

Fine structural investigation of *Malassezia furfur*. II. The envelope of the yeast cells

Feinstrukturelle Untersuchung von *Malassezia furfur*. II. Die Zellwand der Hefezellen

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Key words. *Malassezia furfur*, yeast phase, electron microscopy, ultrastructure, cell envelope.

Schlüsselwörter. *Malassezia furfur*, Hefephase, Elektronenmikroskopie, Ultrastruktur, Zellwand.

Summary. The general ultrastructure of the *Malassezia furfur* cell envelope is shown in a schematic model. Distinct layers such as the outer lamella, the cell wall and the plasma membrane and special structural elements of the cell wall are described and presented in electronmicrographs. Special attention was paid to the outer lamellar layer that seems to be equivalent to a capsule. It probably contains lipids as well as the cell wall, and it seems to participate in attachment processes.

Zusammenfassung. Der charakteristische feinstrukturelle Aufbau der Zellhülle von *Malassezia furfur* läßt sich mittels eines Schemas zeigen. Die Schichten der gesamten Hülle, darunter die lamellose Außenschicht, die Zellwand und die Plasmamembran, sowie spezielle Strukturelemente in der Zellwand werden bezogen auf das Modell beschrieben und in elektronenmikroskopischen Aufnahmen gezeigt. Besondere Berücksichtigung hat die Außenlamelle gefunden, die wahrscheinlich, wie auch die Zellwand, Lipide enthält. Es wird angenommen, daß die Außenlamelle funktionell einer Kapsel entspricht und Adhärenzprozesse der Pilzzellen begünstigt.

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Dedicated to Professor Dr M. Rupec, Marburg.

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Introduction

The ultrastructure of the *Malassezia furfur* cell envelope, a structure which in yeasts generally consists of the plasma membrane, the periplasmic space, cell wall and sometimes a slime layer [1], appears unique. In *Pityrosporum orbiculare*, considered to be one form-type of *Malassezia furfur* [2], Barfatani *et al.* 1964 [3] observed a relatively thick wall. It consisted of two separate layers, exhibiting indentations on the inner layer. No significant differences were found in the appearance of *Pityrosporum ovale*, which is another form-type of *Malassezia furfur* [2], and *Malassezia pachydermatis* (*Pityrosporum canis*); the organisms had relatively thick, multi-layered cell walls with corrugations in a concentric arrangement at the inner surface [4]. Kreger van Rij and Veenhuis 1970 [5] studied isolated cell walls of *Pityrosporum ovale*; Simmons and Ahearn 1987 [6] paid special attention to the wall substructure of *Malassezia* spp.

Malassezia furfur/*Pityrosporum* organisms, grown in the human skin have a fine structured cell envelope similar to that *in vitro*. This was recognized in pityriasis versicolor [7, 8] and in follicles and comedones [9].

Freeze-fracturing [10] and freeze-etching with subsequent scanning electron microscopic examination of the organisms [11, 12] revealed grooves and ridges on the surface of the plasma membrane. Furthermore, "circumvallate bulgings" of the plasma membrane towards the exterior were noticed in certain areas [10, 11], a structure that seems to be identical with the "cone-shaped pits" described by Kreger van Rij and Veenhuis (1970) [5] and the "markedly thin" area of the yeast cell

wall mentioned by Abou-Gabal and Fagerland (1979) [13].

These data were supplemented by personal investigations to present a model of the general structure of the *Malassezia furfur* cell envelope.

Materials and methods

Malassezia furfur strains CBS 1878 and CBS 6001 (Centraalbureau voor Schimmelcultures, Yeast Division, Delft, The Netherlands) were cultured on peptone-glucose-agar or Sabouraud-glucose-agar. A thin layer of either filtered olive-oil or different types of the emulgator Tween (polyethylen(20)sorbitan-Monolaurat = Tween 20, -Monopalmitat = Tween 40, -Monostearat = Tween 60, -Monooleat = Tween 80) was spread on the surface of the agar, control cultures were left without a supplement (Fig. 1). Colonies cultivated for 3 to 8 days were considered to be "young" cultures, colonies cultivated for a month were considered to be "old" cultures. The micro-organisms were harvested at the mentioned times and suspended in the fixatives, a mixture of 3% glutaraldehyde, 1.5% paraformaldehyde, 1.5%

acroleine (4 h room temperature/16 h 4°C) followed by 1.5% potassium permanganate (1.5 h, room temperature) and 1% osmium tetroxide (3 h, 4°C). Furthermore, scales of pityriasis versicolor were fixed in 2% glutaraldehyde (4 h, room temperature), 1.5% potassium permanganate (25 min) and 1% osmium tetroxide (3 h, 4°C). The fixed materials were dehydrated by a series of graded alcohols, embedded in EPON, sectioned with an ULTRATOME (LKB), and poststained with uranyl acetate and lead citrate. The sections were examined with the electron microscope EM 9S (ZEISS, Oberkochen, Germany). Light microscopic observations were performed on fungal cells stained with ink, Gram's stain, and nileblue sulfate.

