SCRIPTA MEDICA (BRNO) – 76 (3): 173–186, *June 2003*

UNUSUAL ULTRASTRUCTURAL CHARACTERISTICS OF THE YEAST *MALASSEZIA PACHYDERMATIS*

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A b s t r a c t

Morphological characteristics of the potentially pathogenic yeast *Malassezia pachydermatis* were studied by transmission electron microscopy (TEM) in specimens prepared by both ultrathin sectioning and freeze-fracturing. *M. pachydermatis* cells were small in size, not exceeding 5.0 μm, with relatively thick cell walls. The broad base for bud emergence, a structure substituting the bud neck in other budding yeasts, was associated with the formation of a characteristic collar. In freeze-fracture replicas, the inner surface of the cell wall (exoplasmic fracture face of plasma membrane) showed right-handed spiral ridges corresponding to left-handed spiral grooves on the plasma membrane (protoplasmic fracture face of plasma membrane) that directed to the growing pole of the daughter cell. In ultrathin sections, these structures appeared as a serrated arrangement of the innermost wall surface and invaginations of the plasma membrane. In the vicinity of a ring-like swelling observed on the plasma membrane at the site of cytokinesis, there were circumvallate bulgings on both the mother and daughter cells visualised by TEM in freeze-fracture replicas. The corresponding structures appeared as pits in ultrathin sections. These unusual ultrastructural findings, which are in agreement with previously reported observations, will further be studied in relation to the organisation of cytoskeletal structures.

K e y w o r d s

Malassezia pachydermatis, Lipophilic yeast, Ultrastructure, Morphology, Transmission electron microscopy

INTRODUCTION

The yeast *Malassezia pachydermatis* (*1,2)*, originally *Pityrosporum pachydermatis*, is one member of the group of lipophilic yeast. This group, now including only the genus *Malassezia,* (*3,4,5*), has been established for taxonomical reasons by classing *Pityrosporum* within *Malassezia.* In contrast to other species in this group, *M. pachydermatis* does not require the presence of lipids in growth medium.

When grown on solid media, *M. pachydermatis* first produces white and later yellow or light brown colonies with convex surfaces that are smooth and dry. The cells are small in size and varying in shape; they may be ellipsoid, circular or bottle-shaped (*6, 7*).

Lipophilic yeasts constitute a monophyletic group separated from the other yeasts a long time ago. The genus *Malassezia* is at present classed within basidiomycetes (*8, 9*).

No sexual reproduction has been reported in either *M. pachydermatis* or any other member of this genus. They proliferate only by enteroblastic budding, with the bud arising from a broad base always present on the same cell pole (monopolar budding).

M. pachydermatis is commonly isolated from the skin of warm-blooded vertebrates, such as dogs, cats, foxes, ferrets, pigs, primates or rhinoceroses. It is present as a commensal but may also turn pathogenic and cause various disorders (*10–13*). Although, in comparison with other species of this genus, its occurrence in humans is less frequent, it may be responsible for dangerous epidemics in neonatal intensive care units (*14–17*).

Several previous studies have reported essential information on the ultrastructure of *M. pachydermatis* (*18–23*). In this work, its morphology was investigated in the light of our previous unsuccessful visualisation of the cytoskeleton, with the objective to find out reasons for this failure and, if possible, to relate the data to a future study of the cytoskeleton. The morphology of *M. pachydermatis* at the ultrastructural level was studied in freeze-fracture replicas and ultrathin sections by transmission electron microscopy (TEM).

MATERIALS AND METHODS

CELL CULTIVATION

M. pachydermatis, the CCY 85-1-4 strain provided by the Collection of Yeast Cultures, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia, was used in this study. The culture was maintained on 2.5% agar with malt extract (7.0 %, w/v) at room temperature. The yeast cells for experiments were grown in malt extract liquid medium (7.0 %, w/v) on a shaker at 37 °C for 24 h.

FREEZE-FRACTURING

The living cells of *M. pachydermatis* were prepared and frozen in Freon 22 and liquid nitrogen by the standard procedure using a BA360 Balzers apparatus (*24*).

ULTRATHIN SECTIONING

Cells were fixed in 3 % glutaraldehyde in cacodylate buffer (200 mM at pH 7.4) for 2 h, post-fixed in 1 % OsO_4 diluted with cacodylate buffer for 1 h, and subsequently dehydrated in a graded series of alcohol (10 to 100 %) and embedded in LR White medium. After polymerisation for 2 days at 60 °C, ultrathin sections were made and contrasted with 2.5 % uranyl acetate for 30 min and Reynold's solution for 6 min (*25*).

