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ULTRASTRUCTURE OF *FELLOMYCES FUZHOUENSIS* – NEW, POTENTIALLY PATHOGENIC YEAST REPRODUCING BY CONIDIOGENESIS

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A b s t r a c t

The potentially pathogenic basidiomycetous yeast *Fellomyces fuzhounesis* reproduces asexually by conidiogenesis. The conidiogenous mother cell produces a thin tubular sterigma, at the end of which an asexual conidium differentiates. While the sterigma remains attached to the mother cell, the conidium separates, grows and gives rise to a new conidiogenous cell. This process was studied by transmission electron microscopy of ultrathin sections and freeze-fracture replicas and by fluorescence microscopy. The conidiogenous cell had a multilamellar cell wall above the plasma membrane; the cytoplasm, which contains one large mucleus, was rich in large mitochondria, endoplasmic reticulum and numerous, small membraneous vesicles, potassium permanaganate-stained granules, and also comprised vacuoles. Conidiogenesis was initiated by production of a sterigma. This had a thin, electron-dense wall enclosing the cytoplasm with endoplasmic reticulum cisternae, membraneous vesicles, electron-dense granules and mitochondria. The sterigma grew in length and its terminal end differentiated to produce an ovoidal conidium. The nucleus moved into the sterigma and elongated inside it. In the next step, postmitotic distribution of the nuclei, one into the conidiogenous cell and the other into the conidium, was observed as well as an actin ring situated at the base of the conidium. From this region, an electron-dense wall closure arose that continued with the inner wall layer of the conidium. Subsequently, the cytoplasm began to retract into the mother cell, leaving the sterigma empty. The cell wall at the base of the sterigma on the mother cell became thick and felt-like in appearance and the surrounding region showed numerous small membraneous vesicles and electron-dense granules. The mother cell separated from the empty sterigma by an electron-dense wall partition continuing with its inner wall layer. The conidium broke off, the sterigma remained attached to the mother cell and, with sterigmata from previous conidiogenous processes, gave the cell a sea-mine appearance. These results show that, in the basidiomycetous yeast *Fellomyces fuzhouensis*, the asexual reproduction by conidiogenesis has many distinct features that distinguish it from the asexual forms of reproduction by budding and fission known in ascomycetous yeasts.

K e y w o r d s

Fellomyces fuzhouensis, basidiomycetous yeast, ultrastructure, conidiogenesis

INTRODUCTION

"Long-neck yeasts" were first isolated by Fell from the Indian Ocean water (at a depth of 110 m), then from the air at Virginia Key in Biscayne Bay, Florida, and from a case of keloid blastomycosis (Jorge Lobo's disease) in Brasil; they were described as " a new fungal genus *Sterigmatomyces"* (*1, 2, 3*). Yamada, Banno and others suggested to exclude some of these yeasts and include them in the genus *Fellomyces* (*4, 5, 6*). This genus now has six species (*7, 8, 9, 10, 11*) taxonomically related to the human pathogen *Cryptococcus neoformans.* Both *Fellomyces* and *Cryptococcus* have been classified, on the basis of molecular taxonomy, as the *Tremellales* clade (*9*).

Fellomyces fuzhouensis, similarly to other "long neck yeasts", is characterised by a unique manner of yeast vegetative reproduction by means of asexual spores, i.e., conidia. While, in the majority of yeast species, vegetative asexual reproduction is carried out by budding, fission or by "bud-fission" (*12*), conidiogenesis, discovered in the genus *Sterigmatomyces,* involves the development of a sterigma, a tube-like, slender projection, that bears a single, spherical or ovoidal conidium (*1*). In the genus *Sterigmatomyces,* conidia separate from sterigmata by cleavage at the midpoints, whereas in the genus *Fellomyces* they separate at the distal ends of sterigmata (*4, 5, 6, 7, 8*).

The increasing incidence of immunodeficiency diseases in the population brought pathogenic and potentially pathogenic yeasts and fungi, which are frequently the final cause of mortality, to the forefront of interest in a wide range of specialists. In contrast to the model systems of budding and fission yeasts (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*), most of the pathogenic and potentially pathogenic yeasts have not yet been studied in detail. We present here the first results of a study concerning the morphology of *Fellomyces fuzhouensis* and its reproduction by conidiogenesis, as demonstrated by transmission electron microscopy in ultrathin sections and freeze-fracture replicas, which was a part of comprehensive research into the morphogenesis, structure and function of the cytoskeleton with the objective to search for new targets of specific inhibitors in some fungi potentially pathogenic for humans (*Cryptococcus neoformans, Aureobasidium pullulans* and *Fellomyces fuzhouensis*).

