INTRODUCTION

Biofilms are populations of micro-organisms embedded in an extracellular polymeric substance (EPS) (Costerton et al., 1987). The biofilm mode of growth is beneficial for micro-organisms, as it provides a higher degree of stability than a planktonic existence, and organisms within a biofilm are more resistant to environmental challenges (Hall-Stoodley et al., 2004), such as low nutrient availability, high fluid shear and antibiotic and antimicrobial agents (Baker & Banfield, 2003; Battin et al., 2001; Donlan & Costerton, 2002; Elasri & Miller, 1999; Stewart & Costerton, 2001). The damage caused by the formation of biofilms is a widespread problem, ranging from pipeline corrosion and biofouling of ship hulls and food-processing equipment to clinical infections, such as endocarditis and cystic fibrosis pneumonia (Beech & Gaylarde, 1999; Callow & Callow, 2002; Costerton et al., 1999; Kumar & Anand, 1998). Many clinical infections due to biofilms are implant-related and occur when micro-organisms adhere to the surfaces of biomaterials used in, for example, prosthetic heart valves, voice prostheses, joint replacement, vascular grafts and urinary catheters (Costerton et al., 1999; Donlan, 2001).

Biofilms can consist of bacterial or fungal species or a mixture of both. For instance, in the case of vascular catheter-related infections or voice prosthetic biofilms, the most commonly isolated microbial species are Gram-positive Staphylococcus epidermidis and Staphylococcus aureus, and the fungus Candida albicans (Hampton & Sherertz, 1988), in addition to streptococci found on voice prostheses. Numerous studies have been carried out to investigate bacterial biofilms and their role in infection (Donlan, 2001; Donlan & Costerton, 2002), but fungal biofilms have been given much less attention, despite the fact that they rank fourth in frequency among species causing bloodstream infections (Douglas, 2003). C. albicans is the most frequently isolated fungal species in clinical infections. Other Candida species, such as Candida tropicalis, Candida parapsilosis and Candida dubliniensis, which are comparatively less pathogenic than C. albicans, recently emerged as clinically important pathogens (Moran et al., 2002).

Most Candida species grow as unicellular yeasts, but they are also capable of producing multicellular, filamentous forms of growth, pseudohyphae and pseudymycelium. Two species, C. albicans and C. dubliniensis, can form true
hyphae (Calderone, 2002). The morphological transition from the yeast to the hyphal growth form is an important factor in virulence (Lo et al., 1997) and biofilm formation (Richard et al., 2005). Temperatures above 35 °C and pH values of 6.5–7.0 are known to be favourable for germ-tube formation, whilst serum and Lees medium can also induce hyphal growth (Berman & Sudbery, 2002). At a high cell density of 10^6 cells ml\(^{-1}\) or above, hyphal formation is suppressed. This cell density-dependent effect is known as the inoculum-size effect (Odds, 1988) and resembles quorum sensing (QS) in bacteria. Farnesol is a signalling molecule involved in QS in C. albicans (Hornby et al., 2001). A two-component histidine kinase, Chk1p, has been implicated in the farnesol-mediated response (Kruppa et al., 2004).

What role hyphae play in maintaining fungal biofilm integrity remains unknown. Studies on the role of morphogenesis in the development and pathogenicity of C. albicans biofilms demonstrated that hyphal-defective mutants colonized polyurethane catheters poorly (Shin et al., 2002) and were unable to inhabit plastic surfaces or form biofilms (Krueger et al., 2004). In another study, biofilms of a yeast-negative mutant did not produce any yeast cells and were detached easily from catheter discs, whilst biofilms of a hypha-negative mutant were thin and densely packed with yeast cells (Baillie & Douglas, 1999). In our experience while working with C. albicans biofilms, we observed that mature biofilms of a strain lacking both copies of CHK1 were more resistant to washing. This prompted a further investigation into the factors that influence mechanical properties of C. albicans biofilms, which so far have received no attention. Mechanical properties are important for selection of treatment or dispersal of biofilm organisms due to a bodily fluid flow. In general, the mechanical properties of a biofilm determine the deformation of a biofilm due to an applied force, such as shear originating from blood or urinary flow. Strength is one of the parameters used to describe mechanical properties. It is defined as a material’s ability to resist applied forces. Increased fluid shear (Dunsmore et al., 2002; Stoolley et al., 2002), presence of divalent cations (Ca\(^{2+}\)) (Korstgens et al., 2001), increased EPS production (Flemming et al., 2000), EPS composition (Wloka et al., 2005) and QS (Davies et al., 1998) are known to affect the strength of bacterial biofilms. For fungal biofilms, the factors affecting biofilm strength still remain to be determined.