ELECTRON MICROSCOPY

The specimens were viewed and photographed with a TESLA BS 500 transmission electron microscope.

RESULTS

The small size of *M. pachydermatis* cells, not exceeding 5.0 μm in length and 2.5 μm in width, was confirmed by both techniques (*Figs. 1a, 1b, 1c, 2*).

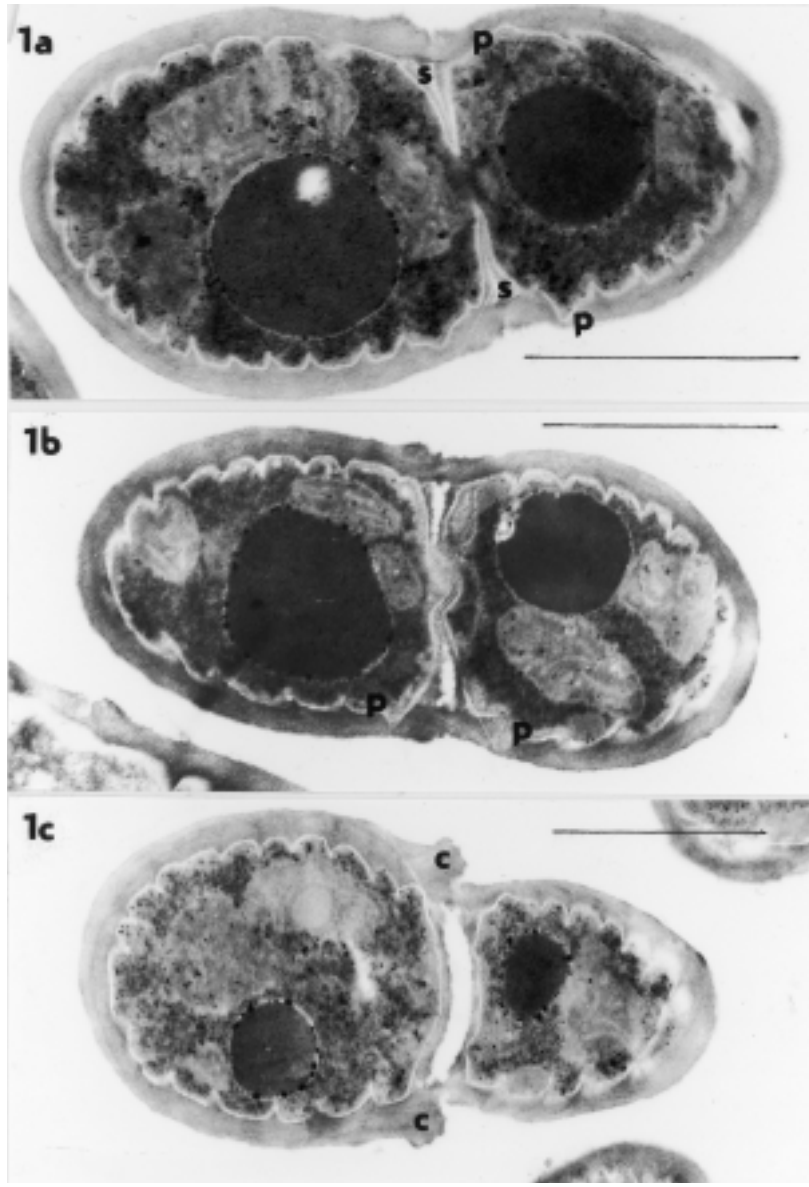

Fig. 1

Ultrathin sections through cells of *M. pachydermatis.* 1a: Developing septum is composed of several layers (s); wall thickness is reduced over the pits (p). 1b: Bud emerges from a broad base, more than 1µm in thickness; pits (p) are present on both mother and daughter cells. 1c: Collar (c), a structure arising from repeated unipolar budding, is seen on the mother cell wall. The wall in its vicinity is multilayer in appearance.

Bar, 1 µm.