MATERIALS AND METHODS

Yeast strain. *Fellomyces fuzhouensis*, CBS 8243 (Yeast Culture Collection, The Netherlands), was kindly provided by Professor H. J. Phaff, University of California, Davis, U.S.A.

Media and cell cultivation. *F. fuzhouensis* was maintained on plates at 25° C for 2 to 7 days in YPD medium consisting of 1% each of glucose, yeast extract and polypeptone or potato-dextrose-broth (PDB; Difco) or 3% malt

extract supplemented with 1.5 % agar. The cells were cultivated in liquid YPD or PDB medium on a shaker at 25° C for about 18 hours, usually overnight. In some experiments, 40-hour or 64-hour cultivation was used.

Phase contrast and fluorescence microscopy. Light and fluorescence microscopy was used to observe the cells. Fixation, staining of cell walls and nuclei, and visualisation of actin and microtubular cytoskeletons were carried out as described previously *(13, 14, 15)*.

Electron microscopy.

Ultrathin sections. Membrane structures, cell walls, sterigmata and conidia were fixed and stained with $KMnO_4$. Growing cells were washed once with distilled water, resuspended in 5 ml of a freshly-made, 2% $KMnO_4$ solution and allowed to fix for 60 min. The fixed cells were washed three times with distilled water, dehydrated in an alcohol series for 2 hours and embedded in the LR White resin and allow to polymerise for 2 days at 60° C. Ultrathin sections were made and contrasted with 2.5 % uranyl acetate for 30 min followed by exposure to lead citrate for 6 min.

Freeze-fracturing. A suspension of living cells in PDB or YPD medium was frozen in Freon 22/liquid nitrogen and processed in a BA 360 M Balzers apparatus as described elsewhere *(16)*.

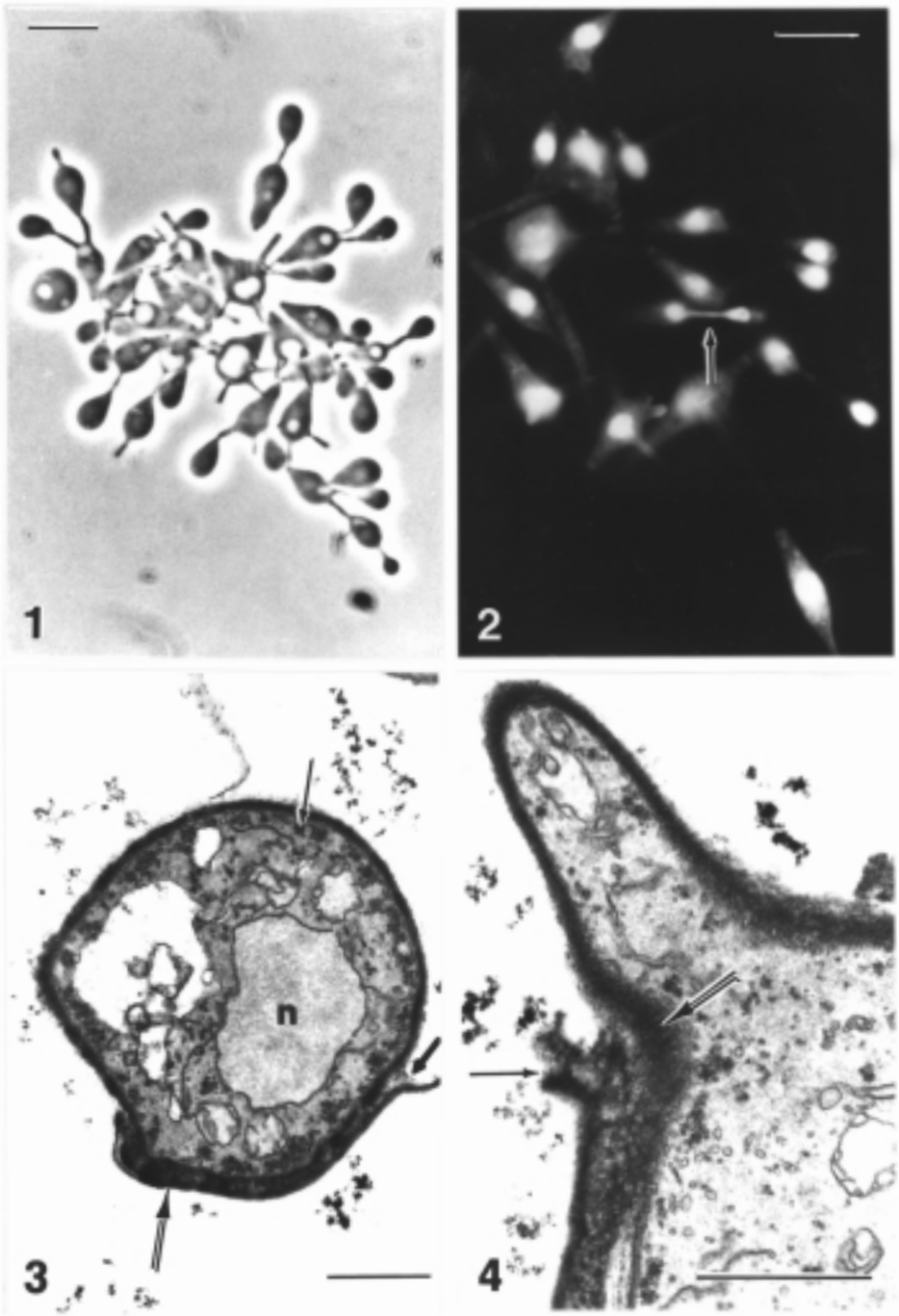
Ultrathin sections and freeze-fracture replicas were viewed and photographed in a Tesla BS 500 electron microscope.