Results

Electron microscopy

Scheme of the cell envelope (Fig. 2). The cell envelope of *Malassezia* organisms is composed of a lamellar layer on the surface [A], a multi-layered wall [B] and a closely attached plasma membrane [C] on the inside. These main structural elements of the *Malassezia furfur* yeast cell envelope are demon-

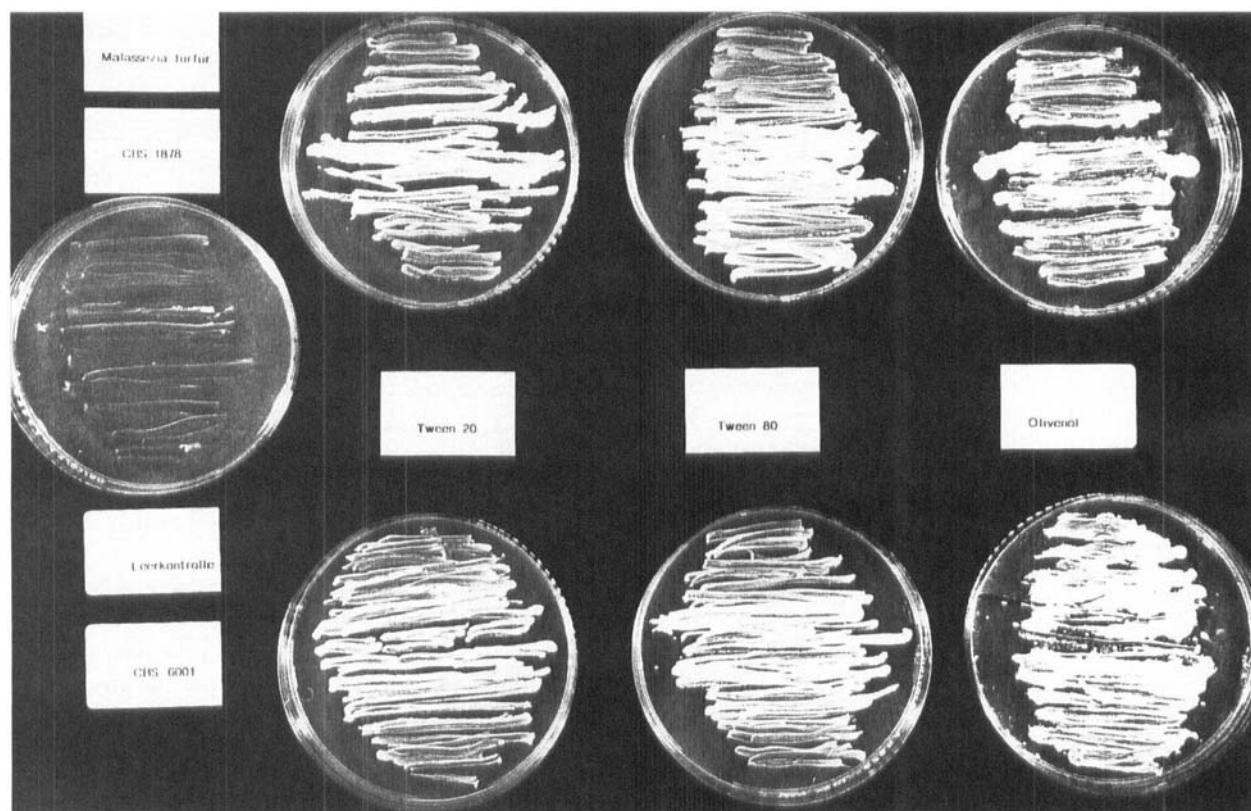


Figure 1. *In vitro* cultivation of *Malassezia furfur*/*Pityrosporum* strain CBS 1878 (upper lane) and CBS 6001 (lower lane) on different lipid sources (from left to right): 1st plate: no lipid; 2nd plates: Tween 20; 3rd plates: Tween 80; 4th plates: olive oil.

strated in a scheme (Fig. 2) and in comparable micrographs of the yeasts *in vitro* (Fig. 3a, b, c) and *in vivo* (Fig. 4d).

