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DYNAMICS OF CELL COMPONENTS DURING BUDDING OF *CRYPTOCOCCUS ALBIDUS* YEAST CELLS

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Phase-contrast and freeze-substitution microscopy were used to study cellular division of the pathogenic yeast Cryptococcus albidus in vitro. It has been shown that the mother and daughter cells (during exponential growth) varied in vacuolar contents and there were little storage compounds in the cytosol. Ultrastructural signs of mother cells for transitioning to bud formation were a migration of the nucleus from distal to lateral, increasing the level of chromatization and nucleolus sizes, proliferation of mitochondria and changes in topography including formation of a «sheath» around the nuclear envelope. Specific feature of cellular division in cultures was permanent nucleolus presence. For the first time, we have described the nucleolus material being uniformly distributed between mother and daughter cells during cellular division. The septum formation of separating the daughter cells have been investigated in detail.

Key words: cell components dynamic, cellular division, *Cryptococcus albidus,* exponential growth phase, freeze-substitution, ultrastructure

ДИНАМИКА КЛЕТОЧНЫХ КОМПОНЕНТОВ В ХОДЕ ПОЧКОВАНИЯ ДРОЖЖЕВЫХ КЛЕТОК *CRYPTOCOCCUS ALBIDUS*

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Методы фазового контраста и замораживания-замещения использовали для изучения деления выращенных in vitro клеток патогенного гриба Cryptococcus albidus. Показано, что характерной чертой строения материнской и дочерней клетки (в экспоненциальной стадии роста) было варьирование содержимого вакуолей и отсутствие запасных веществ в цитозоле. Ультраструктурными признаками перехода материнской клетки к формированию почки были: миграция ее ядра из базальной части клетки в латеральную, возрастание уровня его хроматизации, а также увеличение размеров ядрышка, пролиферация митохондрий и изменение их топографии с формированием «футляра» вокруг ядра. Характерной особенностью деления клеток культуры было постоянное присутствие ядрышка. Впервые показано, что в ходе деления клеток C. albidus материал ядрышка равномерно распределяется между материнской и дочерней клетками. Детально описано формирование отделительной септы между материнской и дочерней клетками.

Ключевые слова: деление клеток, динамика компонентов клетки, замораживание-замещение, *Cryptococcus albidus*, ультраструктура, экспоненциальная фаза роста

INTRODUCTION

During the last 40 years, infection caused by *Cryptococcus albidus* was considerably increased [1]. *C. albidus* is asexual (anamorphic) saprophyte pathogenic fungus, which wide-spread in soil. This fungus together with another yeast species was the member of soil microbiota associated with truffle [2]. *C. albidus* was isolated from the outdoor and indoor air [3], water, wine, plants leaves and food [4, Саттон В. и др., 2001]. This fungus causes the genital infection [Codazza D., et al, 1973] and keratitis in horse [Desbrosse A.M., 1996], systemic infection in dogs [3] and cats [5]. It was revealed in patients with tubercular lungs [Fonseca A., et al., 2000], AIDS [Kordossis T., et al., 1998], leukemia [6] and lymphoma [7]. *C. albidus* has caused the cutaneous infection [8], osteomyelitis [Vasilyeva N.V., et al., 1999], meningitis [Melo J.C., et al., 1980], pulmonary cryptococcosis [Krumholz R.A., 1972], genital cryptococcosis in women [Batista A.C., et al., 1960] and revealed in renal transplant recipient [9]. There are fragmentary data about ultrastructure of mature *C. albidus* yeast mother cells in literature [Brown T.A., et al., 1977]. The peculiarity of the budding process with special attention to cell components transition and nucleolus fate of this fungal species has not been studied before, which was the main goal of this research.

MATERIALS AND METHODS

Preparation of cultures. We have investigated the *C. albidus* cells of the strain 5763 from Culture Collection of the Research Center of Pathogenic Fungi (Chiba University, Japan) which cultivated for 24 hour in YPD medium (1% (w/v) yeast extract, 2% (w/v) bactopepton and 2% (w/v) glucose on a shaker at 30 °C.

Preparation for phase-contrast microscopy. To determinate the morphological characteristics of cultures we made the temporary preparation of living cells and have investigated them under the phase-contrast microscope (Olympus BH-2RFCA).

