Cell surface hydrophobicity-associated adherence of *Candida dubliniensis* to human buccal epithelial cells

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Summary

Microbial adherence to mucosal surfaces is an important first step in the initiation of the pathogenic process in the oral cavity. *Candida albicans*, the most adherent and pathogenic *Candida* species, utilizes a variety of mechanisms to adhere to human tissues. Although the strongest mechanism of adherence involves mannoprotein adhesins on *C. albicans*, cell surface hydrophobicity (CSH) plays an important role in the adherence process by providing hydrophobic interactions that turn the initial attachment between the yeast and a surface into a strong bond. Recent cell wall analytical and comparative studies showed that, *Candida dubliniensis*, unlike *C. albicans*, possesses cell surface variations that allow it to be constantly hydrophobic, regardless of growth temperature. Based on these observations, the present study was designed to compare the adherence abilities of *C. dubliniensis* and *C. albicans* to pooled human buccal epithelial cells (BEC), in regards to their cell surface hydrophobicity. Ten *C. albicans* and nine *C. dubliniensis* isolates, as well as the *C. albicans* hydrophobic variant A9V10 were evaluated for adherence with BEC using visual aggregation in the wells of a microtiter plate and microscopic examination. All 11 *C. albicans* isolates failed to show adherence to BEC, visually or microscopically, when grown at 37°C. The same isolates, however, showed significant increase in aggregation and microscopic adherence to BEC when grown at 25°C. All *C. dubliniensis* isolates tested and the A9V10 *C. albicans* hydrophobic variant resulted in visual aggregation and adhered to BEC when grown at either temperature. The findings from this study show that, based on comparative adherence results and growth temperature changes, *C. dubliniensis* seems to have greater adherence to BEC than do typical *C. albicans* strains and that hydrophobic interactions seem to be the mechanism of adherence involved. Although many questions remain to be answered regarding the clinical implications of this observed *in vitro* enhanced adherence of *C. dubliniensis* to human BEC, these findings support the establishment of this novel species as a clinically significant yeast.

Key words *Candida dubliniensis*, Adherence

Adhesión de *Candida dubliniensis* a células humanas del epitelio oral asociada a la hidrofobicidad de la superficie celular

Resumen

La adhesión microbiana a las superficies mucosas es un primer paso importante en el inicio del proceso infeccioso en la cavidad oral. *Candida albicans*, la especie de *Candida* más adherente y patógena, utiliza diversos mecanismos para adherirse a los tejidos humanos. Aunque el mecanismo más fuerte de adhesión implica a adhesinas manoproteicas de la superficie de *C. albicans*, la hidrofobicidad de la superficie celular (HSC) juega un importante papel al aportar interacciones hidrofóbicas que fortalecen la unión inicial de la levadura a la superficie. Recientes estudios analíticos y comparativos de la pared celular han mostrado que *Candida dubliniensis*, al contrario que *C. albicans*, posee variaciones en su superficie celular que le permiten mantener su hidrofobicidad, independientemente de la temperatura de crecimiento.

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Aceptado para publicación el 22 de Diciembre de 2000

En base a estas observaciones, se planteó el presente estudio para comparar las capacidades de adhesión de *C. dubliniensis* y de *C. albicans* a un pool de células epiteliales humanas (CEH) en relación con su hidrofobicidad de superficie celular. Se evaluó, mediante lectura visual de la agregación en los pocillos y examen microscópico, la adhesión a CEB de diez aislamientos de *C. albicans* y nueve de *C. dubliniensis*, así como de la variante hidrófoba de *C. albicans* A9V10.
Ninguno de los aislamientos de *C. albicans* mostó adhesión a CEB, ni visual ni microscópicamente, cuando crecieron a 37°C. Sin embargo, mostraron un incremento significativo de agregación y de adhesión microscópica al crecer a 25°C. Todos los aislamientos de *C. dubliniensis* así como la variante hidrófoba de *C. albicans* A9V10 presentaron agregación visual y se adhirieron a CEB a ambas temperaturas.
Los hallazgos de este estudio demuestran que, en base a los resultados comparativos de adhesión y los cambios según la temperatura de crecimiento, *C. dubliniensis* parece tener una mayor adhesión a CEB que las cepas típicas de *C. albicans* y que el mecanismo de adhesión parece implicar interacciones hidrófobas. A pesar de que quedan sin resolver muchas preguntas sobre las implicaciones clínicas de este aumento de la adhesión de *C. dubliniensis* a células epiteliales bucales observado *in vitro*, estos hallazgos apoyan el establecimiento de esta nueva especie como una levadura clínicamente significativa.

