Oral Diseases (2008) 14, 582–590. doi:10.1111/j.1601-0825.2007.01424.x

http://www.blackwellmunksgaard.com

REVIEW ARTICLE

Biofilm lifestyle of *Candida*: a mini review

CJ Seneviratne, L Jin, LP Samaranayake

Oral Bio-Sciences, Faculty of Dentistry, The University of Hong Kong, Hong Kong

***Candida* is the major fungal pathogen of humans causing a variety of afflictions ranging from superficial mucosal diseases to deep seated mycoses. Biofilm formation is a major virulence factor in the pathogenicity of *Candida*, and *Candida* biofilms are difficult to eradicate especially because of their very high antifungal resistance. Consequently, research into the pathogenicity of *Candida* has focused on the prevention and management of biofilm development, their architecture, and antifungal resistance. Although studies have shed some light, molecular mechanisms that govern biofilm formation and pathogenicity still await full clarification. This review outlines the key features of what is currently known of *Candida* biofilm development, regulation and antifungal resistance and, their proteomics.**
Oral Diseases (2008) **14**, 582–590

Keywords: *Candida*; biofilm; antifungal resistance

Introduction

Candida is perhaps the archetypal opportunistic human pathogen, having been identified as early as the era of Hippocrates. Today, we know that *Candida* is a commensal fungus that harmlessly inhabits various niches of the human body, including the oral cavity, gastrointestinal tract, vagina, and skin of healthy individuals (Samaranayake and MacFarlane, 1990). Under certain circumstances, however, *Candida* can cause infection, or candidiasis, which can range from superficial mucous membrane infection to life-threatening systemic disease (Samaranayake and MacFarlane, 1990).

Candida is now regarded as a major human pathogen in clinical settings. Candidiasis is the third or fourth leading cause of nosocomial infection in the United States, ranking even higher than some common bacterial infections (Banerjee *et al*, 1991; Edmond *et al*, 1999). Moreover, mortality rates among patients with candidiasis have been increasing, and reported to be as high as 40% to 60% (Wenzel and Gennings, 2005; Colombo *et al*, 2006). The transition of innocuous commensal *Candida* to the disease-causing 'parasitic' form can depend on the immune status of the host. For example, immunocompromised populations, such as patients with HIV/AIDS, transplant recipients, and patients receiving cancer chemotherapy, have increased tremendously in the past decade, and a parallel escalation of candidiasis has been observed (Odds, 1998). Candidiasis is, in fact, the most common fungal infection in both child and adult HIV/AIDS patients (Samaranayake *et al*, 2002). *Candida* is also the major pathogen to infect recipients of solid organ transplants, and the mortality rate due to candidiasis among these patients can be as high as 50% (Patterson, 1999; Hagerty *et al*, 2003). Furthermore, *Candida* infections have the highest crude mortality rate among vascular catheter-related infections (Beck-Sague and Jarvis, 1993). An understanding of the mechanisms underlying the pathogenicity of this ubiquitous fungus is thus a medical priority.

One of the major factors contributing to the virulence of *Candida* is its versatility in adapting to a variety of different habitats for growth and, formation of surface-attached microbial communities known as 'biofilms'. Biofilms are defined as microbial communities encased in a matrix of extracellular polymeric substances (EPS) and, displaying phenotypic features that differ from their planktonic or free-floating counterparts (Costerton *et al*, 1995). Indeed, most microbes in their natural habitat are attached to surfaces within a structured biofilm ecosystem (Costerton *et al*, 1995; Donlan and Costerton, 2002; Mukherjee *et al*, 2006), and at least 65% of all microbial infections are related to biofilm growth (Potera, 1999). *Candida* biofilms are especially widespread and have been observed in most, if not all, medical devices in current use, such as stents, shunts, implants, endotracheal tubes, pacemakers, and various types of catheters (Ramage *et al*, 2006). *Candida* biofilms are difficult to eradicate because of their very high antifungal resistance; an infected implant must be removed if biofilm removal poses an impossible task (Tunney *et al*, 1996). Because *Candida* biofilm-associated infections have many clinical and economic

Correspondence: Dr LP Samaranayake, Oral Bio-Sciences, Faculty of Dentistry, The University of Hong Kong, Prince Philip Dental Hospital, 34 Hospital Road, Hong Kong. Tel: (852) 2859 0480, Fax: (852) 25476133, E-mail: lakshman@hku.hk
Received 29 May 2007; revised 22 July 2007; accepted 26 August 2007

consequences, recent research into the pathogenicity of *Candida* has focused on the prevention and management of biofilms. This review outlines the key aspects of what is currently known of *Candida* biofilm development and regulation.

Candida biofilm structure

Our understanding of *Candida* biofilm formation and development has relied on observations made using various microscopic techniques such as scanning electron microscopy, fluorescence microscopy, and confocal scanning laser microscopy. A biofilm typically develops over four sequential steps: first, the adhesion of a microorganism to a surface, second, discrete colony formation, and organisation of cells; third, secretion of EPS; maturation into a three-dimensional structure; and lastly, dissemination of progeny biofilm cells (Figure 1).

Candida biofilms form after the adherence of free-floating *Candida* cells in suspension to a surface; this process takes 1–2 h (Samaranayake and MacFarlane, 1981; Samaranayake *et al*, 1995). Adhesion is first mediated by non-specific interactions, such as hydrophobic and electrostatic forces, between the cells and the substratum (Samaranayake and MacFarlane, 1990; Donlan and Costerton, 2002). Then, specific adhesion molecules are expressed to facilitate stronger adhesion. Amongst these specific adhesion molecules, one well known group belongs to cell-surface glycoproteins that are encoded by the agglutinin-like sequence gene family (Zhao *et al*, 2005). Once the blastospores adhere, they form a complex spatially sequenced divisions leading to an aggregate of cells that is well organized. Morphogenesis of these cells in the nascent community depends on various factors such as carbon source, substratum, and species.

