore morphogenesis in \textit{C. albicans} (Fig. 4). Nuclear division...
occurred within the suspensor cell itself, and then one of the daughter nuclei subsequently moved from the suspensor cell into the immature chlamydospore. This contrasts with budding cells of *S. cerevisiae* and *C. albicans* in which the nucleus moves to a position adjacent to the bud neck and then divides across the neck region between the mother cell and bud (26). Similarly, during hyphal morphogenesis in *C. albicans*, the nucleus migrates from the mother cell to the future site of septum formation and divides across the developing septum (5, 39). Thus, the pattern of nuclear division seen in suspensor cells represents a dramatic departure from the nuclear dynamics observed in either budding or hyphal cells and suggests that suspensor cells and chlamydospores are subject to different cell cycle control. Following maturation, a single nucleus was detected inside of each mature chlamydospore.

Chlamydospores formed by wild-type cells never showed any evidence, based on DNA staining, to indicate that these cells were undergoing meiosis. Multinucleate chlamydospores and suspensor cells were observed in the *cdc11Δ* strain. However, this result is consistent with previous observations of multinucleate cells in septin mutant strains of *S. cerevisiae* and *C. albicans*. In *S. cerevisiae*, microtubule capture by the septin ring is necessary for proper nuclear partitioning between mother and daughter cells (37), and in *C. albicans* microtubule localization is defective in septin mutant strains (65).

The altered pattern of nuclear division observed in *C. albicans* suspensor cells shares some similarities to more distantly related fungi. For example, mating in some basidiomycetes (e.g., mushrooms), such as *Coprinus cinereus*, results in the formation of a heterokaryon in which the incoming nucleus migrates to a hyphal tip and then through a specialized structure known as a clamp connection to ensure that heterokaryon status is maintained (6). In addition, in some filamentous fungi, such as *Aspergillus nidulans*, germinating spores undergo several rounds of nuclear division before septum formation separates the hyphae into distinct compartments. Nuclear mitoses occur in the hyphal tip compartment in the absence of cytokinesis, producing cell compartments with up to three nuclei (25, 48).

**Septin function in chlamydospores.** At early stages, a septin ring was detected at the suspensor cell-chlamydospore junction that appeared to be similar to the rings that form at bud necks. However, at later stages the ring on the chlamydospore side disappeared, and instead the septins were detected as an array of filaments that extended along the periphery of the chlamydospores. These filaments were difficult to detect in cells carrying a GFP-tagged septin gene under control of its own promoter but were readily detected in cells overexpressing *CDC10-GFP* from the *ADH* promoter. The filaments were only detected in chlamydospores and were not detected in budding or hyphal cells (67). The septin filaments observed in chlamydospores are reminiscent of the septin bars associated with the prospore membrane during sporulation in *S. cerevisiae* (12, 14, 51, 62). These bar-like structures are thought to play a role in guiding development of the specialized cell wall that forms around spores (62). The specific role of septins is unclear since septin mutations caused only mild effects on spore morphogenesis and on the localization of other septin proteins. Nonetheless, these data suggest that the septin filaments inside the chlamydospores may function in an analogous manner to promote the formation of the thick, three-layered chlamydospore wall. This is also supported by the observation that septins are most readily detected in mature chlamydospores at the time at which cell wall thickening occurs (Fig. 5).

The septins were required for septation in chlamydospores, since the *cdc11Δ* mutant displayed obvious defects in forming a septum at the neck of chlamydospores. The defects observed in the *cdc11Δ* mutant may be due in part to the larger diameter of the septin rings at the suspensor-chlamydospore junction relative to septin rings in budding cells (1.91 ± 0.22 μm versus 1.34 ± 0.15 μm).

**Model of chlamydospore development.** A model for chlamydospore development is shown in Fig. 9. Chlamydospores are produced from the tips of suspensor cells, where a septin

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**FIG. 9. Model of chlamydospore development.** The figures show a cartoon of the stages of a chlamydospore developing on top of a suspensor cell. Nuclei are shown in blue and septins are shown in red. Note that in contrast to budding, nuclear division occurs within the suspensor cell and then one nucleus migrates into the chlamydospore. Stages of development are as described in the legend of Fig. 4. See Discussion for additional details.
collar defines the suspensor-chlamydospore boundary (Fig. 9, stages 1 to 2). As the chlamydospore enlarges, nuclear division occurs within suspensor cells, and one daughter nucleus subsequently migrates into the immature chlamydospore (Fig. 9, stages 3 to 4). During maturation, a septum separates the chlamydospore from the suspensor cell, and septin filaments are elaborated throughout the chlamydospore (Fig. 9, stage 5). At maturity chlamydospores develop thick, three-layered cell walls (Fig. 9, stage 6). Chlamydospore development in C. albicans therefore represents a program of cellular differentiation that is distinct from bud or hyphal morphogenesis. Further analysis will elucidate the biological role of chlamydospores and the mechanisms governing their development.

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