Preparation for transmission electron microscopy. The cells in exponential growth phase were collected

by centrifugation and sandwiched between two copper grids. The samples were then freeze-substituted in 2% osmium tetroxide/aceton at –80 °C for 48 hour and embedded in epoxy resin according the method described before [Kopecká M., et al., 2000]. Ultrathin sections (70 nm thick) were cut with diamond knife and stained with uranyl acetate and lead citrate. Then sections were covered with Super support films (Nisshin EM, Tokyo, Japan) and observed with JEM-1400 (JEOL, Tokyo, Japan) transmission electron microscope.

RESULTS AND DISCUSSION

Pictures in the phase-contrast microscope. *C. albidus* yeast cells (or blastoconidia) in exponential phase of growth had spherical or ellipsoid shape (3.5-8.0 x 5.5-9.2 μm in diameter) and showed different developmental stages of budding (Fig. 1a).

The hyphae and pseudo-hyphae were not typical for this fungus [4]. Figure 1 b shows the general view in vitro growing cells at different developmental stages in the transmission electron microscope.

Mother cells before budding. The most volume of mother cell was occupied by both the single nucleus and vacuole. Spherical nucleus (at the average 2.0 μm) localized near wall in the basal part of cell and opposite the budding scar (Fig. 1 c, d). The nucleus had a typical lower level of uniformly distributed condensed chromatin which is probably consistent with low number of chromosome typical for fungal species. On outer nucleus membranes numerous dark evently dispersed ribosomes were present. In nucleoplasm single excentrically localized small (average 0.8 μm in diameter) spherical nucleolus (Fig. 1 d) with lower electron density was visible. The amount of granular and fibrillar components was similar.

The mother cells cytoplasm contained one large and several small vacuoles. The sizes of large vacuole, as a rule, were bigger than the nucleus (Fig. 1 c, d). The big vacuole localized between the budding scar and nucleus. Contact between tonoplast and outer membrane of nucleus envelope was commonly observed. The vacuolar contents were very variable («pleomorphic») inside the one cell or different (Fig. 1 b, c, d) ones. This feature might determine the ultrastructural «image» of mother, subsequently developing bud and daughter cells of fungal species and perhaps may be «fine» indicator of differences in its metabolic activity. The number of mitochondria on median sections varied from 4 to 7. They uniformly distributed at cell periphery (Fig. 1 c, d), and had spherical (from 0.6 to 0.8 μm) or ellipsoid (from 0.8 to 1.2 μm) shape.

Storage compounds for mother and bud cells were absent in exponential growth phase. Similar peculiarity was specific for cells *C. neoformans* [10, Yamaguchi M., et al., 2002] in similar phase of growth. The components of endomembrane system were represented by several short or long cisterns of granular endoplasmic reticulum (ER) and numerous secretory vesicles. The cisterns of ER were visible near nucleus envelope and cell wall. Numerous small (from 30 to 60 nm) light vesicles were localized separately or in small groups near cell wall (Fig. 1 d) often in close contact with plasma membrane. The presence of vesicles was also revealed by Brown T.A and Smith D.G. (1977) after permanganate fixation in mother cells of *C. albidus.* They were also typical for *C. neoformans* cells [Kopecká M., et al., 2000]. Cytosol has moderate electron density and rich with uniformly distributed free ribosomes in form of mono- or polysome.

Plasma membrane situated in tight contact with cell wall. The lateral cell wall (from 200 to 220 nm) showed light electron density (Fig. 1c, d) and sparce microfibrils with lower contrast, which revealed in its upper half part. For comparison, according to the data in literature [Brown T.A., et al., 1977] the cell walls thickness in mother cells of *C. albidus* after chemical fixation was 100 nm. For *C. albidus* cells, the presence of one budding scar was typical (Fig. 1 c, d), which localized strongly on the apical cell pole and considered as the integral part of wall.

Budding. Unipolar type of budding at *C. albidus* was typical. But it was not clear, it may be only once budding from one scar or several? Directly during very early phase of bud formation (Fig. 1 e, arrow), nucleus change its position and localized near wall in middle part of cell on one line with large vacuole. The level of condensed chromatin in nucleus increased significantly at this time (Fig. 1 e, g, i). Perhaps, the increasing in level of chromatization was correlated with initiation of chromosome duplication (DNA synthesis). Similar situation also took place with in vitro growing cells in *C. neoformans* before the initiation of budding [11].