Candida biofilm formation has been described on polymethylmethacrylate strips and occurs essentially in three overlapping phases: early (0–11 h), intermediate (12–30 h), and maturation (38–72 h) phases (Chandra *et al*, 2001). The early stage is characterized by adherence and development of blastospores into distinct microcolonies. By 18–24 h, the *Candida* biofilm community can be seen as a bilayered structure comprising a mixture of yeasts, germ tubes, and young hyphae; this intermediate phase is distinguished by the production of EPS. During maturation, the biofilm becomes a thick EPS layer in which a dense network of yeasts, pseudohyphae, and hyphae are embedded (Figure 2).

In a typical biofilm on flat, hydrophobic surfaces, such as silicone elastomer and polyvinyl chloride, there is a biphasic distribution of components—namely, an adherent blastospore layer covered by a sparse layer of hyphal elements embedded in EPS (Baillie and Douglas, 1999; Chandra *et al*, 2001). Water channels between hyphal cells facilitate the diffusion of nutrients from the environment through the biomass to the bottom layers and also permit waste disposal (Figure 3) (Ramage *et al*, 2001a).

Candida biofilms that are formed in *in-vivo* models seem to follow the same sequence (Andes *et al*, 2004). However, maturation is faster and thickness is greater in these biofilms than in those grown in *in-vitro* systems. The thickness of a biofilm grown *in vitro* can range from 25 μm to 450 μm (Chandra *et al*, 2001; Ramage *et al*, 2001a; Kuhn *et al*, 2002a), whereas it usually exceeds 100 μm in *in-vivo* models (Andes *et al*, 2004).

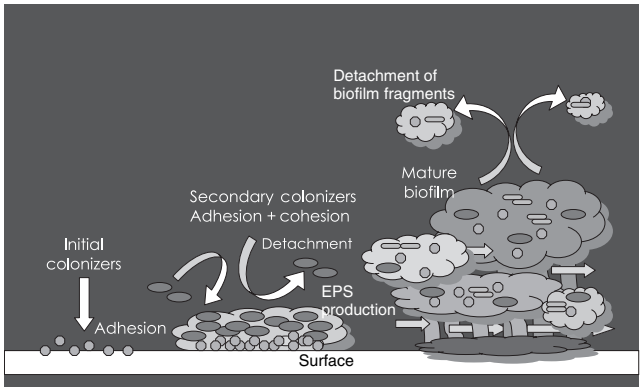

Figure 1 Sequence of biofilm development is depicted: A biofilm typically develops in four sequential steps: first, adhesion of a microorganism to a surface, second, individual colonisation, and organisation of cells; third, secretion of extracellular polymeric substances (EPS); maturation into a three-dimensional structure; and finally, dissemination of progeny biofilm cells

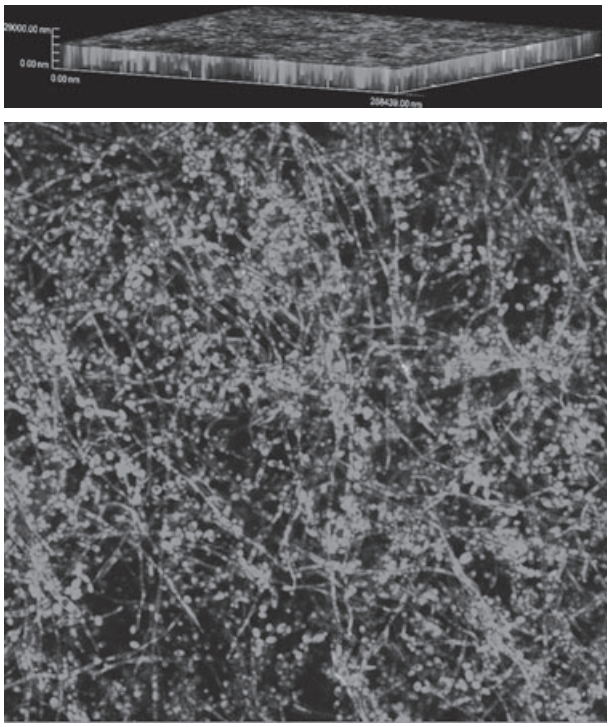

Figure 2 Confocal Scanning Laser Microscopy (CSLM) images of 24 h *Candida* biofilm stained with Live/Dead Backlight viability kit (Molecular probes, Invitrogen, CA, USA). (**a**) Three dimensional view (XYZ) of the biofilm showing the lateral sections. Note that red fluoresce represent the dead whist green fluorescence represent live cells. Most of the dead cells are sandwiched between upper and lower layers. (**b**) Horizontal section (XY) of the middle layer of *Candida* biofilm

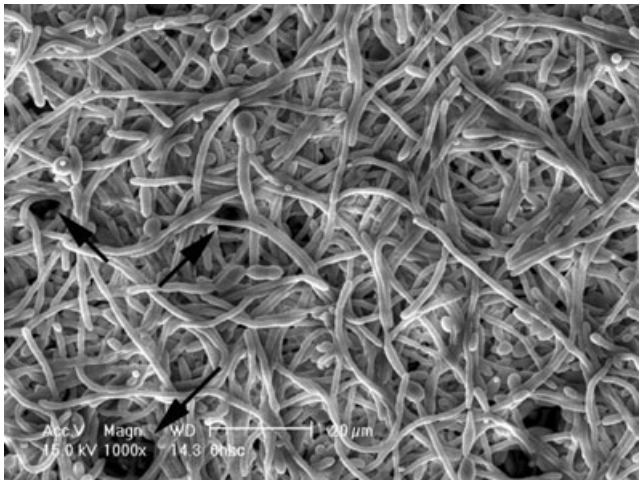

Figure 3 Scanning Electron Microscopy (SEM) analysis of *C. albicans* biofilm. Note the heterogeneous nature of biofilm, showing a mixture of blastospore, pseudohyphae, and hyphae embedded in extracellular polymeric substance (EPS). Arrows indicate the presence of water channels among the cellular component

Factors affecting *Candida* biofilm formation

Numerous factors that can affect *Candida* biofilm formation have been identified such as substratum, nutrient, presence of saliva, availability of oxygen, EPS and *Candida* species. Studies reporting such factors, however, should be interpreted with caution because of the diverse nature of protocols used.