RESULTS

Phase-contrast microscopy of cells during conidiogenesis

After inoculation of *F. fuzhouensis* cells from a stationary culture (containing mostly old and vacuolised cells) onto the surface of a solid medium, a growing group of cells and conidia connected through protrusions, termed sterigmata, into a network was observed. (*Fig. 1)*. This group represented a clone arising from a single cell; the cells ranged from 3 to 5 μm in size.

In liquid nutrient media, mostly single conidiogenous cells producing ovoidal, spherical or pear-shaped conidia were detected. The mature conidia developed into conidiogenous cells that gave rise to a new generation of cells. A new cell started by the formation of a narrow, polarly growing prostrusion, sterigma, whose terminal end differentiated into an ovoidal or spherical conidium. For some time, this remained attached to its conidiogenous cell by the sterigma. Subsequently, a region of fainter contrast, which expanded towards both the conidiogenous cell and the conidium, appeared in the sterigma. In some phase-contrast micrographs, it was apparent that this region was free of the cytoplasm, which was due to the fact that the cytoplasm gradually moved back to the conidiogenous cell, leaving the sterigma empty inside the cell wall in the form of a cylindrical tube. The conidium was later freed by breaking off at the proximal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region of the sterigma and started its own reproduction by conidiogenesis. The sterigma remained attached to its mother conidiogenous cell that, however, closed its connection with the sterigma by forming a new cell wall. All cells reproducing by conidiogenesis had several empty sterigmata on their surfaces, which showed how many times conidiogenesis took place.

Nuclear division visualised by phase-contrast and fluorescence microscopy

The nucleus was observed as a light, ovoidal structure or a bright, fluorescent organelle in the cell centre when viewed in the phase-contrast and fluorescence microscopes, respectively *(Figs. 1, 2).* During the development of both sterigma and conidium, the nucleus remained in the conidiogenous mother cell. After differentiation of the conidium, the elongated nucleus appeared also in the sterigma *(Fig. 2,* arrow*).* The process of nuclear division could not be demonstrated by either of the methods used. The nuclei were detectable only when they appeared one in the conidiogenous mother cell and the other in the conidium.

Fig. 1

A culture of *Fellomyces fuzhouensis* cells growing on the surface of a solid medium. Old, vacuolised, conidiogenous cells with several tube-like sterigmata can be seen in the centre. A drop-shaped conidium developed at the end of each sterigma. (seen at the periphery of the colony). Phase-contrast. Magnification, x 900. Bar 10 μm.

