Journal of Medical Microbiology (2006), **55**, 999–1008 **DOI** 10.1099/jmm.0.46569-0

Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance

Mohammed A. Al-Fattani and L. Julia Douglas

Division of Infection and Immunity, Institute of Biomedical and Life Sciences, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, UK

Correspondence
L. Julia Douglas
J.Douglas@bio.gla.ac.uk

Matrix material was extracted from biofilms of *Candida albicans* and *Candida tropicalis* and analysed chemically. Both preparations contained carbohydrate, protein, hexosamine, phosphorus and uronic acid. However, the major component in *C. albicans* matrix was glucose (32 %), whereas in *C. tropicalis* matrix it was hexosamine (27 %). Biofilms of *C. albicans* were more easily detached from plastic surfaces by treatment with the enzyme lyticase (β-1,3-glucanase) than were those of *C. tropicalis*. Biofilms of *C. albicans* were also partially detached by treatment with proteinase K, chitinase, DNase I, or β-*N*-acetylglucosaminidase, whereas *C. tropicalis* biofilms were only affected by lipase type VII or chitinase. To investigate a possible role for the matrix in biofilm resistance to antifungal agents, biofilms of *C. albicans* were grown under conditions of continuous flow in a modified Robbins device (MRD). These biofilms produced more matrix material than those grown statically, and were significantly more resistant to amphotericin B. Biofilms of *C. tropicalis* synthesized large amounts of matrix material even when grown statically, and such biofilms were completely resistant to both amphotericin B and fluconazole. Mixed-species biofilms of *C. albicans* and a slime-producing strain of *Staphylococcus epidermidis* (RP62A), when grown statically or in the MRD, were also completely resistant to amphotericin B and fluconazole. Mixed-species biofilms of *C. albicans* and a slime-negative mutant of *S. epidermidis* (M7), on the other hand, were completely drug resistant only when grown under flow conditions. These results demonstrate that the matrix can make a significant contribution to drug resistance in *Candida* biofilms, especially under conditions similar to those found in catheter infections *in vivo*, and that the composition of the matrix material is an important determinant in resistance.

Received 9 February 2006
Accepted 18 April 2006

INTRODUCTION

Candida albicans and a small number of related *Candida* species are known to be important agents of hospital-acquired infections. Many of these are implant-associated infections in which the micro-organisms form adherent biofilms on the surfaces of catheters, joint replacements, prosthetic heart valves and other medical devices (Donlan, 2001; Douglas, 2003). *Candida* septicaemias, for example, now rank as the fourth most common type of nosocomial bloodstream infection and are usually catheter-related (Calderone, 2002). Biofilm cells on implants are organized into structured communities embedded within a matrix of extracellular material. They are phenotypically distinct from planktonic or suspended cells; in particular, they are significantly less susceptible to antimicrobial agents (Donlan & Costerton, 2002; Gilbert *et al.*, 2002). As a result, implant infections are difficult to treat and usually the implant must be removed (Costerton *et al.*, 1999).

The matrix is one of the most distinctive features of a microbial biofilm. It forms a three-dimensional, gel-like, highly hydrated and locally charged environment in which the micro-organisms are largely immobilized (Flemming *et al.*, 2000). Matrix-enclosed microcolonies, sometimes described as 'stacks' or 'towers', are separated by water channels which provide a mechanism for nutrient circulation within the biofilm (Donlan & Costerton, 2002). The composition of the matrix varies according to the nature of the organisms present. Matrix polymers of bacterial biofilms are primarily exopolysaccharides, and many are negatively charged due to the presence of carboxyl, sulphate or phosphate groups. Smaller amounts of proteins, nucleic acids and lipids can also be present. Two of the best-characterized matrix polysaccharides in bacteria are alginate (a polymer of mannuronic acid and guluronic acid) produced by *Pseudomonas aeruginosa*, and poly β-1,6-linked *N*-acetylglucosamine secreted by *Staphylococcus epidermidis*

Abbreviations: MRD, modified Robbins device; PIA, intercellular polysaccharide adhesin; SEM, scanning electron microscopy; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

and *Staphylococcus aureus* (Starkey *et al.*, 2004; Gotz, 2002). Synthesis of both polysaccharides has been related to bacterial virulence.

The recalcitrance of biofilms to antimicrobial agents is often attributed to the failure of these agents to penetrate the biofilm matrix. However, a number of studies have demonstrated that reductions in the diffusion coefficients of antibiotics within biofilms are insufficient to account solely for the observed changes in susceptibility (Gilbert *et al.*, 2002). Drug access is also assisted by the presence of water channels in the biofilm structure. Nevertheless, matrix components could retard access to such an extent that cells lying deep within a microcolony escape exposure. This would occur via drug adsorption or neutralization, and would depend on the thickness of the biofilm and on the chemical nature of both the antimicrobial agent and the matrix material. It is known, for example, that fluoroquinolones penetrate *P. aeruginosa* biofilms readily, whereas penetration by positively charged aminoglycosides is retarded (Drenkard, 2003). Similarly, fluconazole permeates single-species *Candida* biofilms more rapidly than flucytosine (Al-Fattani & Douglas, 2004). Rates of drug diffusion through biofilms of *Candida glabrata* or *Candida krusei* are faster than those through biofilms of *Candida parapsilosis* or *Candida tropicalis*, while drug diffusion through mixed-species biofilms of *C. albicans* and *S. epidermidis* is very slow.