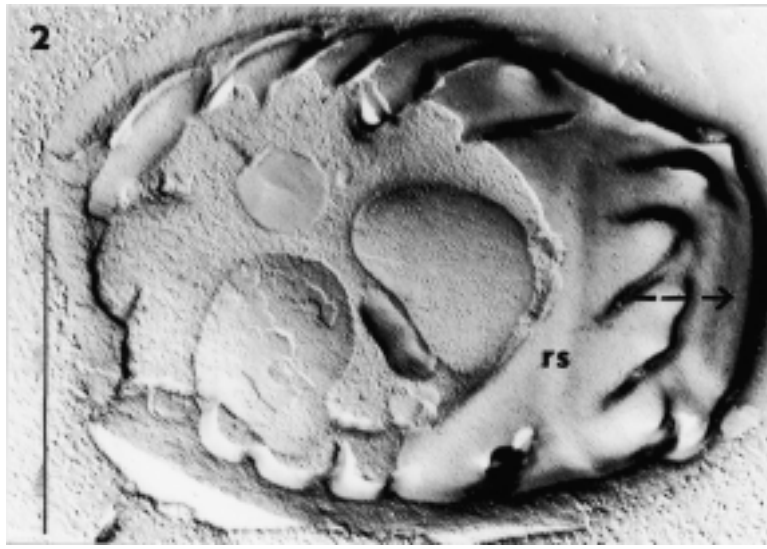

Fig. 2
Freeze-fracture replica of a *M. pachydermatis* cell. Protoplasmic fracture face (PF) shows a ring-like swelling (rs) on the plasma membrane. Grooves seen on the plasma membrane are not present on the bud apex (dashed arrow).
Bar, 1µm.

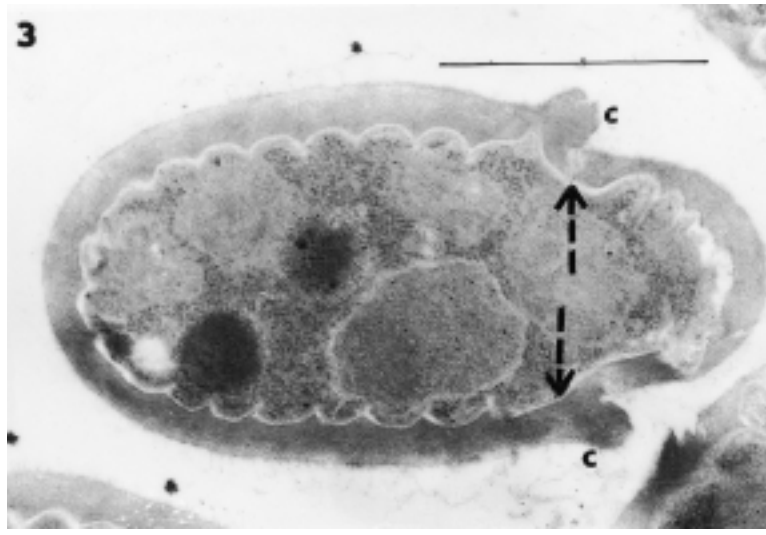

Fig. 3
Ultrathin section through a *M. pachydermatis* cell. Distinct collar (c) on the mother cell; the absence of a serrated arrangement of the wall at the site of bud emergence (dashed arrows).
Bar, 1 µm.

Fig. 4
Freeze-fracture replica. Exoplasmic fracture face (EF) of the plasma membrane shows a spiral pattern of ridges (full arrows).
Bar, 1 μm.

Fig. 5
Protoplasmic fracture face of the plasma membrane in a freeze-fracture replica; spiral arrangement of grooves (full arrows); bud apex free from grooves (dashed arrow).
Bar, 1μm

In comparison with the overall size of the organism, the cell wall was relatively thick, between 0.2 and 0.3 μm in certain areas (*Fig. 1c*). Also the base from which a bud emerged was broad, with up to 1.2 μm in width (*Fig. 1b*). At this site in freeze-fracture replicas, a ring-like swelling of the plasma membrane was observed (*Fig. 2*). The collar, a cell wall structure left on the mother cell after bud separation, was poorly visualised in freeze-fracture replicas but distinct in ultrathin sections (*Fig. 3*). In these specimens, a multilayer structure of the cell wall was well discernible, particularly in dividing cells, in the region adjacent to the collar (*Fig. 1c*) and on the developing septum (*Fig. 1a*).

The inner surface of the cell wall revealed by freeze-fracturing showed a spiral pattern of ridges corresponding to the spiral pattern of plasma membrane grooves (*Fig. 4*); these are also shown in *Fig. 5,* where the left-handed character of the spiral is clearly seen. While on the protoplasmic fracture face (PF) of the plasma membrane, the grooves have a left-handed arrangement (*Fig. 6*), on the

exoplasmic fracture face (EF) they have a right-handed appearance (*Fig. 7)* in relation to the longitudinal axis. The ridges are probably identical with the serrated arrangement of the innermost wall surface detected in ultrathin sections (*Fig. 8*). These structures were visualized by these two techniques on the cell wall of both the mother and the daughter cell and their gradual development was also demonstrated on the growing bud. They were missing only on the broad bud's base (*Figs. 3, 6*) and on the bud apex (*Figs. 2, 5*).

