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Influence of serum and polystyrene plate type on stability of *Candida albicans* biofilms

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ABSTRACT

Adhesion is a crucial initial step in microbial biofilm formation. Firm attachment to a target surface subsequently ensures successful colonization and survival despite turbulent conditions. In the laboratory, polystyrene plates are commonly used in biofilm experiments and the 'washing/rinse steps' before staining are critical for assaying biofilm viability. However, these rinse steps risk the removal (partially or entirely) of the formed biofilm, resulting in inconsistent results. The aim of the present study was to optimize conditions for firmer biofilms, less prone to disruption and thus significantly reducing well-to-well variability. *Candida albicans* SC5314 was used in five different polystyrene 96-well plates from four different manufacturers. Irrespective of how gently we performed the rinse, biofilms came off certain polystyrene plates more easily compared to others. Importantly, preconditioning the polystyrene surfaces with foetal bovine serum (FBS) had a negative impact on firm biofilm attachment. Costar® plates provided the most suitable surface for firm biofilm attachment, both in the presence and absence of FBS. Substratum properties even among seemingly identical synthetic materials may influence biofilm attachment and its subsequent sturdiness, affecting experimental results.

1. Introduction

The term 'biofilm' refers to the attachment onto surfaces of microbial communities of cells enclosed in self-produced extracellular polymeric matrices (Cegelski et al., 2008). In in vitro systems, different types of synthetic materials have been used as substrate for biofilm attachment and formation. They include plastics, silicones, ceramics, glass and also metals (Coad et al., 2014; Nwaugo et al., 2007; Chandra et al., 2001; Webb et al., 1999). Attachment to these substrates is greatly influenced by physical and chemical interactions between the cell surface and the substratum. In bacteria and *C. albicans*, mainly hydrophobic interactions and to some extent electrostatic forces are responsible for the initial attachment to these surfaces (Varghese et al., 2013; Donlan, 2002; Matz and Jürgens, 2001; Gristina et al., 1987). Additionally, substrate surface roughness has been shown to play a critical role in cell adhesion (Biazar et al., 2011, Radford et al., 1999).

Adhesion of *C. albicans* to substrates is multifactorial and has been associated with key factors such as formation of an extracellular matrix (Chandra et al., 2001), adhesins (De Groot et al., 2013), yeast-hyphae transition (Beaussart et al., 2012) and type of growth media (Hoyer et al., 1995). Additionally, in order to closely mimic in vivo conditions, some authors have preconditioned substrates with biological substances such as saliva, human serum, FBS, urine, etc. (Kucharı'ková et al., 2015; Silva et al., 2009; Cannon and Chaffin, 1999; Thein et al., 2007).

Standard biofilm assays to determine the fraction of surviving cells after treatment with antimicrobials, typically use multi-well 96 plates as this provides an inexpensive, rapid, and simple format (O'Toole, 2011; Pierce et al., 2010; Ramage et al., 2001b). Moreover, in these assays, gentle rinse/washing steps typically precede a colorimetric analysis (Delattin et al., 2014; Peeters et al., 2008; Pettit et al., 2005). This ensures removal of non-adherent cells, enabling accurate estimation of surviving biofilm cells and hence effectiveness of an antimicrobial compound. This rinsing, however, can also loosen and subsequently remove attached biofilms, depending on their degree of attachment to a substrate (Pitt et al., 1993), resulting in inaccurate results. While great care should be taken during the washes (Stepanović et al., 2007), biofilms will inevitably detach (partly or completely) if cell-substrate adhesion is weak. Though not widely discussed, biofilm assays in polystyrene plates often yield variable results (Mizan and Jahid, 2016; Nett et al., 2011) and a lack of firm adhesion to polystyrene surfaces is likely to play an important role in this.

Thus the aim of this study was to optimize conditions for firmer (more robust) biofilms, so as to reduce well-to-well variability significantly. To ameliorate the variations in our experiments due to loss

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(partial or complete) of biofilms during rinse steps, we compared the adhesion and retention patterns of *C. albicans* in five brands of polystyrene plates in the presence and absence of FBS to select optimal conditions for firm biofilms.

2. Materials and methods

C. albicans strain SC5314 was a gift from Prof. Patrick van Dijck (KU Leuven, Belgium). Stocks were stored in yeast-extract peptone dextrose (YPD) broth and 20% glycerol at − 80 °C in cryovials. Roswell Park Memorial Institute (RPMI) powder with L-glutamine without sodium bicarbonate, 4-morpholinepropanesulfonic acid (MOPS) and FBS were purchased from Sigma (St. Louis, MO, USA). Resazurin dye was purchased from Acros Organics (New Jersey, USA). Five flat (F)-bottom polystyrene 96-well plates were compared: F-bottom, black (ref 655090) and F-bottom, clear (ref 655101) from Greiner Bio-one, F-bottom (ref 3370) from Costar®, F-bottom (ref 10062-900) from VWR International, Belgium, and tissue culture plates, F-bottom (ref 92096) from TPP Techno Plastic Products AG, Switzerland.

