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

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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 manoprotein 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.

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

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 fortalezcan 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.

**Key words** *Candida dubliniensis*, Adherence

**Resumen**

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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

Palabras clave
Candida dubliniensis, Adhesión
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 tested with 5% BEC suspension in the wells. Two negative control wells containing 50 µl of PBS were run with each microtiter plate. Testing for each isolate 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. 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), plates 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

(−) 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-

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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).
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
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...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.