The lamellar layer [A]. The lamellar layer (Figs 3a, c, 4a, b, d) on the surface of the cell envelope is electron-dense. Sometimes it appears "membrane-like" with two dark lines enclosing an electron-transparent middle line (Fig. 3c). The lamellar

layers sometimes fuse in adjacent fungal cells (Fig. 4b). Microfibrillar structures or an amorphous, sometimes granular material seem to be connected with the outer part of the lamellar layer (Fig. 3b, c). The state of preservation of the lamellar layer varies; differences in its structure are evident between olive-oil- or Tween-80-supplemented cultures (Fig. 3c, d). This suggests that lipids are present in the lamellar layer.

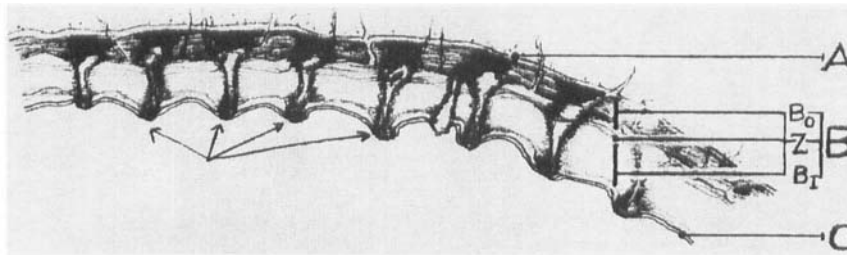


Figure 2. Schematic model of the *Malassezia* cell envelope with an outer lamella [A], a cell wall [B] and the plasma membrane [C]. The cell wall [B] is subdivided in an outer main layer [B₀], an inconsistently present intermediate zone [Z] and an inner main layer [B₁]. (↑) Lomasome-like structures are connected with the plasma membrane and radiate stripes. The latter cross the inner main wall layer [B₁] and are associated with holes in the intermediate zone [Z].