Substratum
Several *in vitro* model systems, such as acrylic, silicone elastomer catheter disks, cellulose cylindrical filters, polymethylmethacrylate, plastic, and glass, have been used to develop *Candida* biofilms (Hawser and Douglas, 1994; Nikawa *et al*, 1996; Baillie and Douglas, 1999; Chandra *et al*, 2001; Jin *et al*, 2004). Biotic surfaces, such as those in an engineered human oral mucosa model, have been also used as a substrate (Mukherjee *et al*, 2006). The physiochemical properties of the substrate can influence *Candida* adhesion and subsequent biofilm formation. For instance, soft lining materials of dentures are better for adhesion of *Candida* than acrylic surfaces (Radford *et al*, 1998). Likewise, different catheter materials permit varying amounts of *Candida* biofilm development (Hawser and Douglas, 1994): the most extensive biofilm was observed for latex urinary catheters, followed by PVC and polyurethane. In contrast, 100% silicone was capable of significantly less biofilm formation. Hence, surface topography is an important factor, with the smoothest surfaces minimising biofilm formation. Modification of surface properties of biomaterials, including the contact angle of the surface, can change the ability of *C. albicans* to form biofilms (Chandra *et al*, 2005).

Nutrient: carbon source
Studies on the effect of carbon source on *Candida* biofilm have yielded conflicting results. Hawser and Douglas (1994) showed a higher degree of *C. albicans* biofilm formation in 500 mM galactose than in 50 mM glucose, whereas Nikawa *et al* (1997) observed the reverse. Other researchers have shown that glucose, fructose, and lactose favour *C. albicans* biofilm formation compared with other dietary sugars such as sucrose and maltose (Parahitiyawa *et al*, 2006).

Presence of saliva
Once implant devices are placed in the body in contact with surrounding body fluid, such as saliva or serum, they are instantly coated with a 'conditioning film' or an 'acquired pellicle'. In saliva, for example, a salivary pellicle has been shown to develop and significantly enhance biofilm formation of *Candida* (Nikawa *et al*, 1997).On the other hand, Jin *et al* (2004) have shown that the presence of a salivary coating does not significantly influence biofilm formation, regardless of dietary sugar supplementation. These contrasting observations may be because of differences in the nature (e.g. the quality of saliva from different individuals), method of sample collection, and application of saliva. The quality control of saliva in comparative studies was stressed previously (Samaranayake *et al*, 1984). It is conceivable that the presence of a saliva or serum pellicle, together with the type of carbon source in the environment, acts in a complex manner to modulate *Candida* biofilm development.

Availability of oxygen
Candida is able to grow either aerobically or anaerobically. However, only a few studies have so far specifically focused on *Candida* biofilm formation in anaerobic conditions (Biswas and Chaffin, 2005; Thein *et al*, 2007). We have recently demonstrated that *Candida* species are able to form biofilms under anaerobic conditions (Thein *et al*, 2007). In contrast, a previous study suggested the inability of *Candida* to form biofilms under strict anaerobic conditions (Biswas and Chaffin, 2005). The aforementioned study used only a single strain of *C. albicans* (SC-5314) and showed that typical multilayered biofilms are not seen on denture material, plastic, or glass coated with poly-L-lysine. However, in our study (Thein *et al*, 2007) we used multiple *Candida* strains and species, which are important in validating the general effect of anaerobic milieu on yeast biofilm formation. Rigorous anaerobic conditions starting from the subculturing step, as in the study of (Biswas and Chaffin, 2005), seem to preclude the biofilm-forming ability of *Candida* cells. Another possible explanation for this discrepancy could be inter-strain variations. Interestingly, it is noted that non-*albican Candida* species are also able to form biofilms under anaerobic conditions (Thein *et al*, 2007). As it has been shown that *Candida* growth can occur in anaerobic milieus such as root canal systems and periodontal pockets leading to polymicrobial infections (Reynaud *et al*, 2001; Siqueira and Sen, 2004), it would be intriguing to explore further candidal biofilm formation in these niches.

Extracellular polymeric substances (EPS)

The production of EPS is a unique feature of the biofilm mode of growth. The secreted substances form a three-dimensional, gel-like, highly hydrated, and locally charged environment in which microorganisms are immobilised. Biofilm EPS has been proposed to play a pivotal role in *Candida* adhesion and biofilm formation. EPS may potentially serve several special functions in the growing biofilm, such as defence against phaygocytic cells, providing a scaffold to maintain biofilm integrity, limiting active drug diffusion, or a combination of all these.

Matrix polymers of bacterial biofilms are primarily exopolysaccharides. Similarly, *Candida* biofilms consist of nearly 40% carbohydrate (Baillie and Douglas, 1998; Al Fattani and Douglas, 2006), with the major carbohydrate component varying between *Candida* species. For example, in the latter study, the total carbohydrate content was found to be 39.6% in a *C. albicans* biofilm but only 3.3% in *C. tropicalis*; the major EPS carbohydrate was glucose (32.2%) and hexosamine (27.4%). Proteins seemed to be a minor component: 5.0% in *C. albicans* and 3.3% in *C. tropicalis*. Apart from the latter two components, there were also small amounts of phosphorus, hexosamine, and uronic acid (Al Fattani and Douglas, 2006). Recent evidence suggests that planktonic cultures grown in the laboratory environment may be able to produce components of biofilm EPS. Protein profiles of *Candida* EPS and liquid supernatant of *in vitro* planktonic *Candida* cultures seemed to share a high degree of similarity (Thomas *et al*, 2006a).

