Candida biofilms and their role in infection

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Pathogenic fungi in the genus *Candida* can cause both superficial and serious systemic disease, and are now recognized as major agents of hospital-acquired infection. Many *Candida* infections involve the formation of biofilms on implanted devices such as indwelling catheters or prosthetic heart valves. Biofilms of *Candida albicans* formed *in vitro* on catheter material consist of matrix-enclosed microcolonies of yeasts and hyphae, arranged in a bilayer structure. The biofilms are resistant to a range of antifungal agents currently in clinical use, including amphotericin B and fluconazole, and there appear to be multiple resistance mechanisms. Recent studies with mixed biofilms containing *Candida* and bacterial species suggest that extensive and striking interactions occur between the prokaryotic and eukaryotic cells in these adherent populations.

In most natural environments, microorganisms exist predominantly as biofilms rather than as planktonic or free-floating cells. Biofilms are structured microbial communities that are attached to a surface. Individual microorganisms in biofilms are embedded within a matrix of – often slimy – extracellular polymers, and characteristically display a phenotype that is markedly different from that of planktonic cells. Crucially, they are significantly less susceptible to antimicrobial agents [1–3]. The first example of a biofilm to be recognized in medical systems was dental plaque on tooth surfaces, but recent estimates suggest that a substantial proportion of human infections involve biofilms [4]. Many of these are implant-related infections in which adherent microbial populations are found on the surfaces of devices such as catheters, prosthetic heart valves and joint replacements [5]. Matrix-enclosed biofilm microorganisms can also be detected in tissues taken from non-device-related chronic infections such as cystic fibrosis [6]. Biofilm infections can be caused by a single microbial species or by a mixture of bacterial or fungal species [7,8]. Bacterial biofilms and their role in disease have been studied intensively in recent years and there is now a considerable amount of information available on their structure and properties, particularly those of Gram-negative bacteria [9,10]. Much less is known about fungal biofilms. This article reviews biofilm formation by pathogenic fungi in the genus *Candida*, the fungal system that is currently receiving most attention.

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Candida infections and biofilms

A relatively small number of *Candida* species are pathogenic for humans. These organisms are capable of causing a variety of superficial and deep-seated mycoses that are distributed worldwide. All are opportunistic pathogens, liable to attack immunocompromised hosts or those debilitated in some other way. The principal pathogen of the genus is the fungus responsible for thrush, *Candida albicans*, which can grow either as oval budding yeasts, as continuous septate hyphae or as pseudohyphae; all of these morphological forms are usually seen in infected tissue [11]. *Candida* species are now recognized as major agents of hospital-acquired infection. Their emergence as important nosocomial pathogens is related to specific risk factors associated with modern medical procedures, notably the use of immunosuppressive and cytotoxic drugs, powerful antibiotics that suppress the normal bacterial flora, and implanted devices of various kinds. Recent data from the US National Nosocomial Infections Surveillance System rank these organisms as the fourth most common cause of bloodstream infection, behind coagulase-negative staphylococci, *Staphylococcus aureus* and enterococci. Mortality rates are high (35%), and treatment is costly [12]. *Candida* spp. are also frequently identified as agents of nosocomial pneumonias and urinary tract infections. Almost invariably, an implanted device such as an intravascular or urinary catheter, or endotracheal tube, is associated with these infections and a biofilm can be detected on the surface of the device [13–15]. Other devices totally implanted into the body, such as prosthetic heart valves, cardiac pacemakers and joint replacements (e.g. hip or knee), are also liable to candidal infection, usually at the time of surgical placement.

The surgically implanted device that is most commonly infected is the central venous catheter, which is used to administer fluids and nutrients as well as cytotoxic drugs. Infections can arise at any time during the use of this type of catheter. The infusion fluid itself, or the catheter hub, can be contaminated but, more often, organisms are introduced from the patient's skin or from the hands of nursing staff. Sometimes the distal tip of the catheter is contaminated at the time of insertion; alternatively, organisms can migrate down the catheter wound [16,17]. However, if *Candida* spp. colonizing the gastrointestinal tract as commensals are able to penetrate the intestinal mucosa and invade the bloodstream, circulating yeasts can 'seed' the catheter tip endogenously [16]. This could be a

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common entry mechanism for cancer patients receiving chemotherapeutic regimens that cause serious damage to the intestinal mucosa [18].

Superficial *Candida* infections associated with implanted devices are much less serious, but are encountered frequently and can be troublesome. The commonest is probably denture stomatitis, which is a *Candida* infection of the oral mucosa that is promoted by a close-fitting upper denture. A biofilm is formed on the surface of the acrylic denture and contains large numbers of bacteria, particularly streptococci, in addition to yeasts [19]. Silicone rubber voice prostheses which are fitted in laryngectomized patients can also become contaminated by polymicrobial biofilms containing *Candida* spp. These devices often fail within months because the biofilm causes a malfunction of the valve mechanism [20].

Non-device-related infections, too, can involve biofilms. *Candida* endocarditis, for example, can result from the formation of biofilms on damaged vascular endothelium of native heart valves in patients with pre-existing cardiac disease. The primary lesion is a thrombus, largely consisting of fibrin and platelets, which develops on the surface of the heart valve. Such thrombotic lesions then become colonized with fungal cells and this can lead to the production of emboli [6]. Although direct evidence is lacking, it seems likely that certain infections on epithelial surfaces also involve *Candida* biofilms. In *Candida* vaginitis, for instance, some fungal cells on the mucosal surface could exist within a mixed-species biofilm mainly comprising members of the bacterial vaginal flora. The recalcitrance of such biofilm cells to conventional antifungal therapy would prevent complete eradication of the organisms from the vaginal lumen and might explain the frequent recurrence of symptomatic candidal infection.

