Biofilm formation by zygomycetes: quantification, structure and matrix composition

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Most studies on fungal biofilms have focused on *Candida* in yeasts and *Aspergillus* in mycelial fungi. To the authors' knowledge, biofilm formation by zygomycetes has not been reported previously. In this study, the biofilm-forming capacity of *Rhizopus oryzae*, *Lichtheimia corymbifera*, *Rhizomucor pusillus* and *Apophysomyces elegans* was evaluated. At appropriate seeding spore densities, *Rhp. oryzae* (10^5 c.f.u. ml⁻¹), *L. corymbifera* (10^4 c.f.u. ml⁻¹) and *Rhm. pusillus* (10^4 c.f.u. ml⁻¹) produced highly intertwined, adherent structures on flat-bottomed polystyrene microtitre plates after 24 h at 37 °C. The adhered fungal hyphae were encased in an extracellular matrix, as confirmed by phase-contrast and confocal microscopy. The thickness of *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* biofilms was 109.67 ± 10.02 , 242 ± 23.07 and 197 ± 9.0 µm (mean ± SD), respectively. Biochemical characterization of the biofilm matrix indicated the presence of glucosamine, constituting 74.54–82.22 % of its dry weight, *N*-acetylglucosamine, glucose and proteins. Adherence and biofilm formation were not observed in *A. elegans*. Although *A. elegans* spores germinated at all three seeding densities tested (1×10^7 , 1×10^6 and 1×10^5 c.f.u. ml⁻¹), no significant difference was observed ($P > 0.05$) between the A_{490} of wells inoculated with *A. elegans* and the cut-off A_{490} for biofilm detection. This study highlights the potential for biofilm formation by at least three medically important species of zygomycetes.

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INTRODUCTION

Biofilms are dense, highly hydrated cell clusters that are irreversibly attached to a substratum, to an interface or to each other, and are embedded in a self-produced gelatinous matrix composed of extracellular polymeric substances (EPS) (Harding *et al.*, 2009). The micro-organisms in these biofilms exhibit an altered phenotype with respect to growth rate, gene transcription, and resistance to physical, chemical and biological stresses (Chandrasekar & Manavathu, 2008; Harding *et al.*, 2009; Mowat *et al.*, 2009). Biofilm formation has been widely implicated in the pathogenesis of implant-associated and chronic infections (Hall-Stoodley *et al.*, 2004).

Most of the work describing biofilm genesis, architecture, chemical composition, genetic regulation and antimicrobial drug resistance has focused on bacteria and yeasts (Chandrasekar & Manavathu, 2008; Harding *et al.*, 2009; Mowat *et al.*, 2009). Occasionally, filamentous fungi have also been reported to form biofilms (Chandrasekar & Manavathu, 2008; Harding *et al.*, 2009; Mowat *et al.*, 2009).

Although filamentous fungi often penetrate the substrates that they grow on, and this invasive growth may not accurately represent the term biofilm (Harding *et al.*, 2009), the yeasts *Candida albicans* and *Trichosporon asahii* have been shown to require differentiation to hyphal forms during biofilm formation (Harding *et al.*, 2009; Ramage *et al.*, 2009). Filamentation in fungi may therefore be a prerequisite for robust biofilm development and virulence, and fungal biofilms perhaps represent much more than a mere biological coating (Harding *et al.*, 2009). Biofilm formation is claimed to be involved in the pathogenesis of localized as well as invasive diseases caused by *Aspergillus fumigatus* (Beauvais *et al.*, 2007; Chandrasekar & Manavathu, 2008; Harding *et al.*, 2009; Lousert *et al.*, 2010; Mowat *et al.*, 2007, 2008, 2009). Aspergilloma, a localized infection, has been shown to consist of highly agglutinated hyphae encased in an extracellular matrix (Lousert *et al.*, 2010; Mowat *et al.*, 2009). A similar exopolysaccharide matrix is also produced at the surface of fungal hyphae during invasive pulmonary aspergillosis (Lousert *et al.*, 2010).

