Mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*

BERIT ADAM, GEORGE S. BAILLIE and L. JULIA DOUGLAS

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

A simple catheter disk model system was used to study the development *in vitro* of mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*, two organisms commonly found in catheter-associated infections. Two strains of *S. epidermidis* were used: a slime-producing wild type (strain RP62A) and a slime-negative mutant (strain M7). In mixed fungal-bacterial biofilms, both staphylococcal strains showed extensive interactions with *C. albicans*. The susceptibility of 48-h biofilms to fluconazole, vancomycin and mixtures of the drugs was determined colorimetrically. The results indicated that the extracellular polymer produced by *S. epidermidis* RP62A could inhibit fluconazole penetration in mixed fungal-bacterial biofilms. Conversely, the presence of *C. albicans* in a biofilm appeared to protect the slime-negative staphylococcus against vancomycin. Overall, the findings suggest that fungal cells can modulate the action of antibiotics, and that bacteria can affect antifungal activity in mixed fungal-bacterial biofilms.

Introduction

Medical implants, such as catheters, prosthetic heart valves and joint replacements, can be colonised by micro-organisms that form an adherent biofilm on the surface of the device [1, 2]. Biofilm cells are organised into structured communities enclosed within a matrix of extracellular material. They are phenotypically different from planktonic or suspended cells; notably, they resist host defences and display a significantly decreased susceptibility to antimicrobial agents [3, 4]. It is likely that multiple mechanisms are involved in drug resistance. These may include an inability of the agent to penetrate the biofilm matrix, phenotypic changes resulting from a low biofilm growth rate, and specific, surface-induced gene expression by biofilm cells [3, 5].

The majority of implant-associated infections are caused by staphylococci, especially *Staphylococcus epidermidis* and *S. aureus* [1]. Some strains of *S. epidermidis* secrete mucoid extracellular polymers (polysaccharides and teichoic acid) that may play a role in the pathogenesis of these infections by promoting biofilm formation. Fungal implant infections are less common than bacterial infections but tend to be more serious and are an increasing problem [1, 6]. They are most frequently caused by the pathogenic *Candida* species, particularly *C. albicans*. *Candida* septicaemias now represent c. 10% of all nosocomial bloodstream infections [7] and are usually catheter-related. Urinary catheters, prosthetic heart valves and cardiac pacemakers are also often associated with candida infections.

Although most implant infections are caused by a single pathogen, polymicrobial infections involving catheters and orthopaedic prostheses have been reported [8, 9]. For example, Costerton *et al.* [9] presented scanning electron micrographs of a mixed species biofilm consisting of bacteria and yeasts that had formed on the plastic surface of an intracardial Hickman catheter. Laboratory cultures yielded *S. epidermidis* and *C. albicans*; the same organisms were isolated from blood cultures when the patient developed septicaemia. Mixed bacterial-fungal biofilms are also associated with infections of endotracheal tubes, biliary stents, silicone voice prostheses and acrylic dentures [3, 8].

Processes involved in adhesion and biofilm formation by staphylococci have been investigated in considerable detail [10]. Recently, several model systems for study-
ing Candida biofilm formation in vitro have also been described [11]. However, there have been no reports of mixed species biofilms consisting of Candida and staphylococci. In this investigation, a simple catheter disk model system [11, 12] was used to follow the development of biofilms containing C. albicans and S. epidermidis. Two strains of S. epidermidis were used: a slime-producing wild type and a slime-negative mutant [13]. The effects of the antifungal agent, fluconazole, and the antibiotic, vancomycin, on mixed species biofilms produced with C. albicans and either the wild-type or mutant strain of S. epidermidis were also studied.

**Materials and methods**

**Organisms and growth media**

Two strains of S. epidermidis (RP62A and M7) were used. Both were kindly provided by Professor C. G. Gemmell, Glasgow Royal Infirmary, and were maintained on Columbia 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 [13]. 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 [13]. C. albicans GDH 2346 (NCYC 1467) was originally isolated at Glasgow Dental Hospital from a patient with denture stomatitis, and was maintained on Sabouraud Dextrose Agar (Difco).

