Candida species biofilm and Candida albicans ALS3 polymorphisms in clinical isolates

Ariane Bruder-Nascimento1, Carlos Henrique Camargo1, Alessandro Lia Mondelli2, Maria Fátima Sugizaki1, Terue Sadatsune1, Eduardo Bagagli1

1Instituto de Biociências de Botucatu, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Botucatu, SP, Brazil.
2Faculdade de Medicina de Botucatu, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Botucatu, SP, Brazil.

Submitted: May 27, 2013; Approved: April 17, 2014.

Abstract

Over the last decades, there have been important changes in the epidemiology of Candida infections. In recent years, Candida species have emerged as important causes of invasive infections mainly among immunocompromised patients. This study analyzed Candida spp. isolates and compared the frequency and biofilm production of different species among the different sources of isolation: blood, urine, vulvovaginal secretions and peritoneal dialysis fluid. Biofilm production was quantified in 327 Candida isolates obtained from patients attended at a Brazilian tertiary public hospital (Botucatu, Sao Paulo). C. albicans ALS3 gene polymorphism was also evaluated by determining the number of repeated motifs in the central domain. Of the 198 total biofilm-positive isolates, 72 and 126 were considered as low and high biofilm producers, respectively. Biofilm production by C. albicans was significantly lower than that by non-albicans isolates and was most frequently observed in C. tropicalis. Biofilm production was more frequent among bloodstream isolates than other clinical sources, in urine, the isolates displayed a peculiar distribution by presenting two distinct peaks, one containing biofilm-negative isolates and the other containing isolates with intense biofilm production. The numbers of tandem-repeat copies per allele were not associated with biofilm production, suggesting the evolvement of other genetic determinants.

Key words: Candida spp., biofilm, ALS3 gene.

Introduction

Candida species are human commensals that can cause since superficial infections to systemic disease (Kojic and Darouiche, 2004) and have emerged as important agents of opportunistic infections worldwide, primarily in immunocompromised persons (Aperis et al., 2006; Tumbarello et al., 2007; Chaves et al., 2013; Kwamin et al., 2013). Although Candida albicans is considered the most common fungal pathogen, an increasing number of non-albicans Candida species infections have been described (Redding, 2001; Krcmery and Barnes, 2002; Nucci et al., 2013). Although Candida albicans is considered the most common fungal pathogen, an increasing number of non-albicans Candida species infections have been described (Redding, 2001; Krcmery and Barnes, 2002; Nucci et al., 2013). Candida species can colonize human tissues and medical devices, such as central venous catheters, prosthetic heart valves and other devices, resulting in biofilm formation and biofilm-related infections (Andes et al., 2004; Kojic and Darouiche, 2004; Douglas, 2013). Candida infections can also be attributed to the use of invasive procedures or endogenous source (Douglas, 2002).

Biofilms are microbial communities of surface-attached cells embedded in a self-produced extracellular polymeric matrix (Donlan and Costerton, 2002). They can cause significant problems in many areas, mainly in medical settings as persistent and recurrent device-related infections (Kumar and Anand, 1998; Flemming, 2002; Fux et al., 2005). It is remarkable that biofilms are more resistant than planktonic cells, and in most cases, antimicrobial ther-

Send correspondence to A. Bruder-Nascimento. Instituto de Biociências de Botucatu, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Botucatu, SP, Brazil. E-mail: ariane@ibb.unesp.br; arianeoli@yahoo.com.br.
apy is not effective (Kumamoto, 2002; Douglas, 2003; Tobudic et al., 2012).

Biofilm formation in Candida spp. is a complex process involving multiple regulatory mechanisms (Nobile and Mitchell, 2006) and once established, Candida biofilms serve as a persistent reservoir of infection and, in addition, offers greater resistance to antifungal agents compared to planktonic phase yeasts (Chandra et al., 2001a, 2001b; Samaranayake et al., 2005; Parahitiyawa et al., 2006).

Several in-vitro systems have been developed to study and quantify biofilm, including yeast development on intravascular catheter discs, acrylic discs, cylindrical cellulose filters, microtiter plates and others (Douglas, 2002; McLean et al., 2004). Crystal violet staining, a basic dye that binds to negatively charged surface molecules and polysaccharides in the extracellular matrix, is commonly utilized to quantify biofilms formed by a broad range of microorganisms, including yeasts (Christensen et al., 1985; Jin et al., 2003; Li et al., 2003; Cobrado et al., 2013; Cooper, 2013), with highly correlation with other methods (Peeters et al., 2008).