Fig. 2

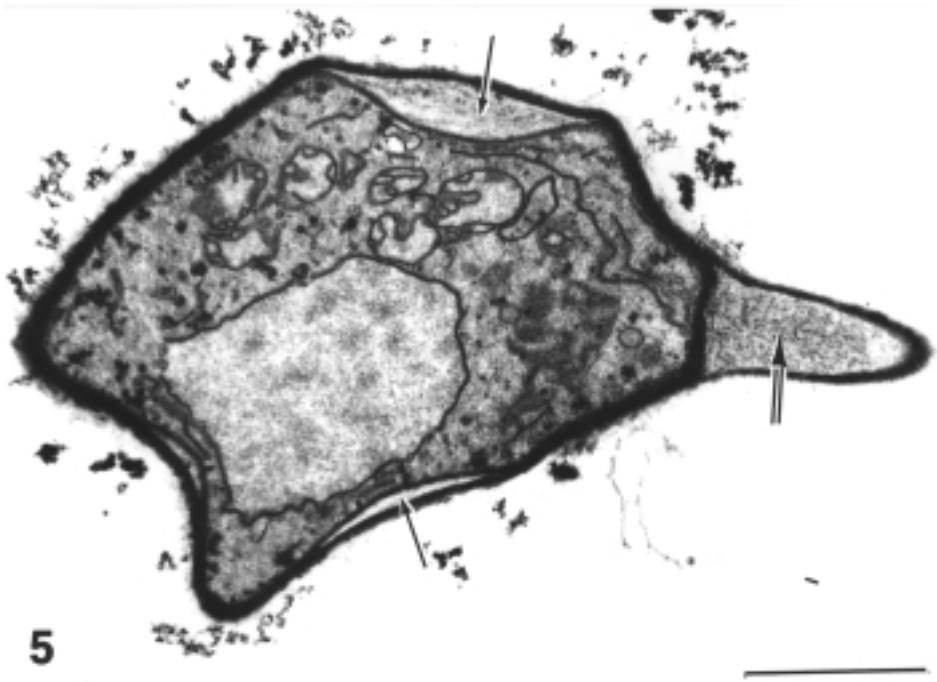
A culture of *F. fuzhouensis* cells. Nuclei are spherical or ovoidal in shape and relatively large. Nuclear division takes place inside the tube-like sterigma. The ends of the dividing nucleus are located in both the conidiogenous cell and the conidium (arrow). Fluorescence microscope. Nuclei visualised by DAPI staining. Magnification, x 1000. Bar 10 μm.

Fig. 3

An ultrathin section across a conidiogenous cell. Cytoplasm contains endoplasmic reticulum, large mitochondria, the nucleus (n) and vacuoles. Dense granules are situated near the plasma membrane (arrow). Around the whole periphery of cell, an inner wall layer, stained with $KMnO_4$ is visible. A thicker outer wall layer is visible at the bottom part (double arrow). Between the outer and inner dark wall layers, a middle electron- transparent layer is visible (thick arrow) in which separation of both dark layers occurs. Fixed with $KMnO_4$, contrasted with uranyl acetate and lead citrate. Magnification 14 000x. Bar 1 μm.

Fig. 4

An ultrathin section across a part of the cell with a sterigma. A remnant of the thick cell wall is apparent (arrow). The inner layer of the wall produces a thickened, felt-like protrusion at the base of the sterigma (double arrow). Invaginations in the plasma membrane can be seen. Near the plasma membrane, there are small membraneous vesicles reminiscent of a disintegrated endoplasmic reticulum, and dark, $KMnO_4$-stained granules. It is suggested that this electron micrograph illustrates the situation before the cytoplasm retracts from the sterigma into the conidiogenous cell and a wall closure is formed. Fixed with $KMnO_4$, contrasted with uranyl actetate and lead citrate. Magnification, x 24 000. Bar 1 μm.



