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Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents

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Extracellular polymeric material (EP), comprising the matrix of *Candida albicans* biofilms, was isolated and its composition was compared with that of EP obtained from culture supernatants of planktonically grown (suspended) organisms. Both preparations consisted of carbohydrate, protein, phosphorus and hexosamine, but biofilm EP contained significantly less total carbohydrate (41%) and protein (5%) than planktonic EP. It also had a higher proportion of glucose (16%) and contained galactose, suggesting that it might possess components unique to biofilms. To investigate whether the EP 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 for 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, indicating that drug resistance is unrelated to the extent of matrix formation. However, biofilms formed on two different types of polyvinyl chloride catheter, obtained from different manufacturers, showed differences in susceptibility to amphotericin B, suggesting that drug resistance may arise as a result of highly specific, surface-induced gene expression.

Introduction

Implanted devices, such as catheters, prosthetic heart valves and joint replacements, provide pathogenic microorganisms with a surface on which they can form an adherent biofilm.[1–3] Biofilms consist of layers of cells embedded within a matrix of extracellular polymeric material (EP). Detachment of microorganisms from the biofilm can result in septicaemia, which may respond to conventional drug therapy. However, the biofilm itself is resistant both to host defence mechanisms and to antimicrobial agents, and so represents an ongoing source of infection. Consequently, implant infections are difficult to treat and usually the implant must be removed.[4,5] Although the majority of these infections are caused by bacteria, notably staphylococci, fungal infections are becoming increasingly common. They are most often caused by pathogenic *Candida* spp., particularly *Candida albicans*, which is now recognized as one of the most important agents of hospital-acquired infection.[6]

Several model systems for studying *Candida* biofilm formation *in vitro* have been developed recently, including the perfused biofilm fermenter[7] and the cylindrical cellulose filter.[8] The simplest system[9] involves growing adherent cell populations on the surfaces of small discs cut from catheters.[10] Growth can be monitored quantitatively by dry weight measurements and by colorimetric or radioisotope assays.[9,10] Biofilms of *C. albicans* produced by this method consisted of yeasts, hyphae and pseudohyphae, arranged in a bilayer structure.[11] A matrix of EP surrounded the biofilm cells and the synthesis of matrix material increased markedly when developing biofilms were subjected to a liquid flow.[12] The biofilms were resistant to the action of five clinically important antifungal agents, including amphotericin B and fluconazole.[13]

The mechanisms by which *Candida* biofilms resist the action of antifungal agents are not known. Possible resistance mechanisms include drug exclusion by the biofilm matrix and phenotypic changes resulting from nutrient limitation or a low growth rate.[14] In a previous investigation,[7] a perfused biofilm fermenter was used to generate *C. albicans* biofilms at different growth rates, and the susceptibility of the biofilm cells to amphotericin B was compared with that of planktonic organisms grown at the

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same rates in a chemostat. The results showed that biofilms were resistant to the drug at all growth rates tested whereas planktonic cells were resistant only at low growth rates. A subsequent study using a different model system[8] demonstrated that glucose-limited and iron-limited biofilms grown at the same low rate were equally resistant to amphotericin B.

In this investigation, we have explored the possibility that the matrix of EP might act as a barrier to the diffusion of antifungal agents and so limit the access of drugs to organisms deep in the biofilm. We exploited the earlier finding[12] that synthesis of matrix material could be dramatically increased by incubating catheter discs with gentle shaking to produce a flow of liquid over the surface of the developing biofilm. Biofilms showing minimal matrix synthesis (grown statically) and maximal matrix synthesis (grown with shaking) were tested for their susceptibility to a range of antifungal agents. In addition, the chemical composition of biofilm EP was investigated and compared with that of EP isolated from culture supernatants of planktonic cells.