The nucleolus become larger in size (at the average 1.2 μm) and electron dense. The number of granular component in its compound was distinctly increased. Another specific feature was changes in mitochondrial number and topography. The number of mitochondria in mother cell increased up to 11-12 and they changed its orientation from cell periphery in direction to nucleus and formed specific «sheath cover» near outer membrane envelope. Mitochondria, as a rule, were in tight contact with nucleus outer membrane. Perhaps, this changes might be indicator of the giant mitochondrium formation. Similar peculiarities in transformations of chondriome (proliferation and concentration around nucleus) was correlated with formation one giant organelle (mitochondrial reticulum) and was typical for cells of strong virulent *C. neoformans* strains which infected mouse brain of after 7 days of beginning experiments [12].

We found nucleolus and chondriome activations in budding cell that indirectly demonstrate that synthesis of cytosol, free ribosomes, proliferation of mitochondria, vesicles and another cell components took place at first and then this components filled developing bud. Perhaps, numerous secretory peripheral vesicles in growing bud involved in increasing its plasma membrane surface and also new cell wall and polysaccharide capsule formations. But functional role of this components in fully developed

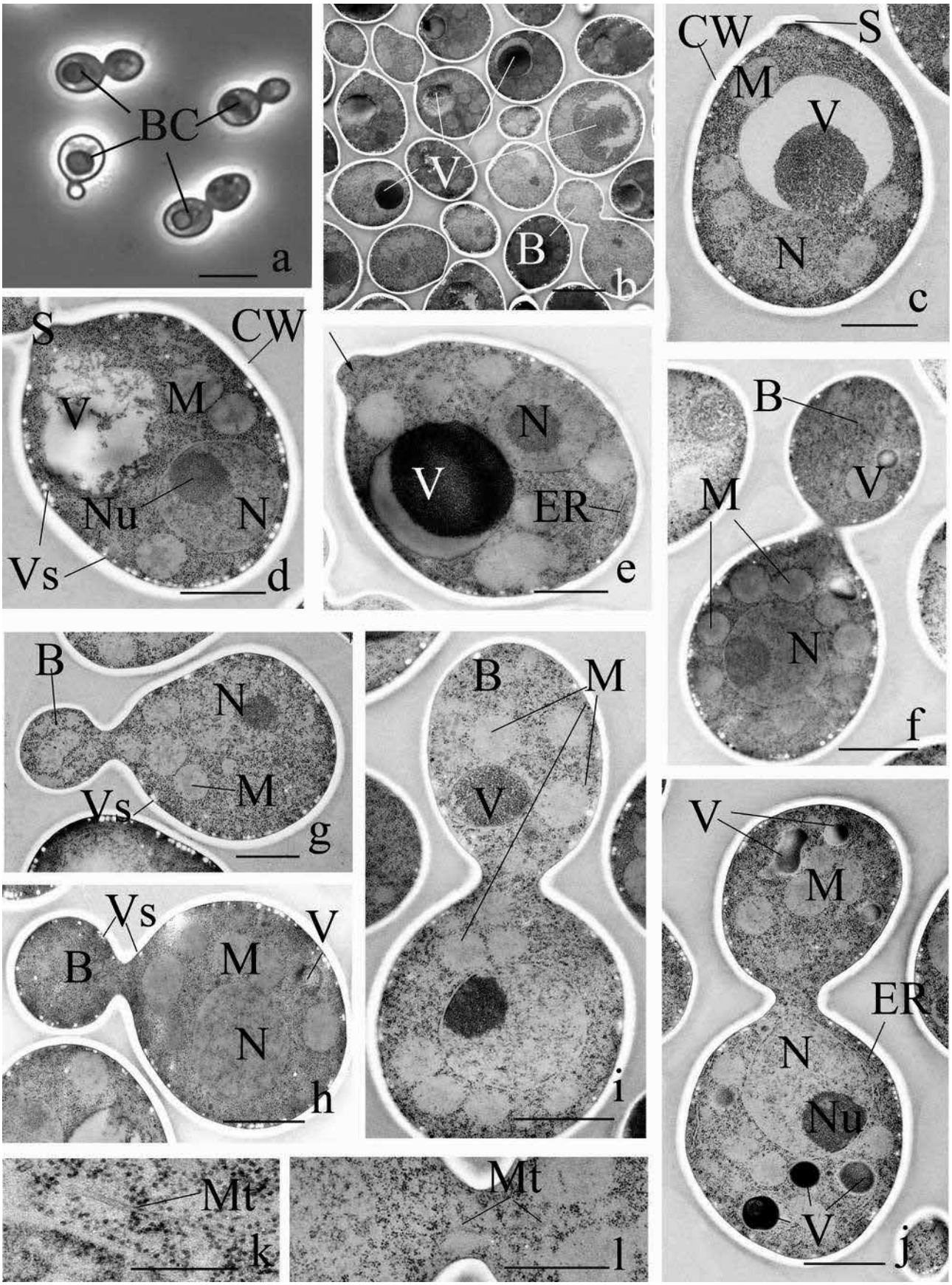

Fig. 1. Phase-contrast (a) and transmission electron microscopy (b-l) of *in vitro* growing *C. albidus* cells.
Explanation for this and another figures: B – bud, BC – budding cells; CW – cell wall, ER – endoplasmic reticulum, M – mitochondrium(ia), Mt – microtubule, N – nucleus, Nu – nucleolus, PC – polysaccharide capsule, S – scar, Sp – septum, V – vacuole, Vs – vesicles. Scale: a = 5 μm, b = 2 μm, c – j = 1 μm