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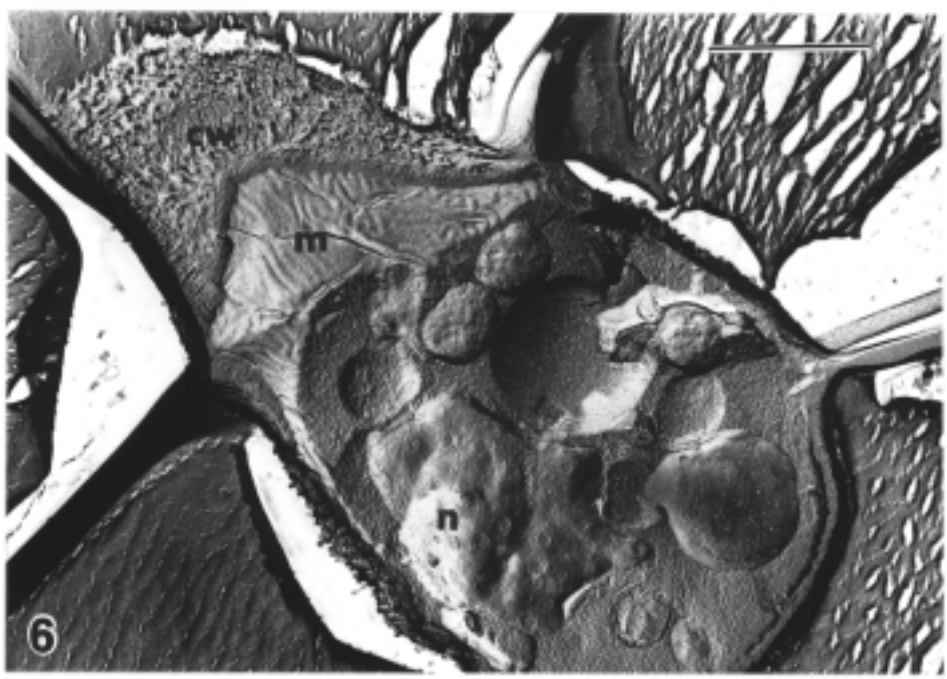

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Cells and the process of conidiogenesis observed by transmission electron microscopy

In ultrathin sections, conidiogenous cells were spherical, ovoidal or irregular in shape with cytoplasm-filled *(Figs. 4, 8)*or empty *(Figs. 5, 9, 10)* sterigmata of variable length and width. Conidia were small, spherical or club-shaped *(Fig. 9).*

In freeze-fracture replicas, the fracture face showed unusual wrinkling of the plasma membrane surface and the presence of invaginations typical of yeast cells (*Fig. 6*).

All three morphological forms were characterised by large numbers of membrane structures *(Figs. 3, 5, 7, 9).* Conspicuous, large mitochondria, rich in cristae mitochondriales, were present in young cells, conidia and cytoplasm-containing sterigmata. In old conidiogenous cells (characterised by a large vacuole) large mitochondria with only a few cristae were present. The high frequency and the length of cisternae of endoplasmic reticulum were striking features. Small membrane vesicles (average size, 0.06 μm) were detected at the base of a developing, not yet closed sterigma *(Fig. 4,* arrow). Electron-dense granules, 30 to 50 nm in size, were observed in these three morphological forms. They were usually located near the plasma membrane *(Figs. 3, 5, 9, 10).*

Nuclei sectioned near their equator were large in relation to the size of their cells. However, the structural differentiation of their content could not be detected by the method used. In longitudinal, ultrathin sections, the first step of nuclear elongation into the sterigma was observed in the conidiogenous cell *(Fig. 7)* but a sterigma containing the elongated nucleus was not yet found.

The cell wall of a conidiogenous cell appeared as an electron-dense structure. Over the plasma membrane, there was a thin electron-dense layer, about 0.1 μm in width, and another, thicker outer layer. The total thickness of the wall was about 0.3 μm. The outer layer was often separated from the inner layer either partially or completely *(Figs. 3, 4).* These two wall layers seemed to consist of several other layers, but potassium permanganate staining failed to distinguish them clearly. In areas where the outer thick layer was separated, the outer surface of the

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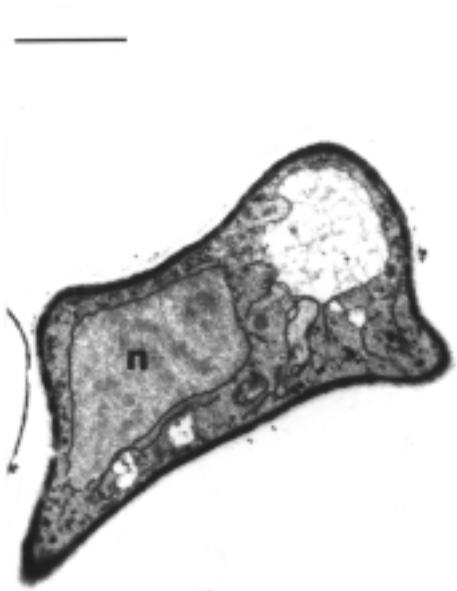
Fig. 5