During a previous investigation in this laboratory, the matrix of *C. albicans* biofilms was isolated and its composition compared with that of extracellular polymeric material obtained from culture supernatants of planktonically grown organisms (Baillie & Douglas, 2000). Both preparations contained carbohydrate, protein, phosphorus and hexosamine, but the matrix had significantly less carbohydrate (41 %) and protein (5 %). It also had a higher proportion of glucose (16 %) than mannose, unlike planktonic extracellular material (McCourtie & Douglas, 1985). To investigate whether the matrix plays a role in the resistance of biofilms to antifungal agents, susceptibility profiles of biofilms incubated statically (which have relatively little matrix) were compared with those of biofilms incubated with gentle shaking (which produce much more matrix material). Biofilms grown with or without shaking did not exhibit significant differences in susceptibility to any of the drugs tested, suggesting that drug resistance is unrelated to the extent of matrix formation (Baillie & Douglas, 2000). On the other hand, earlier studies with a perfused biofilm fermenter (Baillie & Douglas, 1998a) and a cylindrical filter model system (Baillie & Douglas, 1998b) showed that resuspended biofilm cells (which presumably had lost most of their matrix) were some 20 % less resistant to amphotericin B than intact *C. albicans* biofilms, indicating that the matrix could have a contributory role in drug resistance. These findings with resuspended biofilm cells were subsequently confirmed elsewhere (Ramage *et al.*, 2002).

In the study described here, we have isolated and chemically analysed matrix material from biofilms of both *C. albicans* and *C. tropicalis*. Further characterization of matrix composition was achieved by enzymic digestion of biofilms. In a series of experiments designed to investigate biofilm drug resistance, *Candida* biofilms were grown statically and under flow conditions in a modified Robbins device (MRD) to model catheter infections; the susceptibilities of both types of biofilm to antifungal agents were then tested. Mixed-species biofilms of *C. albicans* and *S. epidermidis* were also assayed for antifungal susceptibility after growth under the same static and flow conditions. *S. epidermidis* is the organism most frequently isolated from bacterial implant infections and has been found in polymicrobial infections with *C. albicans* (Jenkinson & Douglas, 2002).

METHODS

Organisms. Two *Candida* species were used in this study. *C. albicans* GDH 2346 (NCYC 1467) was originally isolated at Glasgow Dental Hospital from a patient with denture stomatitis. *C. tropicalis* AAHB 73 was isolated from a patient with a line infection at Crosshouse Hospital, Kilmarnock, Scotland. Both strains were maintained on slopes of Sabouraud dextrose agar (Difco) and subcultured monthly. Every 2 months, cultures were replaced by new ones freshly grown from freeze-dried stocks.

Two strains of *S. epidermidis* (RP62A and M7) were maintained on Colombia blood agar (Oxoid). Strain RP62A (ATCC 35984) is a known slime producer; strain M7 is a slime-negative mutant obtained after chemical mutagenesis of *S. epidermidis* RP62A with mitomycin C (Schumacher-Perdreau *et al.*, 1994). The growth rate, initial adherence, cell-wall composition, surface characteristics and antimicrobial-susceptibility profile of strain M7 are indistinguishable from those of the wild-type (Schumacher-Perdreau *et al.*, 1994).

Medium and culture conditions. Both *Candida* species were grown in yeast nitrogen base (YNB) medium (Difco) containing 50 mM glucose. Batches of medium (50 ml, in 250 ml Erlenmeyer flasks) were inoculated from fresh slopes and incubated at 37 °C for 24 h in an orbital shaker at 60 r.p.m. Cells were harvested and washed twice in 0·15 M PBS, pH 7·2. Before use in biofilm experiments, all washed cell suspensions were adjusted to OD_{520} 0·8.

Tryptic soy broth (Difco) was selected as the liquid medium best able to support the growth of both fungi and bacteria. *C. albicans* GDH 2346 and the two strains of *S. epidermidis* (RP62A and M7) grow at similar rates in this medium (Adam *et al.*, 2002). Cultures were inoculated from fresh slopes and incubated with shaking at 37 °C for 24 h. Cells were harvested, washed twice in PBS and suspended to OD_{520} 0·8 prior to use in biofilm experiments. For mixed-species biofilms, equal volumes of the standardized suspension of each organism were mixed immediately before use.

Isolation of matrix material. Biofilms grown for the extraction of matrix material were formed on sections (4 cm long) of polyvinyl chloride (PVC) Faucher tubes (French gauge 36; Vygon) that had been cut into three equal concave strips. The strips were sterilized by exposure to ultraviolet radiation for 15 min on each side. Standardized cell suspension was added to the concave surface of each strip, and the strips were incubated for 1 h at 37 °C. After removal of non-adherent cells by washing, the strips were transferred to wide-neck 250 ml Erlenmeyer flasks (six strips per flask)

containing YNB (50 ml) with 50 mM glucose. They were then incubated at 37 °C for 48 h in an orbital shaker operating at 60 r.p.m. for biofilm formation.