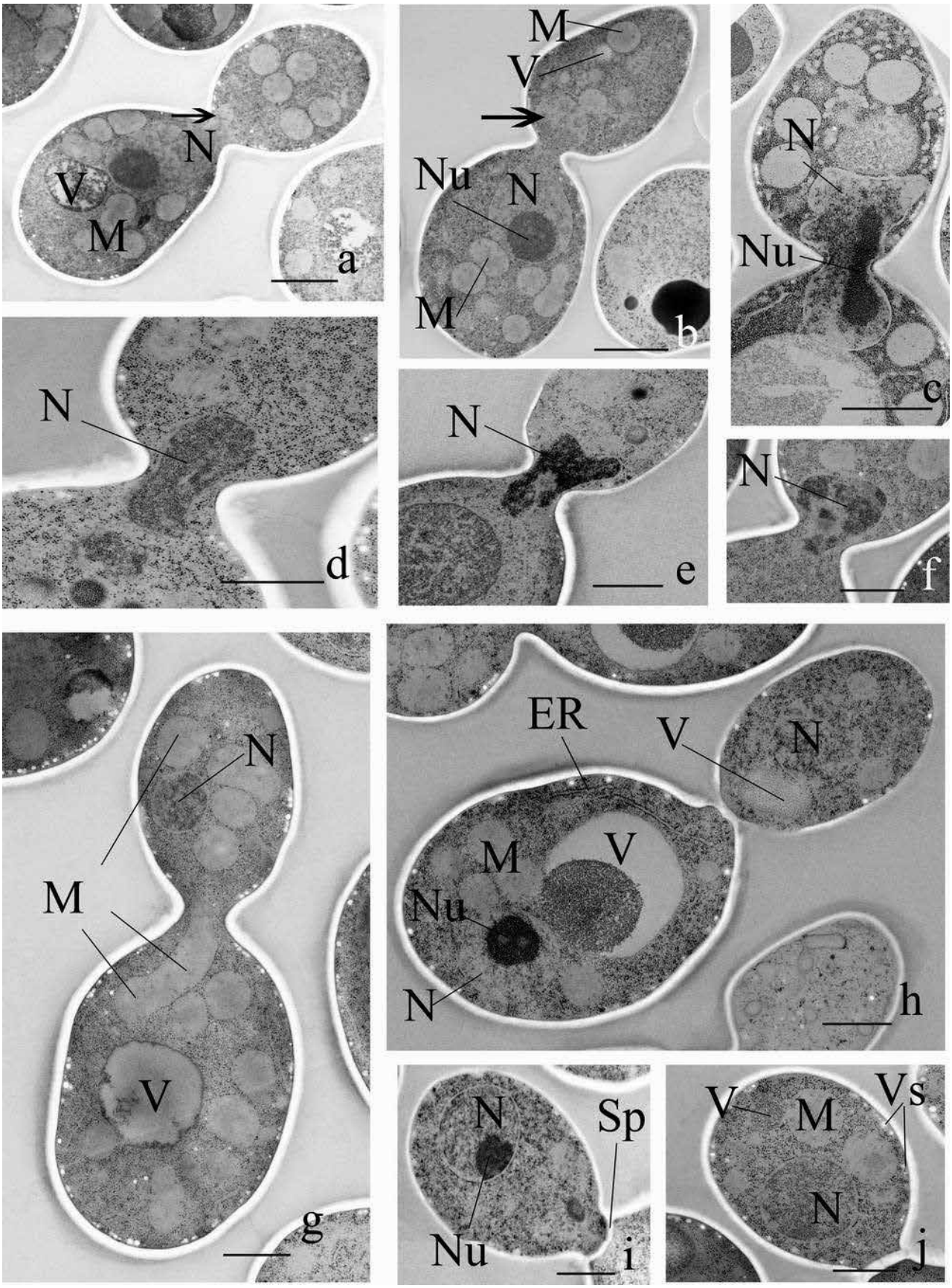

Fig. 2. Ultrastructure of in vitro growing *C. albidus* cells. Scale: a – j = 1 μm

mother cells remain unclear and its presence may interpret as «starting» for subsequent necessity of bud formations.

The bud development was start in scar area by the way of isodiametrical growth (Fig. 1 g, h, f). We did not reveal the sterigma-like protrusion which was typical for *C. neoformans* [Kopecká M., et al., 2000] in early stage of bud formation and last feature in our opinion may be species-specific peculiarity.

In early stage of bud formation, moderate electron density cytosol and numerous free ribosomes (Fig. 1 g) were visible. Later free (from 1 to 3 on median section) single small mitochondria, vacuoles (from 1 to 2) with different contents, rare short ER cisterns and numerous peripheral oriented small light single or in small groups secretory vesicles were observed (Fig. 1 h, i). At this stage in basal part of mother cells large vacuole was localized, in central - nucleus and then opposite the isthmus several mitochondria (Fig. 1 i). It was obvious the pictures of transition across the isthmus of mother→developing bud cell system cytosol, mitochondria, small vacuoles, free ribosomes and cisterns of ER from mother cell in bud content. Similar organellography inside both cell types was obvious during the next stage - apical growth of developing bud during which its shape become ellipsoid (Fig. 1 i). Around the mother cell nucleus exactly before its transition in bud cytoplasm, several long microtubules were revealed in cytosol (Fig. 1 k). Several long