Review

Adherence and biofilm formation of non-*Candida albicans Candida* species

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Most cases of candidosis have been attributed to *Candida albicans*, but recently non-*C. albicans Candida* species have been identified as frequent human pathogens. *Candida* pathogenicity has been attributed to several factors, including adhesion to medical devices and/or host cells, biofilm formation, and secretion of hydrolytic enzymes (proteases, phospholipases and haemolysins). Although 'new' *Candida* species are emerging, there is still a lack of information about their pathogenicity. This review discusses recent advances in our knowledge of *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis* virulence factors, specifically those of adhesion and biofilm formation, which are key components in *Candida* pathogenicity.

Emergence of non-*Candida albicans Candida* species

Twenty years ago *Candida albicans* accounted for 70–80% of the *Candida* isolates recovered from infected patients [1]. Despite the majority of candidosis cases being attributed to *C. albicans*, an increasing number of infections caused by non-*C. albicans Candida* (NCAC) species, specifically *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*, have been reported over the past two decades [2–4].

For many years, *C. glabrata* was considered a relatively non-pathogenic saprophyte of the normal flora of healthy humans, and was not readily associated with serious infection. However, *C. glabrata* can rapidly disseminate throughout the body, and infection with this species is associated with a high mortality rate. Moreover, *C. glabrata* is of added concern because of its inherent resistance to certain antifungal agents [1]. *C. parapsilosis* is generally regarded as one of the least virulent yeast species, although it is now a frequent cause of candidaemia, commonly related to poor hand hygiene of healthcare workers [2]. *C. tropicalis* has emerged as the second or third most common agent of candidaemia, mainly in oncology patients, and is often associated with nosocomial urinary-tract infections [4].

The apparently increasing involvement of NCAC species in human candidosis could be related in part to improvements in diagnostic methods, such as the use of primary agars with the ability to differentiate between *Candida* species, and the introduction of molecular diagnostic techniques into routine use [5]. A number of other factors have been implicated in the increasing prevalence of *Candida* species, including the introduction and more widespread use of certain medical practices, such as immunosuppressive therapy, the use of broad-spectrum antibiotics, and an increase in the number of invasive surgical procedures, such as organ transplantations [6,7]. Furthermore, the increasing number of *Candida* species in candidosis could also be a reflection of species selection in the presence of certain antifungals, given the higher level of resistance demonstrated by several NCAC species [8].

The pathogenesis of invasive candidosis is facilitated by a number of factors, including the ability to adhere to medical devices and/or host cells and to form biofilms. It is also important to highlight the ability of some *Candida* species to switch from yeast to filamentous growth forms, with the latter thought to increase the ability of the organism to invade host tissues. *C. albicans* is a true polymorphic organism, able to grow as hyphae and/or pseudohyphae, and as blastospores (yeast). *C. tropicalis* produces oval blastospores and pseudohyphae, and, according to some reports, true hyphae. In the case of *C. parapsilosis*, blastospore growth is prevalent, and although this species does not produce true hyphae, it can on occasion generate pseudohyphae. By contrast, *C. glabrata* is not polymorphic, growing only as blastospores [9].

Despite significant research aimed at identifying the pathogenic factors of fungi, particularly in *C. albicans*, relatively little is known about the virulence determinants of NCAC species. This review aims to provide a contemporary overview of the virulence factors associated with three of these clinically important NCAC species (*C. glabrata*, *C. parapsilosis* and *C. tropicalis*), with particular focus given to aspects of their adhesion and biofilm formation.

Adhesion ability of NCAC species

The first event in *Candida* infection is its adherence of the organism to host and/or medical-device surfaces, often leading to the formation of biofilms [10]. Thus, adhesion is an extremely important step in the infection process, and the extent of adhesion is dependent on microbial, host and abiotic surface properties, such as cell-surface hydrophobicity and cell-wall composition [11,12].