Biofilm matrix material was isolated using a slight modification of a protocol described previously (Baillie & Douglas, 2000). Catheter strips with their adherent biofilms were transferred to universal bottles (six strips per bottle) each containing 10 ml distilled water. The bottles were sonicated for 5 min in an ultrasonic water bath and vortexed vigorously for 1 min to disrupt the biofilms. Cell suspensions were then pooled and centrifuged. The supernatants were concentrated to one-tenth of the original volume using an Amicon DC2 hollow-fibre system with a 3·0 kDa molecular weight cut-off filter (Millipore) and dialysed at 4 °C for 3 days (3·5 kDa molecular weight cut-off dialysis membrane; Pierce) against five changes (5 l each) of distilled water. The retentates were freeze-dried.

Chemical analysis of matrix material. Protein was determined by the Lowry method, phosphorus by the method of Chen *et al.* (1956), and uronic acid by the method of Bitter & Muir (1962). Total carbohydrate was estimated according to the procedure of Dubois *et al.* (1956), using glucose as a standard. Glucose content was determined enzymically using a glucose oxidase/peroxidase assay kit (Sigma) after hydrolysis of samples in 0·5 M HCl at 100 °C for 5 h. Hexosamine was estimated by the method of Blumenkrantz & Asboe-Hansen (1976) using glucosamine as a standard; before analysis, samples were hydrolysed in 4 M HCl at 100 °C for 12 h.

Enzymic detachment of biofilms. Eight enzymes (all from Sigma) were tested for their ability to detach *Candida* biofilms from plastic surfaces. The enzymes used were: proteinase K extracted from *Tritirachium album*; protease type XIV from *Streptomyces griseus*; deoxyribonuclease 1 type IV from bovine pancreas; *N*-acetylglucosaminidase from *Canavalia ensiformis* (Jack bean); chitinase from *Strep. griseus*; lipase type VII from *Candida rugosa*; phospholipase A2 from bovine pancreas; and lyticase from *Arthrobacter luteus*. All enzyme solutions were prepared immediately before use. Proteinase K, protease type XIV and lyticase were in Na_2HPO_4/NaH_2PO_4 buffer, pH 7·5; deoxyribonuclease 1 type IV and *N*-acetylglucosaminidase were in citric acid/Na_2HPO_4 buffer, pH 5·0; lipase type VII was in Na_2HPO_4/NaH_2PO_4 buffer, pH 7·2; phospholipase A2 was in Tris/maleate/NaOH buffer, pH 8·0; and chitinase was in citric acid/Na_2HPO_4 buffer, pH 6·0.

The detachment assay used was based on that reported by Kaplan *et al.* (2004) for *S. epidermidis* biofilms. Aliquots (100 µl) of standardized *Candida* cell suspension were added to the wells of 96-well polystyrene microtitre plates, and the plates were incubated at 37 °C for 48 h to allow biofilm formation. The growth medium was removed from each well and replaced by an equal volume (100 µl) of test enzyme used at a final concentration of 50 µg ml^{-1}. Control wells received an equal volume of buffer without enzyme. Plates were incubated for 2 h at 25 or 37 °C according to the temperature optimum for the enzyme being tested. Following incubation, biofilms were stained with crystal violet (2 g crystal violet, 0·8 g ammonium oxalate, and 20 ml ethanol per 100 ml) for 2 min, and then twice washed gently with 200 µl distilled water and left to dry. The optical densities of the wells were determined with a Bio-Rad Benchmark microplate reader set to 570 nm.

Biofilm formation under static conditions on PVC catheter disks. Biofilms were formed on small disks (diameter, 0·8 cm) cut from PVC Faucher tubes (French gauge 36; Vygon), as described previously (Hawser & Douglas, 1994; Baillie & Douglas, 1999). Sterile disks were placed in wells of 24-well Nunclon tissue culture plates, and 80 µl of standardized cell suspension was added to each one. After incubation for 1 h at 37 °C (adhesion period), non-adherent organisms were removed by washing with PBS. The disks were then incubated in the wells of fresh plates containing 1 ml YNB with 50 mM glucose, or 1 ml TSB, for 48 h at 37 °C for biofilm formation.

Biofilm formation under flow conditions using the MRD. The MRD is one of the most widely used systems for studying biofilm growth under conditions of continuous flow. It is an artificial multiport sampling catheter, constructed of a perspex block, 41·5 cm long, with a rectangular lumen containing 25 evenly spaced sample ports (Lappin-Scott *et al.*, 1993). The sample studs, also made of perspex, fit tightly into the ports. Each stud has at its bottom end a 1 mm rim into which a catheter disk can be inserted. During incubation, biofilms are formed on these disks and can be removed aseptically by simply taking out the sample studs.

In the experiments described here, a reservoir containing a standardized suspension of the test organism(s) was connected to a peristaltic pump and the MRD via silicone tubing. The entire apparatus was incubated at 37 °C. Cell suspension was pumped through the MRD at a flow rate of 60 ml h^{-1} for 1 h to allow cells to adhere to each of the 25 catheter disks attached to the sample studs. Upon leaving the MRD, the cell suspension was collected in an effluent container. Fresh growth medium (either YNB with 50 mM glucose, or TSB) was then continuously pumped through the MRD at the same flow rate for 48 h. After this time, biofilms formed on the catheter disks could be retrieved by removing the sample studs from the MRD. Following the completion of each experiment, the MRD was sterilized with 0·05 % hibitane, which was pumped through at 60 ml h^{-1} for 1 h. Sterile distilled water was finally pumped through at a rate of 200 ml h^{-1} for 1 h to remove any traces of hibitane.

