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Effects of the Myosin ATPase Inhibitor, 2,3-Butanedione-2-Monoxime, on Growth and Dimorphic Switches of *Candida albicans*

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Abstract Dimorphic yeast *Candida albicans* reversibly switches between the form of yeast and hyphae depending on external conditions. We investigated possible roles of the myosin family in the growth and dimorphic switches of *C. albicans* with a general myosin ATPase inhibitor, 2,3-butanedione-2-monoxime (BDM). Transition to hyphae as well as proliferation by budding was completely inhibited by BDM at 16 mM. Presence of 16 mM BDM did not affect hyphae-to-bud transition but it blocked budding. The effects of BDM on yeast growth and dimorphic switches were reversible. More than 70% of the BDM-treated cells demonstrated defects in the amount and the polarized localization of F-actin as well as in the shape and migration of the nucleus, suggesting that myosin activities are needed in these cellular processes of *C. albicans*.

Key words: *Candida albicans*, BDM, dimorphic switch, myosin, polarized localization of F-actin

The dimorphic yeast *Candida albicans* grows by budding or filamentous hyphae, depending on the external conditions such as temperature, pH, or nutrient composition of the medium [26]. Reversible transformations to filamentous hyphae have been postulated to contribute to colonization and dissemination within the host tissues, and thereby to promote the pathogenicity of this particular fungus [8]. However, the mechanism of the dimorphic switch is difficult to elucidate, since *C. albicans* has no known sexual cycle and is known to be at least diploid [26]. Recently, the mechanisms of the dimorphic switch in *C. albicans* have been studied with its homologous genes for pseudo-hyphal growth in the budding yeast *Saccharomyces cerevisiae*. The gene products of *CPH1*, *HST7*, and *CST20* are the *C. albicans* homologues of the *S. cerevisiae* *STE12*, *STE7*, and *STE20* products that are required for filamentous growth [18]. *TUP1*, which encodes a transcriptional repressor, also controls filament formation in *C. albicans* [5].

Cytoskeleton regulates polarized cell growth and morphogenesis in all eukaryotes including fungi. Actin has been known to be the most important regulator for the polarized growth and the morphological transition in yeast and in filamentous fungi [14, 27]. In both yeast and filamentous fungi, F-actin accumulates at the growing tips and in the septa [1, 14]. Microtubules, another well-characterized component of the cytoskeleton, also play an important role in the polarized growth of yeast and filamentous fungi by interacting with the actin cytoskeleton, although the mechanism has not been elucidated [7, 22]. Studies of the role of cytoskeleton in *C. albicans* with specific anti-actin and anti-microtubule drugs indicate that both actin and microtubule cytoskeletons are necessary for the control of polarity in hyphal growth [2, 29].

Many cellular functions mediated by actin need actin filament motor proteins such as the myosin family. In the budding yeast *S. cerevisiae*, five myosins are present and they are as follows: *MYO3* and *MYO5* (class I) have been implicated in endocytosis, *MYO2* and *MYO4* (class V) in polarized growth and asymmetry between mother and daughter, and the conventional myosin *MYO1* (class II) in cytokinesis [6]. In *C. albicans*, partial sequences of the three classes of putative myosin genes have been reported by the genome sequence efforts and the myosin immuno-analogue has been detected with the antibody generated against the vertebrate muscle myosin [11]. However, the functions of the myosin family have rarely been characterized in *C. albicans*.

2,3-Butanedione-2-monoxime (BDM) is a well-characterized inhibitor of myosin ATPase activity that reversibly represses the actin-myosin interaction [14, 15, 28]. In this study, we examined possible roles of the myosin family proteins in

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the growth and dimorphic switches of *C. albicans* with BDM, a general myosin inhibitor.

MATERIALS AND METHODS

Culture

C. albicans strains CAI4 [10] and ATCC 10231 were used in this study. These strains grow by budding at 25°C and form filamentous hyphae at 37°C, in both YPD [1% yeast extract (w/v), 1% polypeptone (w/v), 2% glucose (w/v)] and Spider [1% nutrient broth (w/v), 0.2% K_2HPO_4 (w/v), 1% indicated sugars (w/v)] media. Less than 30 min after the temperature shift, more than 95% of the cells generated germ tubes (data not shown). The yeast form was cultured in YPD medium at 25°C from a fresh single colony on YPD agar. For the transition to hyphal growth, fresh *C. albicans* culture incubated at 25°C for bud growth then diluted with fresh YPD or Spider medium, then transferred to 37°C, and incubated for 3 h. No difference in the efficiency of hyphal transition was observed in different media. Transition from hyphal growth to bud formation was induced by shifting the same number of yeast cells incubated for 4 h at 37°C back to 25°C.