Materials and methods

Organism and growth medium

C. albicans GDH 2346, a denture stomatitis isolate, was used in all experiments. It was maintained on Sabouraud dextrose agar (Difco Laboratories, Detroit, MI, USA) and was grown in liquid culture, or as biofilms, in yeast nitrogen base (Difco) containing glucose or galactose, as described previously.[10]

Biofilm formation

Biofilms were formed on small discs (surface area, 0.5 cm^2) cut from polyvinyl chloride (PVC) Faucher tubes (French gauge 36; Vygon, Cirencester, UK), as reported elsewhere.[10] In some experiments, discs were cut from PVC vena cava catheters (French gauge 40; Jostra, Hirrlingen, Germany). The discs were placed in wells of 24-well Nunclon tissue culture plates (Nalge Nunc International, Rochester, NY, USA), and a washed cell suspension (80 μL), standardized to an optical density of 0.8 at 540 nm, was applied to the surface of each one. After incubation for 1 h at 37°C (adhesion period), non-adherent organisms were removed by washing with 0.15 M phosphate-buffered saline (PBS) pH 7.2. The discs were then incubated in the wells for 48 h at 37°C, submerged in growth medium (1 mL, containing 50 mM glucose), to allow biofilm formation. In some experiments, biofilms were formed on catheter discs incubated with gentle shaking, at 15 rpm,[12] instead of statically.

Biofilms grown for EP extraction were formed on sections (4 cm long) of Faucher tube that had been cut aseptically into three equal concave strips. Standardized cell suspension was added to the 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 a Petri dish (five strips per dish) containing yeast nitrogen base supplemented with 500 mM galactose and incubated at 37°C for 5 days to allow biofilm formation. A 5 day incubation period in medium containing 500 mM galactose was used to facilitate comparison with planktonic EP, the production of which is optimal under these conditions.[15]

Isolation of EP

Planktonic cultures were grown for 5 days at 37°C in yeast nitrogen base containing 500 mM galactose, and EP was prepared by freeze-drying dialysed culture supernatants, as described previously.[16] Biofilm EP was isolated using a slight modification of this protocol. Briefly, catheter strips with their adherent biofilms were transferred to universal bottles (five strips per bottle) each containing 10 mL of distilled water. The bottles were sonicated for 5 min 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 (Millipore Ltd, Watford, UK) and dialysed at 4°C for 3 days against five changes (5 L each) of distilled water. The retentates were freeze-dried.

Analysis of EP

EP preparations from planktonic and biofilm cultures were analysed quantitatively for total carbohydrate (with mannose as a standard), phosphorus, protein (with bovine serum albumin as a standard), glucose and hexosamine (with glucosamine as a standard) using procedures described elsewhere.[15] Samples of EP were also assayed for uronic acids,[17] and for pyruvyl[18] and acetyl[19] substituents. Before analysis for carbohydrate, EP preparations were treated with detergent to remove any green pigment that might cause interference in the assay.[15] EP was dissolved in 0.1% Triton X-100 and the solution was stirred continuously at room temperature for 5 h. Detergent-treated EP was recovered by precipitation with acetone (2 volumes; 2 h at 4°C), washed with ether, evaporated to dryness *in vacuo* and redissolved in water as required. EP was completely soluble in water.

Acid hydrolysates of biofilm EP (2 M HCl at 100°C for 3 h) were also analysed by high-performance anion-exchange chromatography using a Binary Gradient high-performance liquid chromatography (HPLC) system (Gilson Medical Electronics, Villiers-Le-Bel, France), eluent degas module (Dionex Corporation, Sunnyvale, CA, USA) and PAD electrochemical detector (Dionex). The column used in conjunction with this equipment was a Carbopak PA-100 (4 × 250 mm; Dionex).

