Candida dubliniensis and *Candida albicans* display surface variations consistent with observed intergeneric coaggregation

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Summary

Adherence of yeasts to other microorganisms and epithelial cell surfaces is important in their colonization. Comparative studies based on the coaggregation of *Candida dubliniensis* versus *Candida albicans* with *Fusobacterium nucleatum* and other oral bacteria suggested differences in the surfaces of these yeasts. Transmission electron microscopy was used to test the hypothesis that there are morphologic variations in the cell surface of these two species. *C. dubliniensis* type strain CD36 and *C. albicans* ATCC 18804 were grown on Sabouraud's dextrose agar at various growth temperatures. In some experiments suspensions of yeast cells were treated with dithiothreitol. Fixation for transmission electron microscopy was accomplished using dimethylsulfoxide and alcian blue added to 3% paraformaldehyde and 1% glutaraldahyde in cacodylate buffer. The cell wall of both species was predominantly electron lucent and was visibly differentiated into several layers. A thin electron dense outer layer was seen with clearly visible fibrillar structures, closely associated to the cytoplasmic membrane. The length of the fibrils of the *C. albicans* cells grown at 37°C was approximately two times greater than those of the cells grown at 25°C. The fibrils of the 37°C-grown cells were thin, distinct and tightly packed whereas those of the 25°C-grown cells appeared blunt, loosely spaced and aggregated. *C. dubliniensis* demonstrated short, blunt fibrils appearing similar to those of the 25°C-grown *C. albicans* cells. *C. dubliniensis* showed no difference in the density, length and arrangement of fibrils between the 25°C and 37°C growth temperatures. The shortest and most aggregated fibrils seen were of the 45°C-grown *C. albicans* cells. Dithiothreitol-treated 37°C-grown *C. albicans* cells revealed a distorted and partially destroyed fibrillar layer.

In this investigation *C. dubliniensis,* unlike *C. albicans,* displayed an outer fibrillar layer that did not vary with variations in growth temperature. In addition, the fibrils on the *C. dubliniensis* cells were similar to those of the 25°C-grown *C. albicans* in that they were considerably shorter and less dense than those of the 37°C-grown *C. albicans* cells. It can be postulated, that *C. dubliniensis* exhibits constant cell surface characteristics consistent with hydrophobicity and that this property may give this species an ecological advantage. Therefore, *C. dubliniensis* may compete well in oral environments via enhanced attachment to oral microbes and other surfaces, perhaps even more efficiently than *C. albicans*.

Key words *Candida dubliniensis*, *Candida albicans*, Cell wall, Electron microscopy

Candida dubliniensis y *Candida albicans* presentan variaciones en su superficie compatibles con la coagregación intergenérica observada

Resumen

La adhesión de las levaduras a otros microorganismos y a las superficies epiteliales es importante para su colonización. Estudios comparativos basados en la coagregación de *Candida dubliniensis* frente a *Candida albicans* con

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Aceptado para publicación el 1 de octubre de 1999

1130-1406/99/5.00 Euros

Fusobacterium nucleatum y otras bacterias orales han sugerido que existen diferencias en las superficies de estas levaduras. Se ha utilizado la microscopía electrónica para determinar la existencia de variaciones morfológicas en la superficie celular de estas dos especies. Las cepas CD36 de *C. dubliniensis* y la ATCC 18804 de *C. albicans* se cultivaron en agar glucosado de Sabouraud a diferentes temperaturas. En algunos experimentos, las suspensiones de levaduras se trataron con ditiotreitol. La fijación para microscopía electrónica de trasmisión se realizó utilizando dimetilsulfóxido y azul alcián en tampón cacodilato con 3% de paraformaldehído y 1% de glutaraldehído. La pared celular de ambas especies era predominantemente electrolúcida y se diferenciaba visiblemente en varias capas. Se observaba una fina capa externa electrodensa con estructuras fibrilares en íntima asociación con la membrana citoplásmica. La longitud de las fibrillas de las células de *C. albicans* cultivadas a 37°C era aproximadamente el doble que la de las células cultivadas a 25°C. Las fibrillas de las células cultivadas a 37°C eran finas, nítidas y densamente agrupadas, mientras que las segundas aparecían romas, espaciadas y agregadas. *C. dubliniensis* presentaba fibrillas cortas y romas, similares a las observadas en las células de *C. albicans* cultivadas a 25°C. No se apreciaron diferencias en la densidad, longitud ni disposición de las fibrillas de las células de *C. dubliniensis* obtenidas a 25°C o a 37°C. Las fibrillas más cortas y agregadas se observaron en las células de *C. albicans* cultivadas a 45°C. Las células de *C. albicans* cultivadas a 37°C y tratadas con ditiotreitol presentaban una capa fibrilar distorsionada y parcialmente destruída.
En este estudio, *C. dubliniensis*, al contrario que *C. albicans,* presentó una capa fibrilar externa invariable independientemente de la temperatura de crecimiento. Además, las fibrillas de las células de *C. dubliniensis* eran similares a las de *C. albicans* obtenida a 25°C, ya que eran sensiblemente más cortas y menos densas que las de *C. albicans* cultivada a 37°C. Se puede postular que *C. dubliniensis* presenta características constantes en su superficie celular compatibles con la hidrofobicidad y que esta propiedad puede aportarle una ventaja ecológica. De este modo, *C. dubliniensis* puede competir en la cavidad oral gracias a una mayor adhesión a microorganismos o a otras superficies, quizás de manera más eficiente que *C. albicans*.