EPS production and composition are known to play an important role in maintaining the integrity of bacterial biofilms (Starkey et al., 2004). EPS consists of polysaccharides, proteins and extracellular DNA (eDNA). Polysaccharides represent the most abundant and widely studied component of bacterial EPS. Proteins in EPS have been studied less and it has been suggested that they serve to hold bacteria in a biofilm together via aggregation. Very little is known about the role of eDNA in fungal biofilms. eDNA can be released into EPS by cells or can occur as a result of cell lysis. It has been shown that DNase I can dissolve established bacterial biofilms (Whitchurch et al., 2002). For fungal biofilms, the composition and role of EPS in maintaining biofilm integrity have been studied less than for bacterial biofilms. Al-Fattani & Douglas (2006) showed that EPS of C. albicans and C. tropicalis consists mostly of polysaccharides (30–41 %) with a small amount of protein (3–5 %). Upon application of DNase I, biofilms of C. albicans can be only partially detached from surfaces. In a manner similar to bacterial biofilms, Candida EPS is known to play an important role in drug resistance.

In this study, biofilms of mutant chk1/chk1 (Chk24), the gene-reconstructed chk1/CHK1 (Chk23) and wild-type CHK1/CHK1 (Caf2-1) C. albicans strains, along with two non-albicans Candida species, C. tropicalis and C. parapsilosis, isolated from used voice prostheses, were subjected to compression forces in a uniaxial low-load compression tester (LLCT) (Paramonova et al., 2007). Effects of increasing the shear duration during growth were evaluated only for C. albicans Caf2-1. LLCT evaluates the resistance of biofilms to compression and allows accurate determination of biofilm thicknesses. In addition, cell morphologies within the biofilm, cell-surface hydrophobicities and EPS composition were determined.

**METHODS**

**Microbial strains, growth conditions and biofilm formation.** C. albicans Caf2-1 (CHK1/CHK1), Chk24 (chk1/chk1) and Chk23 (chk1/CHK1) (Kruppa et al., 2004), C. tropicalis GB 9/9 and C. parapsilosis GB 2/8 grown on tryptone soya broth (Oxoid) agar plates were used to inoculate 10 ml yeast nitrogen base (YNB; Difco) without amino acids (BD) containing 50 mM glucose, prepared according to the manufacturer’s instructions with the pH set to 7.0 by using KOH. Cultures were grown at 30 °C for 16 h in ambient air while shaking at 120 r.p.m. Cells were harvested by centrifugation at 5000 g for 10 min, washed once and resuspended in sterile PBS (NaCl, 8.76 g l\(^{-1}\); K\(_2\)HPO\(_4\), 0.87 g l\(^{-1}\); KH\(_2\)PO\(_4\), 0.68 g l\(^{-1}\); pH 7.0).

Biofilms were grown on 1.5 × 1.5 cm polymethylmethacrylate (PMMA) slides in six-well tissue-culture plates. Prior to growing biofilms, the slides were sterilized with 70 % ethanol, rinsed with sterile, demineralized water and coated with 50 % fetal bovine serum (PMMA) slides in six-well tissue-culture plates. Prior to growing biofilms, the slides were sterilized with 70 % ethanol, rinsed with sterile, demineralized water and coated with 50 % fetal bovine serum (Sigma-Aldrich) for at least 30 min to enhance adhesion (Krom et al., 2007), washed once with sterile PBS and placed into the wells. Three millilitres of cell suspension with a density of 1 × 10\(^{7}\) cells ml\(^{-1}\) in sterile PBS was added to each well and cells were allowed to adhere at 37 °C for 90 min while shaking at 60 r.p.m. The suspension was removed by aspiration and the slides were rinsed with PBS. Biofilm growth was propagated by adding 3 ml YNB (pH 7.0) per well and incubating at 37 °C (or 30 °C in the case of Caf2-1 grown at 30 °C) while shaking at 60 r.p.m. for 72 or 144 h. For increased shear-grown biofilms, biofilms of C. albicans Caf2-1 were incubated while shaking at 90 r.p.m. for 72 h at 37 °C. Afterwards, the medium was discarded and biofilms were washed once with PBS.