BDM Treatment

A 5 M stock solution of BDM (2,3-butanedione-2-monoxime, Sigma) was prepared in DMSO at 50°C and stored in the dark at 4°C. The 5 M BDM stock solution was diluted directly into the medium depending on the desired concentrations of BDM. DMSO, the solvent for BDM, was added to BDM-free samples to check the solvent effect, and was found to have no effect on yeast growth or hyphae formation. The effects of BDM on hyphae formation were examined by counting cells and assorted by their morphologies. The effects of BDM on cell proliferation by budding were measured by counting the cell number at every 30 min and by OD_{600} at every hour for a total of 18 h in the presence or absence of 16 mM BDM. The reversibility of BDM effect was examined by washing BDM-treated cells with double distilled H_2O three times followed by incubation in a BDM-free medium for 24 h at each growth temperature. The reversible effects of BDM on hyphal growth were plotted as hyphal growth rates. Total cells and the cells in hyphal growth were counted at every 30 min after the removal of BDM, and hyphal growth rates were calculated by dividing the cells in hyphal growth by total cells. To analyze the reversible effects of BDM on yeast growth, cell growth rate was calculated by counting the cells at every 30 min after washing out BDM and by dividing the cell number at each given time by the initial cell number.

Fluorescence Microscopy

Cells were harvested for 1 h at each growth temperature and fixed by incubating in a fixing solution (5% DMSO and 4% formaldehyde in PBS, pH 8.0). To stain F-actin, fixed cells were washed twice with 5% DMSO in PBS (pH 8.0), permeabilized with PBS containing 1% Triton-X 100 (pH 8.0), and resuspended in PBS (pH 8.0). Fixed and permeabilized cells were stained with 5 μg/ml rhodamine-conjugated phalloidin (Sigma) for 30 min at room temperature. Cells with no F-actin dots or fewer F-actin dots were counted. To visualize nuclei, fixed cells were stained with 1 μg/ml DAPI (4',6'-diamidino-2-phenylindole). Stained cells were examined using an Axioscope (Zeiss) with ×100 objective. Photographs were taken with the T-max 400 (Kodak, Rochester, U.S.A.) and the negatives were scanned by using the Proimager 8200 (Pixelcraft).

Detection of Actin by Immunoblotting

Cells were cultured and treated with BDM as described above. *C. albicans* cell extracts were prepared essentially by following the protocols for *S. cerevisiae* described by Sherman *et al.* [17, 23]. Briefly, 2×10^8 cells were harvested by centrifugation and washed with lysis buffer containing 10% glycerol, 20 mM HEPES, pH 7.9, 10 mM EDTA, 1 mM DTT, phosphatase inhibitors (1 mM NaN_3, 1 mM NaF), and protease inhibitors (1 mM PMSF, 10 μg/ml each aprotinin, antipain, chymostatin, leupeptin, and pepstatin). Cells were lyzed in 300 μl lysis buffer by beadbeating in 1 ml of glass beads (Biospec Products, Inc., U.S.A.), and the extracts were collected. Protein concentrations of the extracts were determined by the dye-binding assay (Pierce, U.S.A.). Equivalent amount of proteins for each sample was analyzed by 8% SDS-PAGE, and blotted onto nitrocellulose membranes. Actin was detected by anti-actin monoclonal antibody (Sigma, U.S.A.) followed by anti-mouse IgG-HRP (Jackson Lab., U.S.A.) and by ECL (Amersham, U.S.A.).

RESULTS AND DISCUSSIONS

Effects of BDM on Dimorphic Switches of *C. albicans*

We investigated possible roles of the myosin family in dimorphic switches of *C. albicans* using a general myosin ATPase inhibitor, 2,3-butanedione-2-monoxime (BDM), as a probe for the myosin function. *C. albicans* cells incubated at 25°C for bud growth were transferred to 37°C in the presence of different concentrations of BDM. Treatment of cells with 20 mM or higher concentrations of BDM was lethal. Germ tube formation and its growth were severely inhibited at 16 mM or at higher concentrations of BDM. About 66% of cells did not form any germ tube and only 1.0% of cells formed hyphae in the presence of 16 mM BDM (Fig. 1A). When the cells were washed to remove BDM and re-incubated at 37°C, they resumed their hyphal growth, which suggests that the effects of BDM on hyphal growth are reversible (Fig. 1B). Nuclear shape was