Palabras clave *Candida dubliniensis*, Adhesión

Adherence is an essential first step in microbial colonization and is a key event in the initiation of the pathogenic process. Microbial attachment to mucosal surfaces has been shown to be an important step in infectious disease processes, particularly in the oral cavity [1-5]. *Candida albicans*, the most frequent cause of candidosis, is the most adherent *Candida* species and the most successful yeast in colonizing the oral cavity [6]. The mechanisms of adherence of *C. albicans* to human tissues are varied and as a result the *Candida*-host cell recognition systems are extremely complex and involve a variety of ligand-receptor components [7]. Depending on the types of receptors involved, different kinds of interactions between the fungus and host tissues have been described, such as protein-protein interactions, protein-carbohydrate interactions and *Candida* mannoprotein ligands which are recognized by unknown host receptors [6,8]. In addition, molecules such as aspartyl proteinases (Saps) and phosphatases, have been shown to play a crucial role in the ability of the yeast to adhere to human buccal epithelial cells (BEC) and other substrates [6,7]. Evidence has recently been provided that certain lipid classes such as phospholipids are also involved in adherence, with a glycosphingolipid of BEC shown to be the adherence target for *C. albicans* [6]. The phenomenon of phenotypic switching is also associated with changes in antigen expression and adherence to epithelial cells [6,7,9]. Although the strongest mechanism for adherence involves a mannoprotein adhesin on *C. albicans*, cell surface hydrophobicity (CSH) has been described by many investigators as involved in adherence [6,8,10].

The cell wall of *C. albicans* is the site of contact for adherence of the fungus to its environment [8,10-12]. Initial attachment of *C. albicans* to surfaces is primarily mediated by mannoprotein adhesins on the fibrils of the outermost fibrillar layer of the yeast cell surface [10,13]. Hydrophobic proteins, however, embedded in the matrix of the *C. albicans* cell wall beneath the fibrillar layer, provide the hydrophobic interactions needed to turn this initial attachment between the fungus and the surface into a strong bond [10]. Hydrophobic interactions, therefore, can significantly increase the number of successful contacts between the two surfaces. Growth temperature changes affect the hydrophobicity of the *C. albicans* yeast cell by influencing the length of the fibrils of the cell wall outermost layer, which masks the hydrophobic proteins in the cell wall matrix [14-16]. Although *C. albicans* is typically hydrophilic when grown at 37°C, indigenous *C. albicans* can become rapidly hydrophobic under appropriate stimulation. It is suggested that *C. albicans* may be hydrophilic as a commensal of the human host and converts to the alternate surface phenotype during parasitism, since cell surface hydrophobicity (CSH) has been shown to precede yeast-to-hypha conversion [6,16]. Studies investigating the effects of the different growth temperatures on the binding strength of *C. albicans* cells, found that hydrophobic 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 addition to enhanced adherence to tissue cells, *C. albicans* cells grown at 25°C were shown to be more virulent than hydrophilic cells grown at 37°C. Mice challenged with hydrophobic yeast cells died faster than those challenged with hydrophilic cells [18]. Furthermore, hydrophobic cells grown at 25°C germinated more rapidly than hydrophilic cells after engulfment by polymorphonuclear neutrophils, providing them with an escape mechanism, making them less susceptible to killing by phagocytes [11,15,17].

Recent electron microscopic comparative studies of the cell surfaces of *C. albicans* and the closely related new species, *Candida dubliniensis*, revealed significant morphologic variations between these two species. Using transmission electron microscopy (TEM) it was shown that *C. dubliniensis*, unlike *C. albicans*, displayed an outer fibrillar layer that did not vary with variations in growth temperature. In addition, the length and arrangement of the fibrils of cells grown at 25 and 37°C were consistent with those that result in a hydrophobic cell phenotype [19]. The data from this study suggested that *C. dubliniensis* is a novel species that exhibits constant CSH. This observation was confirmed when CSH levels for 45 *C. dubliniensis* isolates were determined using the hydrophobicity microsphere assay (HMA) devised by Hazen *et al*. [10,14-16,18]. The results of this study gave percentages for each *C. dubliniensis* isolate that were similar for 25- and 37°C- grown cells of the isolate and within the hydrophobic range (70-100%), according to the guidelines of Hazen *et al*. [10,14,18] (submitted; Jabra-Rizk *et al*.).