An ultrathin section across a conidiogenous cell. Membrane systems, the nucleus and dark granules are visible.The plasma membrane is separated from the wall (plasmolysis-like phenomenon) and this region shows thin layers of wall material (arrows). On the right, an ”empty“ sterigma is visible (double arrow) separated by a wall partition developing from the inner wall layer. The empty sterigma is filled with wall-like materials. Fixed with $KMnO_4$, contrasted with uranyl acetate and lead citrate. Magnification, x 24 000. Bar 1 μm.

Fig.6

An oblique freeze-fracture through a conidiogenous cell. Nuclear membrane (n), mitochondria, vacuole and wrinkled plasma membrane (m) are visible. The outer surface of the cell wall (cw) had an filamentous appearance. Freeze-fracturing. Magnification, x 20 000. Bar 1 μm.



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remaining inner wall layer appeared loose to filamentous in structure *(Figs. 3, 4, 6).* The methods used did not allow us to distinguish whether this filamentous interlayer linked the inner layer to the relatively easily separable outer layer.

The cell walls of sterigmata and conidia were also electron-dense. Their thickness was about 0.1 μm and their surfaces were not clearly delineated. In ultrathin sections, in regions of transition between the conidiogenous cell and its sterigma, the cell wall was conspicuously thickened, electron-dense and felt – like in appearance *(Fig. 4, 8).*

In sterigmata, wall closures from the side of both the conidiogenous cell and the conidium were formed by an electron-dense layer growing from the inner wall layers of the cell and the conidium *(Figs. 5, 9, 10).* Although the wall had closed both ends of the sterigma, the empty cylindrical sterigmata remained as a connection between the conidiogenous cell and the conidium. In the next step, the conidium detached itself from the sterigma *(Fig. 9*) that remained connected to the mother conidiogenous cell *(Figs. 5, 10).* The inner content of all "empty" sterigmata revealed a very fine and thin filamentous structure *(Figs. 5, 9, 10).* Although an actin ring was found in the area of transition between sterigma and coinidium (not shown) and the formation of a centripetal septum was presumed here, typical septum formation was not detected.

Fig. 7

An ultrathin section through a young conidiogenous cell. The nucleus (n) elongates to reach the sterigma. Fixed with $KMnO_4$, contrasted with uranyl acetate and lead citrate. Magnification, x 14,000. Bar 1 μm.

Fig. 8

An ultrathin section through a young conidiogenous cell that includes two sterigmata. At the base of the upper sterigma, there is a typical, felt-like wall thickening (double arrow), at the base of the bottom sterigma, partitioning between the cell and the sterigma content commences (arrow). Fixed with $KMnO_4$, contrasted with uranyl acetate and lead citrate. Magnification , x 10 000. Bar 1 μm.

Fig. 9

An ultrathin section through an apparently growing, drop-shaped conidium. Mitochondria are rich in cristae mitochondriales, near the cell surface there are dense granules, the nucleus (n) is relatively distant from the base of the sterigma. Separation between the cell wall and the plasma membrane already covered by a dark wall layer is apparent. In this cavity, fine layers of wall-like material are visible (double arrow). The sterigma wall becomes the outer layer of the conidium wall, while the wall partition continues with the inner wall layer. Fixed with $KMnO_4$, contrasted with uranyl acetate and lead citrate. Magnification, x 14 000. Bar 1 μm.

Fig. 10

An ultrathin section through a conidiogenous cell with an empty sterigma free from the cytoplasm. The empty sterigma is filled with wall-like materials. The cytoplasm of the conidiogenous cell is separated from the empty sterima by a wall partition continuous with the inner wall layer (arrow). Fixed with $KMnO_4$, contrasted with uranyl acetate and lead citrate. Magnification, x 10 000. Bar 1 μm.

A local, disc-like separation of the plasma membrane from the wall, resembling plasmolysis, was observed frequently in conidiogenous cells and very rarely in conidia. Between the separated plasma membrane and the cell wall, there was an electron-transparent space filled with occasional, very fine and thin longitudinal fibrils or fibrillary layers *(Figs 5, 9)*. Whether any supporting structures were present on the outer plasma membrane surface could not be distinguished at the magnification used.