Production of EPS by *Candida* biofilms itself depends on factors such as species/strain, carbon source, and flow rate of the medium. In general, *C. albicans* produces copious EPS matrix under favourable conditions, whereas *C. glabrata* produces less (Parahitiyawa *et al*, 2006). *C. tropicalis* also forms a large amount of EPS matrix (Al Fattani and Douglas, 2006). Studies on the effect of the carbon source on EPS production have produced mixed results. Although some studies have found that the amount of EPS produced is similar whether candidal biofilms are grown with glucose or galactose (Hawser *et al*, 1998), some have argued that it is not (Jin *et al*, 2004). The flow rate of nutrients can affect EPS production by *C. albicans* biofilms. Compared with static conditions, a medium flow rate can markedly increase EPS synthesis (Hawser *et al*, 1998) and a high flow rate favours extensive matrix formation (Al Fattani and Douglas, 2006). Because nutrient flow is relatively rapid in nature, studies with *in vivo* models may shed more light on this intriguing phenomenon.

Candida species

Candida species differ in their ability to form biofilms. Some of the common features seen among different species are summarized in Table 1. In general, biofilms of different species vary in overall morphology, EPS composition, and antifungal resistance. Some workers claim that *C. albicans, C. dubliniensis,* and *C. krusei* biofilms are more confluent than those of other *Candida* species (Ramage *et al*, 2001a; Samaranayake *et al*, 2005; Parahitiyawa *et al*, 2006). Nevertheless, it should be borne in mind that relevant studies have not been performed with similar strains of the same species; hence, their results should be interpreted cautiously. Indeed, different strains of the same *Candida* species are dramatically different in their ability to form biofilms (Hawser and Douglas, 1994), indicating that 'strong' and 'weak' biofilm-forming strains may exist within each *Candida* species and, this has been confirmed in our own studies (Jin *et al*, 2003; Thein *et al*, 2007).

Wild-type *Candida* strains have been shown to be more 'healthy and confluent' biofilm formers than laboratory reference strains. This phenomenon was observed previously among *C. albicans* strains (Jin *et al*, 2003). Hawser and Douglas (1994) also reported different biofilm-forming abilities among *C. parapsilosis* strains. Interestingly, some studies have pointed out that biofilm-forming ability is greater for non-*albicans* species than for *albicans* species (Shin *et al*, 2002; Kumar and Menon, 2006). This area warrants future research, as non-*albicans Candida* species are considered emerging pathogens.

Metal ions and *Candida* biofilms

Although the human commensal and pathogenic *Candida* rarely encounter metal toxicity, *Candida* species prevail in other niches such as soil and aquatic environments are frequently exposed to highly toxic, water-soluble metal ions. Rapid industrialization and contamination of natural water resources have disseminated water-soluble metal ions into our environment, and it is likely that we encounter some of these metal ions in daily life. Therefore, it is interesting to look into the role of metal ions in modulating biofilm formation of *Candida* species.

There are few interesting studies, which have examined the role of metal ions in *Candida* biofilm formation (Harrison *et al*, 2006, 2007b). Harrison *et al* (2006) observed that *C. tropicalis* biofilms were up to 65 times more resistant to killing by metal ions than corresponding planktonic cultures. Of the most toxic heavy metals tested, only very high concentrations of Hg^{2+}, CrO_4^{2-} or Cu^{2+} killed surface-adherent *Candida*. This group extended these studies further by examining the role of metal ions in the cell type interconversion, drug and metal resistance in *Candida* biofilms (Harrison *et al*, 2007a). *Candida* biofilms were grown either in microtitre plates containing gradient arrays of metal ions or in the calgary biofilm device. This approach identified that many of the sub-inhibitory concentrations of certain metal ions (CrO_4^{2-}, Co^{2+}, Cu^{2+}, Ag^{+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+}, AsO_2^{-} and SeO_3^{2-}) function as environmental cues, which block or trigger a transition between yeast and hyphal cell types. Thus, metal exposure resulted in specific biofilm structure types which authors described as 'domed', 'layer cake', 'flat', and 'mycelial'. The aforementioned studies highlight the functional role of metal ions in modulating *Candida* biofilm morphogenesis and spatial structure. As the biofilm maturation

Table 1 Factors that affect biofilm development of different *Candida* species [i.e. Growth, morphology, extracellular polymeric substance (EPS) and antifungal resistance] as reported from different studies

Species	*Biofilm growth*	*Morphology*	*EPS*	*Antifungal resistance*	*Ref*
C. albicans	Good	Multilayered; mixture of yeast, pseudohyphae and hyphae	Thick	High	Hawser and Douglas, 1994
	Good	Basal blastospores and thick hypahl layer on top	Thick	High	Kuhn *et al*, 2002a
	Good	Multilayered structure	Thick	ND	Samaranayake *et al*, 2005
	Good	Basal blastospore layer	Thin	ND	Parahitiyawa *et al*, 2006
C. krusei	Good	ND	ND	ND	Hawser and Douglas, 1994
	Good	Large blastospore layers	NM	ND	Samaranayake *et al*, 2005
	Good	Large blastospores	Thick	ND	Parahitiyawa *et al*,2006
C. dubliniensis	Poor	ND	ND	ND	Hawser and Douglas, 1994
	Good	Multilayered; yeast and filamentous forms	Thick	High	Ramage *et al*, 2001a
	Good	Similar to *C. albicans*	Thick	ND	Parahitiyawa *et al*,2006
C. glabrata	Poor	ND	ND	ND	Hawser and Douglas, 1994
	Poor	Scant population of blastospores	Thin	ND	Kuhn *et al*, 2002a
	Poor	Only blastospores	Thin	ND	Parahitiyawa *et al*, 2006
C. parapsilosis	Poor	ND	Thin	High	Hawser and Douglas, 1994
	Poor	Only basal blastospore layer	Thin	High	Kuhn *et al*, 2002a
	Good	Blastospores aggregations	NM	ND	Samaranayake *et al*, 2005
C. tropicalis	Poor	Thin hyphal layer, no visible blastospore layer	Thin	ND	Kuhn *et al*, 2002a
	Poor	Only blastospores	Thick	ND	Parahitiyawa *et al*, 2006
	Good	NM	Thick	High	Al Fattani and Douglas, 2006

ND, not done; NM, not mentioned in the article.

involves emergence of drug resistance in parallel with multiple cellular morphogenesis, authors postulate that metal may alter susceptibility to antifungal defences. This area certainly warrants further research.