A

BDM conc.	Shape (%)		
	No germ tube	Small germ tube	Hyphae
BDM-free	11.1±0.66	44.2±1.86	44.7±2.17
BDM-16 mM	63.1±1.17	33.5±0.95	1.0±0.25

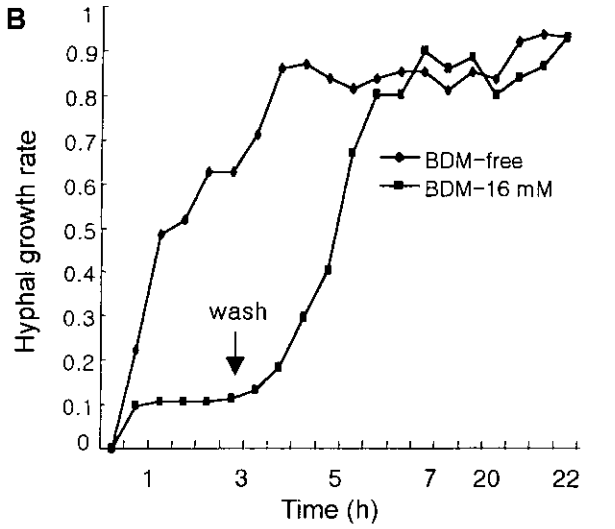


Fig. 1. BDM inhibits the hyphae formation of *C. albicans*.
Hyphae formation was induced in *C. albicans* CAI4 as described in Materials and Methods in the presence or absence of 16 mM BDM. (A) The effects of BDM on the hyphae formation of *C. albicans*. Cells were categorized and counted depending on their morphologies after incubating for 3 h with BDM. The average of three independent experiments was presented. Total of 969 cells were counted for BDM-free and 986 cells for 16 mM BDM-treated. (B) The reversible effects of BDM on the hyphae formation of *C. albicans*. *C. albicans* CAI4 cells treated with 16 mM BDM for 3 h were washed and incubated for 24 h in BDM-free YPD. The arrow represents the time at which BDM was removed. Cells were collected and counted every 30 mins. The cells in hyphal growth divided by the total cells were plotted as the hyphal growth rate, (◆) for BDM-free and (■) for 16 mM BDM-treated cells. (C) F-actin distribution in BDM-treated *C. albicans* cells. (a) BDM-free cells and (b) 16 mM BDM-treated cells were collected, fixed, and stained with DAPI and rhodamine-conjugated phalloidin to visualize nucleus and F-actin, respectively. For (a) and (b), the top panels show DNA by DAPI staining and the bottom panels show F-actin staining. Bar denotes 5 μm and applies to all images.