Besides phenotypical assays to study biofilm formation in Candida species, some genotypical techniques have been used to characterize this phenomenon. The Als (agglutinin-like sequence) proteins have long been considered excellent candidates for biofilm adhesions (Hoyer et al., 1998; Zhao et al., 2003, 2004; Green et al., 2004). Eight ALS genes (ALS1 to ALS7 and ALS9) encode large, cell surface glycoproteins, some of which promote adhesion to host surfaces (Gaur and Klotz, 1997; Hoyer, 2001; Hoyer and Hecht, 2001; Fu et al., 2002; Zhao et al., 2003, 2007). Although ALS genes share a similar three-domain structure, sequence differences among the Als proteins can be large, suggesting that the proteins may present different functions (Hoyer, 2001). Much of the allelic variation in ALS genes occurs within the tandem repeat domain (the central domain of gene) and is manifested as differing numbers of the 108-pb tandem repeats in ALS alleles. It has been suggested that ALS1 is one of the most important genes associated with C. albicans biofilm production (Hoyer, 2001; Zhao et al., 2006; Hoyer, 2008).

The aims of the present study were to quantify and to compare biofilm production in a collection of different Candida species, isolated from different clinical sources, as well as to detect the polymorphisms in the ALS tandem repeat domain and their possible correlation with the biofilm production profiles.

**Materiais and Methods**

**Microorganisms**

A total of 327 Candida species isolates recovered from clinical specimens as part of routine diagnostic procedures, stored in vial tubes containing Brain Heart Infusion plus 10% glycerol, frozen at -80 °C, were re-cultured and tested for biofilm production. The isolates were obtained from patients from the Clinical Hospital, Botucatu Medical School, Sao Paulo State University (CH/UNESP), between 1998 and 2005. Candida species and their sources are summarized in Table 1. The identification of Candida species was conducted by chlamydospore formation, sugar assimilation and fermentation patterns as well as chromogenic agar (CHROMagar Candida, Difco).

**Biofilm formation assay**

Tests for biofilm quantification were performed according to previous protocols (Jin et al., 2003; Li et al., 2003), with slight modifications. Isolates were streaked onto Yeast-Extract Peptone Dextrose agar (YPD) plates and incubated at 37 °C for 48 h. Next, a large loop of actively growing cells was transferred to sterile Yeast Nitrogen

| Species identification      | Blood % (n) | Urine % (n) | Vaginal* % (n) | Peritoneal* % (n) | Total % (n) |
|-----------------------------|-------------|-------------|----------------|-------------------|-------------|
| **C. albicans**             | 22.5 (23)   | 34.1 (29)   | 80.9 (93)      | 32.0 (8)          | 46.8 (153)  |
| All non-**C. albicans**     | 77.5 (79)   | 65.9 (56)   | 19.1 (22)      | 68.0 (17)         | 53.2 (174)  |
| **C. glabrata**             | 4.9 (5)     | 23.5 (20)   | 3.6 (4)        | -                 | 8.9 (29)    |
| **C. guilliermondii**       | 5.9 (6)     | 1.2 (1)     | -              | 8.0 (2)           | 2.8 (9)     |
| **C. lusitaniae**           | 1.0 (1)     | -           | -              | -                 | 0.3 (1)     |
| **C. parapsilosis**         | 43.1 (44)   | 8.2 (7)     | 4.5 (5)        | 40.0 (10)         | 20.2 (66)   |
| **C. pelliculosa**          | 1.0 (1)     | -           | -              | -                 | 0.3 (1)     |
| **C. tropicalis**           | 2.9 (3)     | 32.9 (28)   | 0.9 (1)        | 20.0 (5)          | 11.3 (37)   |
| **C. krusei**               | -           | -           | 10.4 (12)      | -                 | 3.7 (12)    |
| **Candida spp.**            | 18.6 (19)   | -           | -              | -                 | 5.8 (19)    |
| **Total**                   | 102         | 85          | 115            | 25                | 327         |