microtubules was also revealed in isthmus of budding cells (Fig. 1 l) which was typical for *C. neofomans* cells after using freeze-substitution methods [Yamaguchi M., et al., 2002]. The functional role of these components consist in regulation of process of cytosol, nucleus, free ribosomes and cellular components translocation during bud formation. Notice that regarding with visual evaluation the free ribosomes density in mother and bud cells before and after nucleus division and cell separation was equal.

Migration of nucleus from mother cells into the bud started when the daughter cell becomes of sizes 2.7-3.0x3.5-4.0 μm. At first, nucleus migrates from lateral part (Fig. 1 e) to apical and localized directly opposite isthmus (Fig. 1 j).

At this time the part of nucleus with chromatin and its intact envelope penetrate into bud through isthmus (from 0.70 to 0.75 μm, fig. 2 a). Then, the part of nucleus, which localized inside mother cell with the large (at the average 1.2 μm) electron-dense nucleolus, moved toward the bud through isthmus (Fig. 2 b).

When half of nucleus was in mother cell and another half was in bud (Fig. 3 a), the dark large nucleus was visible in mother cell in close contact with nucleus envelope, and asymmetry in level of chromatization was visible (Fig. 3 a). Higher chromatin condensation which was obvious in bud content may interpret as premetaphase, which perhaps, in metaphase pass in the bud. Later equal nucleolus separation were observed (Fig. 2 c). At this period, the part of nucleus in bud was greater in comparison with mother cell and irregular in form.