Fungi belonging to the class Zygomycetes and the order Mucorales, including *Rhizopus*, *Rhizomucor*, *Mucor*, *Lichtheimia*, *Apophysomyces*, *Cunninghamella* and *Saksenaia*, often cause opportunistic infections which are similar to those caused by *Aspergillus* and are characterized by angioinvasion

Abbreviations: CLSM, confocal laser scanning microscopy; Con-A, concanavalin A conjugated to Alexa Fluor; ELLA, enzyme-linked lectinsorbent assay; EPS, extracellular polymeric substance; G6PDH, glucose-6-phosphate dehydrogenase; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; OG, Oregon green; ROC, rhino-orbito-cerebral; WGA, wheatgerm agglutinin.

and fungal-ball formation (Chakrabarti *et al.*, 2001, 2006, 2009; Goodnight *et al.*, 1993; Kirkpatrick *et al.*, 1979; Lahiri *et al.*, 2001; Robey *et al.*, 2009). Mucormycosis is categorized into rhino-orbito-cerebral (ROC), pulmonary, gastro-intestinal, cutaneous and disseminated types depending upon the clinical presentation and the anatomical sites involved (Chakrabarti *et al.*, 2001, 2006, 2009). While the ROC type is often associated with individuals having uncontrolled diabetes and diabetic ketoacidosis, the pulmonary, gastro-intestinal and cutaneous types occur in patients with haematological malignancies or neutropenia, severe malnutrition, and trauma or burns, respectively (Chakrabarti *et al.*, 2001, 2006, 2009). The incidence of mucormycosis has increased globally over the last two decades, with a phenomenal rise in the number of cases reported from India (Chakrabarti *et al.*, 2001, 2006, 2009). Three case series on mucormycosis have been reported from our tertiary-care centre: 129 cases over 10 years (1990–1999), 178 cases during the subsequent five years (2000–2004) and then 75 cases in an 18 month period during 2006–2007 (Chakrabarti *et al.*, 2001, 2006, 2009). *Rhizopus oryzae* and *Apophysomyces elegans* (*sensu lato*) were the predominant isolates in these series (Chakrabarti *et al.*, 2001, 2006, 2009).

Since more than 99 % of microbes have been reported to grow as biofilms, and these surface-attached communities have also been implicated in the pathogenesis of both localized as well as invasive diseases caused by *Aspergillus*, the present study was planned to elucidate the biofilm-forming potential of four common pathogenic zygomycetes: *Rhizopus oryzae*, *Lichtheimia corymbifera*, *Rhizomucor pusillus* and *Apophysomyces elegans*.

METHODS

Fungal strains. *Rhp. oryzae* NCCPF 710004, *L. corymbifera* NCCPF 700002, *Rhm. pusillus* NCCPF 720004 and *A. elegans* NCCPF 102033 were used in this study. The strains were obtained from the National Culture Collection of Pathogenic Fungi (NCCPF), Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, and were preserved in 20 % glycerol at -70°C until use.

Isolation of sporangiospores. *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* were grown on Sabouraud dextrose agar (SDA) at 37°C for 4–5 days. *A. elegans* was cultured on water agar at 37°C for 7 days. The plates were flooded with 10 ml PBS (0.1 M, pH 7.2) and sporangiospores were harvested by repeated washing of the mycelia with the added buffer. The resulting suspensions were centrifuged at 6000 r.p.m. at 4°C for 10 min; the pellets were washed twice with PBS and finally suspended in 1 ml PBS. Spores were counted using a Neubauer haemocytometer and counts expressed as c.f.u. ml^{-1} .