2.1. Biofilm formation, rinsing and visualization

Material from the frozen stock was streaked out on YPD agar plates, which were incubated overnight at 37 °C. A few colonies were inoculated in YPD broth and incubated overnight at 37 °C in a shaking incubator. This overnight culture was then centrifuged at 164 rcf for 3 min and the supernatant was discarded. The pellet was then resuspended in RPMI-MOPS, pH 7 and used in the experiments. Different seeding cell densities were used in the presence and absence of FBS using a standard biofilm protocol (De Brucker et al., 2016), with some adjustments. To this end, 100 μL of FBS was added to each well (for the FBS-coated plates) and incubated at 37 °C for 24 h; the FBS was then removed and the wells were washed once with 100 μL phosphate-buffered saline (PBS). The optical density (OD) of the resuspended overnight culture in RPMI-MOPS was measured in a Perkin Elmer spectrophotometer (MA, USA), and the OD_{600} was adjusted to 0.1, 0.2, 0.5, 1.0 or 2.0 in RPMI-MOPS medium before dispensing 100 μL of the cell suspension per well. After 90 min incubation at 37 °C, the medium was gently aspirated and the wells were rinsed once with 100 μL PBS. Thereafter, 200 μL of RPMI-MOPS medium was gently added, and plates were incubated at 37 °C for 24 h. Following this incubation period, the medium was aspirated and the wells were washed twice with 200 μL PBS. Hundred μL of freshly-prepared resazurin dye (0.01% *w/v*) in PBS was added to each well and the plates were incubated for 1 h at 37 °C. Accurate quantification by fluorescence spectroscopy was not possible as masses of biofilms, peeled off during the washes, could not be completely aspirated and remained lingering on the sides of the wells. When the stain was added, these floating films of cells resulted in false positives as there were no attached biofilms in these wells. We thus opted for visible light microscopy. Biofilms were visualized and photos taken using a fluorescence stereomicroscope (Leica M165FC) connected to a Leica CLS 150 XD light source and a 3iCube - USB3.0 camera from New Electronic Technology (NET) GmbH. The experiment was repeated on three different occasions with at least 16 biofilms for each cell density tested.

2.2. Statistical analysis

A scoring system with five levels was used to evaluate the state of the biofilms after the washes: 1: no attached biofilm, 2: few patches of attached and severely distorted biofilm, 3: more patches of attached and distorted biofilm, 4: almost fully attached and slightly distorted biofilm and 5: fully attached and intact biofilm (see photographs below for representative states of biofilms). Scores of the biofilms originating from the five seeding cell densities were compiled for every plate type in the absence or presence of FBS to determine the most suitable plate for sturdy biofilm formation. Error bars represent standard deviation of the mean scores from three independent experiments.

3. Results and discussion

To counter the challenge of biofilms either peeling off entirely or breaking apart irregularly during washing steps, we set out to identify suitable polystyrene microplates that offer firm attachment substrates for *C. albicans* biofilms. For five polystyrene plates, in the presence and absence of FBS and seeded at different cell densities, we determined how firmly attached the biofilms were after the washing steps.

3.1. Effect of FBS

Prior to washing, we observed that biofilms in 4 of the 5 FBS-coated plates were thicker than in the non FBS-coated plates. Similar observations of FBS enhancing *C. albicans* biofilm formation have been reported in the literature (Ramage et al., 2001a; Samaranayake et al., 1980; Frade and Arthington-Skaggs, 2011; Řičicová et al., 2010). This may be attributed to the presence of serum proteins in FBS, such as albumin, that *C. albicans* cells efficiently bind to, increasing adhesion (Ramage et al., 2001a). However, in the FBS-coated clear Greiner plates, we observed masses of floating cells. Biofilms in the other plates were visibly attached.

Upon performing the first rinse, it became apparent that biofilms in the FBS-coated plates were typically more susceptible to peeling off than their counterparts in non FBS-coated plates, with the exception of Costar plates (Figs. 1 and 2). We want to emphasise that the washing was performed as gently as possible, always pipetting PBS slowly against the side of the wells, and letting it flow down to the bottom of the wells covered with biofilms; this eliminated high fluid shear forces which have been shown to cause detachment of bacterial biofilms (Sharma et al., 2005) in dynamic flow chambers. This step was followed by gently aspirating the fluid overlying the biofilm with tips held against the side of the wells, avoiding direct suction perpendicular to the bottom of the wells that may detach biofilms (Busscher and van der Mei, 2006). In this way, a thin layer of liquid always covered the biofilm, even at the end of aspiration. Thus, liquid-air interfaces such as air bubbles or simply air was not allowed to flow over our biofilms since these have been shown to disrupt biofilms in flow chambers (Gómez-Suárez et al., 2001; Pitt et al., 1993).

The FBS-coated black Greiner plates had severely distorted biofilms after the washes. The FBS-coated TPP plates were also affected particularly at the low cell densities which are commonly used (De Brucker et al., 2016; Ramage et al., 2001b). Despite gentle washes and slow fluid aspirations from the wells, biofilms in these plates consistently displayed poor attachment in the presence of FBS. Interestingly, in the absence of FBS, biofilms in the black Greiner and TPP plates

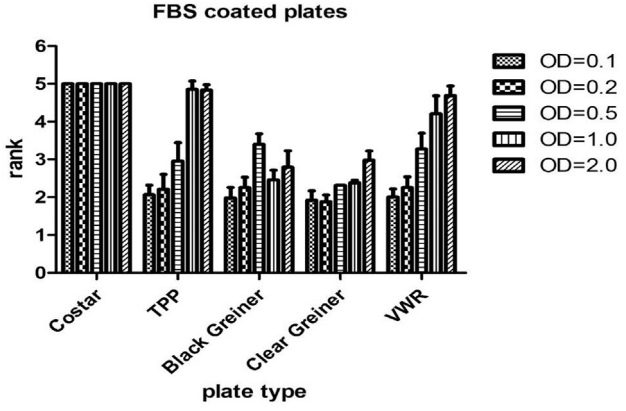

Fig. 1. Effect of FBS on *C. albicans* biofilm stability. The error bars throughout represent the standard deviation of mean scores from three independent experiments.