Susceptibility of biofilms to antifungal agents. After growth under static or flow conditions, *Candida* biofilms and *Candida*/*Staphylococcus* biofilms were treated with amphotericin B (Sigma) or fluconazole (Pfizer) by a procedure described earlier (Hawser & Douglas, 1995; Adam *et al.*, 2002). Freshly prepared stock solutions of the drugs were diluted in growth medium (YNB with 50 mM glucose, or TSB) buffered to pH 7 with 0·165 M MOPS buffer (Sigma). Biofilms (48 h) grown statically or under flow conditions on catheter disks were transferred to wells of 24-well Nunclon plates containing 1 ml of this buffered medium with the test antifungal agent, and incubated for 5 or 24 h at 37 °C. Two different concentrations of amphotericin B (6·5 and 39 µg ml^{-1}; 5 and 30 times the MIC) were used for biofilms of *C. albicans* GDH 2346. Biofilms of *C. tropicalis* AAHB 73 and *Candida*/*Staphylococcus* biofilms were treated with a single concentration of amphotericin B and fluconazole (39 and 12 µg ml^{-1}, respectively; 30 times the MIC for *C. albicans* GDH 2346). Following the drug treatment, biofilms were washed in PBS and biofilm activity was assessed by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay (Baillie & Douglas, 1999; Adam *et al.*, 2002) after transfer of the disks to new wells. The effect of an antifungal agent was measured in terms of XTT reduction by biofilms as compared with values obtained for control biofilms incubated for 5 h in the absence of the agent.

Scanning electron microscopy (SEM). Biofilms were examined by SEM after processing of samples by a freeze-drying technique (Hawser *et al.*, 1998; Baillie & Douglas, 1999), which gives improved preservation of the biofilm matrix. Biofilms formed on catheter disks were fixed with glutaraldehyde (2·5 %, v/v, in 0·1 M cacodylate buffer, pH 7·0), washed gently three times in distilled water, and then plunged into a liquid propane/isopentane mixture (2:1, v/v) at −196 °C before freeze-drying under vacuum (10^{-6} torr, $1{\cdot}3 \times 10^{-4}$ Pa). Samples were finally coated with gold with a Polaron coater and viewed under a Philips 500 scanning electron microscope.

RESULTS AND DISCUSSION

Isolation of matrix material from *Candida* biofilms

Matrix material was prepared from biofilms of two different *Candida* species, *C. albicans* GDH 2346 and *C. tropicalis* AAHB 73. There is no standard extraction procedure for biofilm matrix, and a variety of physical and chemical extractions have been reported (Liu & Fang, 2002; Azeredo *et al.*, 1999). Many of these methods promote leakage of intracellular material (Azeredo *et al.*, 1999). In this study, a physical extraction process was used in an attempt to minimize leakage. This involved gentle sonication, vortexing and centrifugation of biofilms formed on sections of catheter tubing. Two separate preparative procedures were carried out for each organism to provide sufficient material for chemical analysis. The yield from biofilms of *C. tropicalis* (16·5 and 23·6 mg) was much higher than that from biofilms of *C. albicans* (10·7 and 9·4 mg).

Chemical composition of the biofilm matrix

Preparations of biofilm matrix material were analysed for carbohydrate, glucose, protein, hexosamine, phosphorus and uronic acid by colorimetric or enzymic methods. Matrix isolated from *C. albicans* biofilms consisted of carbohydrate (39·6 %, including 32·2 % glucose), together with small amounts of protein (5·0 %), hexosamine (3·3 %), phosphorus (0·5 %) and uronic acid (0·1 %; Table 1). These values largely confirm those reported in an earlier analysis from this laboratory which also revealed the presence of small amounts of mannose and galactose in the matrix (Baillie & Douglas, 2000). Both studies demonstrate that glucose is the major sugar component of *C. albicans* matrix material. However, glucose accounted for a larger proportion of the matrix dry weight in the present investigation. This could be due to a difference in the growth medium: galactose was used as the carbon source in the previous study but was replaced here by glucose.

By contrast, matrix from *C. tropicalis* biofilms consisted mainly of hexosamine (27·4 %), with smaller amounts of carbohydrate (3·3 %, including 0·5 % glucose), protein (3·3 %) and phosphorus (0·2 %; Table 1). The *C. tropicalis* matrix also contained slightly more uronic acid (1·6 %) than that of *C. albicans*. The major difference between the two preparations, however, was that in *C. tropicalis*, hexosamine appeared to replace glucose as the main identifiable sugar component in the matrix (Table 1). As far as we are aware, this is the first reported analysis of the biofilm matrix of *C. tropicalis*. However, a number of bacteria are known to produce similarly large amounts of hexosamine as a matrix component. The best-studied example is *S. epidermidis*, in which hexosamine is present as a polysaccharide of β-1,6-linked *N*-acetylglucosamine residues containing some deacetylated amino groups, and succinate and phosphate substituents (Mack *et al.*, 1996). This polymer, which is sometimes referred to as the intercellular polysaccharide adhesin (PIA), mediates cell–cell interaction within the biofilm (Gotz, 2002) and its production has been related to *S. epidermidis* virulence in catheter-infection models in animals.

Table 1. Analysis of matrix material extracted from biofilms of *C. albicans* GDH 2346 and *C. tropicalis* AAHB 73

The data are mean ± SEM for two independent experiments (with two different matrix preparations) carried out in duplicate or triplicate.