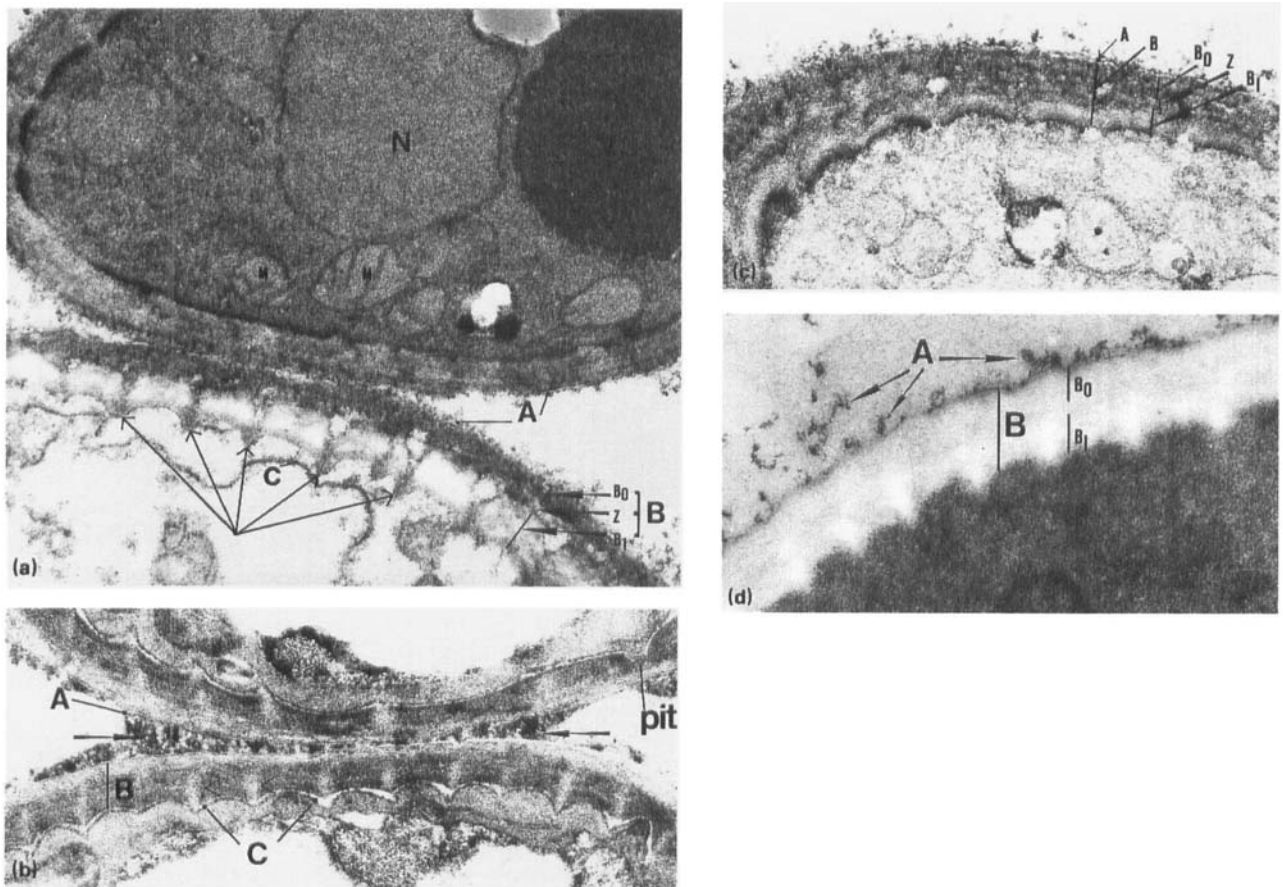


Figure 3. Microphotographs of the *Malassezia* cell envelope, demonstrating the structural elements mentioned in the schematic model (for abbreviations see Fig. 2). Pit: Focal area of reduced wall-thickness; N: nucleus; V: vacuole; M: mitochondrion. Magnification 3a, b: 26 000 ×, 3c, d: 29 000 ×. (a) Cell envelope of a deteriorating cell (old culture). (↑) Lomasome-like structures. (b) Cell envelopes of adjacent cells (young culture). Indentations, stripes [←], plasma membrane [C] and a pit are well visible. A: Lamella. B: Cell wall. (→) Granular material connecting the neighbouring cell envelopes. (c) Cell envelope in an old culture supplemented with olive oil. Lamella [A] is well recognizable. Note the multi-layered substructure of the cell wall [B], especially in its outer part [B₀]. (d) Cell envelope in a young culture supplemented with Tween 80. Outer lamella [A] fragmented, cell wall [B] electron transparent, details become visible only by overexposure of the negatives.