Palabras clave *Candida dubliniensis*, *Candida albicans*, Pared celular, Microscopía electrónica

The clinical significance of *Candida dubliniensis*, a novel species [1-8] can be attributed to many of the virulence characteristics that were once considered exclusive to *Candida albicans*. It differs in its ability to readily develop resistance to fluconazole [8-12]. This property has been related to a trend of increased incidence of candidiasis caused by other *Candida* species such as *Candida krusei* and *Candida lusitaniae* [10,13-16]. The virulence factors exhibited so far by *C. dubliniensis* include germ tube formation [8,10,17-19], exoenzyme production [17,19-21], phenotypic switching [21] and antigenic variation [17,22]. These and many other phenotypic similarities that *C. dubliniensis* share with its closest relative, *C. albicans*, have resulted in a substantial amount of confusion when differentiating these two species.

It is well known that adherence is an essential first step in microbial colonization and thus is a key event in the initiation of the pathogenic process [19,21,23-26]. As a result, any cell wall changes would be expected to affect disease development, ultimately determining virulence [25]. The cell wall of *C. albicans* is essential to its success as a pathogen for several reasons: it provides rigidity to the cell, protects against osmotic changes and penetration of drugs, is important in antigenicity and is involved in the secretion of hydrolytic enzymes necessary for tissue invasion. Most importantly, however, it is the site of contact for adherence of the fungus and its environment [20,21,27].

A major functional difference that has been observed between *C. dubliniensis* and *C. albicans* is based on their coaggregation behavior with the oral anaerobic bacterium, *Fusobacterium nucleatum* [26,28]. *C. dubliniensis* strains when grown at 37°C were found to visibly and turbidometrically coaggregate with *F. nucleatum*. However, when the yeast isolates were grown at 25°C, *C. albicans* as well as *C. dubliniensis* coaggregated. When the 37°C-grown *C. albicans* strains were treated with dithiothreitol (DTT) and then mixed with *F. nucleatum*, they coaggregated in a manner similar to the *C. dubliniensis* isolates. Through inhibition studies, it was found that the coaggregation reaction involved a heat-labile component on the *F. nucleatum* and a mannan-containing heat-stable receptor on both *Candida* species. This latter receptor appeared to be masked by a heat-labile material on the surface of the *C. albicans* cells, that interfered with the coaggregation of the 37°C-grown *C. albicans* with *F. nucleatum*. The *C. dubliniensis* cells, on the other hand, were able to coaggregate with the *F. nucleatum* regardless of growth temperature. This finding suggests that there may be differences in the structure of the cell walls of these two *Candida* species supporting the observation of Bikandi *et al.* [29], who reported the existence of antigenic differences between *C. dubliniensis* and *C. albicans*.