Standardization of biofilm formation. Biofilm formation was determined according to the method of Pierce *et al.* (2008). The spores were adjusted to the required density (*Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* – 1×10^7 , 1×10^6 , 1×10^5 , 1×10^4 and 1×10^3 c.f.u. ml^{-1} ; *A. elegans* – 1×10^7 , 1×10^6 , 1×10^5 c.f.u. ml^{-1}) in RPMI 1640 (pH 7.2) buffered with MOPS (165 mM). Two hundred microlitres of this suspension was inoculated per well in 96-well, flat-bottomed polystyrene microtitre plates. Media-only blanks were also

set up. The plates were incubated at 37°C for 24 h (up to 48 h for *A. elegans*) and the resulting biofilms were washed twice with PBS, fixed with 200 μl 95 % ethanol at 37°C for 15 min and stained with 200 μl 0.1 % safranin for 5 min. Biofilm formation was observed under an inverted microscope (Olympus CKX 41) with a $\times 40$ objective lens and was quantified by measuring the absorbance of the bound safranin, eluted with 200 μl 30 % glacial acetic acid, at 490 nm. Experiments were performed in quadruplicate. The cut-off absorbance was calculated as the mean absorbance of the media-only blanks plus three times their SD ($A_{490}=0.075$).

Adhesion kinetics. Two hundred microlitres of the standardized spore suspension was inoculated per well in 96-well, flat-bottomed polystyrene microtitre plates and incubated at 37°C for 4, 8, 12 and 24 h. The experiments were performed in quadruplicate and media-only blanks were set up in parallel. At each time point, the biofilms were washed twice with PBS and were quantified as described previously.

Confocal laser scanning microscopy (CLSM) of biofilms. Three millilitres of the standardized spore suspension was inoculated in 35 mm Petri dishes and incubated at 37°C for 24 h. The biofilms formed were washed twice with PBS, and then stained with wheatgerm agglutinin conjugated to Oregon green (WGA-OG, 0.1 mg ml^{-1} ; Invitrogen BioServices India) for 15 min (Singh *et al.*, 2010) or with concanavalin A conjugated to Alexa Fluor 488 (Con-A, 50 μg ml^{-1}) and 10 μM FUN-1 (Chandra *et al.*, 2008) (Invitrogen BioServices India) at 37°C for 30 min in the dark. The biofilms were then washed twice with PBS. CLSM was performed with an LSM 510 Meta (Carl Zeiss MicroImaging) attached to an Axioplan II microscope using a $\times 10/0.3$ objective lens.

During CLSM using WGA-OG, HFT 488 was selected as the excitation laser and WGA-OG was detected by fluorescence in the green spectrum using BP505–530. Fungal hyphae were detected by refraction of light in the red spectrum using LP560. NFT 545 was used as the beam splitter and the images were acquired in multitrack mode. For CLSM using dual staining with Con-A/FUN-1, the excitation wavelengths were set to 488 nm and 543 nm for Con-A and FUN-1, respectively. NFT 490 (Con-A) and NFT 545 (FUN-1) were used as the beam splitters, and LP 505 (Con-A) and LP 560 (FUN-1) were used as the emission filters. Image analysis was done using z-series image stacks from four randomly chosen spots of each biofilm and the biofilm architecture and mean thickness were elucidated using LSM image browser version 4.2.0.121 and ZEN 2009.

Extraction of biofilm matrix. Ten millilitres of the standardized spore suspension was inoculated per flask in five 50 ml polystyrene tissue-culture flasks and incubated at 37°C for 24 h. The biofilms formed were washed twice with PBS, flooded with 10 ml sterile MilliQ water per flask and vortexed mildly for 30 s. The suspensions were then pooled and centrifuged at 6000 r.p.m. at 4°C for 20 min. The supernatant was collected, filter-sterilized and treated with 2 volumes of chilled 95 % ethanol overnight at 4°C . The resulting precipitates were collected by centrifugation at 6000 r.p.m. at 4°C for 1 h, washed with chilled 95 % ethanol and dried. The EPS matrix was dissolved in water and centrifuged at 6000 r.p.m. at 4°C for 10 min. The water-soluble fraction (supernatant) was collected and the water-insoluble material (pellet) was then dissolved in trichloroacetic acid (TCA). The experiment was performed three times and the matrix was characterized biochemically each time.