The yeast cell wall is the site for physicochemical interactions between the microorganism and host surfaces, leading to its adherence. The cell surface of *C. glabrata* reportedly exhibits a degree of hydrophobicity comparable

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with that of *C. albicans*; however, these results were obtained in a study of a limited number of *C. glabrata* strains [13]. Interestingly, whereas the hydrophobicity of *C. albicans* appears to be extremely sensitive to specific growth conditions, the cell-surface hydrophobicity of numerous isolates of *C. glabrata* was found to be comparatively stable under those same growth conditions [14]. In addition, Camacho *et al.* [15] did not find a correlation between the level of hydrophobicity and adherence of *C. albicans*, *C. tropicalis* and *C. parapsilosis* to siliconized latex catheters, demonstrating that cell hydrophobicity alone was not a predictor of adhesion. However, Panagoda *et al.* [16] demonstrated that the initial adhesion of *C. parapsilosis* was associated with the hydrophobicity of acrylic surfaces. Recently Silva *et al.* [17] showed that *C. glabrata* adhered to silicone in the presence of urine at higher levels than did either *C. tropicalis* or *C. parapsilosis*. Additionally, *C. glabrata* has also been reported to have a two-fold greater tendency to adhere to denture-acrylic surfaces compared with *C. albicans* [18]. Furthermore, *C. glabrata* has been shown to have higher adherence to urinary epithelial cells than do other NCAC species [19]. *C. tropicalis* has the ability to colonize urinary epithelial cells [20]; however, the extent of adhesion is strain-dependent. In terms of epithelial and endothelial adhesion ability, few studies have been undertaken with *C. parapsilosis*.

An important factor that has correlated with the adhesion ability of *Candida* species is the presence of specific cell-wall proteins, often referred to as adhesins. Groot *et al.* [21] identified 23 *C. glabrata* cell-wall adhesins, which were deemed to be involved in adherence to human epithelia and in biofilm formation. A major group of adhesins of *C. glabrata* is encoded by the *EPA* gene family [21–23]. The ability of *C. glabrata* to adhere to different biomaterials and epithelia could be a reflection of epithelial adhesin (EPA) expression on the surface of cells, which is induced by the presence of nicotinic acid [24]. Although there are relatively few studies concerning *C. glabrata* Epa adhesins, it is known that Epa1p is a Ca^{2+}-dependent lectin [25]. Furthermore, despite the large number of *EPA* genes, it has been shown that deletion of *EPA1* alone reduces adherence *in vitro* [25]. Furthermore, although *EPA6* is not expressed *in vitro*, its expression increases during *in vivo* urinary infection, suggesting that *C. glabrata* can adapt with environmental conditions to enhance its adherence [26]. A bioinformatics search of pathogen-specific gene families of *Candida* species revealed a number of genes encoding cell-wall proteins in *C. parapsilosis*. This study included five genes for agglutinin-like sequence (ALS) proteins and six genes for predicted glycophosphatidylinositol-anchored protein 30 (Pga30) [27]. Unfortunately, there has been no further work in studying the role played by these proteins in *C. parapsilosis* adhesion. Concerning proteins of the *C. tropicalis* cell wall, at least three ALS-encoding genes have been identified [28], but to our knowledge, no further work has been undertaken in this area.

The pathogenesis of mucosal candidosis has mainly been investigated using animal models, but recently, reconstituted human epithelium (RHE) was successfully used to study *in vitro* invasion by *Candida* species. In this