Previous investigators have described a growth temperature-dependent outer fibrillar layer on the surface of *C. albicans* walls consisting largely of mannoproteins [18,19,22, 24,30-33]. The fibers of this outermost layer are longer and more tightly packed on the 37°C-grown *C. albicans* than on the 25°C-grown *C. albicans* [25,34]. The fibers of this "hydrophilic" outermost layer seem to be responsible for the initial attachment of *C. albicans* to other cells and surfaces. This initial association is followed by a tight interaction through a mannoprotein adhesin on *C. albicans* [22,24,30]. Hydrophobic proteins in the matrix of the *C. albicans* cell wall contribute to the strength of this bond. These hydrophobic proteins are pre-

sent in both 25°C and 37°C-grown blastoconidia and are covered by the outer fibrillar layer [18,22,31,33]. The scarcity of these fibrils on the *C. albicans* cells grown at 25°C, however, allows the exposure of these underlying hydrophobic proteins causing the cells to be hydrophobic and more adherent to other cells and surfaces. The 37°C-grown *C. albicans* cells, on the other hand, are considered hydrophilic due to the masking of the hydrophobic proteins by the extensive network of outer surface fibrils [18,27,34].

Many difficulties have been encountered with conventional chemical fixation and embedding techniques for the preservation of yeast ultrastructure [35]. These difficulties are mainly due to the presence of the brush-like outerfibrillar layer on the yeast surface that renders the cell wall of the yeast too thick for adequate penetration of and preservation by fixatives [35,36]. As a result, rapid freeze substitution techniques are often used to achieve adequate preservation of membranous structures of yeast cells. The cell surface of *C. albicans* cells fixed by conventional methods, are revealed as uniformly smooth, whereas freeze-fracture replicas and rapid freezing techniques showed the surfaces to be very rough with fibrillar networks [24,30,32,35]. These techniques, however, are complicated, tedious and difficult to perform. An alternative chemical fixation approach has been devised to preserve the native ultrastructure of yeasts and the delicate precise fibrils. This method utilizes the addition of 0.1% dimethylsulfoxide (DMSO) to glutaraldehyde and osmium tetraoxide-dichromate for fixation of yeasts for electron microscopy [36]. Significant improvement in the study of cell morphology, especially in cell wall detail and overall structure of the yeast, was reported with the addition of DMSO. In addition, preservation of the outer fibrillar layer was further enhanced by the use of alcian blue [36]. This novel yet simple method of chemical fixation for electron microscopy was adapted in this study to compare the cell walls of *C. albicans* grown at 25°C, 37°C, 37°C -DTT treated and 45°C and of *C. dubliniensis* cells grown at 25°C and 37°C (*C. dubliniensis* does not grow at 45°C). The hypothesis tested was that a major difference between *C. dubliniensis* and *C. albicans* lies in the abundancy, length and distribution of the fibers that make up the fibrillar outermost layer of the yeast and that unlike *C. albicans*, these fibrils are not subject to growth temperature changes with *C. dubliniensis.*

MATERIALS AND METHODS

Cells. C. dubliniensis (type strain CD36) was grown on Sabouraud's dextrose agar (SDA) plates at 25° and 37°C; *C. albicans* (ATCC 18804) was grown on SDA at 25°, 37° and 45°C. Cells were washed four times for 10 min each in deionized water and pelleted by centrifugation at 180 x *g* between washes. The final fluid contained 0.1% DMSO [36].

Dithiothreitol Treatment. C. albicans cells were grown on SDA plates at 37°C for 24 h. Cells were treated with DTT (50 mM), as described by Hazen and Hazen [22]. Cells were washed three times with cold deionized water and then adjusted to a cell concentration of 2 x 10^8 cells per ml of deionized water. Two ml of yeast cell suspension was mixed with 2 ml of 50 mM DTT. The mixture was incubated for 60 min at 37°C with occasional shaking. Cells were then washed three times with cold deionized water before being processed for electron microscopy [22].

Electron Microscopy. Cells were prefixed in 3% paraformaldehyde, 1% glutaraldehyde, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% DMSO and 0.1% alcian blue (Polysciences, USA) in 0.1 M sodium cacodylate buffer for 3 h at room temperature and washed three times in the same buffer for 10 min. Samples were incubated for 3 h at room temperature in a solution of 1% OsO_4, 1% $K_2Cr_2O_7$, 0.85% NaCl and 0.1% DMSO in the same buffer. After three, 10 min washes in saline, cells were then dehydrated in an ethanol series with uranyl acetate (SPI-CHEM, USA) added to 70% ethanol [36]. Cells were embedded in 100% Epon 812 and sections were cut on a Reichert-Jung Ultracut E microtome, post-stained in aqueous 2% uranyl acetate and lead citrate (SPI-CHEM) and observed in a JEM-1200 EX II electron microscope. All of these experiments were repeated on two additional occasions.