Susceptibility of biofilms to antifungal agents

Biofilms, grown with or without shaking, were tested for susceptibility to amphotericin B (Sigma, St Louis, MO, USA), flucytosine (5-fluorocytosine; Sigma), fluconazole (Pfizer Ltd, Sandwich, Kent, UK), itraconazole (Janssen Research Foundation, Beerse, Belgium) and ketoconazole (Janssen) using a method described previously.[9,13] Briefly, 48 h biofilms on catheter discs were incubated statically for 5 h at 37°C in wells containing antifungal agents diluted from stock solution in growth medium. The medium contained 50 mM glucose and was buffered to pH 7 with 0.165 M morpholinepropanesulphonic acid (MOPS; Sigma). Amphotericin B was used at ⩽50 mg/L, azoles at ⩽96 mg/L and flucytosine at ⩽250 mg/L. After incubation, biofilms were washed gently in PBS and biofilm activity was measured using assays of [^{3}H]leucine uptake.[9,10] The effect of an antifungal agent was assessed in terms of [^{3}H]leucine incorporation by biofilms calculated as a percentage of that for control biofilms incubated in the absence of the agent.

Results

Composition of the biofilm matrix

EP surrounding fungal cells in biofilms was isolated and its composition was compared with that of EP obtained from culture supernatants of planktonically grown *C. albicans.* The overall composition of planktonic EP was very similar to that reported previously[15] for the same strain grown under identical conditions. It consisted largely of carbohydrate (86%, including 4.7% glucose), together with smaller amounts of protein (8%) and phosphorus (0.3%; Table). By contrast, biofilm EP contained significantly less total carbohydrate (41%) and protein, but had a higher proportion of glucose (16%) than planktonic EP. Although our analyses accounted for all of the dry weight of planktonic EP, approximately half of the dry weight of biofilm EP remained unidentified and may represent one or more unique components. Neither EP preparation contained detectable amounts of uronic acid, or pyruvyl or acetyl residues.

Additional analyses by HPLC confirmed that glucose was the most abundant monosaccharide in biofilm EP, accounting for 19.3% of EP dry weight. Galactose and mannose were present in smaller amounts (3.1% and 9.0%, respectively). Two other unidentified sugars, representing 26% of the total sugar content (11% of EP), were also detected.

Table. Analysis of biofilm and planktonic EP[a]

Component	Percentage composition of	
	biofilm EP	planktonic EP
Carbohydrate	41.1 ± 10.5	86.5 ± 5.3
Phosphorus	0.4 ± 0.1	0.3 ± 0.1
Protein	5.2 ± 0.8	8.3 ± 0.2
Glucose	15.9 ± 5.7	4.7 ± 0.4
Hexosamine	3.4 ± 0.8	0.1 ± 0.1

[a]The data are means ± S.E.M. for three independent experiments (with three different preparations of both biofilm EP and planktonic EP) carried out in duplicate.

Susceptibility to antifungal agents of biofilms incubated under static and flow conditions

To assess the possible role of matrix material in the resistance of *C. albicans* biofilms to antifungal agents, susceptibility profiles of biofilms incubated statically (which possessed a sparse matrix) were compared with those for biofilms incubated with gentle shaking (which produced copious amounts of matrix material). Biofilms grown for 48 h, with or without shaking, were incubated statically with different concentrations of various drugs for a further 5 h at 37°C. The ability of the biofilms to take up [^{3}H]leucine was then determined as a measure of their metabolic activity. Most of the drugs tested had no effect on [^{3}H]leucine uptake by biofilm cells, even at high concentrations (Figure 1). This is in contrast to their effect on planktonic cells as reported previously.[13] For example, flucytosine was ineffective at 1250 × MIC (Figure 1b), fluconazole at 240 × MIC (Figure 1c) and both itraconazole and ketoconazole at 3840 × MIC (data not shown). However, at concentrations around the MIC (1.3 mg/L), amphotericin B inhibited [^{3}H]leucine incorporation into biofilm cells by *c.* 50% (Figure 1a). Biofilms grown with or without shaking did not show significant differences in susceptibility to any of the drugs, indicating that drug resistance was unrelated to the extent of matrix formation. A more detailed investigation of the inhibitory effect of amphotericin B between 0 and 50 min (Figure 2) confirmed that this was also true for shorter incubation periods. However, differences in catheter type produced large differences in susceptibility to amphotericin B: biofilms formed on vena cava catheter discs were more susceptible than those grown on Faucher tubes (Figure 3). Both types are made of PVC, although they are supplied by different manufacturers. The differences in susceptibility were observed for biofilms with or without an extensive matrix, i.e. grown with shaking or statically (Figure 3).