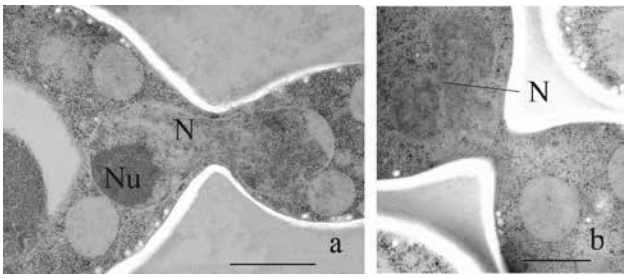

Fig. 3. Budding of *C. albidus* cells. Scale = 1 μm

The nucleus size was reduced and the level of chromatin condensation become bigger (Fig. 2 d-f). Specific features during *C. albidus* cell division - were the constant presence of nucleolus and its separation on equal parts during this process (perhaps in telophase). For comparison, typical for agaricalean fungi extrusion of nucleolus from basidial haploid nucleus during its migration and division through sterigma in basidiospores [Stepanova A.A., Vasilyev A.E., 1994] possible interpret as transition of last in dormant, not active condition. Another pattern of nucleolus behavior (presence during division and exactly after) in the *C. albidus* budding cells may interpret its subsequent growth and maturation without dormant stage. Directly after the nucleus division the nucleus which may be visualized in mother cell content was kidney-shaped and characterized by higher level of chromatization (Fig. 3 b). Exactly after nucleus division, small (0.8 μm) higher chromatizated nucleus was visible inside bud near its lateral cell wall (Fig. 2 g). At this stage the process of mitochondria migration continued and long profiles of this cell components was visible (Fig. 2 g) which indirectly certificate the presence of giant organelle.

Cytokinesis was the final stage of cell division. At this time the sizes of mother cells varied from 4.0 to 8.0 μm and the bud from 3.0 to 6.0 μm respectively. In middle part of isthmus the wedge-shaped electron-transparent septum was simultaneously formed (Fig. 4 a) and centripetal grow before central part when then conjugate. Finally the thick (200-220 nm) continuous light septum was formed (Fig. 2 i, j, 4 b). It was noticed that the secretory vesicles was absent near developing septum. Then in median part of septum separation of mother cell from daughter took place (Fig. 4 c). Soon after this, the mother and daughter cells differ on its size, level of vacuolization (presence of large central vacuole in first one and several little vacuoles in second one) and in nucleus topography (basal under central vacuole in first and central in second one). Ultrastructure of daughter cell wall after cytokinesis was similar with wall of mother cell this stage, but it was thinner (from 160 to 170 nm) which was also typical for *Cryptococcus neoformans* [Kopecká M., et al., 2000].

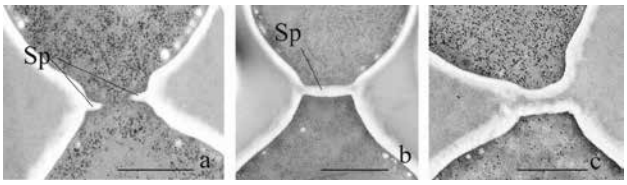

Fig. 4. Septum ultrastructure inside of isthmus in the *C. albidus* cells. Scale = 1 μm

The mother and daughter cell after separation. Exactly after cells separation nucleus has smaller size (form 1.0 to 1.2 μm, fig. 2 h, i) in comparison with similar in mother (at the average 2.0 μm) cell and has higher level of chromatization. Exactly after separation nucleus localized near cell wall and haddark nucleolus (0.4 μm, fig. 2 i). Later nucleus was translocated in the basal part and the number, ultrastructure and topography of organelles in the mother cells were the same as in the cells before budding.

The daughter cell undergo subsequent growth (isodiametrical growth) correlated with formation of central vacuole opposite the budding scar. During this process the nucleus move in the basal part of cell and its size was increased and level of chromatization contrary decreased. Simultaneously, synthesis of cytosol, free ribosomes, proliferation of mitochondria, ER cisterns, secretory vesicles which distributed in the periphery of the cytoplasm (Fig. 2 j) and formation of plasma membrane and polysaccharide capsule took place. The cell wall thickness increased to previous sizes which typical for mother cell.