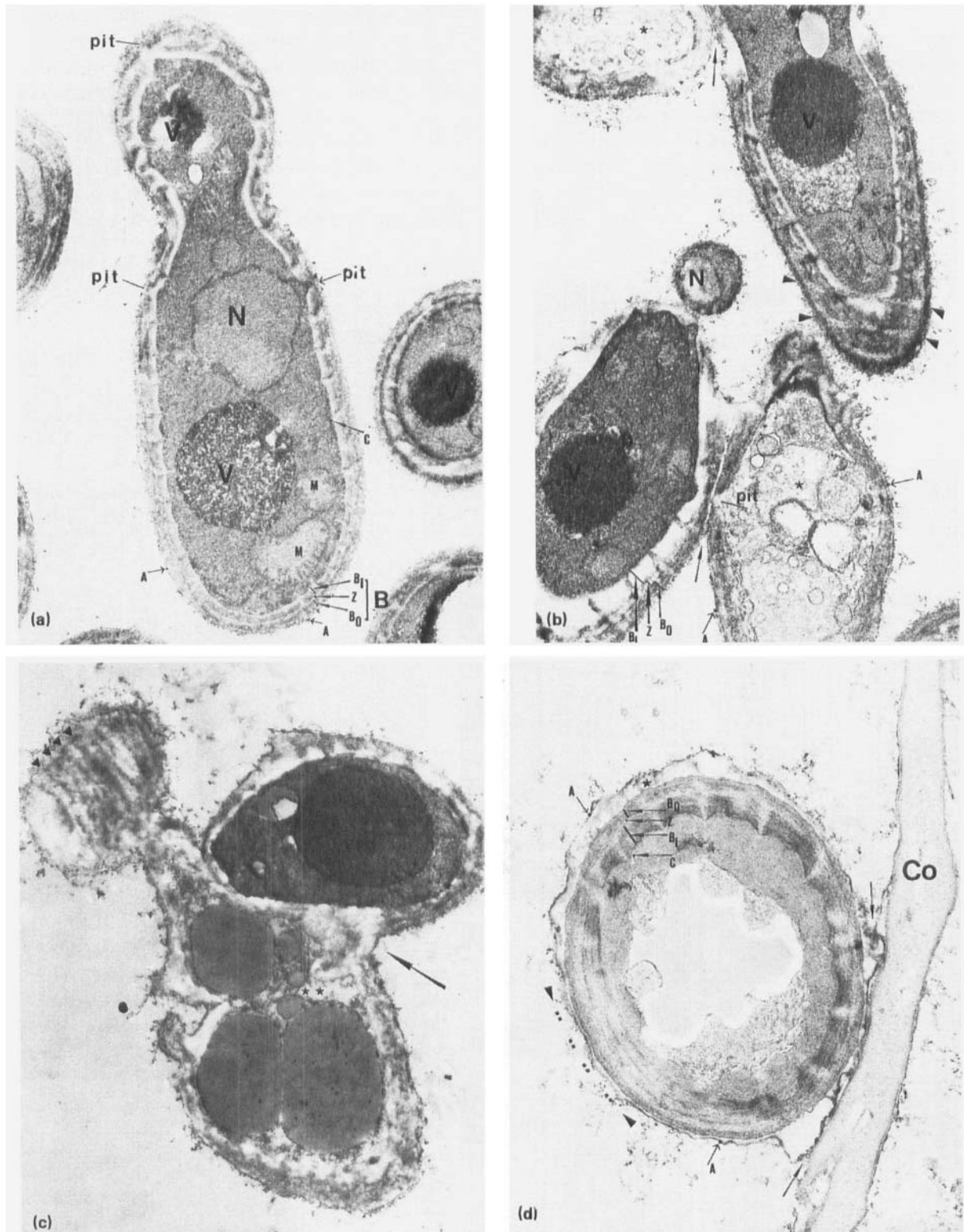


Figure 4. Microphotographs of *Malassezia* yeast cells, demonstrating structural elements of the cell envelope, as mentioned in the schematic model (for abbreviations see Figs 2 and 3). Magnification 4a-d: 15 000 $\times$. (a) Monopolar budding cell of a young culture with olive-oil. "Pits" are present beneath the septal area and in the bud. (†) Pit connected with the nuclear envelope. (b) Cells of an old culture supplemented with olive-oil. (▲) grid-like pattern due to tangentially sectioned stripes; (*) cells with an electron transparent cytoplasmic space with signs of beginning disintegration; (†) fusion of the outer lamella [A] in neighbouring yeasts. (c) Cells of a young culture supplemented with Tween 80 (overexposed negative). (†) atypical attachment of neighbouring cells; signs of a severe intracytoplasmic damage in one of the partners (**); (▲) grid-like pattern in the bud due to tangentially sectioned stripes. (d) *Malassezia furfur* yeast cell in pityriasis versicolor. The fungal cell is located in the horny layer adjacent to a corneocyte (Co). The outer lamella [A] of the *M. furfur* cell envelope is attached to the corneocyte surface (†). (▲...▲) area with a less well developed cell wall substructure; (*) Amorphous material in the space between lamella [A] and cell wall [B].

The cell wall [B]. The cell wall [B] makes up the largest part of the cell envelope. It is subdivided in two main layers, the inner main layer [Bi] and the outer main layer [Bo]. Sometimes these main layers are separated by an intermediate zone [Z]. The main layers stain variably, but at least one main layer exhibits a medium or high electron density in olive-oil supplemented cultures (Figs 3a, b, c, 4a, b) and *in vivo* (Fig. 4d).

The inner main layer of the cell wall [Bi] is about 50–70 nm thick. An inner electron dense rim was found in some organisms (Fig. 3c). Sometimes, a microfibrillar substructure is visible, the microfibrils are orientated parallel to the fungal surface (Fig. 3b, 4d).

The outer main layer of the wall [Bo] is 40–80 nm thick. The microfibrillar substructure is difficult to determine, there are a few microfibrils orientated radially to the fungal surface (Fig. 3a), but there is also a horizontal layering (Figs 3a, c, 4b, d). The lamellated substructure of the main wall layers [Bi, Bo] results in a multi-layered cell wall.

The intermediate zone [Z], which is not always visible, is about 20 nm thick and has a somewhat granular appearance. Within this intermediate layer electron-transparent spaces, giving the impression of holes, are regularly arranged (Figs 3a, c, 4d).

There are indentations on the internal surface of the cell wall (Figs 3b, 4d) and stripes are radially crossing the cell wall (Figs 3a, b, 4d and other electron micrographs) emanating to the fungal poles. In tangential sections the stripes form a grid-like pattern in the cell wall (Fig. 4b). The cell poles reveal a system of 9–14 stripes in a spiral arrangement [8, 11].

The appearance of both structures, indentations and stripes, is influenced by the method of fixation, angle of sectioning (see a partial lack of indentations and stripes in Fig. 4d), size of the cell and the strain type. Yeast cells of strain CBS 6001 usually have less stripes and a wider space of the indentations than strain CBS 1878. In deteriorating yeast cells, the stripes in the inner main wall layer [Bi] together with the holes in the intermediate zone [Z] resemble a channel-system (see scheme in Fig. 2, Fig. 3a).