In light of these findings, the following study was undertaken to compare the adherence of *C. dubliniensis* to pooled human BEC with that of *C. albicans* and to relate these observations to yeast cell surface hydrophobicity.

MATERIAL AND METHODS

*Cell suspensions***.** Pooled human buccal epithelial cells (BEC) were collected from ten healthy adult volunteers (blood group A+), by gently scraping the inside of the cheek with sterile tongue depressors. Exfoliated cells were washed three times with phosphate-buffered saline (PBS) (pH 7.4, 0.1 M), adjusted to a 5% concentration in PBS by centrifugation and refrigerated for up to one week [20].

Ten *C. albicans* clinical isolates of unknown sources recovered from the University of Maryland Hospital Clinical Microbiology Laboratory and nine *C. dubliniensis* isolates recovered from the oral cavities of nine different HIV+ patients, including the type strain (CD36) were grown on Sabouraud dextrose agar for 24 to 48 h at room temperature and at 37°C. In addition to clinical isolates, *C. albicans* ATCC 18804 and *C. albicans* hydrophobic variant A9V10, which was derived by mutagenesis of *C. albicans* strain A9 [21], were also included in the study. Yeast cells were then harvested and washed three times with PBS and adjusted in the same buffer to a concentration of 1% by centrifugation.

Adherence assay. Adherence between BEC and yeast cells was tested by adding 50 µl of 1% yeast cell suspension with 50 µl of 5% BEC suspension in the wells of round-bottom microtiter plates. Plates were then shaken for 2 min (selected as optimal interaction time in preliminary testing), cells allowed to settle at room temperature and then observed for visible aggregation in the bottom of wells. Two negative control wells containing 50 µl of BEC with 50 µl of PBS and 50 µl yeast cells with 50 µl PBS were run with each microtiter plate. Testing for each strain was repeated three times and the complete protocol was repeated on at least four separate occasions. Results were evaluated blindly by multiple readers.

Evaluation of results. The following grading system was used to determine results:

(+): maximum visual aggregation in the bottom of wells
(w): weak, diffuse aggregation in the bottom of wells
(0): no visible aggregation or settling; suspension remained milky.

To observe (+) and (0) visual aggregation microscopically, a drop from each reaction suspension was placed on a clean slide, covered with a coverslip and observed microscopically with a 40x objective. The adherence of each yeast species to BEC was recorded photographically.

RESULTS

Eight *C. dubliniensis* clinical isolates and the *C. dubliniensis* type strain CD36 grown at 25 and 37°C were tested for adherence with 5% BEC. All *C. dubliniensis* isolates grown at either temperature, adhered to BEC with visible aggregation in the bottom of microtiter plate wells (Table 1). In addition to *C. albicans* ATCC 18804, ten clinical isolates of *C. albicans* grown at 25 and 37°C were tested for adherence with 5 % BEC. All 11 37°C-grown *C. albicans* strains failed to adhere to BEC (Table 1). When the 25°C-grown *C. albicans* isolates were tested with 5 % BEC, however, visible aggregation was seen indicating adherence, in a manner similar to that seen with the *C. dubliniensis* isolates and 5% BEC (Table 1). Similar to *C. dubliniensis* isolates, the *C. albicans* hydrophobic variant A9V10, showed adherence with BEC when grown at either 25 or 37°C (Table 1).

A major difference in the degree of adherence to BEC was noted microscopically between *C. dubliniensis* and *C. albicans* yeast cells when grown at 37°C (Figure 1B, 3A, 2B & 3B). When a drop from mixtures of 25 or 37°C-grown *C. dubliniensis* cells (Figure 1A & 1B) or the *C. albicans* A9V10 hydrophobic variant (Figure 4A & 4B) with BEC was observed microscopically, most of the yeast cells were seen adhering to BEC in clumps, linking BEC together. However, when 37°C-grown *C. albicans* cells mixed with BEC were observed microscopically, only few of the *C. albicans* yeast cells were bound to BEC with the majority of yeast cells in the mixture seen floating independently, evenly dispersed in the background (Figure 2B & 3B). When *C. albicans* cells, however, were grown at 25°C, mixed with BEC and observed microscopically, considerable adherence of yeast cells to the BEC was seen (Figure 2A) in comparison to the 37°C-grown *C. albicans* cells (Figure 2B & 3B).