Characterization of biofilm matrix. The intracellular enzyme glucose-6-phosphate dehydrogenase (G6PDH) was used as a marker for detecting cell lysis and thereby a contamination of the harvested matrix with cellular components (Flemming & Wingender, 2010). Ten microlitres of the water-soluble fraction was added to 290 μl of

the assay buffer containing 250 mM glycine buffer (pH 7.4), 60 mM glucose 6-phosphate, 20 mM NADP and 300 mM MgCl_2 , and incubated at 25 °C for 5 min. The production of NADPH was elucidated by measuring the change in absorbance at 340 nm. A molar absorption coefficient of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ was used and 1 U G6PDH was defined as the enzyme activity catalysing the formation of 1 mmol NADPH min^{-1} . Protein concentration was determined in the water-soluble fraction by the bicinchoninic acid method (Smith *et al.*, 1985). Total carbohydrates in the water-soluble fraction were determined by the phenol/sulphuric acid method (Dubois *et al.*, 1951). Glucose was quantified in the water-soluble fraction after acid hydrolysis by using a glucose oxidase kit (Sigma-Aldrich) according to the manufacturer's instructions. The presence of amino sugars in the water-soluble fraction was detected by UV spectroscopy (Kumirska *et al.*, 2010). Briefly, the UV absorption of the fractions was scanned over 190–350 nm in 1 nm step increments. The first-derivative spectra of the samples were elucidated and compared with those of standard solutions containing *N*-acetylglucosamine (GlcNAc) and glucosamine (GlcN): GlcNAc only, GlcN only, and GlcNAc and GlcN mixed in different ratios (4:1, 3:2, 1:1, 2:3 and 1:4). The percentage of acetylated and non-acetylated hexosamine in the samples was determined from standard curves of λ_{min} vs percentage GlcNAc as well as calibrated peak height (*H*) vs percentage GlcNAc. GlcNAc was quantified in the water-soluble and TCA-soluble fractions by enzyme-linked lectinsorbent assay (ELLA) according to the method of Singh *et al.* (2010), using a standard curve of GlcNAc (μg) vs A_{490} . GlcN in the water-soluble fraction was determined by its *N*-acetylation using acetic anhydride in methanol (Levy & McAllan, 1959), followed by quantification of the GlcNAc formed by ELLA. All experiments were performed in quadruplicate.

Statistical analysis. The two-tailed, unpaired *t*-test was used for comparing the adhesion capacity of test strains versus the media-only blanks.

RESULTS AND DISCUSSION

Mucorales usually cause acute angioinvasive ROC, pulmonary, gastrointestinal or cutaneous infections in immunocompromised patients (Chakrabarti *et al.*, 2001, 2006, 2009). In addition, these fungi have been implicated in central-venous-catheter-associated fungaemia (Chan-Tack *et al.*, 2005), peritonitis in patients undergoing continuous ambulatory peritoneal dialysis (Branton *et al.*, 1990; Fergie *et al.*, 1992; Nakamura *et al.*, 1989; Nannini *et al.*, 2003; Nayak *et al.*, 2007; Polo *et al.*, 1989; Serna *et al.*, 2003), pleuritis following implantation of drainage catheters (Kimura *et al.*, 2009), endocarditis on native (Mehta *et al.*, 2004; Mitchell *et al.*, 2010) and artificial heart valves (Gubarev *et al.*, 2007; Sanchez-Recalde *et al.*, 1999), osteomyelitis (Chaudhuri *et al.*, 1992; Eaton *et al.*, 1994; Lopes *et al.*, 1995; Meis *et al.*, 1994; Wilkins *et al.*, 2009), and paranasal fungal balls (Goodnight *et al.*, 1993; Kirkpatrick *et al.*, 1979; Lahiri *et al.*, 2001; Robey *et al.*, 2009). Many of these infections are associated with biofilm formation (Costerton *et al.*, 1999; Hall-Stoodley *et al.*, 2004; Loussert *et al.*, 2010) and were reported to exhibit a chronic course (Eaton *et al.*, 1994; Lahiri *et al.*, 2001; Lopes *et al.*, 1995). Furthermore, surface-attached growth of zygomycetes has often been used for industrial purposes (Cao *et al.*, 1997). However, biofilm formation by zygomycetes has not yet been demonstrated. This is