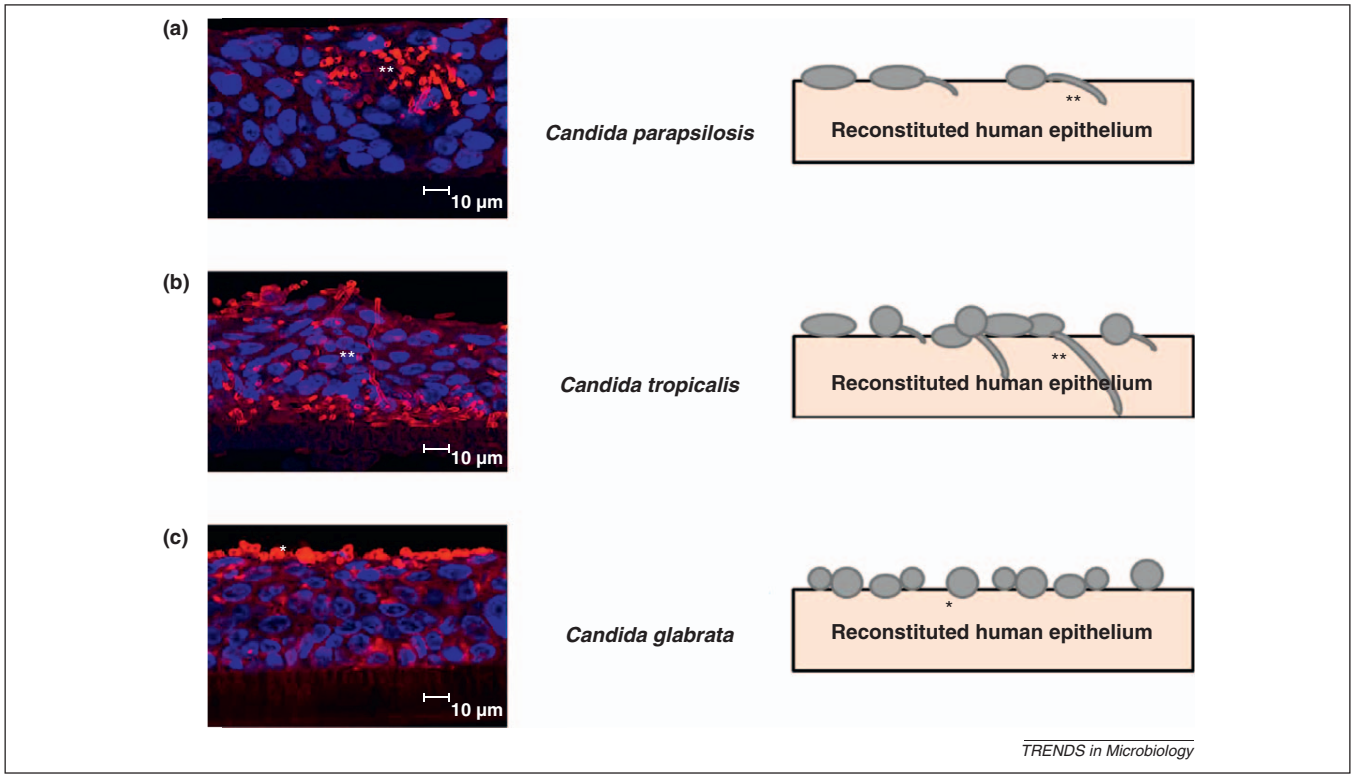

Figure 1. Confocal laser scanning microscopy images and scheme of *Candida* species infecting of a reconstituted human oral epithelium after 12 h of colonization. *Candida* yeast and filaments are shown in red, and the nucleic of the epithelial cells are depicted in blue. **(a)** *C. parapsilosis* showing moderate colonization, and invasion; **(b)** *C. tropicalis* showing extensive colonization and invasion; **(c)** *C. glabrata* showing colonization but no invasion. *Yeast; ** filamentous forms.

model, *C. parapsilosis* exhibited a high ability to colonize the RHE, despite a lower ability to invade this tissue [29,30] (Figure 1a). Furthermore, Gácser *et al.* [31] showed that *C. orthopsilosis* caused similar damage to RHE, whereas *C. metaparapsilosis* had comparatively reduced virulence. Jayatilake *et al.* [32] showed increased ability of *C. tropicalis* (Figure 1b) to colonize and invade RHE, similarly to *C. albicans*, compared with *C. glabrata* (Figure 1c) and *C. parapsilosis* (Figure 1b). *C. glabrata* was shown to be non-invasive in this model [32]. Therefore, given the inability of *C. glabrata* to generate filamentous forms, it might be concluded that the filamentous form is important for host tissue invasion.

Hydrolytic enzyme secretion by NCAC species

Candida species are able to produce and secrete several hydrolytic enzymes, including proteases, phospholipases (PLs) and haemolysins. The activity of these enzymes has been associated with candidal adhesion, cell damage and invasion of host tissue.