The freeze-fracture replicas showed plasma membranes with well discernible circumvallate bulgings situated between neighbouring grooves of the spiral arrangement close to the ring-like swelling (*Fig. 9*). One or two, occasionally three bulgings were present between each two grooves on the mother cell and gradually appeared on the bud during its growth. In ultrathin sections, corresponding structures were seen around the developing septum, in areas where the plasma membrane showed pits in the innermost wall surface. The pits were found on both the mother and daughter cells. The cell wall in these regions was only 0.02 μm in thickness (*Figs. 1a, 1b*).

The intracellular structures were few but relatively large, considering the cell's size. In addition, ultrathin sections (*Fig. 8*) showed a nucleus up to 1.0 μm in size, one to five mitochondria up to 0.7 μm in size, spherical vacuoles varying in size, filled with electron-dense material and small vesicles at the apex of the growing bud. The cytoplasm contained ribosomes. Freeze-fracture replicas also showed the endoplasmic reticulum and Golgi complex (*Fig. 10*).

The specific structures of the cell wall and plasma membrane, as revealed by the two techniques used, are schematically drawn in *Fig. 11*.

DISCUSSION

The small size of *M. pachydermatis* requires electron microscopy for the study of morphological features of the cell. Some authors make distinctions between small and large cell types in this species, with the former measuring 2.5–4.8 μm by 2.6-5.0 μm and the latter 3.8–6.0 μm by 4.8–7.0 μm (*4*). The strain studied here did not exceed 2.5 μm in width and 5.0 μm in length. This yeast species has not been found to produce mycelial hyphae, which may account for an increase in size. *Porro et al.* described the development of hyphae in a related species (*M. furfur*) following supplementation of the culture medium with cholesterol and its esters (*26*).

The relatively thick wall in *M. pachydermatis* may explain the resistance of this yeast to environmental conditions and its persistence on the skin and mucosa of warm-blooded animals. It may also be responsible for interfering with immunofluorescence staining of the cytoskeleton. The maximum of wall thickness in our observations was 0.30 μm and the minimum was 0.02 μm; this was found in regions, where the plasma membrane formed pits. Similar values for

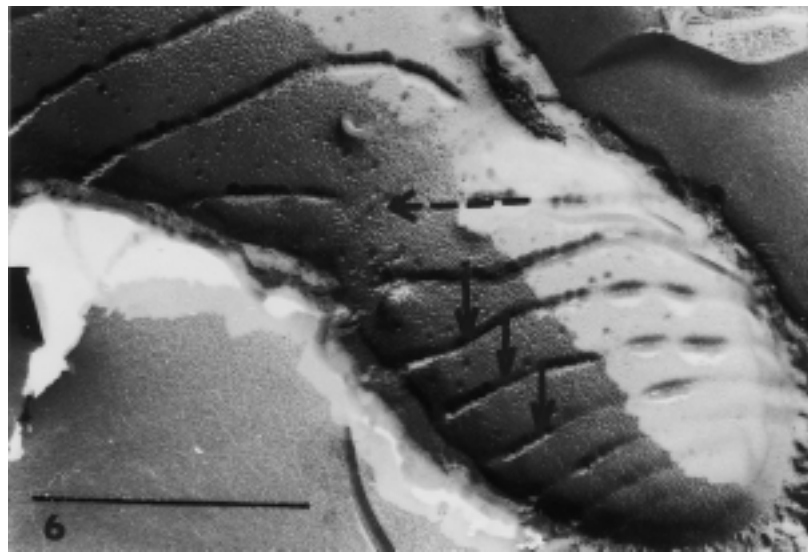

Fig. 6
Protoplasmic fracture face of the plasma membrane in a freeze-fracture replica; spiral arrangement of grooves (full arrows); broad base of the growing bud free from grooves (dashed arrow).
Bar, 1 μm.