Component	Percentage composition of biofilm matrix for:	
	C. albicans	*C. tropicalis*
Carbohydrate	39·6 ± 0·3	3·3 ± 0·0
Glucose	32·2 ± 1·5	0·5 ± 0·0
Hexosamine	3·3 ± 0·6	27·4 ± 0·2
Phosphorus	0·5 ± 0·0	0·2 ± 0·0
Protein	5·0 ± 0·1	3·3 ± 0·0
Uronic acid	0·1 ± 0·0	1·6 ± 0·0

Enzymic detachment of *Candida* biofilms

An assay devised by Kaplan *et al.* (2004) for *S. epidermidis* biofilms was used to investigate whether *Candida* biofilms could be enzymically detached from plastic surfaces by degradation of the matrix polymers. A range of commercially available enzymes of known specificity was tested. Biofilms were grown in the wells of 96-well polystyrene microtitre plates and then treated with different test enzymes at 37 or 25 °C (according to the temperature optimum) for 2 h at a final enzyme concentration of 50 μg ml^{-1}. After washing, the remaining organisms were stained with crystal violet and the OD_{570} measured using a microtitre plate reader.

Biofilms of *C. albicans* were unaffected by lipase type VII, phospholipase A2 and protease type XIV (Table 2). Treatment with proteinase K, chitinase, DNase I or β-*N*-acetylglucosaminidase resulted in a significant decrease in OD_{570}, suggesting that these enzymes partially degraded matrix material and caused some biofilm detachment from the surfaces of the wells. Interestingly, lyticase, which hydrolyses β-1,3 glucan, had by far the greatest effect, causing an 85 % reduction in optical density ($P<0·001$; Table 2). This result suggests that some of the glucose present in the *C. albicans* matrix could be present as β-1,3 glucan, a polysaccharide which is also a major structural component of the cell wall.

Biofilms of *C. tropicalis* responded rather differently to the enzyme treatments. Phospholipase A2, protease type XIV, proteinase K, DNase I and β-*N*-acetylglucosaminidase had no significant effect (Table 2). By contrast, treatment with

Table 2. Detachment of *Candida* biofilms after exposure to different test enzymes

Enzyme*	Biofilm OD_{570} as a percentage of control value†	
	C. albicans	*C. tropicalis*
Lipase type VII	100·0 ± 5·6	73·9 ± 1·9‡
Phospholipase A2	100·0 ± 3·7	92·2 ± 2·5
Protease type XIV	100·0 ± 4·9	88·3 ± 5·4
Proteinase K	69·2 ± 2·8‡	93·1 ± 5·8
Chitinase	77·2 ± 4·7‡	71·3 ± 3·5‡
DNase I	70·8 ± 4·4‡	100·0 ± 2·2
Lyticase	15·4 ± 1·2§	46·2 ± 2·2§
β-*N*-Acetylglucosaminidase	79·3 ± 2·6‡	94·9 ± 2·6

*All enzyme treatments were carried out for 2 h at 37 or 25 °C with a final enzyme concentration of 50 μg ml^{-1}.

†The data are mean ± SEM of two independent experiments each carried out twice with 36 replicates for every enzyme tested. Control OD_{570} values ranged from 0·119 ± 0·01 to 0·169 ± 0·01 for *C. albicans* biofilms, and from 0·274 ± 0·02 to 0·319 ± 0·01 for *C. tropicalis* biofilms.

‡Value significantly different at $P < 0·05$ from that for the control.

§Value significantly different at $P < 0·001$ from that for the control.

lipase type VII and chitinase did appear to produce some biofilm detachment ($P < 0·05$). Chitinase had a similar effect on biofilms of both *C. tropicalis* and *C. albicans*, indicating that most of the hexosamine present in the *C. tropicalis* matrix was unlikely to be in the form of chitin. It could, instead, be in the form of a chitinase-resistant *β*-1,6-linked polysaccharide like that found in *S. epidermidis* and other biofilm-forming bacteria. The greatest effect on *C. tropicalis* biofilms was again observed with lyticase, which caused a reduction in optical density of over 53 % ($P < 0·001$; Table 2). However, lyticase had less effect on these biofilms than on those of *C. albicans*, whose matrix material contains substantially more glucose (Table 1).

Possible lysis of biofilm cells during their exposure to lyticase was investigated by resuspending the cells after enzyme treatment in 1 M sorbitol buffer, and comparing the optical density with that of suspensions of control (untreated) biofilm cells. With *C. albicans*, exposure to lyticase reduced the optical density readings of the suspensions, suggesting that there could have been some cell lysis during the enzyme treatment (results not shown). Alternatively, the reduction in optical density could simply have been due to dissolution of some of the matrix material. The latter explanation seems more likely, since suspensions of *C. tropicalis* showed no such reduction, even though lyticase is known to induce protoplast formation with this organism (Su & Meyer, 1991).

DNA is now known to be a major matrix component in some bacterial biofilms (Starkey *et al.*, 2004). DNase I had no effect on *C. tropicalis* biofilms, but did cause some detachment of *C. albicans* biofilms. The presence of DNA in the *C. albicans* matrix would be consistent with the higher phosphorus content of the matrix of this organism (Table 1). Biofilms of *C. tropicalis*, but not those of *C. albicans*, were partially detached by treatment with lipase type VII, but both were resistant to the action of phospholipase A2. In this context, it is interesting that *C. tropicalis* is capable of producing a fibrillar layer that contains mannoprotein with covalently linked fatty acids (Kappeli & Fiechter, 1977; Kappeli *et al.*, 1984).