*Vaginal secretion; *Peritoneal dialysis fluid.
Base (YNB) broth containing 0.9% D-glucose, and incubated at 37 °C for 24 h. Yeast cells were twice centrifuged (5000 x g for 5 min) and washed with 0.5 mL PBS (0.14 M NaCl, 2.7 mM KCl, 8.5 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4). After, cells were re-suspended in 1 mL YNB broth and adjusted to concentration of 107 cells/mL (0.5 McFarland). Next, 100 µL of each isolate suspension was inoculated into individual wells of polystyrene 96-well plates (TPP), in four repetitions. YNB broth containing no inoculum was used as negative control. Plates were incubated at 37°C for 90 min (adhesion period). Supernatant including planktonic cells and liquid medium was then discarded and wells were gently washed twice with PBS to eliminate any non-adherent cells. For biofilm growth, 100 µL of fresh YNB broth was then added to each well. The plates were incubated at 37 °C for 48 h. After incubation, planktonic cells were discarded through three rounds of washing with 200 µL sterile PBS buffer, and the plates dried at room temperature for 45 min. For staining with Crystal Violet (CV), 150 µL of 0.4% CV, diluted in water, was added to each well, and, after 45 min at room temperature, all the supernatant was discarded before adding 150 µL of 95% ethanol and maintained for 45 min, to dissolve and/or elute the dye from the biofilm cells. Next, 100 µL of each well was transferred to a new 96-well microplate and the absorbance determined using a microplate reader at 540 nm filter (MultisKan EX, Labsystems). The absorbance values were converted into transmittance percentages (%T). The %T values for each test was subtracted from the %T for the reagent blank to obtain a measure of light blocked when passing through the wells (%Tbloc), and the biofilm production scored as either negative (%Tbloc < 10), positive 1+ (%Tbloc 10 to 20), positive 2+ (%Tbloc 20 to 35), positive 3+ (%Tbloc 35 to 50) or positive 4+ (%Tbloc ≥ 50), and the positives further categorized as low-biofilm (1+) or high-biofilm producers (2+, 3+, or 4+) (Tumbarello et al., 2007).

**ALS3 characterization**

The gene ALS3 was studied in all isolates from bloodstream cultures (23 C. albicans and 16 C. non albicans isolates). DNA was extracted according to McCullough et al. (2000) with slight modifications. Colonies of yeasts with growth of 24 h/37 °C onto Sabouraud dextrose agar plates were suspended in 1 mL of 1 M sorbitol and 125 mM of EDTA; suspension was centrifuged (10 min, 13000 x g, 25 °C), the supernatant was discarded, and the pellet was resuspended in 0.5 mL of lysing solution (1 M Tris-HCl, pH 8.0, with 250 mM of EDTA and 5% SDS) plus 10 µL of proteinase K (Invitrogen) and incubated for 1 h at 65 °C. Next, 500 µL 5 M potassium acetate was added, incubated on ice for 2 h and then centrifuged (10 min, 13000 x g, 25 °C). Supernatant was transferred to an Eppendorf tube containing 1 mL of absolute ethanol and mixed by inversion and centrifuged (10 min, 13000 x g, 4 °C). The supernatant was discarded; the pellet was washed with 500 µL of cooled 70% ethanol and centrifuged (10 min, 13000 x g, 4 °C). Finally, supernatant was discarded and the pellet was resuspended in 0.5 mL of sterilized MilliQ water. The size of the tandem repeat domain in each ALS allele was determined by PCR using two independent primer pairs (Oh et al., 2005). When the first pair of primers provides no clear amplification, the second pair was used. PCR products were separated on 0.7% agarose (TBE) gels stained with ethidium bromide. The gels were analyzed in the equipment AlphaEase® FC. To estimate the numbers of motifs present in the tandem repeats in the ALS3 gene, the primers positions were aligned with the deposited genomic sequences of strain SC5314 DNA (GenBank Accession No. AY223552.1), large and small alleles that present twelve and nine motifs, using Mega software. The numbers of motifs for each isolate evaluated were calculated considering 108 bp the mean size for each motif. The amplicon of one homozygous isolate was purified (GFX PCR DNA and Gel Band, GE, Healthcare), sequenced using the DYEnamic ET Dye Terminator Kit (with Thermo Sequenase II DNA Polimerase) in a MegaBACE 1000 DNA Analysis System, and the chromatogram visualized by the Chromas program. The consensus sequence was sent to blastn for comparison with the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST).