Antifungal resistance

Increased antifungal resistance of *Candida,* when displaying the biofilm mode of growth, was first demonstrated by Hawser and Douglas (1995). *Candida* biofilms were 30 to 2000 times as resistant as planktonic cells to various antifungal agents, including amphotericin B, fluconazole, itraconazole, and ketoconazole (Hawser and Douglas, 1995). Subsequent researchers have confirmed these observations (Chandra *et al*, 2001; Ramage *et al*, 2001b; Kuhn *et al*, 2002b; Serefko *et al*, 2006; Jain *et al*, 2007). Resistance to fluconazole, which is used as the major antifungal for HIV/AIDS patients, has been explored with particular interest. *In vitro* fluconazole resistance of *Candida* biofilms can range from 250 to 400 times that of planktonic *Candida* (Chandra *et al*, 2001; Ramage *et al*, 2001b). *In vivo*, *Candida* biofilms also display increased fluconazole resistance: *Candida* biofilms had a minimum inhibitory concentration (MIC) for fluconazole that was 128 times as high as that of planktonic *Candida* (Chandra *et al*, 2001; Andes *et al*, 2004). This property has clinical implications, but a glimpse of hope has emerged with the development of newer antifungal agents, such as echinocandins and liposomal formulations of amphotericin B. Limited studies have suggested that the latter antifungals are particularly effective against *Candida* biofilms (Bachmann *et al*, 2002; Kuhn *et al*, 2002b).

Factors affecting antifungal resistance

Several factors have been proposed for the increased antifungal resistance of *Candida* biofilms. These include altered growth/metabolic rate of biofilm cells, presence of extracellular matrix, expression of resistance genes, and presence of 'persister' cells.

Biofilm growth

Although growth rate has been linked with resistance of bacterial biofilms, Baillie and Douglas (1998) demonstrated that growth rate is unrelated to *Candida* biofilm resistance to amphotericin B. In contrast, however, the susceptibility of planktonic cultures depended on their growth rate (Baillie and Douglas, 1998). Hence, antifungal resistance of *Candida* biofilms is not simply attributable to a reduced cell growth rate. Chandra *et al* (2001) compared the MICs of amphotericin B, nystatin, fluconazole, and chlorhexidine antifungals during early, intermediate, and maturation phases of *Candida* biofilms. Progression of drug resistance of the *Candida* biofilm was associated with parallel increase in metabolic activity of the developing biofilm. This observation further confirmed that increased drug resistance is not because of lower metabolic activity of cells in maturing biofilms, but is more related to the maturation process.

It is important to note that a biofilm consists of a heterogeneous population of cells with different growth rates; therefore, a subpopulation of cells could also confer antifungal resistance because of their slower growth rate (Kumamoto, 2002). Further investigation should confirm or refute this finding and generate

additional data to elucidate fully the relationship between yeast growth and drug resistance.

Extracellular matrix

In general, the EPS may act as a physical barrier that prevents the access of antimicrobials to cells embedded in the biofilm community, in turn contributing to enhanced drug resistance. This hindrance is thought to depend largely on the amount and nature of the EPS, as well as the physicochemical properties of the drug. In bacterial biofilms, EPS enzymes can also digest drugs.

The role of the EPS in *Candida* biofilm resistance is unclear. In one study, *C. albicans* biofilms that formed under a constant flow of liquid were stimulated to increase matrix synthesis to an extent that significantly enhanced resistance to amphotericin B (Al Fattani and Douglas, 2006). In another study, the survival rate of *Candida* cells in biofilms treated with amphotericin B decreased by as much as 20% when the EPS was removed (Baillie and Douglas, 1999). However, poor penetration of drugs through the EPS does not seem to account solely for antifungal drug resistance in biofilms (Al Fattani and Douglas, 2004). Some researchers also demonstrated that *Candida* biofilm cells grown statically in the presence of minimal matrix exhibited the same level of drug resistance to the antifungals such as flucytosine, fluconazole, and amphotericin B as did cells grown in a shaker with a large amount of matrix (Baillie and Douglas, 2000). This area of study is certainly a 'gold mine' for future research work and has clinical implications as EPS or its components may be used as potential drug targets.

Genetic basis of antifungal resistance

Molecular mechanisms conferring superior antifungal resistance in *Candida* biofilms have not been fully elucidated. Studies have shown the involvement of ATP-binding cassette and major facilitator superfamily drug efflux pumps in increased azole antifungal resistance. However, efflux pumps contribute to azole resistance only in the early phase, but not in the later phase, of *Candida* biofilm growth; membrane sterol composition also contributes to azole resistance in the intermediate and mature phases (Mukherjee *et al*, 2003).

In this connection, *MDR1*, *CDR1*, and *CDR2* genes are up-regulated in the biofilm mode of growth. Interestingly, high-level drug resistance of mature *Candida* biofilms was not affected by deletion of these three genes, either singly or in combination (Mukherjee *et al*, 2003). Microarray analysis of *Candida* biofilms under various conditions showed that expression of *MDR* and *CDR* genes in biofilms is phase-specific, contributing to azole resistance only during the early phase, whereas changes in sterol composition are involved in the resistance in the mature phase (Garcia-Sanchez *et al*, 2004). However, a study using an *in vivo* biofilm model has yielded different results, namely, that *CDR1* and *CDR2* expression is significantly up-regulated in biofilms compared with planktonic cells, but *EFG11* and *MDR1* expression is similar in both biofilms and planktonic cells (Andes *et al*, 2004). More sophisticated *in vitro* and *in vivo* genome wide expression analyses are needed to elucidate the role of genes that confer higher antifungal resistance in *Candida* biofilms.