Discussion

Various mechanisms have been proposed to explain the recalcitrance of biofilms to antimicrobial agents. Prominent among these is the suggestion that the matrix of extracellular polymeric material, sometimes known as the glycocalyx, may exclude or limit the access of a drug to organisms

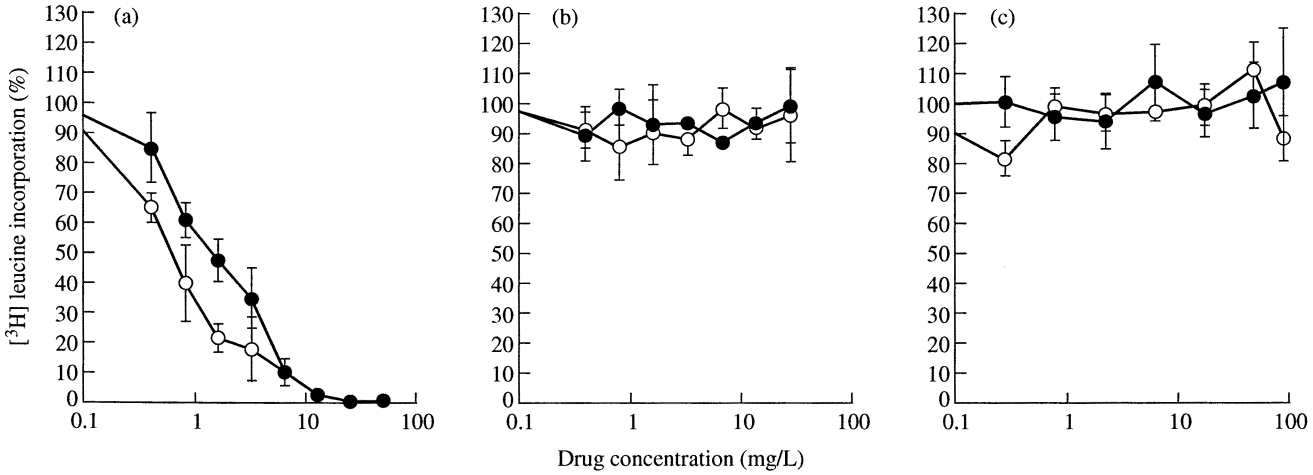

Figure 1. Effect of amphotericin B (a), flucytosine (b) and fluconazole (c) on *C. albicans* biofilms grown statically (●) or with gentle shaking (○). [^{3}H]Leucine incorporation by biofilms was determined as a percentage of that for control biofilms incubated in the absence of the antifungal agent. Results represent mean values (± S.E.M.) from three independent experiments carried out in triplicate.