**Statistical analysis**

Chi-square analysis was used to compare biofilm positivity among different Candida species or among isolates recovered from different sources. The Kolmogorov-Smirnov test was used to compare the numbers of tandem repeat copies between biofilm-positive and biofilm-negative isolates. Differences between groups were considered to be significant for p < 0.05.

**Results**

**Biofilm production**

A total of 198 of 327 (60.6%) Candida species isolates were biofilm-positive. Of these, 72 (36.4%) and 126 (63.6%) isolates were low and high biofilm producers, respectively. Biofilm production by C. albicans isolates was significantly lower than that by non-albicans isolates (43.1% vs. 75.8%, respectively; p < 0.001), and among the biofilm-positive isolates, the non-albicans isolates were classified mainly as high-biofilm producers, with C. albicans isolates defined as low-biofilm producers (p < 0.001) (Table 1).

Considering the species separately, biofilm production was most frequently observed in C. tropicalis isolates (94.6%, p < 0.001), followed by C. parapsilosis (80.3%),
C. glabrata (44.8%), and C. albicans (43.1%). Among biofilm-positive isolates, the highest biofilm production intensity (3+ and 4+) was observed in C. tropicalis isolates (81.1%, p < 0.001) (Figure 1).

In relation to the sources, biofilm production was positive in 79.4% of the isolates obtained from the bloodstream, 63.5% from urine, 37.4% from vaginal secretion and 80% from peritoneal dialysis fluid (Table 2). Biofilm production among bloodstream isolates was more frequent compared to any other source (p < 0.001), except peritoneal dialysis fluid isolates (p > 0.05), which were also prolific biofilm producers. Biofilm production was most frequent in non-albicans isolates (p < 0.001) in all sources, also with the exception of peritoneal dialysis fluid isolates (p > 0.05). In urine, the isolates displayed a peculiar distribution by presenting two distinct peaks, one containing biofilm-negative isolates (36.5%) and the other containing isolates (37.6%) with intense biofilm production (%Tbloc, >35) (Figure 1). Collinearity was not observed between species and sources of Candida isolates (p > 0.05).

**ALS3 characterization**

Polymerase chain reaction was performed on a total of 23 C. albicans isolates from bloodstream infections. In 19 of 23 C. albicans isolates, the expected fragments of the ALS3 gene were amplified by using the first or the second pair of primers (Figure 2). In the 16 non-albicans evaluated, the PCR products were not amplified with both pairs of primers.

The consensus sequence of a fragment containing approximately 700 bp in the Blast analysis presented 93% identity with the C. albicans large allele ALS3 gene (GenBank Accession No. AY223552.1; E value 0.0).

The numbers of copies of the central tandem repeat domain were divergent among the isolates, which three quarters presented homozygosity. Alleles in the examined isolates encoded between 7 and 14 copies of tandemly repeated 108-bp sequence (Table 3). The mean numbers ± standard deviation of tandem repeat copies per allele in biofilm-negative and biofilm-positive isolates were 11.6 ± 1.4 and 10.7 ± 1.7, respectively, which was not significant (p > 0.05).

**Discussion**

Biofilm production in 327 clinical isolates clearly confirms that different Candida species have different abilities to produce biofilm in vitro. Biofilm production was also associated with source of Candida isolation. Biofilm production has been considered an important virulence factor among Candida species (Pitts et al., 2003). A broad range of methods has been employed to evaluate this phenomenon in yeasts, mainly in Candida species (Peeters et al., 2008). Herein, we quantified the biofilm production in 327 clinical isolates of Candida species by the crystal violet assay, a widely used method to quantify biofilm production in several microorganisms, including yeasts (Christensen et al., 1985; Stepanovic et al., 2000; Li et al., 2003; Peeters et al., 2008, Cobrado et al., 2013; Cooper, 2013). Although this method is not able to differentiate between living and dead cells (Peeters et al., 2008, Pitts et al., 2003), the results obtained by the crystal violet assay have correlated highly with other assays that differentiate between living and dead cells, such as the 2,3-bis (2-methoxy-4-nitro-5-sulfo-
Table 2 - Comparison of biofilm production by *Candida* species isolates from blood, urine, vaginal secretion and peritoneal dialysis fluid obtained in a Brazilian tertiary public hospital (HC/UNESP), 1998-2005.