The plasma membrane [C]. The plasma membrane often follows the indentations on the inner surface of the cell wall very closely. It is rarely visible in vital cells as in Fig. 3b. In deteriorating cells round electron dense particles, lomasome-like structures, seem to be detached from the plasma membrane (Fig. 3a), but they are connected with the “stripes” in the inner main wall layer [Bi]. During further

de-arrangement of the yeast cells the lomasome-like structures gather in the periplasmic space located between plasma membrane [C] and cell wall [B].

“Pits” [p]. In certain areas, there are extremely thin zones of the fungal wall, named “pits” [p], an expression mentioned by Kreger-van Rij & Veenhuis (1970) [5]. In these regions, the plasma membrane [C] comes close to the outer lamellar layer [A]. The reduction of cell wall thickness in these areas is associated with an unusually deep protrusion of the protoplast towards the outside (Figs 3b, 4a, b). This “pit”-structure is often found in the cell wall beneath the septal area and beyond the septum in the wall of the bud (Fig. 4a, b). Sometimes, it seems to be connected with the nucleus, especially the outer membrane of the nuclear envelope (Fig. 4a).

Age-related changes. All structural elements of the cell envelope were present in organisms of different age of cultivation (Fig. 3a, b, c). Organisms of the older culture tended to a greater diversity:

- The cells either have a well-contrasted or a rather transparent cytoplasmic substance, the latter cells show signs of a beginning disintegration (Fig. 4b).
- The cells have a wide range of wall-thickness and stratification; multi-layering is often demonstrable in the outer main wall layer [Bo] (Figs 3c, 4b).

Tween-related changes. *Malassezia furfur* standard strains grow on culture plates supplemented with different Tweens or with olive-oil, whereas colony growth ceases on an unsupplemented plate (Fig. 1).

Ultrastructurally, cells of the Tween-80 culture have a granular and fragmented outer lamellar layer [A] and the cell wall [B] appears rather electron transparent in both main layers (Fig. 3d). The details of the wall are hardly recognizable but they may be outlined in overexposed microphotographs (Figs 3d, 4c). Degenerated and destructed cells were more frequently observed by electron microscopy and there were also signs of an atypical attachment of neighbouring cells in the Tween-80-supplemented culture (Fig. 4c).

Results in vivo. In the scales of pityriasis versicolor *Malassezia furfur* yeast cells have a cell envelope similar to that found *in vitro* (Fig. 4d). The micro-organisms in the scales of pityriasis versicolor are covered by the lamellar layer [A] on their surface, but sometimes this layer [A] is detached and a homogenous material of medium electron density fills up the space between the lamellar layer [A]

and the cell wall [B]. The lamellar layer can be found in close apposition to corneocytes (Fig. 4d). The inner and outer main layers of the cell wall substructure [Bi, Bo] and sometimes the intermediate zone [Z] are visible. There are radiate stripes in the fungal cell wall and indentations on its inner surface. As *in vitro*, the plasma membrane [C] is seldom visible.

Light microscopy

Ink stains the outlines of the fungal cells and the cytoplasm, except for the transparent vacuoles and some totally transparent cells. The latter cells are increased in 1-month-old cultures, they are probably deteriorating cells, comparable to the ultrastructurally transparent cells mentioned above, with signs of a beginning devitalization. Gram stain gives varying results; there are stained and unstained cells, the latter being less frequent. In the Nileblue sulfate stain there are many stained and a few unstained cells, surrounded by a delicate purple lamella, probably resembling the lamellar layer [A] of the electron microscopic material. Cells with a rather light-stained cytoplasm reveal a blue-tinted cell wall (Fig. 5).

Discussion

The proposed model of the *Malassezia furfur* cell envelope (Fig. 2) unifies certain developmental differences of the individual fungal organisms' envelopes. This makes it easier to consider details of the general structure of the *M. furfur* cell envelope and to recognize changes induced, for instance, in Tween-80-supplemented cultures (Figs 3d, 4c).

Our observation of the thin outer lamellar layer [A] of *M. furfur* is in accordance with findings on

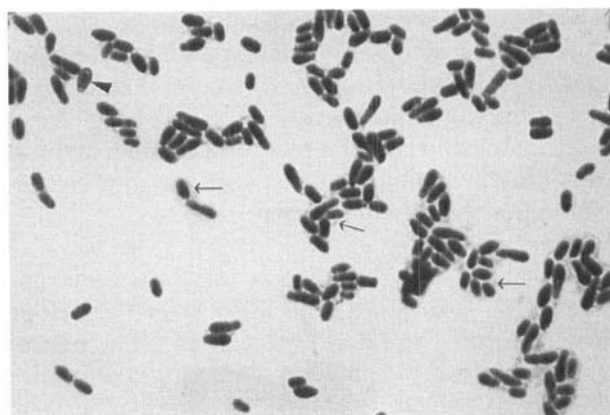


Figure 5. Light microscopy. Nileblue sulfate stain of *Malassezia furfur* strain CBS 6001. (↑) delicate purple stained lamella; (▲) dark blue stained cell wall. Magnification: 530 ×.