believed to be the first study to elucidate the biofilm-forming capacity of *Rhp. oryzae*, *L. corymbifera*, *Rhm. pusillus* and *A. elegans*, and study their adhesion kinetics, biofilm morphology and matrix composition.

Standardization of biofilm formation

The spore density of *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* in the initial inoculum was found to be important for germination of the adhered spores as well as structural integrity of the biofilms formed (Fig. 1). Whilst very high seeding density (*Rhp. oryzae*, 1×10^7 and 1×10^6 c.f.u. ml^{-1} ; *L. corymbifera* and *Rhm. pusillus*, 1×10^7 , 1×10^6 and 1×10^5 c.f.u. ml^{-1}) resulted in poor germination of the adhered spores, low inoculum concentration (*Rhp. oryzae*, 1×10^4 and 1×10^3 c.f.u. ml^{-1} ; *L. corymbifera* and *Rhm. pusillus*, 1×10^3 c.f.u. ml^{-1}) reduced the hyphal density. Similar results have been previously reported for *Aspergillus fumigatus* (Mowat *et al.*, 2007), *Trichosporon asahii* (Di Bonaventura *et al.*, 2006) and various *Candida* species including *C. albicans* (Ramage *et al.*, 2009), and may be associated with the production of quorum-sensing molecules (Ramage *et al.*, 2009). Initial seeding densities of 1×10^5 c.f.u. ml^{-1} (*Rhp. oryzae*) and 1×10^4 c.f.u. ml^{-1} (*L. corymbifera* and *Rhm. pusillus*) produced robust, filamentous adherent structures and were selected for further experiments. At these inoculum concentrations, the mean adherence of *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* on polystyrene plates after 24 h of incubation was 2.06 ± 0.02 , 2.74 ± 0.29 and 1.44 ± 0.05 (A_{490} , mean $\pm$ SD), respectively.

Apophysomyces elegans spores germinated at all the three seeding densities tested but adherence and biofilm formation were not detected. No significant difference ($P > 0.05$) was observed between the A_{490} of wells inoculated with *A. elegans* compared to the cut-off A_{490} for biofilm detection [1×10^7 c.f.u. ml^{-1} , 0.092 ± 0.0007 (24 h) and 0.091 ± 0.0 (48 h); 1×10^6 c.f.u. ml^{-1} , 0.078 ± 0.0 (24 h) and 0.083 ± 0.0007 (48 h); 1×10^5 c.f.u. ml^{-1} , 0.073 ± 0.018 (24 h) and 0.097 ± 0.013 (48 h)]. *A. elegans* is usually associated with superficial infections in immunocompetent hosts, although infections in deep tissue have also been described (Chakrabarti *et al.*, 2003, 2010). We reported an upsurge of such *A. elegans* infections over the past decade in India (Chakrabarti *et al.*, 2003, 2010). These findings indicate that biofilm formation may not play an important role in the pathogenesis of *A. elegans* infections. Species-specific differences in biofilm-forming capacity have also been reported in *Candida*, and have been correlated with the pathogenesis and the associated risk factors (Hawser & Douglas, 1994; Kuhn *et al.*, 2002; Li *et al.*, 2003; Ramage *et al.*, 2009).