| Candida species | Total Blood Urine Vulvov Perit |
|-----------------|-------------------------------|
| *C. albicans*   | 66/153 (43.1)                 |
| All non-albicans species | 132/174 (75.9)% |
| *C. parapsilosis* | 53/66 (80.3)                 |
| *C. tropicalis* | 35/37 (94.6)                 |
| *C. glabrata*   | 13/29 (44.8)                  |
| Other *Candida* species | 31/42 (73.8)                  |
| Total           | 198/327 (60.6)               |

| Biofilm positive (22) | 9.1 (2) | 36.4 (8) | 36.4 (8) | 4.5 (1) | 4.5 (1) | 9.1 (2) |
|-----------------------|---------|----------|----------|---------|---------|---------|
| Percent (n) alleles in each tandem repeat copy group | 10 | 11 | 12 | 13 | 14 | 7 |
| Mean no of repeat copies/allele* | 11.6 ± 1.4 |

| Biofilm production (n) | Percent (n) alleles in each tandem repeat copy group | Mean no of repeat copies/allele* |
|-----------------------|---------------------------------------------------|---------------------------------|
| Biofilm negative (16) | 0 (0) | 31.3 (5) | 25.0 (4) | 0 (0) | 43.8 (7) | 0 (0) |
| Biofilm positive (22) | 9.1 (2) | 36.4 (8) | 36.4 (8) | 4.5 (1) | 4.5 (1) | 9.1 (2) |

*Mean number of repeat copies per allele ± standard deviation. p value not significant.

Table 3 - Distribution of allele per ALS3 gene tandem-repeat copies in *C. albicans* isolates from the bloodstream obtained in a Brazilian tertiary public hospital (HC/UNESP), 1998-2005.

| Biofilm production (n) | Percent (n) alleles in each tandem repeat copy group | Mean no of repeat copies/allele* |
|-----------------------|---------------------------------------------------|---------------------------------|
| Biofilm negative (16) | 0 (0) | 31.3 (5) | 25.0 (4) | 0 (0) | 43.8 (7) | 0 (0) |
| Biofilm positive (22) | 9.1 (2) | 36.4 (8) | 36.4 (8) | 4.5 (1) | 4.5 (1) | 9.1 (2) |

*Mean number of repeat copies per allele ± standard deviation. p value not significant.

We confirmed that non-albicans species are the highest biofilm producers. Among biofilm producers, *C. tropicalis* showed the highest intensity of biofilm production. In other studies involving similar numbers of *Candida* species isolates, *C. tropicalis* was the species with the highest percentages of biofilm positivity, while *C. albicans*, ranked third or fourth among the biofilm-positive isolates (Shin et al., 2002; Tumbarello et al., 2007).

We also evaluated the associations between the source and the biofilm positivity of isolates. Isolates obtained from the bloodstream and peritoneal dialysis fluid typically were prolific biofilm producers. Urine isolates presenting intense biofilm production might be associated with the use of urinary catheter, or, alternatively, be originated from systemic candidiasis and not from urinary tract infections, since the isolates from invasive infections tend to produce more biofilm than those from non-invasive infections (Kuhn et al., 2002). Once that we have not evaluated patient charts, this hypothesis of urinary catheter remains to be proven.