Secreted aspartyl proteinases

The secretion of aspartyl proteinases (Sap1 to Sap10) by *C. albicans* is recognized as an important virulence determinant for this species [33]. Saps facilitate colonization and invasion of host tissues through the disruption of host mucosal membranes [34], and by degrading important immunological and structural defence proteins [35].

With regard to *C. glabrata*, only one study has shown that this species is capable of secreted proteinase production, but the type of proteinase was not specified [36]. In addition, compared with *C. albicans*, *C. parapsilosis* has been shown to possess relatively low Sap activity [37]. Only three *SAP* genes have been identified in *C. parapsilosis* (*SAPP1–3*), two of which remain largely uncharacterized [38]. It has been reported that the expression of *SAPP1–3* genes varies with the clinical isolate of *C. parapsilosis* when grown in contact with an oral epithelium and even in planktonic form [30]. However, an associated trend was also reported, which related Sap production and the site of isolation of the organism; both vaginal and skin isolates of *C. parapsilosis* exhibited higher *in vitro* Sap activity than blood isolates [39]. *C. parapsilosis* poorly invades oral epithelium, but can nevertheless induce significant damage, which was related to specific *SAP* gene expression [30].

As with *C. albicans*, *in vitro* studies revealed that *C. tropicalis* secretes high levels of Saps in culture media containing bovine serum albumin as the sole source of nitrogen. Furthermore, *C. tropicalis* possesses at least four genes encoding Saps (*SAPT1–4)* [40,41], although Sapt1p is the only one that has been purified from culture supernatant, and biochemically characterized and crystallized [42,43]. Sap secretion by *C. tropicalis* has also been reported on the surface of fungal elements penetrating tissues during disseminated infection, and on macrophages following phagocytosis of yeast cells [34]. Sap expression by *C. tropicalis* in the oral environment is not associated with invasion or tissue damage [44], a finding similar to that reported for *C. albicans* [45,46].

Despite the high number of studies concerning the role of Saps on *C. albicans* virulence, little emphasis has been given to NCAC species Saps. Moreover it is important to highlight that although several studies have highlighted the differential expression and potential roles of various *SAP* genes during colonization and infection of host by *Candida* species, there were discrepancies in the results obtained. The reasons for such discrepancies could relate to differences in the sensitivities of the methods used, intrinsic differences even in apparently similar infection models (e.g. RHE and murine), and interspecies and strain variability [45,47].

PLs and lipases

In addition to Saps, enzymes categorized as lipases and PLs are often considered to be factors associated with *Candida* pathogenicity.

PLs are enzymes that hydrolyze phospholipids to fatty acids. Depending on the different and specific ester bonds cleaved, these enzymes have been classified into PLs A, B, C and D [48]. The production of all classes of PLs has been described for *Candida* species, and their presence could conceivably contribute to host cell-membrane damage, which could promote cell damage and/or expose receptors to facilitate adherence of *Candida* [49].

Several studies have indicated that NCAC species are able to produce extracellular PLs [50,51], albeit at lower levels compared with *C. albicans* [45]. There have been a number of contradictory findings, with some investigators reporting PL activity in 51% of strains assayed, whereas others have failed to detect PL activity in assayed strains [45]. Although *C. tropicalis* appears to have a reduced ability to produce extracellular PLs, this is strongly strain-dependent [21,38,52,53]. Furthermore, although a few studies of PL activity have been undertaken for *C. tropicalis* and *C. parapsilosis*, none has been reported for *C. glabrata* [54].

Lipases are involved both in the hydrolysis and synthesis of triacylglycerols. These enzymes are stable at high temperatures and in organic solvents and are resistant to proteolysis. In *C. albicans*, 10 lipase genes have been identified [55]. Gácser *et al.* [56] showed that *C. albicans* mutants deficient in *CaLIP8* were significantly less virulent in a murine intravenous infection model. This work clearly indicated that Lip8p was likely to be an important virulence factor for this particular species. For *C. parapsilosis*, *CpLIP1* and *CpLIP2* have been reported, with the latter known to encode for an active protein [57,58]. Recently, Gácser *et al.* [31] demonstrated that a lipase inhibitor significantly reduced tissue damage during *C. parapsilosis* infection of reconstituted human tissues, and that *CpLIP1*/*CpLIP2* mutants formed thinner and less complex biofilms. Recent genomic DNA sequencing suggests that two additional *CpLIP* genes might exist in *C. parapsilosis* [2]. Sequences similar to *C. albicans* (*LIP1–10*) were also detected in *C. tropicalis*, but not in *C. glabrata* [59]. However, no studies have been performed to investigate the role of these genes in the virulence of *C. tropicalis*.