RESULTS

Preservation of *C. albicans* was achieved using the DMSO method allowing for the observation of detailed ultrastructure of *C. albicans* (Figure 1A). The cell wall was predominantly electron lucent and was visibly differentiated into several layers (Figure 1B) consistent with the descriptions and models of Shepherd and others [20,21] (Figure 1C). In the cytoplasm, abundant ribosomes, round or ovoid nuclei and mitochondria, electron dense vesicles and a smooth contour of membranous systems could be seen (Figure 2A). A thin electron dense outer layer was seen with clearly visible delicate fibrillar structures, closely associated to the cytoplasmic membrane (Figure 2C), often linking adjacent fungal cells (Figure 2B).

Cells of *C. albicans* grown at 25°C and 37°C displayed an outer fibrillar layer, however, the length of the fibrils of the *C. albicans* cells grown at 37°C were at least two times greater than those of the cells grown at 25°C (Figures 3C and 3D). In addition, the fibrils of the 37°C-grown cells were thin, distinct and tightly packed whereas those of the 25°C-grown cells were irregularly spaced and in aggregates. *C. dubliniensis*, on the other hand, showed no difference in the density, length and arrangement of fibrils between the 25°C (Figure 3A) and 37°C (Figure 3B) growth temperatures. At both of these temperatures, *C. dubliniensis* yeast cells had short, blunt fibrils that were very similar to those of the 25°C-grown *C. albicans* cells. The shortest and most aggregated fibrils seen were those of *C. albicans* cells grown at 45°C (Figure 3E). The DTT-treated 37°C-grown *C. albicans* cells revealed a distorted and partially destroyed fibrillar layer (Figure 3F). These results were consistent on each of these experimental occasions and for all of the multiple ultrathin sections.

DISCUSSION

Due to the slow penetrability of common fixatives through the thick, multilayered cell wall of *C. albicans*, routine chemical fixation of the yeast has so far been unable to preserve the ultrastructure of yeast, resulting in the loss of the outermost fibrillar layer or change from its native state [24]. The fibrillar structure of the yeast cell wall is especially sensitive to alcohol dehydration in conventional procedures. Dehydration causes the precipitation of the sugar component of the mannoproteins that make up the fibrillar network, resulting in drastic deformation and disappearance of the fibrillar layer [32]. The use of DMSO has been shown to improve the rate of fixative penetration into the yeast [32,36] allowing good preservation of ultrastructural detail for *C. albicans* and *C. dubliniensis.* The addition of 0.1% alcian blue into the fixation protocol enhances the radially oriented fibrils of