Pityrosporum ovale by Kreger van Rij and Veenhuis (1970) [5]. This lamellar layer is present in earlier microphotographs of *M. furfur*/*Pityrosporum* but it was not mentioned by the authors [4, 8]. Winiarczyk (1992a) [14] referred to it in a study of *Malassezia* (*Pityrosporum*) *pachydermatis*. Lack of the outer dark line in cells lying close together was mentioned by Kreger-van Rij and Veenhuis 1970 [5], our own material also revealed fusion of the lamellar layer (Fig. 4b) or of an attached electron dense material in adjacent cells (Fig. 3b). This suggests that the lamellar layer is important in attachment processes, as seen in the horny layer in pityriasis versicolor (Fig. 4d). *Malassezia furfur*/*Pityrosporum* can attach to human stratum corneum cells [15] as well as to broviac catheters used for parenteral fat emulsions [16]. Scanning electron microscopy failed to resolve special attachment structures in the latter case.

Considering the Nileblue sulfate stain and the fine-structural differences achieved with different supplements during cultivation, it is suggested, as also proposed by Winiarczyk (1992b) [17], that the lamellar layer contains lipid components. Generally, lipids are important in adhesion of yeasts to host cells [18, 19]. In *Candida albicans* a variable cell surface was found with different carbon sources, which were obviously utilized for construction of the capsule-like slime layer [20]. In *M. furfur* the lamellar layer [A] may resemble a capsule of these lipid-dependent yeasts, comparable in function to the slime layer of other fungi. In spite of its sometimes membrane-like appearance, it is no true biomembrane, as it does not divide two different compartments of the micro-organism (for definition of "biomembrane" see [21]).

Ultrastructurally, the outer lamellar layer [A] of *Malassezia* organisms seems to have parallels to the outer "membrane-like" layer of gram-negative bacteria [22, 23]. Due to this structure, the gram-negative bacteria have periplasmic spaces, which contain enzymes and make an electric surface charge possible. With respect to enzymes in *M. furfur*/*Pityrosporum*, Nazzaro-Porro *et al.* (1986) [24] could demonstrate lipoxygenase activity on the cell surface and in the supernatant of the *in vitro* cultured fungal cells. Ran *et al.* (1993) [25] demonstrated a lipase in association with the *Malassezia* wall. Cells of *M. furfur*/*Pityrosporum* however, stain gram positive [26, 27], although this is variable among the individual fungal cells [28, 29, own observations].

Considering the stratification of the cell wall of *Malassezia* organisms, most authors agree on two [3, 8, 10], three [13] or several layers [5, 6] of different electron-density. It is difficult to deter-

mine the exact number of layers, as this seems to depend on the culture conditions, methods of fixation, angle of sectioning and state of preservation of the cells. Therefore, we consider two main layers [Bi, Bo] as an essential feature of the cell wall of these fungi according to the proposed model (Fig. 2). Furthermore, there may be an inconsistently visible intermediate zone [Z], separating the main layers. The main layers may be further lamellated, thus revealing a multi-layered substructure.

In general there seems to be no sharp boundary between individual fungal wall layers, rather, they merge into one another. This multilamellar structure of the cell wall is responsible for the wall's mechanical strength, likened to a concrete construction [30].

There are similarities recognizable in the multi-layered substructure of the *Malassezia* cell wall and the wall architecture of *Rhodotorula glutinis* as described by Marquardt (1962) [31] and Mares (1982) [32]. The multilamellar wall structure of *M. furfur* suggests that—according to Kreger-van Rij and Veenhuis (1971) [33]—this species has to be classified with the basidiomycetes [6].