DISCUSSION

In comparison with ascomycetous yeasts, especially the budding yeast *Saccharomyces cerevisiae* and fission yeast *Schizosaccharomyces pombe*, *Fellomyces fuzhounesis* was rich in membranous organelles, especially long cisternae of the endoplasmic reticulum and large mitochondria. It was apparent that the density of cristae mitochondriales was related to the physiological activity (growth) of each cellular compartment, i.e., conidiogenous cell, sterigma and conidium.

The multilamellar cell wall in conidiogenous, ageing cells is reminiscent of the cell wall in the *Basidiomycetes (17)* and shows that this organism was correctly classified as a basidiomycetous yeast *(4,5,6,7,8,9,10,11)*. Staining with potassium permanganate clearly distinguished the inner wall from the outer, separable, thick wall layer and revealed a layer less densely stained in which separation of the old wall occurred. In freeze-fracture replicas, this layer had a blob-like or fibrillar appearance. Whether the filamentous surface occasionally found on fracture faces or a felt-like appearance of the wall outer line seen in ultrathin sections were characteristic features of any of the three morphological forms studied could no be decided at the magnification used. The distinction of two cell wall layers, outer and inner, made in this study need not be definite in view of the finding of several layers different in intensity of potassium permanganate staining. In the old cell wall of the conidiogenous cell, several separate layers were observed. The inner layer was further divided into an inner part that formed a closure of the sterigma and an outer part continuing as the proper sterigma wall.

We made an unusual finding of dark granules near the plasma membrane and showed that their contrasting by potassium permanganate was very similar to that of the wall material. Their nature was not investigated in this study but, on the basis of their frequency and location we suggest that they may be related to cell wall synthesis.

”Long-neck yeasts“ reveal a unique manner of vegetative, asexual reproduction by conidiogenesis *(1–12)*. One of the morphological features that, besides the differences in biochemical characteristics and chemical composition of the wall *(5, 6, 7)* has led to exclusion of the genus *Fellomyces* from the genus *Sterigmatomyces,* is a different site of breaking off the empty sterigma from the conidiogenous cell. The long, empty sterigma visualised in *Fellomyces fuzhounesis* is due to the fact

that breakage occurs in the narrowest part of the sterigma at the conidium base. This area also showed the presence of an actin ring but to what extent this was associated with wall closure could not be determined at this stage of study. Conidia were easily detached when cultured in liquid media on a shaker but, on solid media, subsequent cell generations remained connected in a network.

Our observations provided no convincing evidence to suggest a typical centripetal septation in this yeast. In contrast, we found indications for partitioning between the conidiogenous cell and its empty sterigma. After segregation of daughter nuclei and their distribution into the mother cell and conidium, the cytoplasm began to retract into the conidiogenous mother cell. In the empty sterigma, some light material was observed. Whether it was amorphous or fibrillar remains to be determined. We presume that this material is synthesised by a naked plasma membrane and may contribute to the stabilisation of this membrane. In the majority of yeast species, the naked plasma membrane, which has been separated from the cell wall by plasmolysis or protoplasting, produces wall materials (*19).* In our experiments, a local thickening of the cell wall was detected at the sterigma base before the cytoplasm was completely retracted into the conidiogenous cell. It had a felt-like appearance and the surrounding area was rich in wall vesicles, probably involved in transport of wall material.

While, in *Sterigmatomyces,* sterigmata break in the middle, liberation of conidia in *Fellomyces* occurred by a typical end-break in the sterigma. Although typical, centripetal septation was not observed it was believed that a study of nuclei during mitosis would provide a clue to an understanding of this process. In contrast to ascomycetous yeasts, where the septum develops perpendicularly to the centre of the dividing nucleus, in basidiomycetous yeast, such as *Cryptococcus neoformans (15),* the nucleus migrates into the bud to commence nuclear division; subsequently, the daughter nucleus returns to the mother cell and the bud is closed by a septum. Our experiments in *F. fuzhouensis*, however, failed to reveal any mechanism determining the site of breakage at either the ultramicroscopic or light microscopic levels. Dividing, elongated nuclei were visualised by DAPI staining in sterigmata and thickened nuclear poles were detected in both conidiogenous cells and conidia. It is suggested that the appearance of an actin ring at the site where the conidium was later detached from the sterigma is related to the continuing, ”drop-like“ growth of the conidium and to a move of the nuclear division centre. However, nothing more specific is known about this ”asymmetrical cytokinesis“ in conidiogenous yeasts.