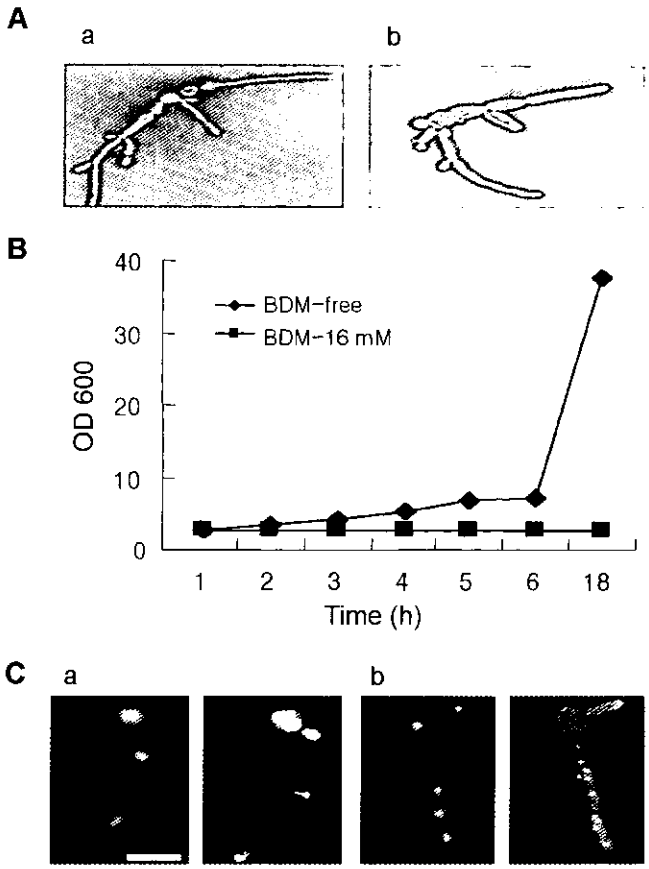


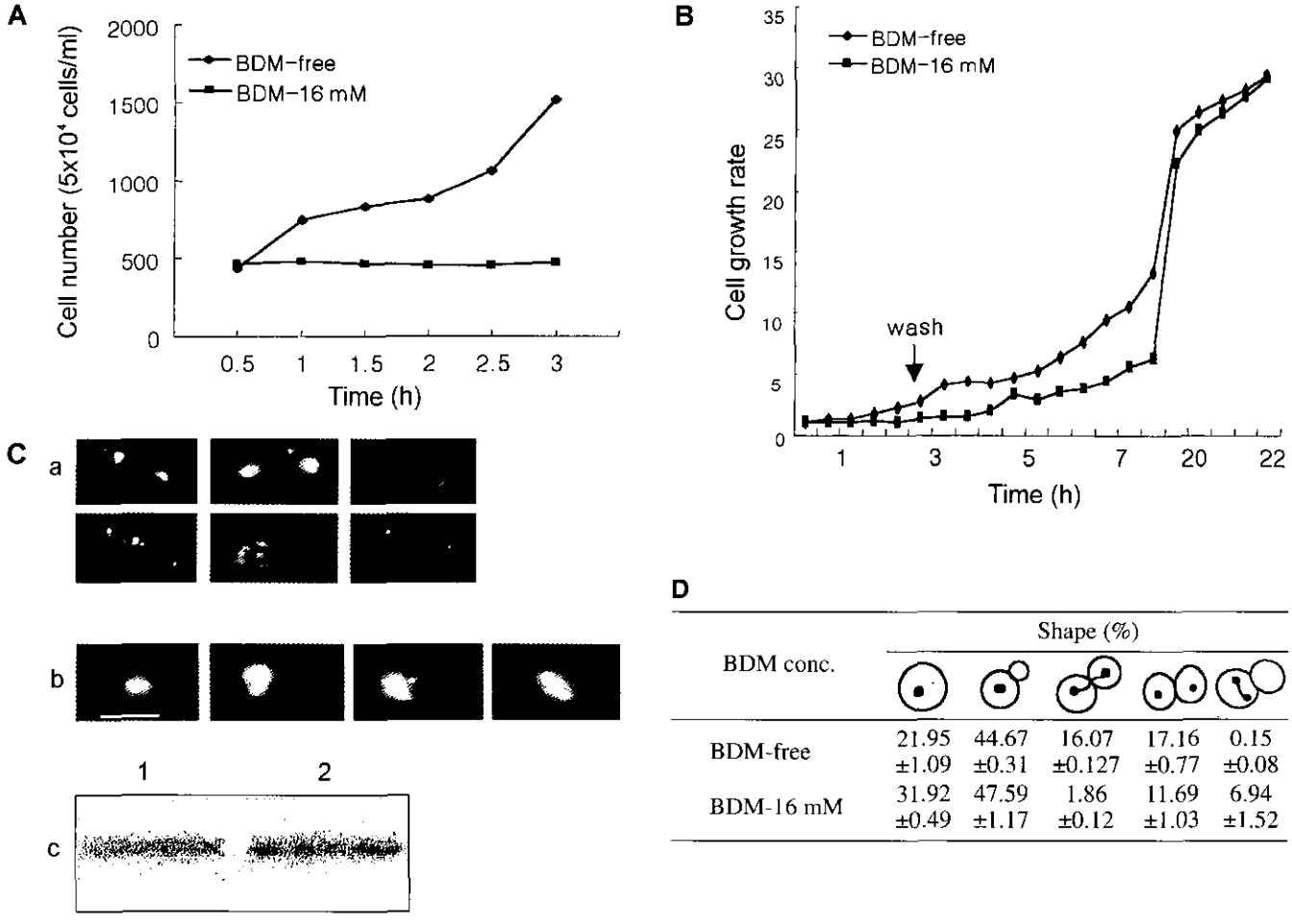
Fig. 2. BDM does not inhibit hyphae-to-bud transition but it blocks budding in *C. albicans*.
Hyphae-to-bud transition of *C. albicans* CAI4 was induced in the presence or absence of 16 mM BDM as described in Materials and Methods. (A) Phase-contrast images of (a) BDM-free and (b) 16 mM BDM-treated cells. (B) Cell proliferation by budding was measured by OD_{600} at every hour for 18 h incubated in the presence (■) or absence (◆) of 16 mM BDM. (C) F-actin localization in (a) BDM-free and (b) 16 mM BDM-treated *C. albicans* cells. For (a) and (b), the left panels show DNA and the right panels show F-actin staining. Bar denotes 5 μm and applies to (A) and (C).