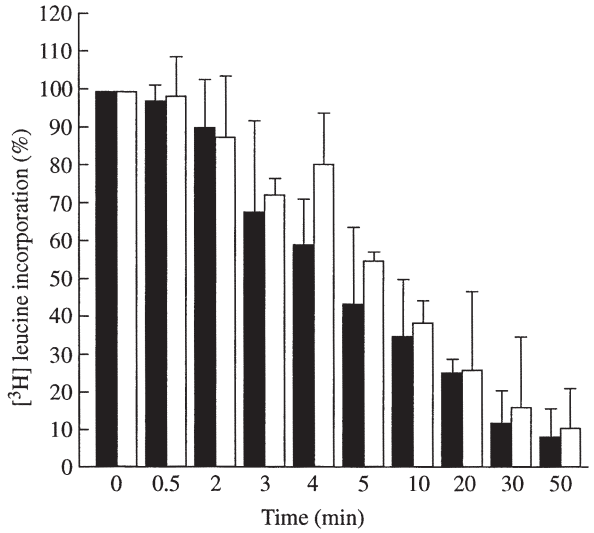

Figure 2. The effect of amphotericin B with time on *C. albicans* biofilms grown statically (■) or with gentle shaking (□). After incubation with the drug for the time period indicated, [^{3}H]leucine incorporation by biofilms was determined as a percentage of that for control biofilms incubated in the absence of the drug. Results represent mean values (± S.E.M.) from three independent experiments carried out in triplicate.

deep in the biofilm. Synthesis of matrix material during the formation of *C. albicans* biofilms on catheter discs is highly dependent on the conditions of incubation. Under static conditions matrix synthesis is minimal, but it can be greatly enhanced by subjecting developing biofilms to a liquid flow.[12] In this study, these findings were exploited to investigate whether the biofilm matrix acts as a barrier to drug penetration. Susceptibility to different antifungal agents was compared after growth of biofilms statically or with gentle shaking to produce a flow of liquid over the surface of the cells. The results clearly show that the extent of matrix formation did not affect the susceptibility of biofilms to any of the drugs. Biofilms, grown with or without shaking, were resistant to flucytosine and to three azole compounds, even at concentrations greatly in excess of the MICs. Similar observations with statically grown biofilms and azoles were made previously using a different model system[7] and may partly reflect the fungistatic nature of these drugs. Amphotericin B, on the other hand, did have an inhibitory effect on the activity of biofilm cells, but this was not abrogated by an increased synthesis of matrix material.

Possible drug exclusion by the matrix of bacterial biofilms seems to depend on a number of factors, including the nature of the antibiotic and the binding capacity of the matrix towards it.[14,20] For compounds such as tobramycin and cefsulodin, reductions in diffusion coefficients across the matrix, relative to liquid media, are insufficient to account for the different susceptibilities of *Pseudomonas aeruginosa* biofilm cells.[21,22] On the other hand, mucoid strains of *P. aeruginosa* grown as biofilms are significantly less susceptible to the quinolone ciprofloxacin than are non-mucoid strains.[23] With *C. albicans* biofilms, our results indicate that the matrix does not constitute a barrier to the penetration of five clinically important antifungal agents of differing chemical structure. Previous studies with a perfused biofilm fermenter[7] and a cylindrical filter model system[8] 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, suggesting that the matrix might play a minor role in drug resistance. However, the detailed investigation of