The plasma membrane detachment discernible in some subcortical regions of conidiogenous cells, and also in large conidia, which resembled the phenomenon of plasmolysis may be explained by movement of the cytoplasm during conidiogenesis. The ”cavities“ thus produced contained a layer, probably composed of wall material, which might indicate some initial steps in separation

of the plasma membrane from the rigid surface structure. Any association of this phenomenon with previous sterigma locations were not proved.

Empty sterigmata, protruding out of conidiogenous cells and giving them a sea-mine appearance, can be considered analogous to bud scars in budding yeasts. On the basis of their number it can be concluded that one conidiogenous cell can undergo about ten processes of condiogenesis. Cells showing eight to ten empty sterigmata also contained vacuoles, gradually ceased to grow and eventually died. While budding in basidiomycetous yeasts is repeatedly initiated from one and the same site of the cell surface *(18)*, in *F. fuzhouensis* cells, the finding of several sterigmata on every conidiogenous cell suggests a different mechanism for the production of progeny.

The unusual features of morphology and dynamics of the cell cycle presented here give support to the view of *Kurtzman (9)* that conidiogenesis is a unique process of yeast asexual reproduction that warrants further, more extensive and detailed studies to be understood.

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ULTRASTRUKTURA FELLOMYCES FUZHOUENSIS – NOVÉ POTENCIÁLNĚ PATOGENNÍ KVASINKY REPRODUKUJÍCÍSE KONIDIOGENEZÍ

S o u h r n

Ultrastruktura nedávno isolované potenciálně patogenní basidiomycetové kvasinky *Fellomyces fuzhouensis* byla studována transmisní elektronovou microskopií ultratenkých řezů a replik mrazového lámání. Tato kvasinka se asexuálně reprodukuje konidiogenesí. Mateřské konidiogenní buňky vytvářejí tenké trubičkovité sterigmata, na jejichž konci se diferencuje jedna asexuální konidie. Zatím co sterigma zůstává na mateřské buňce, konidie se odloučí a vyroste v novou konidiogenní buňku. Elektronová mikroskopie prokázala na kvasinku neobvyklé množství mitochondrií a endoplasmatického retikula. Jádro při dělení se elonguje do trubičkovité sterigmy. Po separaci jader do mateřské buňky a konidie se plasma sterigmy vrací do konidiogenní buňky. Řídkou amorfně-fibrilární hmotou vyplnění sterigmy zůstávají na mateřských buňkách, které mají vzhled „mořských min“. V místě odštěpení konidie, kde byl detegován aktinový prstenec, a kde se pro kvasinky typicky mělo centripetální tvořit septum, byl nacházen stínový uzávěr, přecházející ve vnitřní vrstvu stěny konidie. Obdobně vypadal stínový uzávěr vyprázdnění sterigmy v konidiogenní buňce, kde však byla před uzávěrem detegována lokalisace většího počtu drobných vesiklů a plsťovité ztluštělý val buněčné stěny s výskytem kalium permanganát densních granul. Taková granula stejné density jako stěnové vrstvy byla ve velkém množství lokalizována v blízkosti plasmamembrány. Stínová uzávěr zřetelně vycházel z vnitřní vrstvy buněčné stěny a překrýval plasmamembránu v ústí sterigmy. Mezi buněčnou stěnou a plasmamembránou byly misty nalezeny velké dutiny podobné plasmolyse s řídkou lamelární strukturou, patrné důsledek přelévání plasmy při konidiogenesi. Buněčná stěna starých konidiogenních buněk byla vícevrstevné. Na

plasmamembránu naléhala vnitřní vrstva, překrytá tlustou vnější vrstvou stěny, většinou částečně od vnitřní vrstvy separovanou. Mezi nimi, zejména na replikách mrazového lámání, byla zřetelně řidší, granulárně – fibrilární vrstvička. Obě základní vrstvy stěny se zdály být složeny ze subvrstev. Potvrzuje se, že i na ultrastrukturální úrovni se asexuální reprodukce kvasinek konidiogenesí zásadně odlišuje od reprodukce kvasinek pučením a štěpením.

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