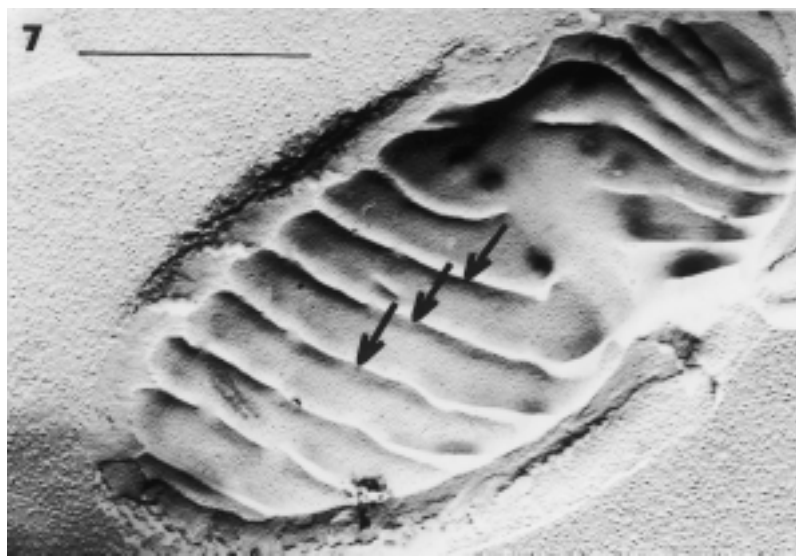

Fig. 7
Exoplasmic fracture face of the plasma membrane in a freeze-fracture replica; ridges have an appearance of a right-handed spiral (full arrows).
Bar, 1μm.

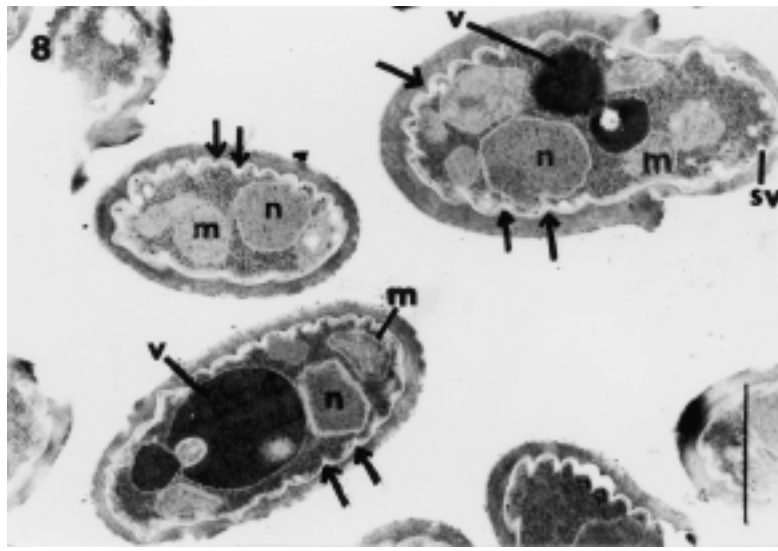

Fig. 8
Ultrathin sections through *M. pachydermatis* cells with a distinct serrated arrangement of the cell wall (full arrows). Intracellular structures include a nucleus (n), mitochondria (m), electron-dense vacuoles (v) and small vesicles (sv) on the bud apex.
Bar, 1 µm.

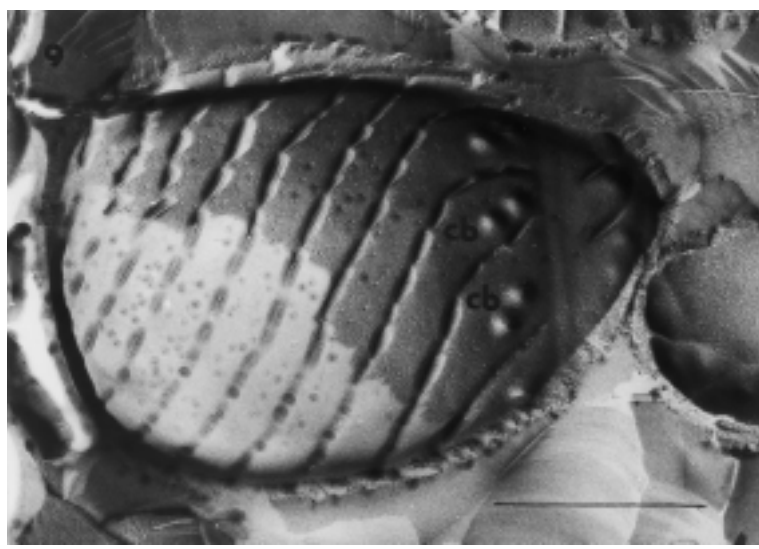

Fig. 9
Protoplasmic fracture face of the plasma membrane in a freeze-fracture replica: circumvallate bulgings (cb) situated between neighbouring grooves, corresponding to the pits seen close to the base of bud emergence in ultrathin sections.
Bar, 1µm

