BACTERIAL CHARACTERISATION

Production of extracellular matrix by *Candida albicans* biofilms

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Growth of *Candida albicans* biofilms and production of extracellular matrix were monitored by dry weight, colorimetric and radioisotope assays, and by scanning electron microscopy. Under static incubation conditions synthesis of matrix material was minimal, but increased dramatically when developing biofilms were subjected to a liquid flow with the result that the cells were enveloped in extracellular polymer. These findings suggest that production of matrix material could contribute to the resistance of biofilm cells to antifungal agents *in vivo*.

Introduction

The major complication associated with the use of medical implants such as intravascular catheters or joint prostheses is infection. Micro-organisms can colonise these devices and form biofilms consisting of layers of cells embedded within a matrix material [1–7]. Implant infections are usually difficult to treat because biofilm micro-organisms are resistant to antimicrobial therapy [2, 7, 8]. Although fungal implant infections are less common than bacterial ones, they tend to be more serious [4, 9]. They are most often caused by the pathogenic *Candida* spp, particularly *C. albicans*, which is now recognised as one of the most important agents of hospital-acquired infection [10].

Recently, a model system was devised for studying candida biofilms growing on the surface of small disks of catheter material [11, 12]. Growth of the biofilms was monitored quantitatively by dry weight measurements and by colorimetric or radioisotope assays. Biofilm formation by 15 different isolates of *C. albicans* and a number of other *Candida* spp. was investigated with this system. Previous studies included examination of biofilm formation by scanning electron microscopy (SEM). This demonstrated that yeast-phase cells of *C. albicans* adhered to the surface of polyvinyl chloride (PVC) catheter disks and, during subsequent incubation for 48 h, formed a dense biofilm consisting

of yeast cells, germ tubes, pseudohyphae and hyphae. Extracellular polymeric material was visible on the surface of some of these morphological forms, but its production under the static incubation conditions used was not extensive [11]. This paper reports that synthesis of the matrix material by growing biofilms can be dramatically increased by incubating the catheter disks with gentle shaking.

Materials and methods

Organism and culture conditions

C. albicans GDH 2346 was grown in Yeast Nitrogen Base Medium (Difco) containing glucose or galactose as described previously [11, 13]. Before use in biofilm experiments, all washed cell suspensions were adjusted to an OD₅₂₀ of 0.8.

Biofilm formation

Biofilms were grown on small disks (surface area, 0.5 cm²) cut from PVC central venous catheters as described previously [11]. Briefly, the disks were placed in wells of 24-well Nunclon tissue culture plates and standardised cell suspension (80 µl) 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 (pH 7.2). The disks were then incubated in the wells for 48 h at 37 °C, submerged in 1 ml of growth medium (biofilm formation). In other experiments, biofilms were formed on catheter disks incubated with shaking (2–60 rpm), instead of statically. After the 1-h adhesion period (static incubation), non-adherent organisms were removed by washing as

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before. Each disk was then transferred to an Erlenmeyer flask (100 ml) containing 20 ml of growth medium and shaken at the appropriate speed for 48 h at 37°C. In control experiments, disks without cells were incubated with or without shaking.

Measurement of biofilm growth

Quantitative measurements of biofilm growth were made by dry weight determinations and assays involving the incorporation of [^3H]leucine or reduction of the tetrazolium salt, MTT, as described previously [11].

Scanning electron microscopy (SEM)

Initially, biofilms were fixed and prepared for SEM by a procedure which involved critical point drying, as described previously [11]. To improve the visualisation of extracellular matrix material, an alternative freeze-drying technique [14] was developed. Here, biofilms formed on catheter disks were fixed with glutaraldehyde 2.5% v/v in 0.1 M cacodylate buffer (pH 7.0), washed 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).

Results and discussion

Scanning electron microscopy of biofilms formed by *C. albicans* GDH 2346 incubated statically confirmed previous observations [11]: after 48 h there was a dense network of yeasts, germ tubes, pseudohyphae and hyphae, with small amounts of extracellular polymeric material present on the surface of some of these cells (Fig. 1A). By contrast, incubation of catheter disks with gentle shaking markedly increased the synthesis of extracellular matrix material. Slow shaker speeds (2–5 rpm) produced biofilms consisting of a mixture of yeasts and hyphae, as before, but the cells were surrounded by a thick extracellular matrix. At higher speeds (10–30 rpm), biofilm cells were largely hidden under an extensive ‘canopy’ of matrix material (Fig. 1B, C). Shaking speeds in excess of 60 rpm virtually precluded biofilm development and very few cells were observed on the catheter surface.