The stripes and indentations on the interior side dominate the fine structure of the cell wall. Ridges, formed by the indentations on the inner cell wall surface, are even visible by light microscopy in *M. furfur* [34, 35, own observations]. The stripes and indentations as well as the ridges were considered to be a characteristic feature of the genus [4, 7, 13, 34]. Nevertheless, electron microscopically similar "stripes" without indentations are visible in the ascospores of *Neurospora dodgei* [36] and "stripes" without a lamellar wall are present in the ascomycete *Piedra nigra* [37]. The stripes of *Malassezia* are arranged in a spiral system on the fungal surface which is clearly visible in the polar region [5, 8, 10, 11]. In all strains tested (*Pityrosporum ovale*, *Pityrosporum orbiculare*, *Pityrosporum pachydermatis*) Takeo & Nakai (1978) [11] found only impression marks of left-handed spirals on the surface of the protoplasts. Our own ultrastructural photographs of *M. furfur* organisms *in vitro* and *in vivo* suggest that the stripes within the wall belong to a channel system. Such a channel system has been demonstrated (though less clearly visible) in the *Candida albicans* cell wall [38, 39].

The areas of markedly reduced wall thickness resemble the "cone-shaped pits" [5] and the "circumvallate bulgings" [10–12]. Tangential sections reveal the "circlets" [11, 12].

The true function of these circumscribed, markedly thin areas in the cell envelope is unknown. Breathnach *et al.* (1976) [5] speculated that they might be equivalent to bud scars. Their partici-

pation in the regular spacing process of the yeast cell surface seemed likely and they were considered to be natural markers, showing how the mother cell and the bud grow independently [11, 12]. An influence of the "pits" on construction and destruction of the cell wall and the septum is supported by their characteristic position in a spatial relationship to the septal area [13]. It is speculative so far, whether they are furthermore involved in the division process of the fungal cell and its nucleus (see Fig. 4a) as well as in extrusion and adherence processes (Fig. 4b). This could implicate parallels to the "adhesion zones" in the gram-negative bacteria [22, 23, 40].

The plasma membrane [C] is not often visible in vital fungal cells, which is probably due to the osmotic pressure of the protoplast contained by the fungal wall [21]. The plasma membrane [C] becomes well recognizable as a regular biomembrane in deteriorating cells (Fig. 3a). Electron dense bodies, probably connecting the "stripes" of the cell wall with the plasma membrane, are lomasome-like. For general information on true lomasomes see Moore 1965 [41], Marchant and Moore 1973 [42], and Rajasingham and Cawson 1984 [43]. In disintegrating cells of *Trichophyton rubrum*, subjected to econazol, similar 'lomasomes' were found [44]. In *M. furfur*, the lomasome-like structures are present in a rather regular spacing (see Fig. 3a). Perhaps they participate in the electron dense rim of the inner layer of the cell wall when compressed in vital fungal cells (Fig. 3c). Considering their position, they might have a pore-like function in vital cells.

The biochemical composition of the *Malassezia* cell envelope is as yet rather unknown. The varying results of the Gram stain suggest a variable composition. High amounts of carbohydrates were found chemically [26] and represented the main portion of an antigen preparation of the micro-organism [45]. A newly developed polyclonal, monospecific *Malassezia furfur*-antibody, however, mainly reacts with proteins, the antigens are of 72 KD and 58 KD [46]. Enzymes acting on glucan could not digest the *M. furfur* wall (Bastide *et al.* 1975 [47], own results), but Ran *et al.* 1993 [25] were successful with zymolyase (a glucanase) after pre-treatment with a strong detergent. In our opinion, this is no convincing proof for a glucan component in the *M. furfur* wall, as the detergent may well have a decisive effect on fungal wall destruction [48, 49]. Thompson and Colvin (1970) [50] demonstrated 50–52% neutral sugars, mainly glucose, 10–12% protein, 10–15% hexosamine, mainly glucosamine and about 15% lipids in the wall of *M. furfur* type *Pityrosporum ovale*. In its constituents this wall differs significantly from the

wall of *Saccharomyces cerevisiae*, which contains about 90% neutral sugars but only 1–2% lipids [50]. Considering the results with the Nile blue sulfate staining, there seem to be lipids in the wall. Our own ultrastructural results with *M. furfur* raised on Tween-supplemented agar further underline that lipids participate in the architecture of the cell envelope. It was generally suggested that lipids may account for a multi-layered cell wall in yeasts [51, 52]. Lipids may contribute to the high osmotolerance found in *M. furfur*/*Pityrosporum* (Brotherton 1967 [53], own experiments). A possible role of the lipid component in attachment processes has already been mentioned.

Proteins seem to be concentrated in the outer part [Bo] of the cell wall of *M. furfur*/*Pityrosporum* [50], an aspect supported indirectly by our own experiments, as the outer main wall layer was less well fixed without aldehydes, which are known for their ability to preserve proteins [54]. The visibility of *M. furfur* by haematoxylin-eosin (HE) (Graham and Barroso-Tobila 1971 [27], own results) could be due to a considerable protein component in the cell wall of *Malassezia* yeasts.

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