Important advances have been achieved in outlining the genetic basis of biofilm production, however, the subject is far from being completely understood. After the *C. albicans* genome was sequenced (Jones et al., 2004), the biofilm production by this yeast was better comprehended, although biofilm formation in non-albicans species remains poorly understood (Weber et al., 2008). Several genes are involved in the biofilm formation by *Candida* spp. (López-Ribot, 2005; Ramage et al., 2005; Nobile and Mitchell, 2006; Yu et al., 2012; Lin et al., 2013; Srikanta et al., 2013). It was demonstrated that the expression of several ALS genes is upregulated during biofilm formation; furthermore, the Als proteins have long been considered excellent candidates for biofilm adhesins (Green et al., 2004; Blakenship and Mitchell, 2006). It was analyzed the association between biofilm production and polymorphisms in the ALS3 central domain. Previous studies showed that ALS3 gene expression is altered in *C. albicans* sessile cells compared to planktonic cells (Chandra et al., 2001a, 2001b; García-Sánchez et al., 2004; Nailis et al., 2006). Maximal ALS3 expression is associated with formation of germ tubes and hyphae (Hoyer et al., 1998); and an overexpression of ALS3 was observed in initial stages of biofilm formation (Nailis et al., 2009). We did not observe association between the number of ALS3 tandem-repeat copies per allele and biofilm production. However, biofilm-negative isolates showed the majority of alleles with 13 tandem-repeat copies, while the majority of alleles in biofilm-positive isolates showed 10 or 11 copies (Table 3).
In conclusion, we showed that *Candida non-albicans* species were more prolific biofilm producers than *C. albicans* and that the source of isolates might influence the biofilm production, in which more invasive isolates (blood and peritoneal dialysis fluid) show greater biofilm production. The polymorphism of the ALS3 central domain in *C. albicans*, detected by the different numbers of tandem-repeat copies, appears not to be directly related to biofilm production.

Acknowledgments

We thank Professor Augusto Cezar Montelli for kindly providing the isolates, and Raquel Cordeiro Theodoro, Sandra Bosco, Severino Assis Macoris and Virginia Richini-Pereira, for their lab assistance. The study was financially supported by FAPESP (grant numbers 07/01946-4, 08/10835-4, 08/09231-7).

References

Andes D, Nett J, Oschel P, Albrecht R, Marchillo K, Pitula A (2004) Development and characterization of an in-vivo central venous catheter *Candida albicans* biofilm model. Infect Immun 72:6023-6031.

Aperis G, Myriounis N, Spanakis EK, Mylonakis E (2006) Developments in the treatment of candidiasis: more choices and new challenges. Expert Opin Investig Drugs 15:1319-1336.

Blakenship JR, Mitchell AP (2006) How to build a biofilm: A fungal persp. Curr Op Microbiol 9:588-594.

Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA (2001) Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. J Bacteriol 183:5385-9534.

Chandra J, Mukherjee PK, Leidich SD, Faddoul FF, Hoyer LL, Ghannoum MA (2001) Antifungal resistance of *Candidal* biofilms formed on denture acrylic in vitro. J Dent Res 80:903-908.

Chaves GM, Diniz MG, da Silva-Rocha WP, de Souza LB, Gondim LA, Ferreira MA, Svidzinski TI, Milan EP (2013) Species Distribution and Virulence Factors of *Candida* spp. Isolated from the Oral Cavity of Kidney Transplant Recipients in Brazil. Mycopathologia 175:255-263.

Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, Beuchey EH (1985) Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol 22:996-1006.

Cobrado L, Silva-Dias A, Azevedo, MM, Pina-Vaz C, Rodrigues AG (2013) In vivo antibiotic effect of ceftriaxone, chlorotacin and hamamelitin in against usual agents of catheter-related bloodstream infections. J Antimicrob Chemother 68:126-130.

Cooper RA (2013) Inhibition of biofilms by glucose oxidase, lactoperoxidase and guaiacol: the active antibacterial component in an enzyme alginoel. Int Wound J (in press May 15)

Donlan RM, Costerton JW (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 15:167-193.

Douglas LJ (2002) Medical importance of biofilms in *Candida* infections. Rev Iberoam Micol 19:139-43.

Douglas LJ (2003) *Candida* biofilms and their role in infection. Trends Microbiol. 11:30-36.

Flemming HC (2002) Biofouling in water systems - cases, causes and countermeasures. Appl Microbiol Biotechnol 59:629-640.

Fu Y, Ibrahim AS, Sheppard DC, Chen YC, French SW, Cutler JE,Filler SG, Edwards JE Jr (2002) *Candida albicans* Als1p: An adhesion that is a downstream effector of the EFG1 filamentation pathway. Mol Microbiol 44:61-72.

Fux CA, Costerton JW, Stewart PS, Stoodley P (2005) Survival strategies of infectious biofilms. Trends Microbiol 13:34-40.

Garcia-Sánchez S, Aubert S, Iraqui I, Janbon G, Ghigo JM, d’Enfert C (2004) *Candida albicans* biofilms: a developmental state associated with specific and stable gene expression patterns. Eukaryot Cell 3:536-545.