Production of matrix material appeared to be unaffected by the nature and concentration of the carbon source used in the growth medium; media containing 4 mM glucose, 50 mM glucose or 500 mM galactose all gave biofilms with similar amounts of extracellular matrix as judged by SEM. However, investigation of various

Fig. 1. Scanning electron micrographs of biofilm formation by *C. albicans* GDH 2346 on PVC catheter disks after critical point drying of samples. Biofilms were incubated for 48 h statically (A), or with shaking at 10–30 rpm (B, C). Bar, 10 μm .

Fig. 2. Scanning electron micrographs of biofilm formation by *C. albicans* GDH 2346 on PVC catheter disks after freeze-drying of samples. Biofilms were incubated for 48 h statically (**A**), or with shaking at 5 rpm (**B**) or 15 rpm (**C**). Control disks were shaken at 5 rpm without cells (**D**). Bar, 10 μ m.

preparative techniques for SEM revealed that the dehydration procedure used markedly affected the extent to which this biofilm structure was preserved. Consistently better results were obtained by a freeze-drying technique [14]. With this procedure even biofilms grown statically were seen to contain cells clearly linked together by strands of extracellular polymer (Fig. 2A). Incubation of the catheter disks with slow shaking (5 rpm) increased the synthesis of this material, which seemed to envelope the cells completely (Fig. 2B). At higher speeds, the matrix was very extensive and completely obscured the cells (Fig. 2C). In control experiments where the catheter disks were incubated with shaking in the absence of *C. albicans*, the PVC surface was clearly visible and devoid of extracellular polymer (Fig. 2D). Other dehydration procedures, including air drying or chemical drying with Peldri II [15] and hexamethyldisilazane [16], resulted in little or no preservation of the matrix structure.

Quantitative measurement of biofilms incubated at low shaking speeds (2–5 rpm) gave values similar to those

obtained with biofilms which had been incubated statically by all methods used (Table 1). However, at

Table 1. Quantitative measurement of 48-h biofilms of *C. albicans* GDH 2346 incubated statically or with shaking*

Shaking speed (rpm)	Dry weight (mg)	[3 H]leucine incorporation (cpm)	MTT formazan formation (A_{540})
0	1.92	9765	1.16
2	1.94	9788	1.14
5	1.97	9887	1.15
10	2.23 [†]	10105 [†]	1.08
20	2.46 [†]	10260 [†]	1.03
30	2.44 [†]	10260 [†]	1.03
45	1.41 [‡]	7075 [‡]	0.78 [‡]
60	ND	ND	ND

ND, not detected.

*Each value is the mean of three independent experiments done in triplicate.

[†]Value significantly different at $p < 0.05$ from that obtained with strain GDH 2346 grown under static conditions.

[‡]Value significantly different at $p < 0.001$ from that obtained with strain GDH 2346 grown under static conditions.

higher speeds (10–30 rpm), values for dry weights and [^3H]leucine incorporation were significantly greater ($p < 0.05$). Interestingly, there were no corresponding increases in MTT formazan formation (Table 1). These differences may reflect the differing sensitivities of the three types of assay to the presence of extracellular matrix material. When biofilms were incubated at a shaking speed of 45 rpm, there were significant decreases ($p < 0.001$) in all three parameters as compared with values obtained in the static system. At the highest shaking speed of 60 rpm, quantitative measurements confirmed the SEM observations that biofilm formation was completely inhibited, presumably due to the intense shear forces generated at this speed (Table 1).

The matrix material of bacterial biofilms, sometimes known as the glycocalyx, appears to consist largely of exopolysaccharides. It is thought to act as a physical barrier to the penetration of certain antibiotics and may partly account for the recalcitrance of biofilms to therapeutic agents [8]. Some bacteria are known to produce copious amounts of slimy matrix; this has been well documented with coagulase-negative staphylococci, which are the commonest bacterial agents of implant infection [17]. Extensive production of matrix material or 'slime' by fungal biofilms has not been reported previously. The present results indicate that gentle shaking to produce a flow of liquid over developing *Candida* biofilms increases the synthesis of extracellular material. Such conditions may resemble those found *in vivo* and biofilms *in vivo* may therefore afford greater protection from both antifungal agents and host defence mechanisms. Preliminary results indicate that candida biofilms grown with shaking are even more resistant to amphotericin B than biofilms grown statically, at least over short time periods (unpublished observations). The chemical nature of the extracellular matrix material and the mechanisms by which it is produced are unknown, although it seems likely that it will contain mannoproteins, cell wall components including adhesins that can be sloughed off the yeast surface in planktonic cultures [18].

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