DISCUSSION

The adherence of *Candida* to epithelial cells is one of the main pathogenic characteristics of the genus [5,22]. Such attachment enables the organisms to avoid elimination by the cleansing action of mucosal secretions, allo-

Table 1. Adherence assay results of 1% *Candida albicans* and *Candida dubliniensis* suspensions with 5% human buccal epithelial cells.

		Isolate #											
	°C	1	2	3	4	5	6	7	8	9	10	11	13
C. albicans	37	0	0	0	0	0	0	0	0	0	0	0[a]	+[b]
	25	+	+	+	+	+	+	+	+	+	+	+	+
C. dubliniensis	37	+	+	+	+	+	+	+[c]	+	+			
	25	+	+	+	+	+	+	+	+	+			

Key: 0 = no visible aggregation or settling; suspension remained milky; + = maximum visual aggregation with settling.
a: *C. albicans* ATCC #18804 grown at 37°C; b: *C. albicans* A9V10 (hydrophobic mutant); c: *C. dubliniensis* British type strain (CD36).

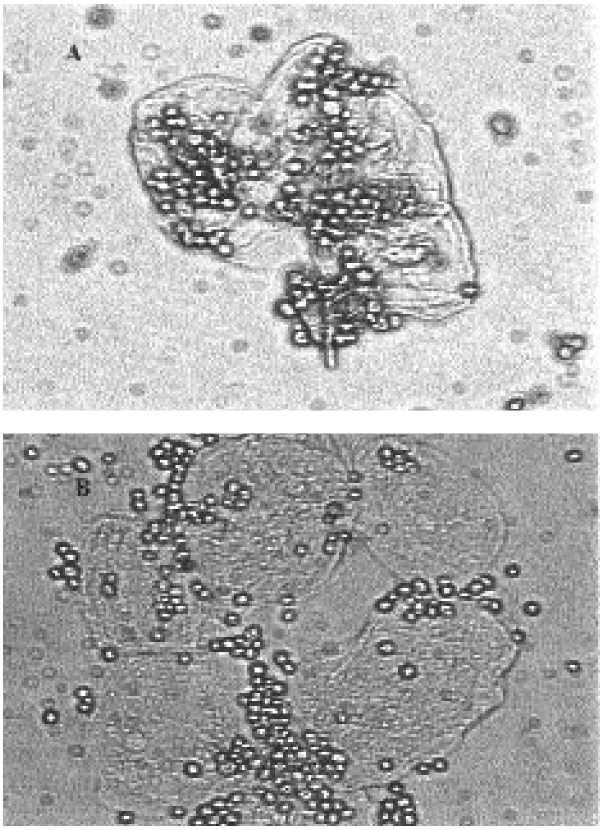

Figure 1. A high magnification view (x320) of the enhanced adherence ability of *Candida dubliniensis* CD36 cells grown at (A) 25°C and (B) 37°C to human buccal epithelial cells.

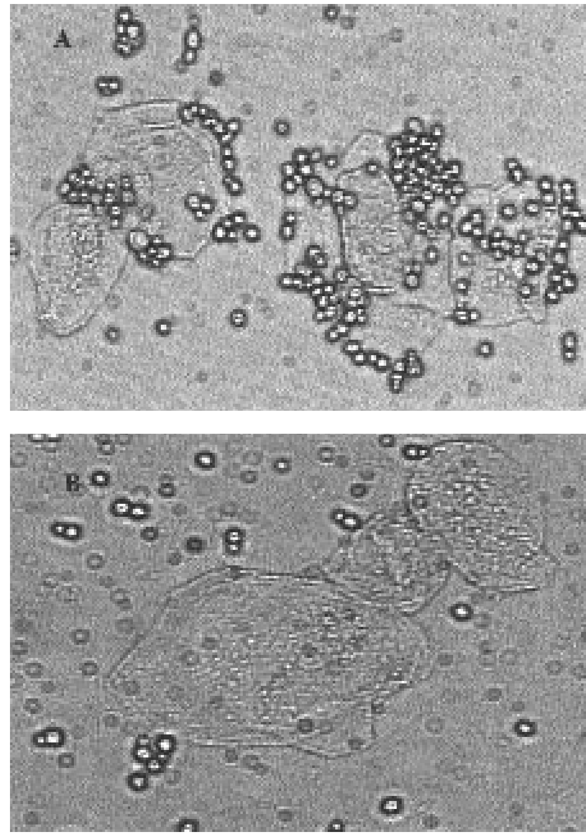

Figure 2. A high magnification view (x320) of adherence of *Candida albicans* ATCC 18804 cells grown at (A) 25°C and (B) 37°C to human buccal epithelial cells.

wing the yeast to colonize [23]. Studies have shown that there are variations in the adherence capabilities of *Candida* species, which explains why some are found to more frequently colonize mucosal surfaces [5,23].