Gaur NK, Klotz SA (1997) Expression cloning, and characterization of a *Candida albicans* gene ALS1, that confers adherence properties upon Saccharomyces cerevisiae for extracellular matrix proteins. Infect Immun 65:5289-5294.

Green CB, Cheng G, Chandra J, Mukherjee P, Ghannoum MA, Hoyer LL (2004) RT-PCR detection of *Candida albicans* ALS gene expression in the reconstituted human epithelium (RHE) model of oral candidiasis and in model biofilms. Microbiology 150:267-275.

Hoyer LL (2001) The ALS gene family of *Candida albicans*. Trends Microbiol 9:176-180.

Hoyer L., Green CB, Oh SH, Zhao X. (2008) Discovering the secrets of the *Candida albicans* agglutinin-like sequence (ALS1) gene family - a sticky pursuit. Med Mycol 46:1-15.

Hoyer LL, Hecht JE. (2001) The ALS5 gene of *Candida albicans* and analysis of the Als5p N-terminal domain. Yeast 18:49-60.

Hoyer LL, Payne TL, Bell M, Myers AM, Scherer S (1998) *Candida albicans* ALS3 and insights into the nature of the ALS gene family. Curr Genet 33:451-459.

Jin Y, Yip HK, Samaranayake YH, Yau JY, Samaranayake LP (2003) Biofilm-forming ability of *Candida albicans* is unlikely to contribute to high levels of oral yeast carriage in cases of human immunodeficiency virus infection. J Clin Microbiol 41:2961-2967.

Jones T, Federspiel NA, Chibana H, Dungan J, Kalman S, Magee BB, Newport G, Thorstenson YR, Agabian N, Magee PT, Davis RW, Scherer S (2004) The diploid genome sequences of *Candida albicans* and analysis of the Als5p N-terminal domain. Yeast 18:49-60.

Kojic EM, Darouiche RO (2004) Candida infections of medical devices. Clin Microbiol Rev 17:255-267.

Krcmery V, Barnes AJ (2002) Non-*albicans* *Candida* spp. causing fungaemia: Pathogenicity and antifungal resistance. J Hosp Infect 50:243-260.

Kuhn DM, Chandra J, Mukherjee PK, Ghannoum MA (2002) Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. Infect Immun 70:878-888.

Kumamoto CA (2002) *Candida* biofilms. Curr Opin Microbiol 5:608-611.

Kumar CG, Anand SK (1998) Significance of microbial biofilms in food industry: A review. Int J Food Microbiol 42:9-27.
Biofilm and ALS3 polymorphism in Candida spp.

Kwamin F, Narrey NO, Codjoe FS, Newman MJ (2013) Distribution of Candida species among HIV-positive patients with oropharyngeal candidiasis in Accra, 15 Ghana. J Infect Dev Cities 15:41-45.

Li X, Yan Z, Xu J (2003) Quantitative variation of biofilms among strains in natural populations of Candida albicans. Microbiology 149:353-362.

Lin CH, Kahrawala S, Fox EP, Nobile CJ, Johnson AD, Bennett RJ (2013) Genetic Control of Conventional and Phero-mone-Stimulated Biofilm Formation in Candida albicans. PLoS Pathog 9:e1003305.

López-Ribot JL (2005) Candida albicans biofilms: more than filamen-tation. Curr Biol 15:R453-R455.

McCullough MJ, DiSalvo AF, Clemons KV, Park P, Stevens DA (2000) Molecular epidemiology of Blastomyces dermatiti-dis. Clin Infect Dis 30:328-335.

McLean RJC Bates, CL Barnes MB McGowin, CL Aron, G.M (2004) Methods of studying biofilms In: Ghannoum M., O’Toole GA (eds) Microbial Biofilms. ASM Press, Washington, DC. pp. 379-413.

Nailis H, Coenye T, Van Nieuwerburgh F, Deforce D, Nelis HJ, Peeters E, Nelis HJ, Coenye T (2008) Comparison of multiple

Parahitiyawa NB, Samaranayake YH, Samaranayake LP, Tsang LW, Cheung BP, Yau JY, Yeung SK (2006) Interspecies variation in Candida biofilm formation studied using the Calgary biofilm device. PLoS One. 8:e59373.