SEM of biofilms grown statically and under conditions of continuous flow

The model system used for static biofilm culture involved the growth of adherent populations for 48 h on the surface of small disks cut from catheters. This model has been well characterized and is known to give reproducible biofilm populations (Hawser & Douglas, 1994; Baillie & Douglas, 1999). To produce flow conditions, an MRD was used.

SEM showed that biofilms formed by *C. albicans* incubated statically on catheter disks consisted of a dense network of yeasts, germ tubes, hyphae and pseudohyphae. As reported previously (Hawser *et al.*, 1998), relatively little matrix material was visible in these biofilms, even when samples were prepared using a freeze-drying technique that gives improved preservation of the matrix. However, biofilms grown in the MRD under flow conditions had an extensive matrix as revealed by SEM (Fig. 1A). This confirmed earlier findings which demonstrate that biofilms subjected to a liquid flow produce substantially more matrix material than those incubated statically (Hawser *et al.*, 1998). In contrast with *C. albicans*, biofilms of *C. tropicalis* synthesized large amounts of extracellular material even during

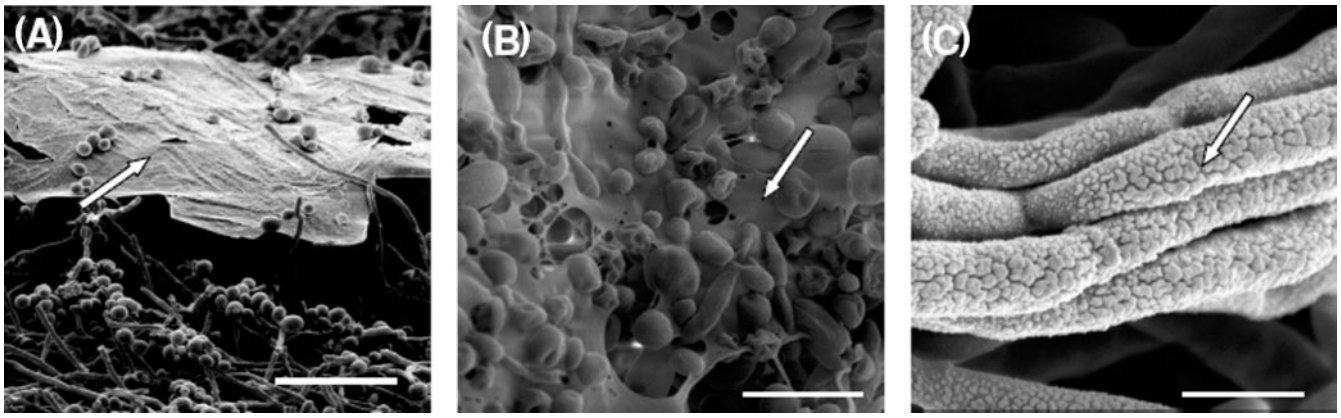

Fig. 1. Scanning electron micrographs of biofilm formation by *C. albicans* (A) and *C. tropicalis* (B, C) on PVC catheter disks. Biofilms were incubated under flow conditions in the MRD (A), or statically (B, C), for 48 h in YNB medium containing 50 mM glucose. Arrows indicate matrix material. Bars, 20 μm (A); 10 μm (B); 2 μm (C).

growth under static conditions, and many of the cells were almost hidden by the enveloping matrix (Fig. 1B). At high magnification, matrix material was clearly visible on the surface of the cells (Fig. 1C).

Drug susceptibility of biofilms grown under static or flow conditions

Biofilms of *C. albicans* grown under static and flow conditions were exposed to different concentrations of amphotericin B at 37 °C for 5 or 24 h. After incubation, the metabolic activity of the biofilms, as measured by XTT reduction, was compared with that of control biofilms incubated in the absence of the drug (Table 3). *C. albicans* biofilms grown under flow conditions were highly resistant to amphotericin B at a concentration five times the MIC; exposure for 24 h had no effect on metabolic activity. At an even higher drug concentration (30 times the MIC), with a shorter exposure time (5 h), the biofilms were rather less resistant. However, for both drug treatments, biofilms formed under flow conditions were significantly more resistant than those grown statically (Table 3). These results differ from those obtained in a previous study in which flow conditions were achieved by gentle shaking of biofilms during incubation, a procedure which promotes the synthesis of matrix material (Hawser *et al.*, 1998). Biofilms grown with or without shaking did not exhibit significant differences in susceptibility to flucytosine, fluconazole or amphotericin B (Baillie & Douglas, 2000). A possible explanation for this is that the shaking procedure, which produced conditions of turbulent flow, was less effective at stimulating matrix synthesis than the laminar flow system provided by the MRD. The morphology and physical properties of some bacterial biofilms are strongly influenced by the magnitude of the shear stresses under which the biofilms are formed (Stoodley *et al.*, 2000). Our present results with *C. albicans* indicate that a constant flow (60 ml h^{-1}) of liquid across the developing biofilm promotes matrix synthesis to an extent that significantly enhances resistance to amphotericin B.