Adhesion kinetics

A direct correlation was observed between biomass, quantified by measuring the absorbance of the bound

Fig. 1. Effect of seeding density on the development of *Rhp. oryzae* NCCPF 710004 biofilms after 24 h at 37 °C. Very high (A, 1×10^7 ; B, 1×10^6 c.f.u. ml⁻¹) or low spore concentrations (D, 1×10^4 ; E, 1×10^3 c.f.u. ml⁻¹) resulted in poor germination and reduced hyphal density, respectively. An initial seeding density of 1×10^5 c.f.u. ml⁻¹ (C) produced robust, filamentous adherent structures and was selected for further experiments. The images were taken at $\times 40$ using an inverted microscope. Scale bar, 10 mm.

safranin, and hyphal development (Figs 2 and 3). Following initial seeding, the spores adhered to the polystyrene surface and began to swell and germinate in 4–6 h. Hyphae were observed within 8–10 h of incubation. These hyphae formed monolayers by 10–12 h, and then further intertwined and increased in density over the next 12 h. This pattern resembles the kinetics of biofilm formation in *Aspergillus fumigatus* (Mowat *et al.*, 2007). The intertwining of the mycelial mass provides stability and integrity to the biofilms and is achieved by extensive branching, followed by elongation of the hyphal branches by apical growth (Chandrasekar & Manavathu, 2008; Mowat *et al.*, 2009).

CLSM of biofilms

Microbial biofilms are characterized by the presence of surface-attached cells enmeshed in a self-produced gelatinous matrix made primarily of polysaccharides (Costerton *et al.*, 1999; Hall-Stoodley *et al.*, 2004). This matrix, also termed slime or EPS, forms a scaffold for the three-dimensional architecture of the biofilm (Flemming & Wingender, 2010; Hall-Stoodley *et al.*, 2004). It provides stability to the biofilm structure, helps in adherence as well as in trapping the nutrients, and also protects the biofilm cells from desiccation, UV radiation, protozoan grazers, antimicrobials and host immune defences (Flemming & Wingender, 2010; Hall-Stoodley *et al.*, 2004).

To detect the presence of polysaccharide matrix and elucidate the biofilm architecture, confocal microscopy was performed using the plant-based lectins WGA and

concanavalin A. These markers bind to GlcNAc (WGA) (Singh *et al.*, 2010), glucose and mannose (concanavalin A) (Chandra *et al.*, 2008), and therefore detect these specific residues in the hyphae and the matrix, if any. Individual fungal hyphae were also detected via the red light refracted by the cells (WGA-OG staining) or FUN-1 (Con-A/FUN-1 staining), and the images obtained were compared with WGA-OG or Con-A fluorescence for differentiation between the binding of WGA or Con-A to the hyphae and the matrix.

Phase-contrast images of the confocal micrographs revealed a haze-like film, suggestive of a polysaccharide matrix, covering the fungal hyphae in the biofilms (Fig. 4). CLSM images also indicated the presence of highly intertwined mycelia mass, with WGA-OG staining many inter-hyphal regions as well (Fig. 4). These results demonstrate the existence of an exopolysaccharide matrix in *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* biofilms and further satisfy the criteria proposed for detecting biofilm formation in filamentous fungi (Harding *et al.*, 2009). Also, the staining of many inter-hyphal regions with WGA-OG suggests the presence of GlcNAc in the matrix. In contrast, Con-A detected the fungal hyphae but inter-hyphal regions were not significantly stained with this marker, indicating that glucans and mannans were not a major component of the matrix. This was further confirmed by biochemical characterization of EPS extracted from the biofilms. The thickness of *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* biofilms was 109.67 ± 10.02 μ m, 242 ± 23.07 μ m and 197 ± 9.0 μ m (mean $\pm$ SD), respectively.

Fig. 2. Hyphal development during adhesion of *Rhp. oryzae* NCCPF 710004 on 96-well, flat-bottomed polystyrene microtitre plates. The plates were incubated at 37 °C for 4 h (A), 8 h (B), 12 h (C) and 24 h (D). The images were taken at $\times 40$ using an inverted microscope. Scale bar, 10 mm.