As a result of preliminary experiments with a range of media, Tryptic Soy Broth (Difco) was selected as the optimal growth medium for both bacteria and yeast. C. albicans and the two S. epidermidis strains grew at similar rates in this medium when incubated with shaking at 37°C, with a stationary-phase optical density of 2.5 at 520 nm (OD520).

**Biofilm formation on catheter disks**

Biofilms of C. albicans, S. epidermidis or mixtures of both organisms were grown on small disks (surface area 0.5 cm²) cut from polyvinyl chloride (PVC) Faucher tubes (French gauge 36; Vygon, Cirencester, UK) as described previously [11]. Briefly, the disks were placed in the wells of 24-well Nunclon tissue-culture plates, and 80 μl of a standardised cell suspension (adjusted to OD520 0.8) were applied to the surface of each one. For mixed species biofilms, equal volumes of the standardised suspension of each organism were mixed immediately before use. After incubation for 1 h at 37°C, non-adherent organisms were removed by gentle washing with 0.15 M phosphate-buffered saline (PBS, pH 7.2). The disks were then submerged in 1 ml of growth medium in the wells and incubated for up to 48 h at 37°C to allow the development of biofilms.

Biofilm growth was quantified by three methods: (a) determination of dry weights; (b) incorporation of [3H]leucine; and (c) reduction of the tetrazolium salt, XTT. The radiolabelling procedure involves incubating 48-h biofilms with [3H]leucine for 4 h at 37°C and then determining the radioactivity present in trichloroacetic acid-precipitable material. The colorimetric XTT assay depends on the formation of a brown XTT formazan product after incubation of 48-h biofilms with XTT for 5 h at 37°C. All these methods are described in detail elsewhere for C. albicans biofilms grown in yeast nitrogen base medium [11]. In the present study, the relatively poor incorporation of label observed in method (b) can be attributed to the selection of the amino acid-rich tryptic soy broth as the optimal growth medium for both bacteria and yeast.

**Scanning electron microscopy**

Biofilms formed on PVC catheter disks were fixed with glutaraldehyde, then treated with osmium tetroxide and uranyl acetate as described previously [12]. After dehydration in a series of ethanol solutions, samples were dried overnight in a desiccator, coated with gold with a Polaron coater and viewed with a Philips 500 scanning electron microscope.

For improved preservation of the biofilm matrix, the freeze-drying procedure of Hawser et al. [14] was used. Biofilms formed on catheter disks were fixed with glutaraldehyde and then plunged into a liquid propane-iopentane mixture at −196°C before freeze-drying under vacuum.

**Susceptibility of biofilms to antimicrobial agents**

After growth for 48 h, single species and mixed species biofilms were treated with fluconazole (Pfizer), vancomycin (Sigma), or with a combination of the drugs by a procedure described earlier [15]. Freshly prepared stock solutions of the drugs were diluted in growth medium buffered to pH 7 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma). After incubation of biofilms on catheter disks for 48 h, the growth medium was removed from each well and replaced with 1 ml of buffered medium containing fluconazole (0.4 or 10 μg/ml), vancomycin (50 or 200 μg/ml), or both drugs. The biofilms were incubated for a further 5 h at 37°C and then washed gently in 5 ml of PBS. Biofilm activity was assessed by the XTT reduction assay, after transfer of the disks to new wells. The effect of an antimicrobial 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.
Results

Quantitative analysis of biofilm growth

The development of single species and mixed species biofilms of *C. albicans* and *S. epidermidis* on catheter disks was monitored either by determining biofilm dry weight or by measuring the uptake of [3H]leucine by biofilm cells. These methods were preferred to viable counts, which fail to give reproducible results with *C. albicans* biofilms [12], presumably because of the significant numbers of hyphae present. The two procedures gave broadly comparable results (Table 1). All three single species biofilms showed maximum growth after 24 h, but mixed species biofilms continued to grow up to 48 h. The slime-producing, wild-type strain (RP62A) of *S. epidermidis* appeared to form a more extensive biofilm than did its slime-negative mutant, M7. However, as the growth rates of these strains are identical in planktonic culture [13], the observed differences could be accounted for by matrix material (slime) in biofilms of the wild type that remains bound to cells during dry weight measurements and is labelled with [3H]leucine; the M7 mutant is known to lack a 140-kDa extracellular protein produced by the wild type [16]. Growth of mixed bacterial-fungal biofilms with *C. albicans* also appeared to be significantly greater with the wild type than with the mutant (Table 1) but again the differences could be attributable to the presence of slime in wild-type biofilms. In subsequent experiments investigating the effect of drugs on biofilm formation, a third type of assay which measures metabolic activity of biofilm cells was used.