also affected by BDM, adapting a round to a deformed shape (Fig. 1C-b). The same effects of BDM on hyphal transition were observed in both *C. albicans* strains used (CAI4 and ATCC 10231), indicating that BDM affects hyphal transition of *C. albicans* in general and not in a strain-specific manner.

We also investigated the effects of BDM on hyphae-to-bud transition of *C. albicans*. As described in Materials and Methods, the same number of yeast cells grown at 25°C was transferred to 37°C and allowed to grow for 4 h to generate hyphae, and then were shifted back to 25°C to induce bud formation in the presence of 16 mM BDM. Regardless of the presence of BDM, more than 96.3% of hyphae cells started budding at the tip and in the septa of the hyphae, as shown in Fig. 2A. However, the presence of BDM inhibited cell proliferation by budding after the transition to bud growth, when OD_{600} was measured every

hour for 18 h after the shift to 25°C, in the presence or absence of 16 mM BDM (Fig. 2B).

Distribution of F-actin in morphological transitions was examined in the BDM-treated cells with rhodamine-conjugated phalloidin (Rh-Ph), which specifically stains F-actin. Without BDM treatment, the cells in hyphal growth showed condensed dots at the growing tips and filamentous actin fibers in the germ tubes (Fig. 1C-a) [29]. Regardless of the arrested morphology, 71.1% of the cells with hyphae transition which was induced by 16 mM BDM contained fewer F-actin dots and lost their polarized localization as well. No F-actin dots were detected in 23.3% of these cells (Fig. 1C-b). F-actin dots should be at the buds or in the bud-neck of the cells in which yeast growth is induced from hyphae (Fig. 2C-a). However, 93.2% of the cells, in which hyphae-to-bud transition was induced in the presence of 16 mM BDM, carried fewer F-actin dots that were not properly localized (data not shown, Fig. 2C-b). The effects of BDM on decrease or loss of polarized localization of F-actin in the dimorphic switches of *C. albicans* suggest that myosins function not only in polymerization but also in polarized localization of F-actin as well. Fewer F-actin dots in BDM-treated cells that underwent morphological transitions imply that BDM might block the assembly of F-actin. Actin protein was present both in the BDM-treated cells and in untreated



D

BDM conc.	Shape (%)				
BDM-free	21.95 ±1.09	44.67 ±0.31	16.07 ±0.127	17.16 ±0.77	0.15 ±0.08
BDM-16 mM	31.92 ±0.49	47.59 ±1.17	1.86 ±0.12	11.69 ±1.03	6.94 ±1.52

Fig. 3. BDM inhibits the yeast growth of *C. albicans*.

(A) BDM inhibits the yeast growth of *C. albicans*. *C. albicans* CAI4 cells were incubated in the presence (■) or absence (◆) of 16 mM BDM for 3 h at 25°C. Cells were collected and counted every 30 min. (B) The reversible effects of BDM on the yeast growth of *C. albicans*. Cells treated with 16 mM BDM for 3 h were washed and incubated for 20 or more hrs in BDM-free YPD. The arrow represents the time at which BDM was removed. Cells were counted every 30 min and cell growth rate was calculated by dividing the cell number at each given time by the initial cell number. (C) The F-actin localizations and nuclear morphologies of the BDM-treated *C. albicans* cells in yeast growth. Top panels of (a) and (b) panels show DAPI staining and bottom panels of (a) show rhodamine-conjugated phalloidin staining. The left panels show BDM-free and other panels show 16 mM BDM-treated cells. Bar, 5 μm. (c) Both in BDM-free cells (lane 1) and in 16 mM BDM-treated cells (lane 2), the presence of actin is shown by immuno-blotting as described in Materials and Methods. (D) The distribution of BDM-treated *C. albicans* cells in the cell cycle stages of yeast growth. CAI4 cells in yeast growth were treated with BDM for 3 h, fixed, and stained with DAPI to visualize the nucleus. This analysis was repeated three times and the average was presented. 1,294 of BDM-free and 1,181 of 16 mM BDM-treated DAPI-stained cells were counted and categorized by cell cycle stages. The cell cycle stage of each cell was determined by its morphology, as depicted on the top of the table.