Characterization of the biofilm matrix

EPS has often been called the ‘dark matter’ of biofilms, owing to the large range of biopolymers it may contain, and the difficulty in analysing them (Flemming & Wingender, 2010). It accounts for a major component of the biofilm biomass and consists primarily of polysaccharides, although proteins (including many enzymes), lipids and extracellular DNA may also be present (Flemming & Wingender, 2010).

G6PDH activity was not detected in the matrices extracted from *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* biofilms,

Fig. 3. Adhesion kinetics of *Rhp. oryzae* NCCPF 710004 (◆—◆), *L. corymbifera* NCCPF 700002 (■- -■) and *Rhm. pusillus* NCCPF 720004 (▲····▲) on 96-well, flat-bottomed polystyrene microtitre plates. The plates were incubated at 37 °C for 4, 8, 12 and 24 h (note the non-linear time scale). Data are represented as A_{490} . Experiments were performed in quadruplicate. Error bars indicate standard deviation (not shown where smaller than symbols).

confirming an absence of contaminating cellular components. Hexosamine and *N*-acetylhexosamine constituted 93.4–95.6 % and 4.4–6.6 %, respectively, of the amino sugars in the water-soluble EPS fraction. GlcN was found to be the primary component of *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* biofilm matrix, constituting 745.38 ± 115.25 , 822.20 ± 178.99 and $803.94 \pm 135.43 \mu\text{g (mg EPS)}^{-1}$ (mean $\pm$ SD), respectively. In contrast, GlcNAc constituted 43.49 ± 6.72 , 47.97 ± 10.44 and $46.91 \pm 7.90 \mu\text{g (mg EPS)}^{-1}$ (mean $\pm$ SD) in *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* biofilms, respectively. These results suggest the presence of the chitin-derivative chitosan, a partially acetylated β -1,4-linked GlcN polymer, which is usually soluble in water at a high degree of deacetylation (Kumirska *et al.*, 2010). In contrast to other fungi, chitin and chitosan constitute a major component of the cell wall in zygomycetes (Bartnicki-Garcia, 1968). Chitosan cements the cell wall, protects chitin from enzymic attack and also adsorbs ionic material by salt or complex formation (Bartnicki-Garcia, 1968). The content of this aminopolysaccharide in zygomycetes' cell wall may be up to three times the amount of chitin (Bartnicki-Garcia, 1968). A similar partially deacetylated β -1,6-linked GlcNAc polymer termed polysaccharide intercellular adhesin (PIA) is the primary component of *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilm matrix and it mediates cell-to-cell interaction in these bacteria (Singh *et al.*, 2010). Hexosamine has also been reported as an important component of EPS in *Candida tropicalis* biofilms (Al-Fattani & Douglas, 2006).

The high concentration of amino sugars interfered with the determination of carbohydrates by the phenol/sulphuric acid method. Upon reaction with this reagent, the samples exhibited a peak absorbance at 405 nm, compared to the usual 490 nm observed in sugars. Glucose content was therefore determined in the water-soluble fraction using a

Fig. 4. Phase-contrast (Aa and Ba) and confocal laser scanning micrographs (Ab and Ac, Bb and Bc, Ca and Cb) of *Rhm. pusillus* NCCPF 720004 biofilms. (A) WGA-OG staining. WGA-OG (Ab) was used to detect GlcNAc in the fungal hyphae and the biofilm matrix. Individual fungal hyphae were also detected via the red light refracted by the cells (Ac) and the images obtained were compared with WGA-OG fluorescence for differentiation between the binding of WGA to the hyphae and the matrix. The arrow indicates a representative inter-hyphal region stained by WGA-OG. Scale bars, 100 μm ; (B) Con-A and FUN-1 staining. Con-A (Bb) was used to detect glucose and mannose in the fungal hyphae as well as in the biofilm matrix, if any. Individual fungal hyphae were also detected via FUN-1 (Bc) and the images obtained were compared with Con-A fluorescence for differentiation between the binding of Con-A to the hyphae and the matrix, if any. Scale bars, 100 μm . (C) Three-dimensional reconstruction of biofilms after staining with WGA-OG. (Ca) Detection of GlcNAc in the hyphae and matrix with WGA-OG; (Cb) detection of hyphae by collecting the red-refracted light.