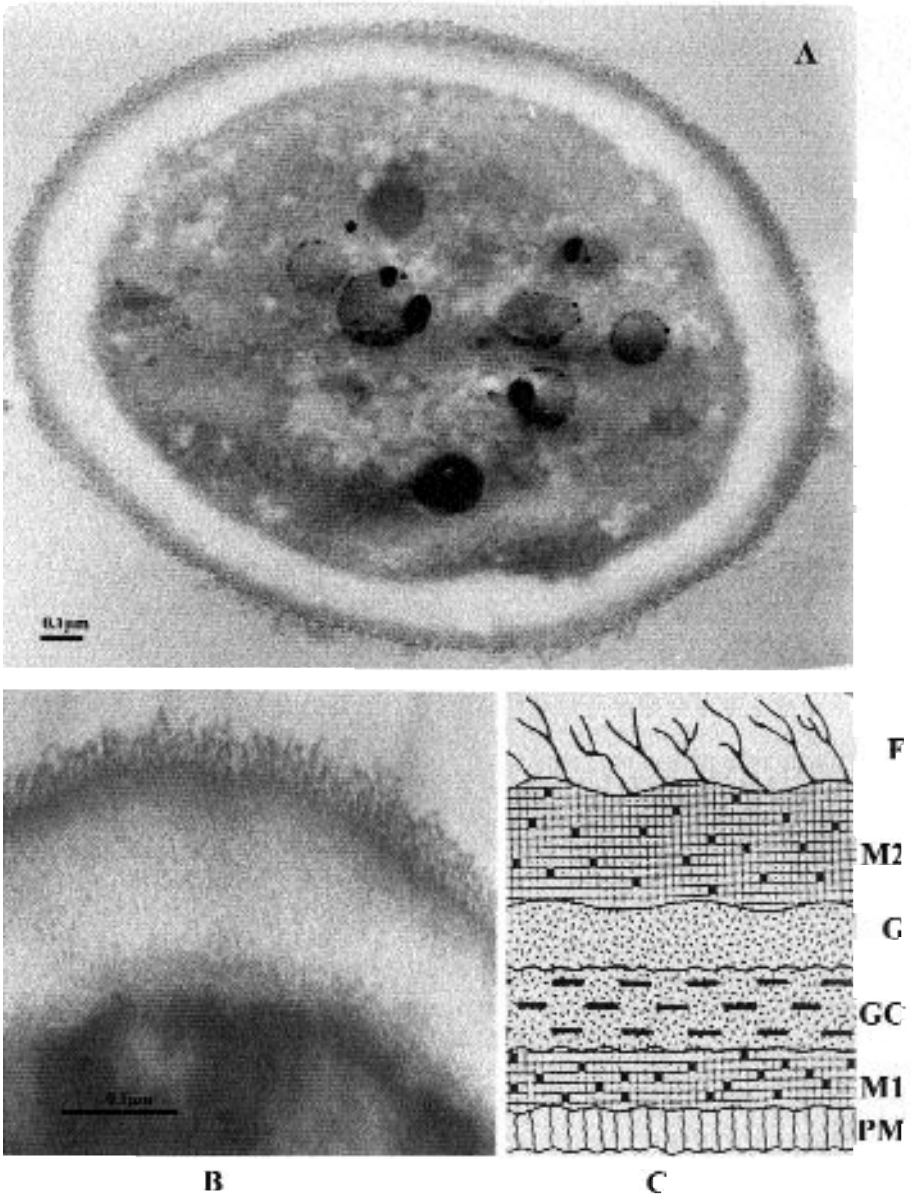

Figure 1. Transmission electron micrographs of ultrathin sections of *Candida albicans* fixed with DMSO: (A) A whole yeast cell grown at 37°C, showing long, regularly radiating fibrils; (B) Section of a yeast cell grown at 25°C, showing details of cell envelope ultrastructure; and (C) Schematic diagram of a typical cell wall derived from Shepherd [20] showing the various layers: plasma membrane (PM), zone of mannoprotein (M1), glucan-chitin (GC), glucan (G), mannoprotein (M2) and outer fibrillar layer (F).

the outer layer by modifying the physiochemical properties of the fibrils allowing their preservation [32,36]. Our studies confirmed these previous observations.

The determination of the accurate ultrastructure of yeasts is very important as the cell wall and its outermost and fibrillar layers may be related to pathogenicity, adherence and virulence of yeasts [17,20,23,27,31,33]. Studies to investigate the effect of different growth temperatures on the binding strength of *C. albicans* cells, showed that the 37°C-grown cells with the longest fibrils were the least avid, followed by the 25°C-grown cells, with the DTT-treated cells being the most avid [34]. Hence, any alterations in the fibrils of the outermost layer of the cell wall, whether due to growth temperature changes or treatment with DTT, may lead to changes in the ability of the cell to interact with other surfaces by exposing hydrophobic proteins which are pivotal to the success of colonizing yeasts in causing infections [18,22,24,31,34,37]. Studies on the effect of growth temperatures on the cell wall of *C. albicans* have provided substantial evidence on the influence of yeast cell growth temperatures on the virulence of the yeast. Yeast cells grown at 25°C produced