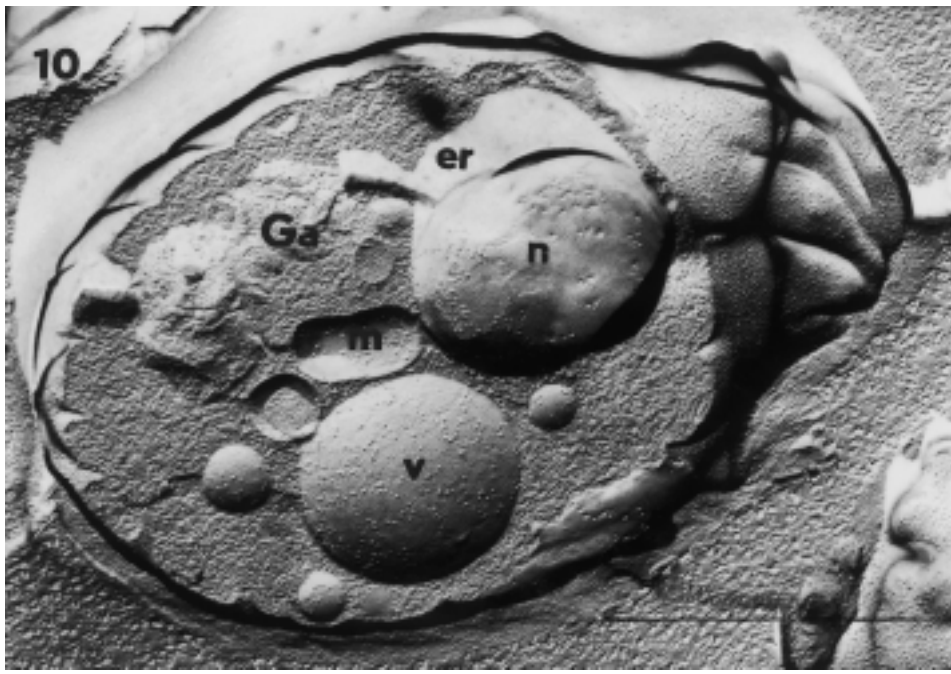

Fig. 10
Intracellular structures visualized by freeze-fracturing: nucleus (n), mitochondria (m), endoplasmic reticulum (er), Golgi apparatus (Ga), vacuole (v).
Bar, 1 μm

wall thickness, i.e., 0,10 to 0,25 μm, have been reported by *Nishimura et al.* (*21*). The cell wall consisted of several layers discernible particularly in the vicinity of the septum and collar. This is in agreement with the previous observations that the cell wall of *Malassezia* cells has two or more layers (*9, 18, 22, 27, 28, 29*). *Guillot et al.* (*30*) described a multilamellar, thick wall (0.30 to 0.45 μm) and distinguished two main layers. However, they noted that the number of layers varied in relation to the conditions of cultivation, fixation or contrasting techniques. *Mittag* (*31*) detected a three-layer wall in *M. furfur*, but the middle layer was visible only under certain circumstances. He also observed a thin lamellar coat on the outer cell wall surface and assumed that this contained lipids. This outer layer showed a different structure in relation to the composition of culture medium and the author suggested that it might play a role in facilitating adhesion of these yeast cells to human skin cells in dermatosis called *pityriasis versicolor*. A layer with membrane characteristics situated on the outside of the cell wall was also described by *Winiarczyk* (*32*). We observed no such layer but

A) ULTRATHIN SECTION THROUGH A CELL;
B) PROTOPLASMIC FRACTURE FACE OF THE PLASMA MEMBRANE IN A FREEZE-FRACTURE REPLICA (PF)