glucose oxidase kit. Although glucose constitutes 32–74 % of the biofilm matrix in *Aspergillus fumigatus* (Beauvais *et al.*, 2007) and *Candida* species (Al-Fattani & Douglas, 2006; Silva *et al.*, 2009), it accounted for only a small proportion of the biofilm-matrix weight in the Mucorales that we studied. *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* biofilm EPS contained 7.42 ± 0.92 , 7.92 ± 1.20 and 7.17 ± 0.71 μg glucose $(\text{mg EPS})^{-1}$ (mean $\pm$ SD), respectively. Proteins were also detected in the matrix preparations, constituting 2.23 ± 0.10 , 7.70 ± 0.31 and 3.17 ± 0.61 μg $(\text{mg EPS})^{-1}$ (mean $\pm$ SD) in *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus*, respectively. However, these results probably underestimate the protein content of the slime. The solubilized matrix harvested from the biofilms was concentrated using ethanol, an organic solvent which preferentially precipitates polysaccharides

(Flemming & Wingender, 2010). The protein content of the suspensions before ethanol precipitation was about sevenfold higher than that of the precipitated EPS.

The TCA-soluble EPS fraction was processed for the detection of chitin, a β -1,4-linked GlcNAc polymer insoluble in water and many acids (Kumirska *et al.*, 2010), and its monomer GlcNAc was quantified by ELLA. The concentration of GlcNAc in the TCA-soluble fraction of *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* biofilm matrix was 59.43 ± 0.80 , 74.28 ± 8.09 and 56.89 ± 4.40 μg $(\text{mg EPS})^{-1}$ (mean $\pm$ SD), respectively.

Thus, the extracellular matrix in *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus* biofilms is primarily composed of amino sugars (GlcN and GlcNAc), with glucose and proteins also

being present in small amounts. By contrast, glucose is the major component of *Candida* and *Aspergillus fumigatus* biofilms (Al-Fattani & Douglas, 2006; Baillie & Douglas, 2000; Beauvais *et al.*, 2007; Lal *et al.*, 2010; Silva *et al.*, 2009). In *A. fumigatus* biofilms, the EPS consists of glucose (74 %), mannitol (18 %), trehalose (3 %), glycerol (5 %), and melanin and proteins (2 %) (Beauvais *et al.*, 2007). Immunolabelling studies have further revealed the presence of galactomannan, galactosaminogalactan and α -1,3 glucans (Beauvais *et al.*, 2007; Loussert *et al.*, 2010). *C. albicans* biofilms usually contain glucose (16–32.2 %), hexosamine (3.3 %), proteins (5 %), phosphorus (0.5 %) and uronic acid (0.1 %) as well as mannose, rhamnose and galactose in the matrix (Al-Fattani & Douglas, 2006; Baillie & Douglas, 2000; Lal *et al.*, 2010). However, *C. tropicalis* biofilm matrix comprises mainly hexosamine (27.4 %), with smaller amounts of other carbohydrates (3.3 %, including 0.5 % glucose), protein (3.3 %) and phosphorus (0.2 %) (Al-Fattani & Douglas, 2006).

In conclusion, our results reveal the biofilm-forming potential of *Rhp. oryzae*, *L. corymbifera* and *Rhm. pusillus*, but not *A. elegans*. At appropriate seeding densities, these fungi produced robust, highly intertwined, filamentous, adherent structures that were encased in an extracellular matrix composed primarily of GlcN and GlcNAc. Although Mucorales are usually implicated in angio-invasive infections, biofilm formation may be involved in the pathogenesis of paranasal fungal balls, endocarditis, osteomyelitis and catheter-based infections caused by these pathogens. It may also be important for the survival of these saprophytic fungi in the environment (Ramage *et al.*, 2009).

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