Persister cells

Persister cells are phenotypic variants of wild-type cells, rather than mutants (Keren *et al*, 2004b), and can survive despite the presence of antibiotics at concentrations well above the MIC. Bacterial biofilms produce dormant persister cells that are known to be responsible for multidrug tolerance (Keren *et al*, 2004a,b), and have been observed in biofilms of *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus*. Persisters can account for 1% of these bacterial biofilm populations. Similarly, a subpopulation of highly antifungal tolerant cells has been found in *Candida* biofilms but not in *Candida* planktonic cultures (LaFleur *et al*, 2006). In the latter study, *Candida* biofilms exhibited a biphasic killing pattern in response to microbial agents. The surviving subpopulation of cells or 'persisters' displayed multidrug tolerance, while other subpopulation was susceptible to the antifungals studied. Reinoculation of surviving cells produced a new biofilm with a new subpopulation of persisters, thereby confirming that persisters are phenotypic variants of the wild type with a heritable genotype. This area of research in biofilm biology warrants interest and many studies could be anticipated to explore this intriguing phenomenon.

Genomics and proteomics of *Candida* biofilms

Genomics and proteomics are broad disciplines, which have emerged as essential sciences for understanding molecular mechanisms governing pathophysiological phenomena. Although a detailed discussion is beyond the scope of this mini review, we outline the most recent findings of these areas in relation to *Candida* biofilms. Much broader reviews can be found elsewhere (Nobile and Mitchell, 2006; Pitarch *et al*, 2006; Thomas *et al*, 2006b).

Genomic studies of *Candida* biofilms have provided invaluable insights into the mechanisms governing biofilm formation. Recent genomic analyses of *Candida* have been fuelled by the completed annotation of the *Candida* genome sequence and the availability of well-established databases (Braun *et al*, 2005; d'Enfert *et al*, 2005). Several approaches have attempted to demystify the genetic control over *Candida* biofilm formation. Investigators have assessed the effect of single deletion mutants, performed systemic searches using collections of mutants (Richard *et al*, 2005), conducted transcriptomic analyses (Garcia-Sanchez *et al*, 2004; Murillo *et al*, 2005), or conducted individual transcription factor analyses (Ramage *et al*, 2002).

It has been shown that trancriptomic changes in the biofilm phase that are distinct from those in the planktonic phase in otherwise similar conditions, take place as early as 30 min of surface contact and *Candida* adhesion (Murillo *et al*, 2005). One of the interesting outcomes of these genomic studies is the generation of substantial evidence of the pivotal role of hyphal

formation in *Candida* biofilms (Ramage *et al*, 2002; Garcia-Sanchez *et al*, 2004; Nobile *et al*, 2006). Most of the genes identified to date, which are critical for biofilm formation, are at least indirectly linked to genes that regulate hyphal development (Nobile and Mitchell, 2006). However, it is still inconclusive whether genes governing hyphal morphogenesis or hyphal cell wall proteins are more critical in the formation of *Candida* biofilms. There is no doubt that future genomic studies will further our understanding of *Candida* biofilm mechanisms.

The term 'proteome' was coined by Marc Wilkins to describe the study of the 'protein complement of the genome' (Wilkins *et al*, 1996). The advancement of technical approaches such as MALDI-TOF (Matrix Assisted Laser Desoprtion ionization – Time of Flight) in mass spectrometry has widened the horizons of proteomics research. However, *Candida* proteomics has largely been confined to cell wall analysis and evaluation of proteomic changes associated with drug response, change in virulence of mutants, and serological response to candidiasis (Pitarch *et al*, 2006; Thomas *et al*, 2006b). There have been only few studies on the proteomics of *Candida* biofilms (Mukherjee *et al*, 2006; Thomas *et al*, 2006a; Vediyappan and Chaffin, 2006). Intriguingly, these proteomic studies suggest that *Candida* biofilm proteome is markedly similar to planktonic proteome (Thomas *et al*, 2006a; Vediyappan and Chaffin, 2006). Vediyappan and Chaffin (2006), for instance, noted that non-glucan attached proteins of the cell surface and extracellular matrix of *C. albicans* biofilms are generally similar to protein profiles of planktonic yeast and germ tubes. In another study of 24 h *Candida* biofilms, Thomas *et al* (2006b) also observed a high degree of similarity between the protein profiles associated with planktonic and biofilm extracts. They identified nine proteins, of which six were up-regulated and two down-regulated in biofilm cells compared with planktonic cells. Among these were glycolytic enzymes such as enolase, alcohol dehydrogenase, and pyruvate decarboxylase. This study identified up-regulation of Adh1p (immunogenic alcohol dehydrogenase) and down-regulation of alcohol dehydrogenase 2 (Adh2p) in biofilms compared to planktonic cells. On the contrary, Mukherjee *et al* (2006) showed that alcohol dehydrogenase (Adh1p) is significantly down-regulated in *Candida* biofilms compared with planktonic cells, and Adh1p restricts the ability of *Candida* to form biofilms. Thus, disruption of ADH significantly enhances the biofilm-forming ability of *Candida in vitro*. This observation was confirmed by an engineered human oral mucosa model and an *in vivo* rat model. A proteomic approach to demystify the process of biofilm development appears to be still in its infancy.

Conclusion

Biofilm formation is a major characteristic that helps *Candida* to cause many different disease variants both in the oral cavity and elsewhere. Biofilm development, architecture, higher antifungal resistance and mechanisms behind antifungal resistance have been the focus of past *Candida* biofilm research. Although available studies have shed some light, molecular mechanisms that govern biofilm formation and pathogenicity await full clarification. Emerging tools in microscopy, genomics and proteomics are likely to elucidate a clearer picture of the mechanistic and molecular basis behind pathogenic processes of *Candida* biofilm formation. Such findings might potentially provide us with invaluable clues to combat the recalcitrant infections caused by this ubiquitous fungus.

Acknowledgements

We wish to acknowledge gratefully the editorial support provided by Dr Trevor Lane PhD (Oxon), and the financial support from the Research Grants Council of Hong Kong CERG grant No HKU 7624/06M of LPS. We are grateful to Dr. Bernard Low and Dr. Thein ZM, Faculty of Dentistry, The University of Hong Kong for Figures 1 and 2 respectively.

References

Al Fattani MA, Douglas LJ (2004). Penetration of *Candida* biofilms by antifungal agents. *Antimicrob Agents Chemother* **48:** 4073.

Al Fattani MA, Douglas LJ (2006). Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *J Med Microbiol* **55:** 999–1008.

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.