Table 3. Effect of amphotericin B on *C. albicans* GDH 2346 biofilms grown under static and flow conditions

Drug treatment*	XTT formazan formation†			
	Static conditions‡		Flow conditions‡	
	OD_{492}	Percentage of control	OD_{492}	Percentage of control
5× MIC (24 h)	1·19±0·03	60·7±1·30	2·95±0·04	99·4±1·2
30× MIC (5 h)	0·94±0·00	39·6±0·10	1·62±0·22	54·6±7·4

*Biofilms were treated with amphotericin B at five times MIC for 24 h or 30 times MIC for 5 h.
†The data are mean±SEM of two independent experiments carried out in quadruplicate.
‡Results for biofilms grown statically were significantly different from those for biofilms grown under flow conditions ($P<0·001$).

Attempts to grow biofilms of *C. tropicalis* AAHB 73 under flow conditions in the MRD were unsuccessful. This organism grew on, and rapidly blocked, the silicone tubing leading to the device, apparently by producing large amounts of slime. Biofilms of *C. tropicalis* grown statically were totally resistant to the action of both amphotericin B and fluconazole when exposed to high concentrations of the drugs for either 5 or 24 h (Table 4). Rates of drug diffusion through statically grown *Candida* biofilms have been determined recently using a filter disk assay (Al-Fattani & Douglas, 2004). Of several *Candida* species and strains tested, the slowest penetration was observed with *C. tropicalis* AAHB 73. In view of our analytical data on matrix preparations (Table 1), this suggests that drug resistance could be affected not only by the overall extent of matrix formation but also by its composition. Biofilms of *C. tropicalis*, with an extensive, hexosamine-rich matrix, were poorly penetrated by antifungal agents. On the other hand, biofilms of *C. albicans*, with a less-extensive glucose-rich matrix, were more readily penetrated by drugs. Several reports indicate that in bacteria, possession of a mucoid phenotype is associated with decreased susceptibility to antibiotics. For example, biofilms of a mucoid clinical isolate of *P. aeruginosa* are substantially less susceptible to the quinolone antibiotic ciprofloxacin than biofilms of a non-mucoid isolate (Evans *et al.*, 1991). Similarly, biofilms produced by an alginate-overproducing strain of *P. aeruginosa* exhibit a highly structured architecture and are significantly more resistant to tobramycin than biofilms formed by an isogenic non-mucoid strain (Hentzer *et al.*, 2001).

Drug susceptibility of mixed fungal/bacterial biofilms grown under static and flow conditions

Previous work with statically grown *C. albicans* biofilms has indicated that the presence of bacteria (*S. epidermidis*) can enhance biofilm resistance to antifungal agents (Adam *et al.*, 2002). In this study, the drug susceptibility of mixed fungal/bacterial biofilms grown under static and flow conditions was compared. As before, two strains of *S. epidermidis* were used: a slime-producing wild-type (RP62A) and a slime-negative mutant (M7). Strain RP62A produces the intercellular adhesin PIA; M7 is a mutant of strain RP62A that also produces PIA but lacks a 140 kDa antigen termed accumulation-associated protein (Hussain *et al.*, 1997; Gotz, 2002). The mutant is able to form biofilms on PVC disks (Adam *et al.*, 2002), although it was originally reported as being unable to accumulate on glass surfaces (Schumacher-Perdreau *et al.*, 1994). However, the extent of biofilm formation (or production of matrix material) by the mutant is less than that of the wild-type strain, as judged by both SEM and quantitative assays (Adam *et al.*, 2002). The M7 mutant is also more easily eradicated *in vitro* and in animal models by various antibiotics than is the wild-type strain (Schwank *et al.*, 1998).

Mixed-species biofilms of *C. albicans* and *S. epidermidis* RP62A grown statically, or under flow conditions in the MRD, were highly resistant to both amphotericin B and fluconazole (Table 5). At exposure times of 5 and 24 h, the drugs had no effect on the metabolic activity of the biofilms, despite the high drug concentration used (30 times MIC). Moreover, biofilms produced statically were just as resistant as those grown under flow conditions (Table 5). These results contrast with those obtained for single-species *C. albicans* biofilms treated with amphotericin B, for which biofilms grown statically were more susceptible to the drug (Table 3). They suggest that the slime produced by *S. epidermidis* RP62A might partially protect *C. albicans* from amphotericin B in these statically grown, mixed-species biofilms. Preparations of matrix material (slime) from clinical isolates of *S. epidermidis* have been shown to reduce the efficacy of some antibiotics when mixed with the drugs in zone-of-inhibition bioassays (Souli & Giamarellou, 1998). Similar results were obtained when staphylococcal

Table 4. Effect of amphotericin B and fluconazole on biofilms of *C. tropicalis* AAHB 73

ND, Not determined.

Antifungal agent*	XTT formazan formation†		
	Static conditions‡		Flow conditions
	OD_{492}	Percentage of control	
Amphotericin B (5 h)	1·98 ± 0·04	109·7 ± 2·4	ND
Amphotericin B (24 h)	1·92 ± 0·06	104·7 ± 3·4	ND
Fluconazole (5 h)	1·92 ± 0·08	106·5 ± 4·7	ND
Fluconazole (24 h)	1·86 ± 0·06	101·8 ± 3·3	ND

*Amphotericin B was used at a concentration of 39 μg ml^{-1} and fluconazole at 12 μg ml^{-1}. Exposure to each drug was for 5 or 24 h.