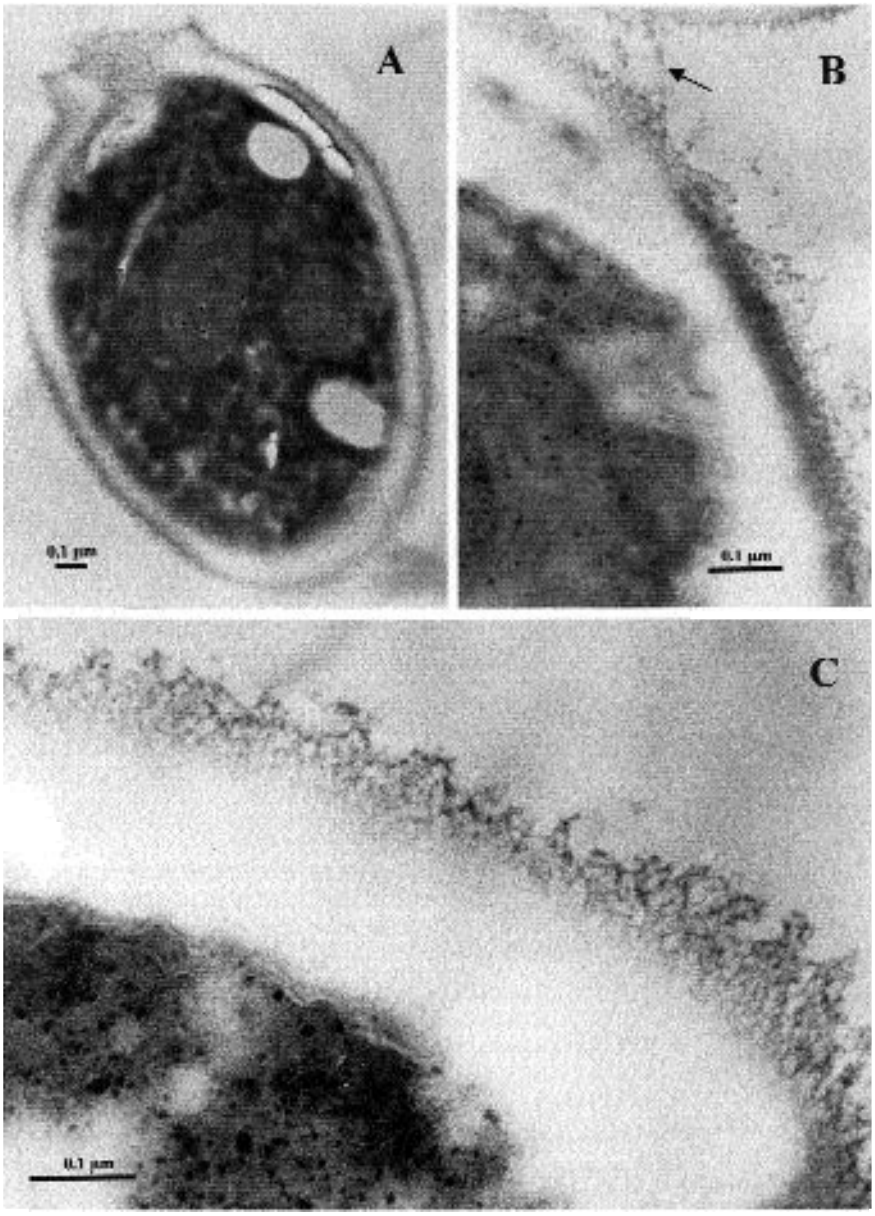

Figure 2. Transmission electron micrographs of ultrathin sections of *Candida albicans* fixed with DMSO: (A) An image of a 25°C-grown cell revealing intracellular organelles; (B) Brush-like fibrillar structure on the outermost surface of a *C. albicans* cell grown at 37°C. Note the interconnections of the long fibrils between two cells (arrow); and (C) A cell grown at 37°C showing distinct, long individual fibrils.

germ tubes faster and more abundantly than 37°C-grown cells after engulfment by polymorphonuclear neutrophils, providing them with an escape mechanism, making them less susceptible to killing by phagocytes [17,27,34]. In addition, cells grown at 25°C adhered to epithelial cells better than cells grown at 37°C and appeared to be less sensitive to toxic substances and growth inhibitors [17].

In this investigation, it was shown that *C. dubliniensis*, unlike *C. albicans*, has an outer fibrillar layer that did not vary when cells were grown at 25°C or 37°C. In addition, the fibrils on the *C. dubliniensis* cells were similar to those of the 25°C-grown *C. albicans*, in that they were considerably shorter and less dense than those of the 37°C-grown *C. albicans* cells. Although further studies need to be performed using more isolates, these preliminary investigations suggest that *C. dubliniensis* exhibits constant cell surface hydrophobicity. This property may enhance colonization of this species via attachment to oral microbes and other surfaces.