Bachmann SP, VandeWalle K, Ramage G *et al* (2002). In vitro activity of caspofungin against *Candida albicans* biofilms. *Antimicrob Agents Chemother* **46:** 3591–3596.

Baillie GS, Douglas LJ (1998). Effect of growth rate on resistance of *Candida albicans* biofilms to antifungal agents. *Antimicrob Agents Chemother* **42:** 1900–1905.

Baillie GS, Douglas LJ (1999). Role of dimorphism in the development of *Candida albicans* biofilms. *J Med Microbiol* **48:** 671–679.

Baillie GS, Douglas LJ (2000). Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. *J Antimicrob Chemother* **46:** 397–403.

Banerjee SN, Emori TG, Culver DH *et al* (1991). Secular trends in nosocomial primary blood-stream infections in the United-States, 1980–1989. *Am J Med* **91:** S86–S89.

Beck-Sague C, Jarvis WR (1993). Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980–1990. National Nosocomial Infections Surveillance System. *J Infect Dis* **167:** 1247–1251.

Biswas SK, Chaffin WL (2005). Anaerobic growth of *Candida albicans* does not support biofilm formation under similar conditions used for aerobic biofilm. *Current Microbiology* **51:** 100–104.

Braun BR, van Het HM, d'Enfert C *et al* (2005). A human-curated annotation of the *Candida albicans* genome. *PLoS Genet* **1:** 36–57.

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:** 5385–5394.

Chandra J, Patel JD, Li J *et al* (2005). Modification of surface properties of biomaterials influences the ability of *Candida*

albicans to form biofilms. *Appl Environ Microbiol* **71:** 8795–8801.

Colombo AL, Nucci M, Park BJ *et al* (2006). Epidemiology of candidemia in Brazil: a nationwide sentinel surveillance of candidemia in eleven medical Centers. *J Clin Microbiol* **44:** 2816–2823.

Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM (1995). Microbial biofilms. *Annu Rev Microbiol* **49:** 711–745.

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

Edmond MB, Wallace SE, McClish DK, Pfaller MA, Jones RN, Wenzel RP (1999). Nosocomial bloodstream infections in United States hospitals: a three-year analysis. *Clin Infect Dis* **29:** 239–244.

d'Enfert C, Goyard S, Rodriguez-Arnaveilhe S *et al* (2005). CandidaDB: a genome database for *Candida albicans* pathogenomics. *Nucleic Acids Res* **33:** D353–D357.

Garcia-Sanchez 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.

Hagerty JA, Ortiz J, Reich D, Manzarbeitia C (2003). Fungal infections in solid organ transplant patients. *Surg Infect (Larchmt)* **4:** 263–271.

Harrison JJ, Rabiei M, Turner RJ, Badry EA, Sproule KM, Ceri H (2006). Metal resistance in *Candida* biofilms. *FEMS Microbiol Ecol* **55:** 479–491.

Harrison JJ, Ceri H, Yerly J *et al* (2007a). Metal ions may suppress or enhance cellular differentiation in *Candida albicans* and *Candida tropicalis* biofilms. *Appl Environ Microbiol* **73:** 4940–4949.

Harrison JJ, Turner RJ, Ceri H (2007b). A subpopulation of *Candida albicans* and *Candida tropicalis* biofilm cells are highly tolerant to chelating agents. *FEMS Microbiol Lett* **272:** 172–181.

Hawser SP, Douglas LJ (1994). Biofilm formation by *Candida* species on the surface of catheter materials in-vitro. *Infect Immun* **62:** 915–921.

Hawser SP, Douglas LJ (1995). Resistance of *Candida albicans* biofilms to antifungal agents in-vitro. *Antimicrob Agents Chemother* **39:** 2128–2131.

Hawser SP, Baillie GS, Douglas LJ (1998). Production of extracellular matrix by *Candida albicans* biofilms. *J Med Microbiol* **47:** 253–256.

Jain N, Kohli R, Cook E, Gialanella P, Chang T, Fries BC (2007). Biofilm formation by and antifungal susceptibility of *Candida* isolates from urine. *Appl Environ Microbiol* **73:** 1697–1703.

Jin Y, Yip HK, Samaranayake YH, Yau JY, Samaranayake LP (2003). Biofilm-forming a 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.

Jin Y, Samaranayake LP, Samaranayake Y, Yip HK (2004). Biofilm formation of *Candida albicans* is variably affected by saliva and dietary sugars. *Arch Oral Biol* **49:** 789–798.

Keren I, Kaldalu N, Spoering A, Wang YP, Lewis K (2004a). Persister cells and tolerance to antimicrobials. *FEMS Microbiol Lett* **230:** 13–18.

Keren I, Shah D, Spoering A, Kaldalu N, Lewis K (2004b). Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. *J Bacteriol* **186:** 8172–8180.

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

Kuhn DM, George T, Chandra J, Mukherjee PK, Ghannoum MA (2002b). Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. *Antimicrob Agents Chemother* **46:** 1773–1780.

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

Kumar CPG, Menon T (2006). Biofilm production by clinical isolates of *Candida* species. *Medical Mycology* **44:** 99–101.

LaFleur MD, Kumamoto CA, Lewis K (2006). *Candida albicans* biofilms produce antifungal-tolerant persister cells. *Antimicrob Agents Chemother* **50:** 3839–3846.

Mukherjee PK, Chandra J, Kuhn DA, Ghannoum MA (2003). Mechanism of fluconazole resistance in *Candida albicans* biofilms: phase-specific role of efflux pumps and membrane sterols. *Infect Immun* **71:** 4333–4340.

Mukherjee PK, Mohamed S, Chandra J *et al* (2006). Alcohol dehydrogenase restricts the ability of the pathogen *Candida albicans* to form a biofilm on catheter surfaces through an ethanol-based mechanism. *Infect Immun* **74:** 3804–3816.

Murillo LA, Newport G, Lan CY, Habelitz S, Dungan J, Agabian NM (2005). Genome-wide transcription profiling of the early phase of biofilm formation by *Candida albicans*. *Eukaryot Cell* **4:** 1562–1573.