Scanning electron microscopy

Examination of single species biofilms by scanning electron microscopy showed that, after 24–48 h, *C. albicans* had formed the typical bilayer structure described previously [17] in which a basal yeast layer is covered by a relatively open network of hyphae. Both staphylococcal strains formed dense biofilms, with clearly defined channels between the microcolonies (Fig. 1a, b), although microcolonies of the mutant strain M7 appeared flatter than those of the wild type. Synthesis of matrix material, or slime, was evident with *S. epidermidis* RP62A, but not with M7, and was clearly visible (Fig. 1c) in biofilms processed by a freeze-drying procedure that is known to improve preservation of *C. albicans* biofilm matrix [14].

A similar examination of mixed species biofilms by scanning electron microscopy demonstrated that growth continued between 24 and 48 h, in accordance with the quantitative analyses (Table 1). Both staphylococcal strains showed extensive interactions with *C. albicans*; these interactions were more easily seen with the slime-negative mutant, or by using a preparative procedure that did not preserve the biofilm matrix (Fig. 1d). Staphylococci were present beneath and above the yeast and hyphal layers, and were clearly adherent to both morphological forms of the fungus.

Effect of fluconazole on biofilm activity

The susceptibility of mature, 48-h biofilms to drugs was determined colorimetrically by measuring the ability of biofilm cells to reduce the tetrazolium salt, XTT, to a formazan product. The metabolic activity of drug-treated biofilms was determined as a percentage of that of control biofilms incubated in the absence of the drug. Previously, *C. albicans* biofilms were shown to be highly resistant to the action of the antifungal agent, fluconazole, when compared with planktonic cells of the same strain [15]. Similarly, in this study, exposure of fungal biofilms to the drug at concentrations of 0.4 μg/ml (the MIC) or 10 μg/ml (25 times the MIC) produced only small but reproducible decreases in XTT reduction (Table 2). Neither bacterial strain was affected by fluconazole. The small decrease in metabolic activity seen with fluconazole-treated *C. albicans* biofilms was also observed with mixed species biofilms containing the slime-negative staphylococcal mutant, M7. However, no such decrease was apparent when *C. albicans* was grown together with the wild-type strain, RP62A, suggesting that bacterial extracellular polymeric material might act as a barrier to fluconazole penetration.

**Table 1.** Quantitative measurement of single species and mixed species biofilms formed on catheter disks after incubation for 24 h or 48 h

<table>
<thead>
<tr>
<th>Biofilm organism(s)</th>
<th>Mean (SEM) dry weight (mg)</th>
<th>Mean (SEM) [3H]leucine incorporation (cpm)</th>
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<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
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<tr>
<td><em>C. albicans</em></td>
<td>0.46 (0.10)</td>
<td>0.31 (0.01)</td>
</tr>
<tr>
<td><em>S. epidermidis</em> RP62A</td>
<td>0.40 (0.04)</td>
<td>0.47 (0.07)</td>
</tr>
<tr>
<td><em>S. epidermidis</em> M7</td>
<td>0.36 (0.02)</td>
<td>0.27 (0.05)</td>
</tr>
<tr>
<td><em>C. albicans</em> + RP62A</td>
<td>0.36 (0.10)</td>
<td>0.80 (0.09)</td>
</tr>
<tr>
<td><em>C. albicans</em> + M7</td>
<td>0.27 (0.03)</td>
<td>0.50 (0.01)</td>
</tr>
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</table>

*Data are means and SEM of three independent experiments done in triplicate.
substantially decreased by these high concentrations of vancomycin. Biofilms of the slime-negative strain, M7, were significantly more susceptible (XTT reduction, 40.9%) than those of the wild type (XTT reduction, 83.0%; p < 0.003) at vancomycin 200 µg/ml, again suggesting that the extracellular polymeric material produced by S. epidermidis might constitute a barrier to drug penetration.