Numerous studies have demonstrated that the adherence of *C. albicans* to host tissues is primarily mediated by a mannoprotein adhesin on the yeast cell surface. They have also demonstrated that although cell surface hydrophobicity (CSH) is not the predominant mechanism, it has been shown to be an important determinant in the adhesion of *C. albicans* [8,10,15,16].

Transmission electron micrographs of the adherence of *C. albicans* with epithelial cells showed that *Candida* often assumes a very close relationship with the epithelial cells [24]. In these pictures, the fibrils of the outer fibrillar layer of the *Candida* appeared to condense or disperse so that contact between the epithelial membrane and the inner layers of the cell wall can take place [24]. These observations support the data that hydrophobic proteins in the matrix of the cell wall of *C. albicans* are responsible for the tight adhesion needed for successful colonization and invasion of host tissue by *Candida* [15,16].

A recent study by de Repentigny *et al.* [25] investigating the *in vitro* binding abilities of several *Candida* species to purified mucin, showed significant differences, closely correlating with their hierarchy of virulence. Interestingly, *C. dubliniensis* was found to be the most adherent species, preceding *C. albicans* and *C. tropicalis*. The investigators attributed the adherence of the *Candida* to mucin, to hydrophobic interactions rather than *Candida* cell surface mannoproteins or electrostatic forces.

In order to determine whether the constant CSH of *C. dubliniensis* results in adherence properties different from *C. albicans*, the adhesion of both species with human buccal epithelial cells (BEC) was studied. Although it was difficult to assess the adherence of yeast cells to BEC quantitatively, it was clearly observed that *C. dubliniensis* adhered more to BEC when grown at either 25 or 37°C, whereas *C. albicans* showed adherence in a manner similar to *C. dubliniensis* cells with BEC only when grown at 25°C. The 37°C-grown *C. albicans* cells failed to show any visible aggregation when mixed with BEC. Similar to *C. dubliniensis*, the hydrophobic *C. albicans* variant, A9V10 adhered to BEC when grown at either temperature. The observations from this adherence study seem to correlate with the previously reported visual coaggregation phenomenon, where only hydrophobic yeast cells (*C. dubliniensis* and *C. albicans* hydrophobic variant cells and 25°C-grown *C. albicans*), were shown to adhere to cells of *Fusobacterium nucleatum*, an oral anaerobic gram-negative bacterium frequently associated with periodontal diseases [26,27]. In those experiments, hydrophilic, 37°C-grown *C. albicans* cells failed to adhere to *F. nucleatum* cells with no coaggregation seen [26].

The variety of oral niches and the complex adherence mechanisms of the yeast make it very hard to understand oral colonization of *Candida*. Which adherence mechanism is operative at any given time is dependent on the host tissue, the surface of the yeast cell and the surface molecules that are exposed.

The *in vitro* results of this study show that, under the described conditions, hydrophobic interactions seem to be the predominant adherence mechanisms of the yeast