Oh SH, Cheng G, Nuesen JS, Jako J, Yeater KM, Zhao X, Pujol C, Soll DR, Hoyer LL (2005) Functional specificity of Candida albicans Als3p proteins and clade specificity of ALS3 alleles discriminated by the number of copies of the tandem repeat sequence in the central domain. Microbiology 151:673-681.

Parahitiyawa NB, Samaranayake YH, Samaranayake LP, Thang PW, Cheung BP, Yau JY, Yeung SK (2006) Interspecies variation in Candida biofilm formation studied using the Calgary biofilm device. PLoS One. 8:e59373.

Peeters E, Nelis HJ, Coenye T (2008) Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. J Microbiol Methods 72:157-65.

Pitts B, Hamilton MA, Zelver N, Stewart PS (2003) A microtiter-plate screening method for biofilm disinfection and removal. J Microbiol Methods 54:269-276.

Ramage G, Saville SP, Thomas DP, López-Ribot JL (2005) Candida biofilms: An update. Eukaryot Cell 4:633-638.

Redding SW (2001) The role of yeasts other than Candida albicans in oropharyngeal candidiasis. Curr Opin Infect 14:673-677.

Richardson MD (2005) Changing patterns and trends in systemic fungal infections. J Antimicrob Chemother 56:i5-i11.

Samaranayake YH, Jin Y, Yao JYY, Cheung BPK, Samaranayake LP (2005) In vitro method to study antifungal perfusion in Candida biofilms. J Clin Microbiol 43:818-825.

Shin JH, Kee SJ, Shin MG, Kim SH, Shin DH, Lee SK, Suh SP, Ryang DW (2002) Biofilm production by isolates of Candida species recovered from nonneutropenic patients: comparison of bloodstream isolates with isolates from other sources. J Clin Microbiol 40:1244-1248.

Srikantha T, Daniels KJ, Pujol C, Kim E, Soll DR (2013) Identification of Genes Upregulated by the Transcription Factor Bcr1 That Are Involved in Impermeability, Impenetrability and Drug-Resistance of Candida albicans a/α. Biofilms. Eukaryot Cell (in press).

Stepanovic S, Vukovic D, Dakic I, Savic B, Svabie-Vlahovic M (2000) A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods 40:175-179.

Tobucic S, Kratzer C, Lassnigg A, Presterl E (2012) Antifungal susceptibility of Candida albicans in biofilms. Mycoses 55:199-204.

Tumbarello M, Posteraro B, Trecarichi EM, Fiori B, Rossi M, Porta R, Donati KG, La Sorda M, Spanu T, Fadda G, Cauda R, Sanguinetti M (2007) Biofilm Production by Candida Species and Inadequate Antifungal Therapy as Predictors of Mortality for Patients with Candidemia. J Clin Microbiol 45:1843-1850.

Weber, K Sohr, R Schulz B, Fleischhacker M, Ruhneke M (2008) Secretion of E.E-Farnesol and Biofilm Formation in Eight Different Candida Species. Antimicrobial Agents Chemother 52:1859-1861.

Yu Q, Wang H, Xu N, Cheng X, Wang Y, Zhang B, Xing L, Li M (2012) Spf1 strongly influences calcium homeostasis, hyphal development, biofilm formation and virulence in Candida albicans. Microbiology 158:2272-2282.

Zhao X, Daniels KJ, Oh SH, Green CB, Yeater KM, Soll DR, Hoyer LL (2005) Candida albicans Als3p is required for wild-type biofilm formation on silicone elastomer surfaces. Microbiology 151:2287-2299.

Zhao X, Oh SH, Jako R, Diekema DJ, Pfaller MA, Pujol C, Soll DR, Hoyer LL (2007) Analysis of ALS5 and ALS6 allelic variability in a geographically diverse collection of Candida albicans isolates. Fungal Genet Biol 44:1298-1309.

Zhao X, Pujol C, Soll DR Hoyer, LL (2003) Allelic variation in the contiguous loci encoding Candida albicans ALS5, ALS1 and ALS9. Microbiology 149:2947-2960.

All the content of the journal, except where otherwise noted, is licensed under a Creative Commons License CC BY-NC.