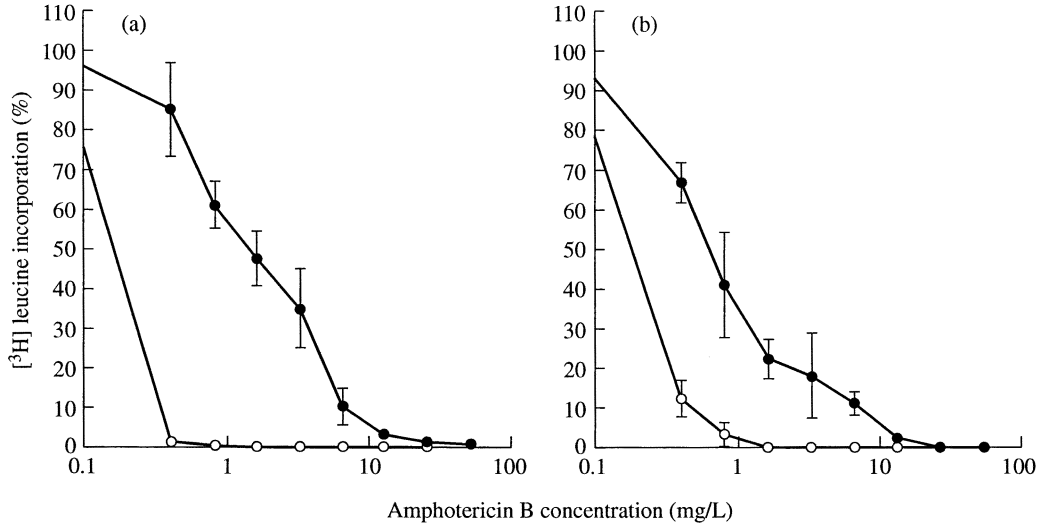

Figure 3. Effect of amphotericin B on *C. albicans* biofilms grown statically (a) or with shaking (b) on PVC discs cut from Faucher tubes (Vygon) (●) or vena cava catheters (Jostra) (○). [^{3}H]Leucine incorporation by biofilms was determined as a percentage of that for control biofilms incubated in the absence of the antifungal agent. Results represent mean values (± S.E.M.) from three independent experiments carried out in triplicate.

amphotericin action on biofilms in experiments reported here provides no support for that conclusion.

Certain bacterial genes are known to be switched on at a surface[24,25] and there is mounting evidence for the involvement of cell–cell signalling in the development of bacterial biofilms.[26–29] Since the drug resistance of *Candida* biofilms cannot be attributed to a matrix barrier effect or to a low growth rate,[7] it seems increasingly likely that contact-induced gene expression may be the mechanism by which drug resistance is acquired. It is already known that synthesis of new proteins occurs after attachment of the yeast to certain surfaces.[30] Moreover, previous work from this laboratory has shown that exclusively yeast-form biofilms are observed with *C. albicans* GDH 2346 growing on cylindrical cellulose filters,[8] but not on cellulose acetate membrane filters,[7] suggesting that morphogenesis is dependent on highly specific contact-induced gene expression. The present study has demonstrated that biofilms formed on two different types of PVC catheter, obtained from different manufacturers, showed significant differences in susceptibility to amphotericin B. It is also noteworthy that antifungal susceptibilities reported here for all five drugs do not wholly correspond with previous values determined for biofilms of the same strain of *C. albicans* using a type of PVC catheter that is no longer produced.[13] Clearly, catheters made of the same material (PVC), but perhaps with a different plasticizer content or composition, can generate *C. albicans* biofilms with varying resistance properties, suggesting that drug resistance may also arise as a consequence of highly specific, surface-induced gene expression.

Matrix polymers of bacterial biofilms are primarily exopolysaccharides and many of them are negatively charged. Smaller amounts of proteins, nucleic acids and various other components may also be present. The polysaccharides vary considerably from species to species according to the nature of the sugar residues involved, their linkages and the presence of uronic acids or acetyl, pyruvyl and succinyl substituents.[31,32] Analytical evidence presented so far indicates that, in the majority of environments, biofilm bacteria produce exopolysaccharides of the same composition as those formed in planktonic cultures and that biofilm-specific polysaccharides are rarely found.[33] However, a recent study by Ruiz and co-workers[34] demonstrated that biofilms of *P. fluorescens* produce a distinct matrix polymer of high molecular mass that is not synthesized by equivalent planktonic cells; interestingly, the analyses reported by these authors, like those presented here, failed to account for the total weight of matrix material. Our analyses of EP isolated from biofilms and planktonic cultures of *C. albicans* revealed both qualitative and quantitative differences in composition. Quantitative analysis of planktonic EP indicated a composition very similar to that reported previously for the same strain.[15] By comparison, biofilm EP contained much less carbohydrate and rather less protein. Both preparations contained glucose, mannose and hexosamine, but galactose was also detected in biofilm EP. Glucose was the most abundant monosaccharide in biofilm EP, whereas planktonic EP contained more mannose, probably present as mannoprotein.[15] These analytical data suggest that *C. albicans*, unlike many bacteria, may produce biofilm-specific EP. If these findings were confirmed, they would provide further evidence for contact-induced gene expression in *Candida* biofilms.

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