**Low-load compression testing.** Biofilm resistance against compression was measured during uniaxial compression in an LLCT (Paramonova et al., 2007). The LLCT apparatus consists of a linear positioning stage (Intellistage M-511.5IM; Physik Instrumente) connected to a cylindrical, moving upper plate with a diameter of 2.5 mm (Fig. 1). A stationary bottom plate is fixed to an automatic
force-compensating balance (SW 50/300; Wipotec). The positioning stage and the load cell are interfaced to a PC. LabVIEW 7.1 software is used to control the positioning stage and to record the induced load. For biofilm-thickness measurements, the substratum with biofilm was placed on the bottom plate and the top plate was moved downwards until it touched the area of the substratum without biofilm. This position was recorded as the bottom of the biofilm. In the second step, the top plate was moved downwards until it touched the biofilm and this position was recorded as well. Subsequently, biofilm thickness was calculated from the difference between both positions. For compression experiments, a deformation rate of 1 μm s⁻¹ was chosen to enable us to register biofilm response to compression as accurately as possible without dehydrating biofilms during the measurements. Both load and strain (ε), defined as the fractional change in biofilm thickness from the initial contact point to the point where the plunger position was detected, were recorded. The induced load was normalized over the cross-sectional area of the plunger area to calculate the stress (σ) exerted, i.e. the force exerted per unit area of biofilm. Biofilms were compressed until 0.5 (50 %) strain was reached, at which point the compression was stopped because further deformation would only lead to compaction of the biofilms. Three measurements were taken for each independently grown biofilm, and the mean of the three measurements was calculated and used for further analysis. Values of strain were plotted against corresponding values of stress to obtain stress–strain diagrams. These diagrams help to acquire data about a material’s strength without regard for its physical size or shape (Hibbeler, 2000). The modulus of elasticity (E) was calculated at an arbitrarily chosen point of 0.2 strain (20 % deformation) by using Hooke’s law:

\[ E = \frac{\sigma}{\varepsilon} \]

Quantification of biofilm wet weight. PMMA slides with Candida biofilms were weighed, after which biofilms were scraped off the slides and the weights of the clean slides were measured. The difference between the two measurements yielded the wet weight of the biofilm.

Cell morphology counts. Biofilm scrapings in PBS were subsequently vortexed for 2 min to break up the matrix. The resulting suspension was sonicated on ice five times for 30 s at 30 W to break up biofilm clumps further. Cooling on ice was done to prevent overheating and lysis. A cell suspension was placed into a Bürker–Türk counting chamber and the numbers of yeast and hyphal cell morphologies were counted by using phase-contrast microscopy (× 40 objective lens; Olympus BH-2). Hyphae-to-yeast ratios were calculated from a minimum of 150 cells counted per experiment.

Hydropobicity measurements. Hydrophobicities of biofilms were assessed by water-contact angle measurements. Biofilms grown on PMMA slides were air-dried at room temperature, while measuring water-contact angles as a function of drying time (Busscher et al., 1984). Water-contact angles became independent of drying time after 110 min, following which stable water-contact angles could be established for at least a further 1 min. A drop of water was placed on the surface of a biofilm and images were taken from the side, using an Olympus MXR 5010 mounted with a 160 mm × 4 macro lens, from which contact angles were calculated by using Contact Angle Measurement System ver. 2.07 software (MT). Measurements were performed in triplicate with independently grown biofilms, with three water droplets measured on each biofilm.

EPS analysis. For determination of the proteins and eDNA content of EPS, EPS was isolated by using a modification of a protocol developed by Baillie & Douglas (2000). Biofilms were scraped from PMMA slides into PBS. The solution was vortexed vigorously for 3 min to disrupt the biofilms. The cell suspension was centrifuged at 5000 g for 10 min at 10 °C and supernatant was collected for further analysis. Quant-IT Protein Assay and dsDNA HS Assay kits (Molecular Probes) were used to quantify proteins and DNA according to the instructions of the manufacturer. Measurements were performed in triplicate with independently grown biofilms, with two samples measured on each biofilm. Amounts of proteins and DNA were normalized per unit of biofilm wet weight.