cells (data not shown), verifying that BDM blocks proper actin polymerization and localization and does not affect other cellular processes such as actin synthesis. Since myosin is involved in protein and vesicle transport, one explanation of fewer F-actin could be reduced transport of actin monomers for proper F-actin formation in the BDM-treated cells. Alternatively, it is also possible that F-actin assembly was directly blocked by BDM. Previous reports that the myosin subfragment induced assembly of F-actin and that BDM suppressed myofibrillogenesis in skeletal muscle cells in culture support the latter possibility [9, 25], but we can not exclude the possibilities of both influences together.

Our findings that the myosin inhibitor BDM decreased the polarized localization of F-actin in the dimorphic switches of *C. albicans* also suggest that actin polarity for polarized growth was maintained by myosins. In the budding yeast *S. cerevisiae*, the class V myosins, Myo2 and Myo4, have been proven to localize at the growing tips [6]. The results of other investigators suggest that Myo2 is involved in the delivery of vesicles to the growing tips and that Myo4 functions in the asymmetric localization of specific mRNAs to regulate polarized growth [3, 12, 16]. Similar myosin functions can be easily deduced in the polarized growth of buds and germ tubes in *C. albicans*. Our results in *C. albicans* are consistent with the reported myosin functions in the polarized growth of *S. cerevisiae*. Cloning and the knock-out of each myosin gene in *C. albicans* will help to elucidate each function of myosins in polarized growth and morphological transitions.

Effects of BDM on the Yeast Growth of *C. albicans*

To understand the effects of BDM on yeast growth, we applied several different concentrations of BDM to *C. albicans* at 25°C. Cells did not proliferate at all in the presence of 16 mM BDM, while cells actively duplicated and doubled their numbers approximately every 70 min in the absence of BDM (Fig. 3A). After BDM was removed, these cells resumed cell cycle and recovered their normal growth rate (Fig. 3B), suggesting that the effects of BDM on the yeast growth of *C. albicans* were reversible.

When polarized localization of F-actin dots in the BDM-treated yeast cells were examined, few F-actin dots were observed in 99% of the cells in which we could observe F-actin, as previously described by the effects of BDM on morphological transitions (Fig. 1C-b). Actin dots were dispersed in the cells arrested in bud growth (data not shown, Fig. 3C-a right panels) and they were not concentrated in the bud neck for cytokinesis as in BDM untreated cells, shown in the left panel of Fig. 3C-a. Presence of actin protein was verified both in the BDM-treated and in untreated cells, as shown by immunoblotting in Fig. 3C-c, indicating that BDM blocks only proper actin polymerization and localization.

The BDM-treated cells also showed defects in nuclear shape as revealed by DAPI staining, indicating that nuclear structures were disorganized in the presence of BDM. Most cells contained nuclei with a fuzzy boundary or a sickle shape, and small DAPI stained fragments were also observed (Fig. 3C-b). Proper localization and migration of the nucleus during the cell cycle were also affected by BDM treatment. Usually, nucleus is present in the middle of the cell or in the vicinity of bud neck depending on the stages of the cell cycle. However, 87% of the BDM-treated cells nuclei were at the edge of the cell and it did not migrate to the neck of a fully-grown bud (Fig. 3C-b). Among the BDM-treated cells, 6.9% of the cells were arrested with the divided nuclei, in which both nuclei were present in the mother cell (Fig. 3D, data not shown). These observations imply that the myosin proteins are needed for proper shape, localization, and migration of the nucleus in *C. albicans*. The functions of F-actin along with cytoplasmic microtubules have been reported to be of proper orientation of mitotic spindle and nuclear positioning in *S. cerevisiae* [4, 20, 21]. Disruption of actin cytoskeleton with cytochalasin D has been shown to produce deformation of nuclear shape in the chondrocyte [13], and cytochalasin J which inhibits actin cytoskeleton has been shown to affect both chromosome congression and spindle microtubule organization at prometaphase in PtK1 cells [24]. These studies suggest the involvement of the actin cytoskeleton in an appropriate nuclear shape or chromosome organization. Our observation that a blockade of myosin functions by BDM induced disorganized nuclear structures and migrations suggests the roles of F-actin and myosin proteins in regulating the proper shape and migration of the nucleus in these processes.

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