Haemolytic activity

Pathogenic microorganisms can grow in the host by using haemin or haemoglobin as a source of iron. Haemolysins

Table 1. *C. tropicalis, C. parapsilosis* and *C. glabrata* biofilm characteristics

Species	Biofilm formation[a]	Matrix composition	Biofilm structure
C. tropicalis	+	Low level of carbohydrates and proteins	Discontinuous compact monolayer
C. parapsilosis	+	High level of carbohydrates and low levels of proteins	Discontinuous monolayer or multilayer
C. glabrata	+/−	High level of carbohydrates and proteins	Compact monolayer or multilayer

[a]+, Robust biofilm growth; +/−, less biofilm growth.

are produced by *Candida* species to degrade haemoglobin and extract elemental iron from host cells. Thus, haemolysins are likely to be key virulence factors as might promote pathogen survival and persistence in the host [60,61]. *C. albicans* has the ability to further utilize the acquired iron to produce a haemolytic factor that can release more haemoglobin by lysing erythrocytes [60,61]. *Candida* haemolytic activity can be concentrated by absorption on a concanavalin-A sepharose matrix. Analysis of the absorbed haemolysin demonstrated that the haemolytic factor involved was mannoprotein in origin [61]. Production of this haemolysin appears to be regulated by the presence of glucose in the growth medium. *C. glabrata*, *C. parapsilosis* and *C. tropicalis* are also able to produce haemolysins *in vitro* and at various levels, inducing partial or total erythrocyte lysis [59]. The genetic expression of haemolytic activity by *Candida* is, at present, poorly understood, but Luo *et al.* [62] showed that the haemolysin-like protein (*HLP*) gene was associated with the hemolytic activity of *C. glabrata*. Clearly, investigations are at an early stage with respect to the haemolysin activity of *Candida* species. This is highlighted by the fact that for pathogenic fungi, cloning, disruption and virulence evaluation has only led to the identification of the *C. albicans* haemolysin. Further investigations aimed at determining the role of haemolysins in the virulence of different NCAC species are required to establish whether these molecules are universal virulence factors of *Candida* species.

Apart from *C. albicans*, our knowledge of fungal adhesion, colonization and invasion of human epithelia and medical devices remains limited. The first contact between microorganisms and host surfaces is generally via the fungal cell wall, and this is therefore believed to play a key role in the pathogenicity of *Candida* species. Therefore, elucidation of the physicochemical properties (e.g. hydrophobicity), including chemical composition, should facilitate better understanding of the pathogenesis of NCAC infections. Furthermore, establishing the role of cell-wall components in the infection process could also enhance management strategies by potentially identifying appropriate and alternative targets for antifungal drugs.

Biofilm formation by NCAC species

Initial attachment of *Candida* to the host or/and medical devices is followed by cell division, proliferation and subsequent biofilm development [63]. Biofilms are surface-associated communities of microorganisms embedded within an extracellular matrix (ECM), and are considered the most prevalent growth form of microorganisms [64]. Biofilm formation is a potent virulence factor for a number of *Candida* species, as it confers significant tolerance to antifungal therapy, primarily by limiting the penetration of substances through the biofilm matrix. Growth as a biofilm also serves to protect the embedded cells from host immune responses [65]. Moreover, *C. albicans*, *C. parapsilosis*, *C. tropicalis* and *C. glabrata* isolates are adept at forming biofilms, and their presence during infection has been linked to higher mortality rates compared with isolates incapable of forming biofilms [66]. It is thought that the formation of mature biofilms and subsequent production of the ECM is strongly dependent upon species, strain, and environmental conditions such as pH, medium composition and oxygen [63,67].