†The data are mean ± SEM of two independent experiments carried out in quadruplicate.

‡Results not significantly different from those of the controls ($P > 0·05$).

Table 5. Effect of amphotericin B and fluconazole on mixed-species biofilms of *C. albicans* GDH 2346 and *S. epidermidis* RP62A grown under static or flow conditions

Antifungal agent*	XTT formazan formation†			
	Static conditions‡		Flow conditions‡	
	OD_{492}	Percentage of control	OD_{492}	Percentage of control
Amphotericin B (5 h)	2·86±0·03	98·9±1·0	2·31±0·16	105·8±7·3
Amphotericin B (24 h)	2·87±0·05	99·8±1·7	2·97±0·06	93·2±1·9
Fluconazole (5 h)	2·76±0·06	95·5±2·1	2·18±0·06	100·1±2·8
Fluconazole (24 h)	2·95±0·02	102·4±0·7	3·04±0·06	95·3±1·9

*The concentrations of the two drugs used in this assay were equivalent (30 times MIC). Exposure to each drug was for 5 or 24 h.

†The data are mean±SEM of two independent experiments carried out in quadruplicate.

‡Results not significantly different ($P>0·05$) for biofilms grown under static and flow conditions with identical drug treatments.

slime was mixed with planktonic bacteria in susceptibility testing using a broth-dilution method (Konig *et al.*, 2001). However, attempts to correlate the hydrophobicity or charge of each antibiotic tested with loss of activity due to the slime were unsuccessful (Souli & Giamarellou, 1998).

Mixed-species biofilms containing the slime-negative mutant M7 grown under flow conditions were highly resistant to amphotericin B and fluconazole at both exposure times (5 and 24 h), despite the high drug concentration used (30 times MIC; Table 6). They were, however, slightly less resistant than biofilms containing the slime-producing *S. epidermidis* RP62A treated in the same way (Table 5). For both drug treatments, mixed-species biofilms containing M7 and developed under static conditions were significantly more susceptible than those grown under conditions of continuous flow. The difference was particularly marked for biofilms treated with fluconazole (Table 6). These findings suggest that under flow conditions, enhanced production of matrix material by either *C. albicans* or M7, or both organisms, might afford some protection against antifungal agents.

Overall, our results indicate that drug resistance of *C. albicans* biofilms may be significantly enhanced by increased production of matrix material under flow conditions in the MRD, or by the presence of one or more matrix polymers of *S. epidermidis* in mixed-species biofilms. Biofilms of *C. tropicalis*, on the other hand, are less susceptible to antifungal agents than *C. albicans* biofilms, even when grown statically, possibly due to the synthesis of a hexosamine-containing matrix polymer similar to *S. epidermidis* PIA. Drug diffusion through *C. albicans*/*S. epidermidis* biofilms grown statically on cellulose filters is slower than that through *C. albicans* biofilms or even *C.*

Table 6. Effect of amphotericin B and fluconazole on mixed-species biofilms of *C. albicans* GDH 2346 and *S. epidermidis* M7 grown under static or flow conditions

Antifungal agent*	XTT formazan formation†			
	Static conditions‡		Flow conditions‡	
	OD_{492}	Percentage of control	OD_{492}	Percentage of control
Amphotericin B (5 h)	2·05±0·03	88·7±1·3	2·73±0·01	99·0±0·4
Amphotericin B (24 h)	1·69±0·09	71·8±3·8	2·94±0·09	91·9±2·8
Fluconazole (5 h)	1·53±0·07	66·4±3·0	2·58±0·11	93·5±3·9
Fluconazole (24 h)	1·59±0·11	67·2±4·6	2·92±0·04	91·5±1·3

*The concentrations of the two drugs used in this assay were equivalent (30 times MIC). Exposure to each drug was for 5 or 24 h.

†The data are mean±SEM of two independent experiments carried out in quadruplicate.

‡Results are significantly different at $P<0·01$ for biofilms grown under static and flow conditions with identical drug treatments.

tropicalis biofilms (Al-Fattani & Douglas, 2004). Interactions between different matrix polymers in these mixed-species biofilms could produce a more viscous matrix. Such a finding was reported by Skillman *et al.* (1999) during a study of *Enterobacter agglomerans*/*Klebsiella pneumoniae* biofilms; increased matrix viscosity was advanced as a possible explanation for enhanced resistance to disinfection. Similarly, rheological interactions between matrix polysaccharides from *Pseudomonas* (now *Burkholderia*) *cepacia* and *P. aeruginosa* have been shown to decrease the rates of diffusion and antimicrobial activities of antibiotics (Allison & Matthews, 1992). Clearly, matrix polymers do contribute towards drug resistance in both single-species and mixed-species biofilms containing *Candida*, especially under the flow conditions which prevail in many implant infections. However, biofilm resistance overall is likely to be multifactorial, involving, in addition, drug-resistant physiologies such as dormant 'quiescent' cells and expression of efflux pumps (Gilbert *et al.*, 2002).

ACKNOWLEDGEMENTS

M. A. A.-F. is the recipient of a research studentship from the Ministry of Health, Saudi Arabia. We are indebted to Margaret Mullin for expert assistance with electron microscopy.

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