We show schematically the ultrastructural pattern of budding *C. albidus* in vitro growing cells (in exponential phase) in fig. 5.

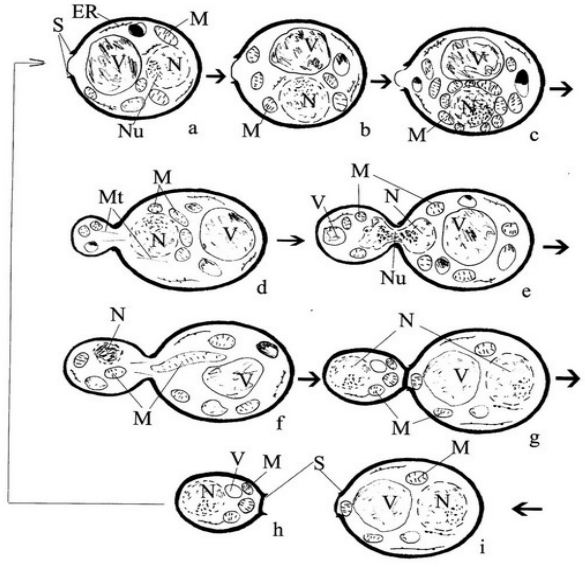

Fig. 5. Schematic drawing of *C. albidus* budding cell: a, b – mother cells before bud formation; c - f – bud formation; g – mother and daughter cells after separating septum formation, h, i – mother and daughter cells after separation

We revealed that during the budding *C. albidus* cells the existence of correlation in the pattern of organelle polarity, topography and dynamics in system mother→bud cell. Mother cell during this process was the places for synthesis of cytosol, free ribosomes, mitochondria, secretory vesicles and activation of nucleolus. Perhaps, formation of little vacuoles and cistern of ER cisterns also take places in this compartment.

The first signal for bud formation would be changes in the nucleus topography which translocated from basal (Fig. 5 a) to lateral part of cells (Fig. 5 b). Then changes of nucleus took place before nucleus division, the content of developed bud cytosol, free ribosomes, small mitochondria and vacuoles move from mother cell (Fig. 5 d). It was not clear the source of secretory vesicles in mother, bud and daughter cells. The developed bud was the places of plasma membrane, cell wall and polysaccharide capsule synthesis and growth. The role of microtubules in process of nucleus, cytosole and another cell componensts migration in system mother→bud cells system was apparent (Fig. 5 d-f). During *C. albidus* cell budding the nucleolus material was uniformly separated (Fig. 5 e) between mother and bud cells. For comparison, in budding *Rhodotorula glutinis* cells new nucleolus was formed in daughter cell [McCully E.K., Robinow C.F., 1972]. It was possible, that in yeast the fate of nucleolus during budding has taxonomical significance. Our data about increasing the number of mitochondria during mitosis in mother cells and decreasing after this process was also correlated with the results obtained for *C. neoformans* [Mochizuki T., et al., 1989]. According to our data, the specific structural feature of the divided mother cell which situated in exponential growth phase was its «not overloading» with storage substance. After septum formation (Fig. 5 g) and subsequent separation of mother cell from daughter ones (Fig. 5 h, i), the sizes and quantity of cell components for exception nucleus were not equal. Eventually daughter cells took place changes during which she obtained the ultrastructural characteristics typical for mother cell (Fig. 5 h→a).

It was obvious that it may be necessary to perform subsequent investigations of this fungal species in tissues to reveal pattern of budding and to clarify the cytological criteria of its pathogenicity.

RESUME

The high level of vacuolization, moderate number of mitochondria and cistern of rough ER, numerous peripheral secretory vesicles and free ribosomes were typical for in vitro growing *C. albidus* mother cells.

Ultrastructural signs for the transition of mother cells to budding were nucleus migration from basal to lateral and then apical part, increasing the level on nucleus chromatization and nucleolus activation, proliferation of mitochondria and changes in its orientation with formation «sheath cover» around nucleus.

The characteristic features of mother and budding cells in exponential phase of growth was variability in vacuolar contents not only inside one cell, but also different ones and absence of storage compounds in cytosol.

The constant presence of nucleolus for *C. albidus* budding cells was typical and it is separated on equal parts during this process.

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