**Fig. 1.** Scanning electron micrographs of biofilms formed on PVC catheter disks: (a) single species biofilm of *S. epidermidis* RP62A; (b) single species biofilm of *S. epidermidis* M7; (c) single species biofilm of *S. epidermidis* RP62A processed by freeze-drying to preserve the biofilm matrix; (d) mixed species biofilm of *C. albicans* and *S. epidermidis* M7. Bars, 10 µm (a, b, d) and 1 µm (c).

**Table 2.** Effect of fluconazole on XTT reduction by 48-h single species and mixed species biofilms

<table>
<thead>
<tr>
<th>Biofilm organism(s)</th>
<th>Mean (SEM) percentage XTT reduction* after treatment with fluconazole at a concentration of</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>0.4 µg/ml</td>
<td>10 µg/ml</td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>87.5 (9.5)</td>
<td>84.2 (12.0)</td>
<td></td>
</tr>
<tr>
<td><em>S. epidermidis</em> RP62A</td>
<td>100.4 (7.2)</td>
<td>101.4 (8.1)</td>
<td></td>
</tr>
<tr>
<td><em>S. epidermidis</em> M7</td>
<td>92.1 (6.3)</td>
<td>100.3 (6.4)</td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em> + RP62A</td>
<td>101.6 (4.9)</td>
<td>102.8 (4.8)</td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em> + M7</td>
<td>83.4 (5.9)</td>
<td>95.0 (5.8)</td>
<td></td>
</tr>
</tbody>
</table>

*XTT formazan reduction by biofilms is expressed as a percentage of that of control biofilms incubated in the absence of the drug. Data are means and SEM of three independent experiments done in triplicate. Control values (A492) ranged from 1.44 SEM 0.17 to 1.98 SEM 0.21.
The results of treatment with a combination of vancomycin and fluconazole are shown in Table 3. Single species biofilms of *S. epidermidis* M7 were significantly less resistant than those of *S. epidermidis* RP62A (p < 0.05 and p < 0.003 for low and high concentrations, respectively). Mixed species biofilms of *C. albicans* and M7 were as resistant to the combined drug treatment as *C. albicans* biofilms, but significantly (p < 0.001) more resistant than single species M7 biofilms. This increased drug resistance was not observed with mixed species biofilms containing the wild-type strain, RP62A. Overall, the results support earlier suggestions that slime production contributes to the drug resistance of *S. epidermidis* biofilms. They also indicate that the resistance of slime-negative strains can be enhanced by the presence, in the biofilm, of an unrelated fungal species.

**Discussion**

Although implant-associated infections involving both bacteria and fungi are not uncommon [3], mixed species biofilms of this type have been studied relatively little. In the present investigation, extensive interactions between *S. epidermidis* and *C. albicans* have been demonstrated in biofilms containing both organisms. Scanning electron microscopy revealed that bacteria bound to yeast and hyphal forms of the fungus. The nature of these interactions is unknown but they are likely to be complex. Co-aggregation studies have shown that *C. albicans* also binds to various oral streptococci [18]. Yeast attachment to *Streptococcus gordonii* appears to be mediated by various adhesion–receptor interactions that require the participation of two families of streptococcal polypeptide adhesins, bacterial cell-wall polysaccharide and as yet unidentified yeast cell-surface components [19].

*S. epidermidis* M7, the slime-negative mutant, was able to form single species biofilms on PVC disks, although it was originally reported as being unable to accumulate on glass surfaces [13]. However, the extent of biofilm formation (or production of matrix material) by the mutant was less than that of the wild-type strain RP62A, as judged by both scanning electron microscopy and quantitative assays. Mutant biofilms were also significantly less resistant to vancomycin than those of the wild type, suggesting that the extracellular slime produced by *S. epidermidis* RP62A acts as a barrier to drug diffusion within the biofilm. Matrix material of *C. albicans* biofilms, on the other hand, is produced in substantial amounts only under conditions of liquid flow and was not readily visible in these experiments. Moreover, the susceptibility of single species *Candida* biofilms to antifungal agents, including fluconazole, seems to be largely independent of the amount of matrix material formed, indicating that this material does not constitute a major barrier to drug penetration [20].