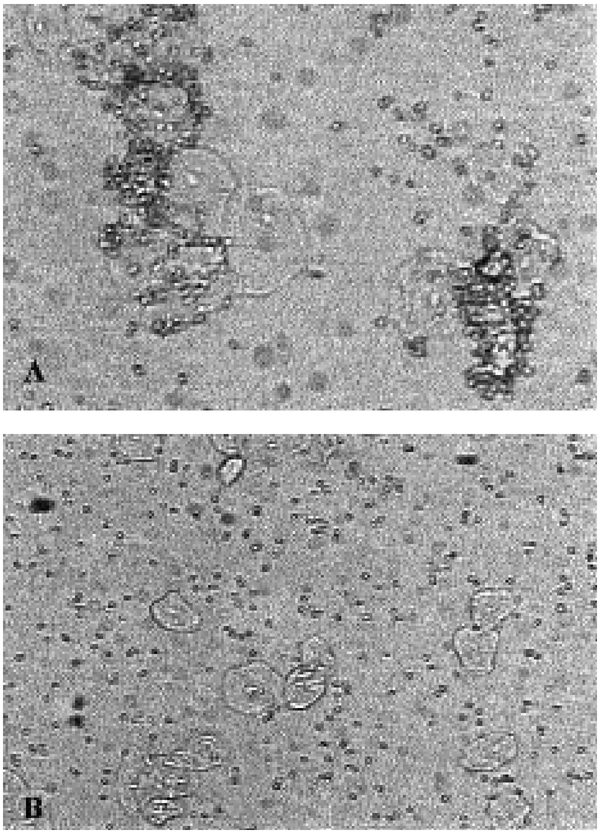

Figure 3. A low magnification view (x100) of adherence between buccal epithelial cells (BEC) and *Candida* yeast cells: (A) *C. dubliniensis* CD36 showing maximum adherence (as compared to *C. albicans*) of yeast cells to BEC, linking BEC together, causing aggregates of BEC and yeast cells; (B) *C. albicans* ATCC 18804 showing no or minimal adherence of yeast cells to BEC.

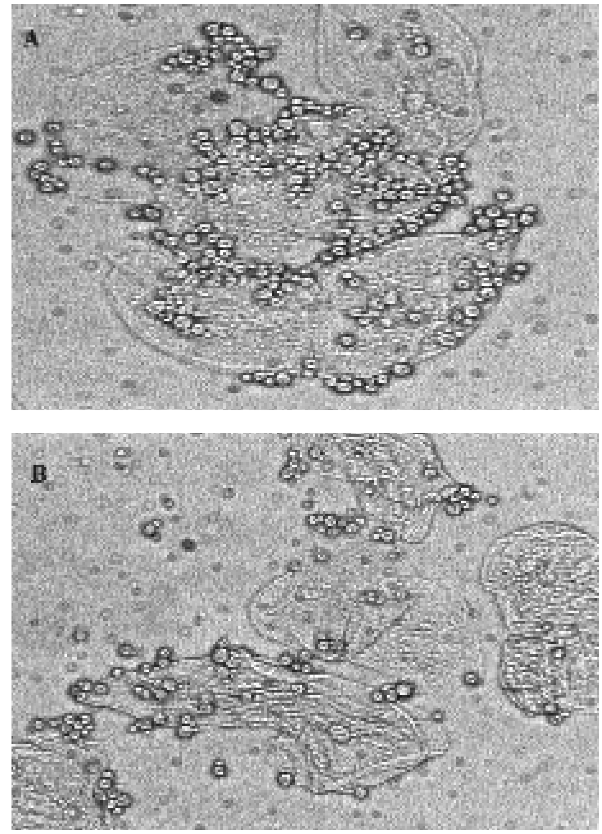

Figure 4. A high magnification view (x320) of the adherence of *Candida albicans* hydrophobic variant A9V10 cells grown at (A) 25°C and (B) 37°C to human buccal epithelial cells.

cells to BEC, although other mechanisms may also be at play. The fact that *C. dubliniensis* is a more hydrophobic species would explain the enhanced adherence ability observed with this species to BEC. Our adherence results are consistent with the observations of McCullough *et al.* [28] and Gilfillan *et al*. [29], who have shown that *C. dubliniensis* isolates have greater adherence to BEC than do typical *C. albicans* strains (Table 1).

Among the multiplicity of virulence factors that *C. dubliniensis* and *C. albicans* possess are extracellular proteolytic enzymes, mainly secreted aspartyl proteinases (Saps) [7,30]. Sap2p, the major isoenzyme of the Sap family, has been shown to degrade mucins, the major constituents of mucous that play a role in protection against invasion by pathogens that colonize mucosal surfaces [25,30]. McCullough *et al.* [28] have also shown that, in addition to greater adherence to BEC, *C. dubliniensis* isolates have a significantly higher proteinase activity. These combined findings regarding the mucinolysis-adhesive, *Candida*-mucin interactions support the hypothesis that the virulence capability of *C. dubliniensis* may be partially explained by increased binding to mucous layers followed by facilitated penetration by degradation of the mucin barrier in the oral cavity and small intestine by Sap2p. Both of these properties may facilitate access of the yeast to host epithelial cells as well as creation of new receptors, promoting colonization and invasion of the fungus within the host.

Despite the establishment of *C. dubliniensis* as a clinically significant yeast species in immunocompromised states [31-37], many questions remain. Specifically, this organism's constant cell surface hydrophobicity [19], enhanced *in vitro* adherence ability to other oral microorganisms [26], mucins [25] and attachment to human BEC must be assessed in the pathogenesis of human infections and oral disease.

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