Total polysaccharides in the EPS were quantified by using the phenol–sulphuric acid method of DuBois et al. (1956). Biofilms were scraped from PMMA slides into 1 ml PBS. The suspension was mixed with 0.5 ml 1 M NaOH, vortexed and incubated while shaking at room temperature for 15 min. Further, the suspension was centrifuged at 10 000 g for 5 min. Supernatant was collected and mixed with 100 % ice-cold ethanol (0.5 ml supernatant : 1.5 ml ethanol) and placed in the freezer for at least 30 min. After freezing, 1 ml sample was combined with 25 μl aqueous 80 % (v/v) phenol solution in a glass test tube, to which 2.5 ml concentrated sulphuric acid was added rapidly. The tubes were vortexed and the mixture was incubated at room temperature for 10 min, followed by incubation in a 30 °C water bath for 20 min. After cooling to room temperature for 5 min, A₅₀₀ of the mixtures was measured (neutral polysaccharides). Measurements were performed in triplicate with independently grown biofilms, with two samples measured on each biofilm. Amounts of polysaccharides measured were normalized per unit of biofilm wet weight.

Statistical analysis. Statistical analysis was performed by using SPSS software (Statistical Package for Social Sciences, version 14.0.0; SPSS). Before analysis, data were tested for distribution by using a Kolmogorov–Smirnov goodness-of-fit test. Post-hoc multiple comparisons were performed to quantify differences between variables, using a Tukey test with a level of significance of P<0.05.

RESULTS

Hyphal content of C. albicans biofilms ranged from 0 to 79 %, depending on the growth conditions, and biofilms of C. tropicalis and C. parapsilosis comprised yeast cells only (Fig. 2). Hyphal content of C. albicans Caf2-1 biofilms increased slightly with higher shear and decreased in older biofilms. When the growth temperature of C. albicans Caf2-1 was reduced to 30 °C, hyphal content dropped to...
zero. There was no change in the hyphal content of *C. albicans* Chk24 biofilms over time.

Analysis of EPS content of the biofilms showed that polysaccharide content was significantly lower in 6-day-old biofilm of *C. albicans* compared with that of other biofilms studied (Fig. 3a). Protein content of EPS was variable across the different fungal biofilms and was highest for 3-day-old *C. tropicalis* biofilms (Fig. 3b). The amount of eDNA was also variable across the biofilms and was higher in 6-day-old biofilms of *C. albicans* than in 3-day-old biofilms. The eDNA content was highest for *C. albicans* CaF2-1 grown at 30°C (Fig. 3c).

Stress–strain curves (Fig. 4) showed that biofilms containing hyphae were able to withstand higher compressive forces than biofilms without hyphae, whilst hyphae content was influenced by growth temperature, shear rate during growth and the specific strain involved.

All biofilms, with the exception of *C. tropicalis*, had similar thicknesses, ranging from 150 to 340 μm. *C. tropicalis* and all *C. albicans* biofilms were hydrophobic, with contact angles >50°. Biofilms of *C. parapsilosis* were hydrophilic (Fig. 5). However, none of these parameters showed a systematic variation with biofilm strength, as can also be seen in Fig. 5.

**DISCUSSION**

Hyphal content was found to be a determining parameter for the strength of fungal biofilms (Fig. 6). Biofilms with a high hyphal content were more resistant to compression and more difficult to disturb by vortexing and sonification than biofilms with a lower hyphal content. The weakest biofilms consisted of yeast cells only. It appears that there was a linear relationship (linear correlation coefficient,
$R^2 = 0.943$) between biofilm strength and hyphal content in 3-day-old Candida biofilms (Fig. 6a). Interestingly, the biofilms without hyphae and with the highest eDNA content, C. albicans Caf2-1 grown at 30°C, possessed the lowest compressive strength.