Table 1 summarizes the most relevant characteristics of *C. parapsilosis*, *C. tropicalis* and *C. glabrata* biofilms. *C. albicans* biofilm formation is associated with the dimorphic switch between yeast and hyphal growth, and biofilms of this species generally have two distinct layers: a thin, basal yeast layer and a thicker, less compact hyphal layer [68]. In contrast to *C. albicans*, *C. parapsilosis* biofilms (Figure 2) tend to be thinner, less structured, and consist almost exclusively of aggregated blastospores [69]. Interestingly, biofilm formation by *C. parapsilosis* is strongly strain-dependent [64]. The selective preference of this species for plastic medical devices is of particular interest, as biofilm formation enhances the capacity of *C. parapsilosis*

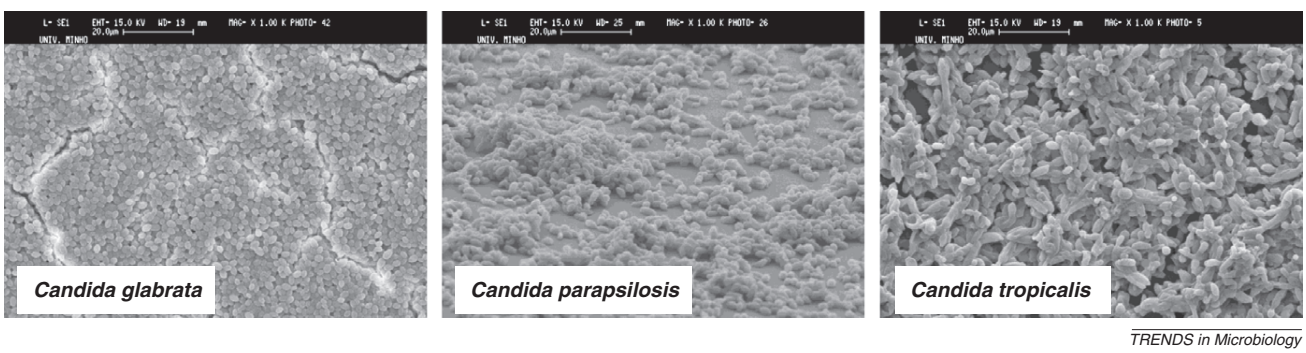

Figure 2. Biofilm structure of *C. glabrata, C. parapsilosis* and *C. tropicalis.* Scanning electron microscopy images taken after biofilm growth in SDB after 48 h.

to colonize catheters and intravascular central lines [2]. *C. tropicalis* clinical isolates have been classified as being prolific biofilm formers [21,64], and their mature biofilms consist of a dense network of yeast cells in addition to evident filamentous morphologies (Figure 2) [64]. A genetic screen of *C. glabrata* biofilm mutants identified four genes involved in biofilm formation, and biofilm growth conditions induced the transcription of *EPA6* and *EPA7* [70]. *C. glabrata* biofilms exhibit a more compact structure than those of *C. tropicalis* and *C. parapsilosis* biofilms (Figure 2). Additionally, two newly identified *Candida* species (*C. orthopsilosis* and *C. metapsilosis*) have also been shown to be capable of forming biofilms [71]. It must be stressed that comparisons of the biofilms formed by *C. glabrata*, *C. tropicalis* and *C. parapsilosis* should always consider the inherent physiological differences between these species, such as relative cell size, morphology and biochemistry. When comparing *Candida* biofilms, future research should carefully define the most appropriate parameters to investigate.