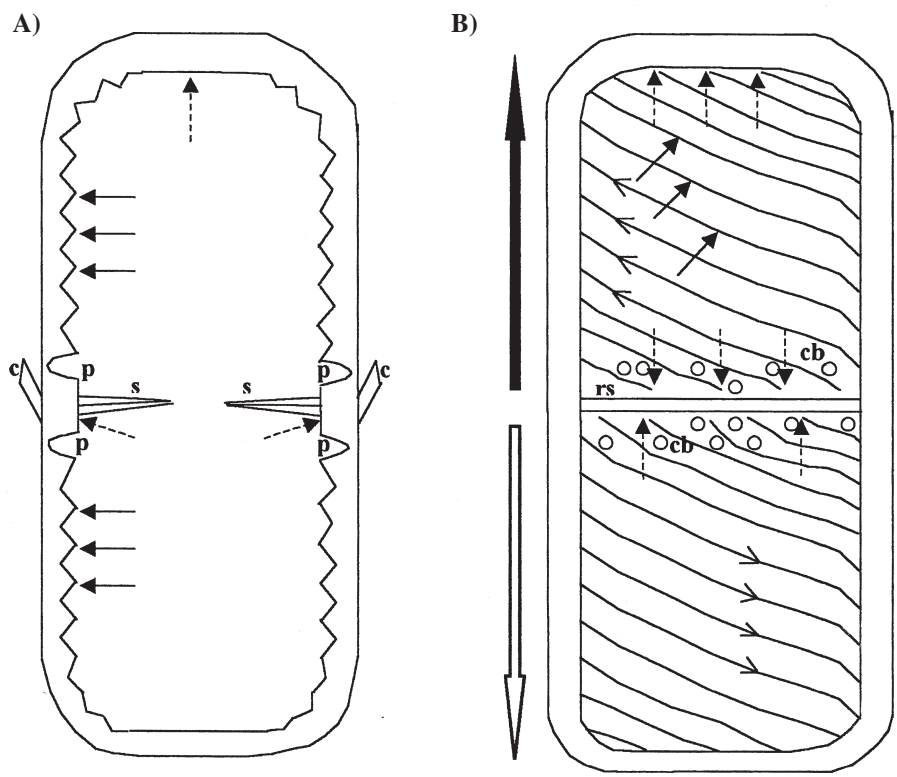

Fig. 11

Diagrams of the specific arrangement of the cell wall and plasma membrane in a *M. pachydermatis* cell, as revealed by A) ultrathin sectioning and B) freeze-fracturing: c, collar; s, septum; rs, ring-like swelling; p, plasma membrane pits; cb, circumvallate bulgings of the plasma membrane corresponding to the pits. The serrated arrangement of the cell wall and plasma membrane invaginations (full arrows) shown by ultrathin sectioning (A) correspond to cell wall ridges and plasma membrane grooves (full arrows) in freeze-fracture replicas. In the PF diagram (B), arrow heads indicate the direction of groove formation on both mother cell and bud. On the protoplasmic fracture face, grooves appear as left-handed spirals in relation to the direction of polarised growth. Dashed arrows indicate sites free from the serrated arrangement and ridges of the cell wall in ultrathin section and freeze-fracture replicas, respectively. Arrows outside the cells indicate the direction of polarised growth: hollow arrow, growth of the mother cell before the last division; full arrow, bud growth. The PF diagram was taken from *Takeo and Nakai* (*23*) and modified.

cell agglutination present in cultures grown in liquid medium may be taken as indirect evidence of the hydrophobic nature of cell surfaces.

In the genus *Malassezia*, the bud develops from a very broad base; this fact may be interpreted as a transition between budding and fission (*8*). The size of this base in our study was about 1.0 μm, which is in agreement with the findings made by *Nishimura et al.* (*21*). Other members of this genus have a narrower base ranging from 0.5 to 0.7 μm, and this serves as one of the taxonomic features for species differentiation (*3*).

In freeze-fracture replicas, we found a ring-like swelling of the plasma membrane in place of the broad bud base. This structure has previously been described by *Takeo* and *Nakai* (*23*). In ultrathin sections, the most prominent structure on the mother cell was a collar that persisted on the wall even after bud separation (*21*). A collar has also been observed in other basidiomycetes that posses multilamellar cell walls (*31*).

Takeo and *Nakai* (*23*) found right-handed spiral ridges on the inside of the cell wall (exoplasmic fracture face) in *M. pachydermatis* cells studied by freeze-fracturing. The ridges corresponded to grooves present in the plasma membrane. In ultrathin sections, the innermost surface of the cell wall showed a serrated arrangement, to which the invaginations of the plasma membrane closely adhered (*9, 12, 22*). Since, in our *M. pachydermatis* specimens, similar images were visualized by both freeze-fracturing and ultrathin sectioning, we can conclude that these structures are corresponding to each other. In *M. furfur* studied by ultrathin sectioning, the serrated appearance of the innermost wall surface (exoplasmic fracture face of the plasma membrane) has also been demonstrated (*9, 19, 20, 31*). *Guillot et al.* (*30*) observed electron-transparent stripes passing within the ridges across the full thickness of the wall. *Mittag* (*31*), who too has found them in *M. furfur*, suggests that they may function as a channel system. The role for the plasma membrane invaginations is not fully understood yet. One of its functions may involve enlargement of the plasma membrane surface area in a small cell. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe,* cells incubated in media with osmotic stabilisers showed enlarged ridges of the cell wall and their penetration into the cytoplasm (*34*). These structures, however, are not arranged in regular patterns as in *M. pachydermatis* cells. The effect of osmotic stabilisers on the ultrastructure of *M. pachydermatis* cells remains open to further investigation. Some insight may be also gained by studying the invaginations during enzymatic degradation of the cell wall and subsequent protoplast production, and their relation to cytoskeletal structures.