In C. albicans, hyphal production can be suppressed not only by reducing the growth temperature (Berman & Sudbery, 2002), but also by the presence of farnesol, a QS molecule (Hornby et al., 2001). Here we see that, in the absence of CHK1, more hyphal cells are produced in the biofilms of Chk24 than in biofilms of the wild-type strain Caf2-1, resulting in stronger biofilms. Interestingly, 6-day-old biofilms for Caf2-1 showed a significant reduction in hyphal content that was not seen for the strain lacking Chk1p. This could therefore illustrate the effect of farnesol inducing yeast-cell growth in wild-type biofilms, whereas mutant biofilms are unable to sense farnesol and maintain a high hyphal content. Growth of C. albicans Caf2-1 under higher shear conditions also induced more hyphal production, although not to the extent that they were significantly stronger than Caf2-1 biofilms grown under lower shear.

Hyphal and yeast cells do not differ qualitatively in cell-wall composition. The main differences, however, are in the proportions of the main components, such as chitin or β-glucans (Chauhan et al., 2002). The cell wall of C. albicans consists of 80–90% carbohydrates, most important of which are β-glucans (β-1,3 and β-1,6 glucose polymers), accounting for 47–60% of cell-wall weight; chitin, which constitutes 0.6–9.0%; and mannan, polymers of mannose bound covalently to proteins. Even though the chitin content of the cell wall is small, it is an important component of the septa, bud scars and the ring around the constriction between mother cell and bud. Hyphal cells contain at least three times as much chitin as yeast cells (Braun & Calderone, 1978; Chaffin et al., 1998). Chitin is a hydrophobic material that is also found in insects, crabs, shrimps and lobsters, where it provides rigidity to the outer skeleton. Chitin has been shown to increase the mechanical and flexural strength of bone substitutes (Chen et al., 2005). A higher amount of chitin, due to the presence of more hyphal cells in biofilms, may be responsible for increased compressive strength of the biofilm.

Furthermore, eDNA content in EPS showed an adverse effect on biofilm strength of C. albicans Caf2-1 and Chk24. EPS amount and composition were previously shown by others to have a significant influence on the structure and

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**Fig. 4.** Averaged stress–strain curves for (a) biofilms of C. albicans containing hyphal cells and (b) biofilms of Candida strains without hyphal cells. Note the different scales. Error bars represent SD over nine independently grown biofilms.

**Fig. 5.** Moduli of elasticity (E) of Candida biofilms with biofilm characteristics, found to be unrelated to biofilm strength. Error bars represent SD over nine independently grown biofilms.
cohesiveness of bacterial biofilms (Flemming et al., 2000). Moreover, the presence of eDNA was shown to affect biofilm formation by Pseudomonas aeruginosa (Whitchurch et al., 2002). In our study, we see that normalized amounts of eDNA increased with biofilm age and shear (Fig. 3c) and contributed to the decrease in strength of Candida albicans biofilms with approximately similar hyphal content, as suggested in Fig. 6(b). In 6-day-old biofilms of Chk24, strength decreased by 33% compared with that of 3-day-old biofilms, while hyphal content remained unchanged. In 3-day-old biofilms of Caf2-1, strength of biofilms grown at higher shear decreased slightly compared with that of biofilms grown at normal shear, while the amount of eDNA increased and, here too, hyphal content remained similar. Six-day-old biofilms of Caf2-1 showed a 23% decrease in hyphal content, concurrent with a 60% increase in the amount of eDNA and a major decrease in biofilm strength. This suggests that eDNA probably does not play the same essential role in Candida biofilms as it does in bacterial biofilms. This is in line with recent observations that bacterial biofilms can be fully removed by DNase I treatment (Nemoto et al., 2003), whereas Candida biofilms were removed by only 30% (Al-Fattani & Douglas, 2006). In addition to changes in eDNA content, polysaccharide and protein contents in EPS varied with biofilm age. Polysaccharide content decreased in ageing biofilms while protein content increased, probably due to cell lysis. Neither polysaccharides nor proteins seemed to contribute significantly to biofilm strength. Additionally, no correlation was found between biofilm thickness and hydrophobicity.

This study contributes to a thin body of knowledge of fungal biofilm mechanics and helps to gain knowledge of which parameters are important for fungal biofilm strength. We show that hyphal cells are the most sturdy components in fungal biofilms and that their presence is determinant for the compression strength of Candida albicans biofilms. However, fungal biofilm strength can be affected adversely by the presence of eDNA. Without hyphae, fungal biofilms are weaker and can be removed more easily. Understanding what factors are important for biofilm integrity may help in the development of new fungal-specific drugs.

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