Nikawa H, Nishimura H, Yamamoto T, Hamada T, Samaranayake LP (1996). The role of saliva and serum in *Candida albicans* biofilm formation on denture acrylic surfaces. *Microb Ecol Health Dis* **9:** 35–48.

Nikawa H, Hamada T, Yamamoto T, Kumagai H (1997). Effects of salivary or serum pellicles on the *Candida albicans* growth and biofilm formation on soft lining materials in vitro. *J Oral Rehabil* **24:** 594–604.

Nobile CJ, Mitchell AP (2006). Genetics and genomics of *Candida albicans* biofilm formation. *Cell Microbiol* **8:** 1382–1391.

Nobile CJ, Nett JE, Andes DR, Mitchell AP (2006). Function of *Candida albicans* adhesion Hwp1 in biofilm formation. *Eukaryot Cell* **5:** 1604–1610.

Odds FC. (1998). *Candida and candidosis*. Bailliere Tindall, London, England.

Parahitiyawa NB, Samaranayake YH, Samaranayake LP *et al* (2006). Interspecies variation in *Candida* biofilm formation studied using the Calgary biofilm device. *Apmis* **114:** 298–306.

Patterson TF (1999). Approaches to fungal diagnosis in transplantation. *Transpl Infect Dis* **1:** 262–272.

Pitarch A, Nombela C, Gil C (2006). *Candida albicans* biology and pathogenicity: insights from proteomics. *Methods Biochem Anal* **49:** 285–330.

Potera C (1999). Microbiology – forging a link between biofilms and disease. *Science* **283:** 1837.

Radford DR, Sweet SP, Challacombe SJ, Walter JD (1998). Adherence of *Candida albicans* to denture-base materials with different surface finishes. *J Dent* **26:** 577–583.

Ramage G, Vande Walle K, Wickes BL, Lopez-Ribot JL (2001a). Biofilm formation by *Candida dubliniensis*. *J Clin Microbiol* **39:** 3234–3240.

Ramage G, Vande Walle K, Wickes BL, Lopez-Ribot JL (2001b). Standardized method for in vitro antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrob Agents Chemother* **45:** 2475–2479.

Ramage G, VandeWalle K, Lopez-Ribot JL, Wickes BL (2002). The filamentation pathway controlled by the Efg1 regulator protein is required for normal biofilm formation and development in *Candida albicans*. *FEMS Microbiol Lett* **214:** 95–100.

Ramage G, Martinez JP, Lopez-Ribot JL (2006). *Candida* biofilms on implanted biomaterials: a clinically significant problem. *Fems Yeast Research* **6:** 979–986.

Reynaud AH, Nygaard-Ostby B, Boygard GK, Eribe ER, Olsen I, Gjermo R (2001). Yeasts in periodontal pockets. *J Clin Periodontol* **28:** 860–864.

Richard ML, Nobile CJ, Bruno VM, Mitchell AP (2005). *Candida albicans* biofilm-defective mutants. *Eukaryot Cell* **4:** 1493–1502.

Samaranayake LP, MacFarlane TW (1981). The adhesion of the yeast *Candida albicans* to epithelial-cells of human-origin in vitro. *Arch Oral Biol* **26:** 815–820.

Samaranayake LP, MacFarlane TW (1990). *Oral Candidosis*. Wright-Butterworth, London.

Samaranayake LP, Hughes A, MacFarlane TW (1984). The proteolytic potential of *Candida albicans* in human-saliva supplemented with glucose. *J Med Microbiol* **17:** 13–22.

Samaranayake YH, Wu PC, Samaranayake LP, So M (1995). Relationship between the cell surface hydrophobicity and adherence of *Candida krusei* and *Candida albicans* to epithelial and denture acrylic surfaces. *Apmis* **103:** 707–713.

Samaranayake LP, Fidel PL, Naglik JR *et al* (2002). Fungal infections associated with HIV infection. *Oral Dis* **8:** 151–160.

Samaranayake YH, Ye J, Yau JYY, Cheung BPK, Samaranayake LP (2005). In vitro method to study antifungal perfusion in *Candida* biofilms. *J Clin Microbiol* **43:** 818–825.

Serefko A, Chudzik B, Malm A (2006). In vitro activity of caspofungin against planktonic and sessile *Candida* sp. cells. *Pol J Microbiol* **55:** 133–137.

Shin JH, Kee SJ, Shin MG *et al* (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.

Siqueira JF, Sen BH (2004). Fungi in endodontic infections. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **97:** 632–641.

Thein ZM, Samaranayake YH, Samaranayake LP (2007). *In vitro* biofilm formation of *Candida albicans* and non-*albicans Candida* species under dynamic and anaerobic conditions. *Arch Oral Biol* **52:** 761–767.

Thomas DP, Bachmann SP, Lopez-Ribot JL (2006a). Proteomics for the analysis of the *Candida albicans* biofilm lifestyle. *Proteomics* **6:** 5795–5804.

Thomas DP, Pitarch A, Monteoliva L, Gil C, Lopez-Ribot JL (2006b). Proteomics to study *Candida albicans* biology and pathogenicity. *Infect Disord Drug Targets* **6:** 335–341.

Tunney MM, Keane PF, Jones DS, Gorman SP (1996). Comparative assessment of ureteral stent biomaterial encrustation. *Biomaterials* **17:** 1541–1546.

Vediyappan G, Chaffin WL (2006). Non-glucan attached proteins of *Candida albicans* biofilm formed on various surfaces. *Mycopathologia* **161:** 3–10.

Wenzel RP, Gennings C (2005). Bloodstream infections due to *Candida* species in the intensive care unit: identifying especially high-risk patients to determine prevention strategies. *Clin Infect Dis* **41:** S389–S393.

Wilkins MR, Sanchez JC, Gooley AA *et al* (1996). Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. *Biotechnol Genet Eng Rev* **13:** 19–50.

Zhao X, Oh SH, Yeater KM, Hoyer LL (2005). Analysis of the *Candida albicans* Als2p and Als4p adhesins suggests the potential for compensatory function within the Als family. *Microbiology-Sgm* **151:** 1619–1630.