In freeze-fracture replicas of *M. pachydermatis* cells, the plasma membrane showed circumvallate bulgings between the grooves situated close to the ring-like swelling, which is in agreement with the observations by *Takeo* and *Nakai* (*23*). These structures seem to correspond to the pits in the plasma membrane

that were demonstrated in ultrathin sections. The cell wall attached to the outer side of these pits was very thin. The pits observed in our study have also been reported by other authors in *M. pachydermatis* (*18, 21*) and by *Mittag* in *M. furfur* (*31*). They have been suggested to be involved in oriented monopolar cell division or in collar development. They may also function as exocytotic vesicles bringing material for septum construction because they are invariably found close to the broad budding base.

The intracellular structures were large in relation to the size of *M. pachydermatis* cells. In ultrathin sections, they included a nucleus, vacuoles, mitochondria, small vesicles next to the bud apex and ribosomes in the cytoplasm. The nucleus was approximately circular in shape and up to 1.0 μm in size. The micronucleus, seen as a dark body in the centre of the nucleus by *Gabal and Fagerland* (*18*), was not differentiated by our preparation technique, nor was it confirmed by other studies (*4*).

In contrast to the findings made by *Kreger van Rij and Veenhuis* (*29*) in *Malassezia,* we did not find any endoplasmic reticulum or Golgi complex in cells processed by ultrathin sectioning but detected them in freeze-fracture replicas, probably because the latter technique involves fixation procedures less harmful to the cell.

The methods of freeze-fracturing and ultrathin sectioning used in this study to visualise cell morphology at the ultrastructural level revealed unusual corresponding images of cell surface structures in *M. pachydermatis* and demonstrated that both are reliable tools for further investigations. These will be concerned with the relation of these surface structures to cytoskeletal structures because no information on this important relationship is available in this species.

A c k n o w l e d g e m e n t

The authors wish to thank Mrs V. Ramíková, Mrs D. Klemová and Mr P. Hnilička for their skillful technical assistance. This study was supported by grant no. 310/03/1195 from the Grant Agency of the Czech Republic.

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NEOBVYKLÉ ULTRASTRUKTURÁLNÍ CHARAKTERISTIKY KVASINKY *MALASSEZIA PACHYDERMATIS*

S o u h r n

Cílem naší studie bylo prozkoumat morfologické charakteristiky potenciálně patogenní kvasinky *Malassezia pachydermatis* pomocí transmisní elektronové mikroskopie (TEM). Pro srovnání byly použity dvě metody přípravy preparátů: ultratenké řezy a mrazové lámání. Byla zaznamenána poměrně malá velikost buněk *M. pachydermatis*, jež v preparátech nepřesahovala 5.0 m a relativně silná buněčná stěna. Dále široká báze pučení, která nahrazuje krček jiných pučících kvasinek, s tvorbou charakteristického límečku. Na preparátech připravených mrazovým lámáním

byly zjištěny na vnitřním povrchu buněčné stěny (EF plasmatické membrány) *M. pachydermatis* pravotočivé spirální hřebeny, které odpovídaly levotočivým spirálním rýhám na plasmatické membráně (PF plasmatické membrány), které směřovaly k růstovému pólu dceřinné buňky. Tyto struktury odpovídají pilovitému uspořádání vnitřního povrchu buněčné stěny a invaginacím plasmamembrány na ultratenkých řezech kvasinkou. Na buňkách zpracovaných mrazovým lámáním dále bylo na plasmatické membráně v místě cytokinese pozorováno prstencovité zduření a v jeho blízkosti jak na mateřské tak na dceřinné buňce byly zaznamenány hrazené výdutě. Ty jsou zřejmě ekvivalentem k jamkám, které vytváří plasmamembrána, zjištěným na ultratenkých řezech. Tyto neobvyklé ultrastrukturální nálezy buněčných povrchů, které potvrzují dřívější nálezy jiných autorů, budou dále studovány ve vztahu k organizaci jejího cytoskeletu.

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