In the experiments with mixed species biofilms, fluconazole appeared to inhibit metabolic activity slightly when *C. albicans* was combined with *S. epidermidis* M7, but not when it was combined with strain RP62A. Although the observed difference was small, this result suggests that the slime produced by the wild-type staphylococcus might act as a barrier to fluconazole penetration even though the *C. albicans* matrix alone does not. Alternatively, interactions between the different matrix polymers might result in a more viscous matrix. Such a finding has been reported by Skillman *et al.* [21] during a study of mixed species bacterial biofilms of *Enterobacter agglomerans* and *Klebsiella pneumoniae*; increased matrix viscosity was advanced as a possible explanation for the enhanced resistance to disinfection of these mixed species biofilms. Rheological interactions between polysaccharides from *Pseudomonas cepacia* and *P. aeruginosa* have also been shown to decrease the diffusion and antimicrobial activity of antibiotics [22]. Differences in matrix viscosity may also explain the increased resistance to vancomycin of mutant M7 biofilms when grown in conjunction with *C. albicans*, although neither organism seems to produce an extensive matrix under the growth conditions used here.

Recent studies with single species *P. aeruginosa* biofilms [3, 23] have demonstrated the involvement of cell-to-cell signalling in biofilm development. A specific signalling mutant, deficient in the synthesis of acylhomoserine lactones, formed flat, thin biofilms that were devoid of microcolonies. The acylhomoserine lactone signals that are produced by *P. aeruginosa* are likely to be complex. Co-aggregation studies have shown that *S. epidermidis* also binds to various oral streptococci [18]. Yeast attachment to *Streptococcus gordonii* appears to be mediated by various adhesion–receptor interactions that require the participation of two families of streptococcal polypeptide adhesins, bacterial cell-wall polysaccharide and as yet unidentified yeast cell-surface components [19].

**Table 3.** Effect of combined fluconazole and vancomycin treatment on XTT reduction by 48-h single species and mixed species biofilms

<table>
<thead>
<tr>
<th>Biofilm organism(s)</th>
<th>fluconazole (0.4 µg/ml) + vancomycin (50 µg/ml)</th>
<th>fluconazole (10 µg/ml) + vancomycin (200 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
<td>76.6 (5.8)</td>
<td>77.0 (4.1)</td>
</tr>
<tr>
<td><em>S. epidermidis</em> RP62A</td>
<td>76.9 (5.8)</td>
<td>72.4 (4.9)</td>
</tr>
<tr>
<td><em>S. epidermidis</em> M7</td>
<td>46.1 (5.1)</td>
<td>37.0 (3.2)</td>
</tr>
<tr>
<td><em>C. albicans</em> + RP62A</td>
<td>81.4 (2.8)</td>
<td>78.8 (3.5)</td>
</tr>
<tr>
<td><em>C. albicans</em> + M7</td>
<td>72.8 (3.0)</td>
<td>69.4 (2.6)</td>
</tr>
</tbody>
</table>

*XTT reduction by biofilms is expressed as a percentage of that of control biofilms incubated in the absence of drugs. Data are means and SEM of three independent experiments done in triplicate. Control values (A492) ranged from 1.63 SEM 0.15 to 2.25 SEM 0.18.*

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lactone type of signalling or quorum-sensing molecule appears to be confined to gram-negative bacteria and, not unexpectedly, attempts to identify such compounds in single species *C. albicans* biofilms have been unsuccessful (R. A. Caddick and L. J. Douglas, unpublished results). To date, little information is available on the role of intercellular signalling in mixed species biofilms although it may differ significantly from that observed in single species biofilms [4]. It could be particularly important where competition for attachment to the surface is fierce, and is likely to be a critical factor in the diversity and distribution of microorganisms within a mixed species biofilm.

We are indebted to M. Mullin for expert assistance with electron microscopy.

References