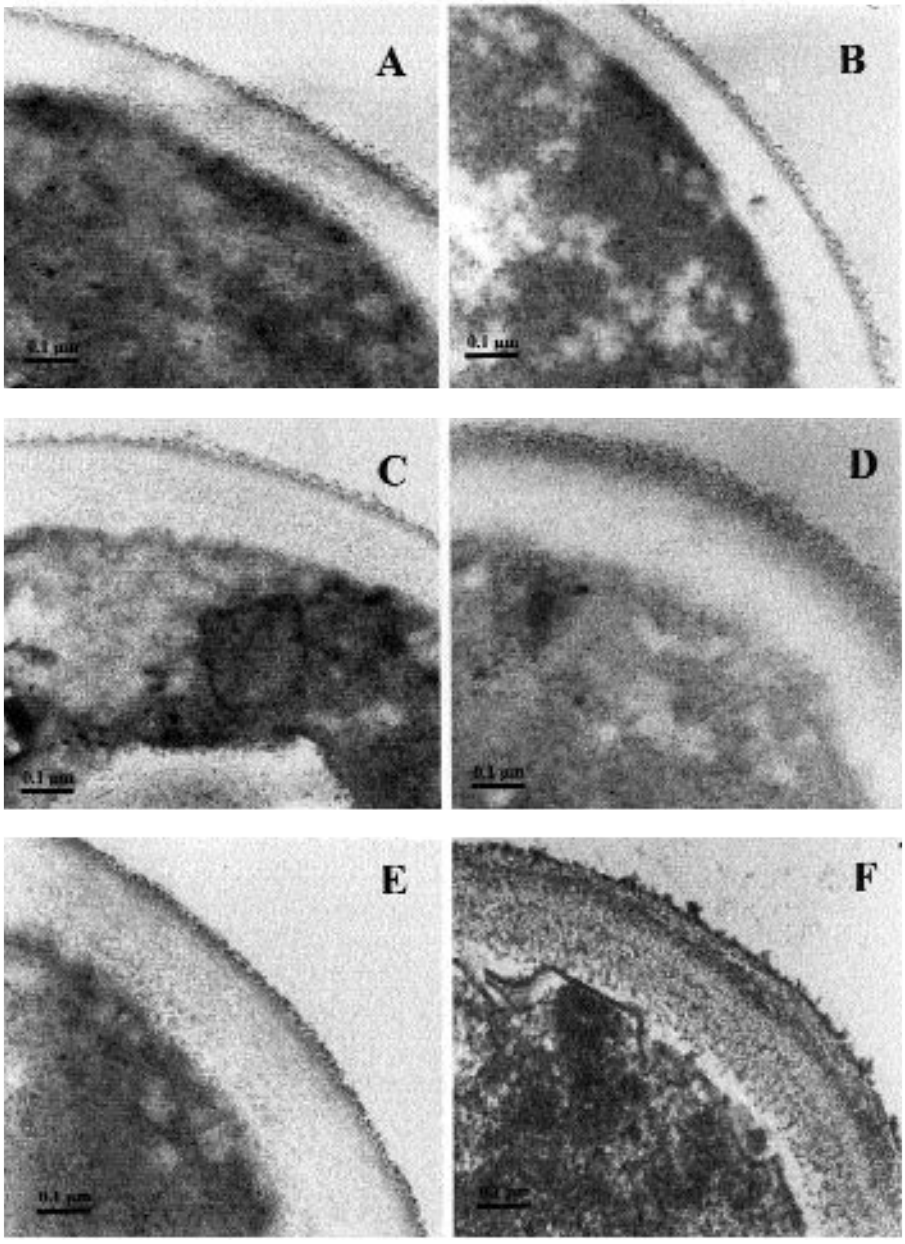

Figure 3. Transmission electron micrographs of ultrathin sections of *Candida albicans* and *Candida dubliniensis* cells fixed with DMSO, showing the effect of different growth temperatures and DTT treatment on cell wall ultrastructure: (A) A cell of *C. dubliniensis* grown at 25°C; (B) A cell of *C. dubliniensis* grown at 37°C showing fibrils similar to those of the 25°C grown cells in length and density; (C) A cell of *C. albicans* grown at 25°C showing short, blunt fibrils; (D) A cell of *C. albicans* grown at 37°C showing long, tightly packed and distinct fibrils; (E) A *C. albicans* cell grown at 45°C with short, aggregated fibrils; and (F) A *C. albicans* cell grown at 37°C and treated with DTT showing fibril loss.

This work was supported in part by NIH grant DE11373. We thank Professor Kushnaryov for his assistance with the methods for specimen preparation for TEM. We also thank Dr. Robert Nauman and Eleanor Wade for their technical assistance.

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