Biofilm formation and matrix composition are strongly dependent on the strain and the environmental conditions (medium composition, pH and oxygen) used [63]. Recently, Silva *et al.* [17] showed that *C. glabrata* produced higher biofilm biomass on silicone in the presence of urine, compared with *C. parapsilosis* and *C. tropicalis*. Conversely, *C. tropicalis* and *C. parapsilosis* yielded a higher biofilm biomass in Sabouraud dextrose broth (SDB) compared with *C. glabrata* [64]. These results are in accordance with Shin *et al.* [72], who reported that biofilm formation by *C. glabrata* was lower than that of other NCAC species, when cultured in nutritionally rich media. *C. glabrata* cells are smaller (1–4 μm) than those of *C. tropicalis* (4–8 μm) and *C. parapsilosis* (2.5–4 μm) and also have a narrower spectrum of carbohydrate utilization. Unlike *C. parapsilosis* and *C. tropicalis*, *C. glabrata* is unable to generate filamentous forms, which might also be expected to exhibit different metabolic activities, and thus this might contribute to the lower amount of *C. glabrata* biomass in rich medium. Such factors undoubtedly reflect the inherent physiological differences between these species, and could have significance for their pathogenic potential. Biofilms are readily formed by *C. parapsilosis* cells grown in media containing high glucose and lipid concentrations, which can be thought of being analogous to bloodstream infections in patients receiving parenteral nutrition. These results highlight the observation that biofilm formation and consequent matrix production are strongly dependent on the source of nutrients available.

The biofilm matrix of *C. albicans* is mainly composed of carbohydrates, proteins, phosphorus and hexosamines [73]. The matrix of *C. glabrata* biofilms produced in SDB has been found to contain high levels of proteins and carbohydrates [64]. The ECM of *C. parapsilosis* biofilms is composed of large quantities of carbohydrates, and the protein content is low compared with biofilms of *C. glabrata* and *C. tropicalis* [64]. The matrix of *C. tropicalis* biofilms also has a notable difference from those of the other NCAC species. Although the *C. tropicalis* matrix contains carbohydrates, proteins, hexosamine, phosphorus and uronic acid [74], it has lower amounts of carbohydrates and protein [64]. Furthermore, the major component in the *C. tropicalis* biofilm matrix is hexosamine (27%). In addition, *C. tropicalis* biofilms can be partially detached after treatment with lipase type VII and chitinase, whereas biofilms of *C. albicans* are detachable after treatment with proteinase K, chitinase, DNase I or β-N-acetylglucosamidase [74]. These findings highlight that detachment and destruction of NCAC biofilms is dependent on matrix composition. This opens the possibility that some medical devices could be coated with specific types of hydrolytic enzymes as a means of preventing biofilm formation by NCAC species.

Several genes and proteins have been reported as essential for *Candida* biofilm formation and matrix composition. Two recent studies showed that *C. parapsilosis LIP* knockout mutants had a decreased ability to form biofilms. *C. parapsilosis* lipase mutants produce significantly less biofilm than wild-type strains [31], and the biofilm and cell wall regulator (*BCR*) gene was found to be necessary for proper biofilm formation [75]. Notably, the biofilm-deficient *C. parapsilosis* lipase mutants were less virulent in tissue culture infection models and also in mice [31]. Little is known about the genes involved in biofilm formation for *C. glabrata* and *C. tropicalis*.

The studies described above highlight the diversity found for *C. glabrata*, *C. parapsilosis* and *C. tropicalis*, in terms of biofilm-forming ability, structure and matrix composition. It must be emphasized that it is clearly very important to continue these studies to elucidate the inherent differences between these and other NCAC species to identify and combat their involvement in infection.

Box 1. Outstanding questions

- Is the emergence of NCAC species related to the development of molecular diagnostic approaches, or to changes in their virulence determinants?
- What determines the switch from harmless commensal into infectious pathogen for NCAC species?
- What are the genes and proteins expressed or regulated during infection with NCAC species?

Concluding remarks

An alteration to host immunity is generally required by opportunistic pathogens, such as *Candida*, to switch from harmless commensal microorganisms to potentially life-threatening human pathogens. *Candida* utilizes several genes, which play an important role in adhesion, biofilm formation and secretion of enzymes, and are consequently involved in virulence. Given these findings and the increased incidence of candidosis caused by NCAC species, especially *C. glabrata*, *C. parapsilosis* and *C. tropicalis,* and the unacceptably high morbidity and mortality associated with them, it is essential to increase our knowledge about the factors associated with adhesion and biofilm formation in NCAC infections. Further studies in this area will also contribute towards the identification of new targets for future therapeutics against these recently emerged pathogens (Box 1).

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