Model biofilm systems

Various model systems (Table 1) have been used to characterize the overall properties of *Candida* biofilms [21]. Almost all of these have been adapted from methods reported previously for bacteria. The simplest, and the first to be described, involves growing adherent populations on the surfaces of small discs cut from catheters [21–23]. Growth is monitored quantitatively by a colourimetric assay that depends on the reduction of a tetrazolium salt, or by [^{3}H]leucine incorporation; both methods give excellent correlation with biofilm dry weight [22]. A similar model system has been used to study the formation of biofilms on strips of denture acrylic [24,25]. Alternatively, for rapid processing of large numbers of samples, biofilms can be grown in 96-well microtitre plates [26]. This method was devised for high-throughput testing of biofilm susceptibility to antifungal agents.

All of these procedures measure biofilm formation under static incubation conditions. *In vivo*, however, developing biofilms are often subjected to a liquid flow. Under laboratory conditions, this can be simply achieved by gentle shaking of the growing biofilm on a catheter disc in liquid medium [27]. More sophisticated flow systems (Table 1) include biofilm formation on cylindrical cellulose filters [21,28,29], or in the perfused biofilm fermenter [21,30]. These systems are more complex to operate than static ones, although several biofilms can be studied simultaneously and with relative ease using cylindrical cellulose filters. The perfused biofilm fermenter is the only model system that allows accurate control of biofilm growth rate.

Various factors affecting biofilm formation *in vitro* are listed in Table 2. There was some correlation between the ability to form biofilms and pathogenicity when different *Candida* species were tested in the catheter disc system. Isolates of *Candida parapsilosis*, *Candida pseudotropicalis* and *Candida glabrata* all gave significantly less biofilm growth than the more pathogenic *C. albicans* [22]. The finding that *C. albicans* isolates consistently produce more biofilm *in vitro* than non-*C. albicans* isolates has been confirmed recently [31]. On the other hand, non-*C. albicans* species, particularly *C. tropicalis* and *C. parapsilosis*, appear to form biofilms quite readily when grown in medium containing 8% glucose [32]. This ability might be important in enabling these species to cause candidaemia in patients receiving total parenteral nutrition, where the solution being administered usually has a high glucose concentration.

The nature of the catheter material used affects biofilm formation by *C. albicans* in the catheter disc model system. Biofilm formation was slightly increased on latex or silicone elastomer, compared with polyvinylchloride (PVC), but substantially decreased on polyurethane or 100% silicone [22]. *In vivo*, catheters and other implants rapidly adsorb host proteins, which form a conditioning film on the implant surface. Preincubation of PVC catheter discs *in vitro* with fibrinogen or collagen enhanced the formation of biofilms by *C. albicans* (S.P. Hawser and L.J. Douglas, unpublished). Similarly, conditioning films of serum or saliva promoted biofilm formation on denture acrylic [24,25].

A defining characteristic of any biofilm is the presence of a matrix of extracellular polymeric material, produced

Table 1. Methods used to study *Candida* biofilms

Method	Refs
Model systems	
Catheter disc	[21–23]
Acrylic strip	[24,25]
Microtitre plate	[26]
Cylindrical cellulose filter	[21,28,29]
Perfused biofilm fermenter	[21,30]
Quantitative analysis of biofilm growth	
Dry weight measurements	[21,22]
Isotope incorporation	[21,22]
Tetrazolium salt reduction	[21,22,25,26]
ATP bioluminescence assay	[24]
Biofilm ultrastructure	
Scanning electron microscopy	[21,22]
Confocal laser scanning microscopy	[35–37]
Raman microscopy	[38]

Table 2. Factors affecting *Candida* biofilm formation *in vitro*

Factor	Refs
Candida species and strain	[22,31,32]
Nature of colonized surface	[22]
Presence of conditioning film	[24,25]
Liquid flow	[21,27]
Bacteria	[8,50,52]

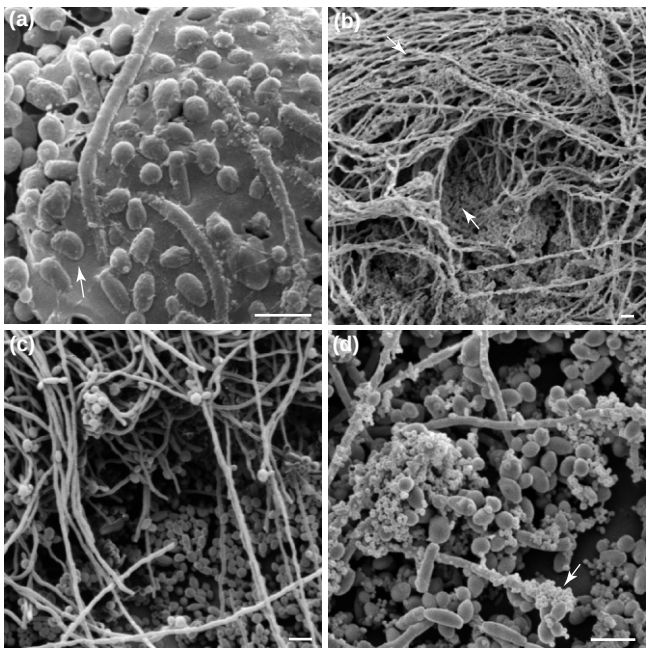

Fig. 1. Scanning electron micrographs of *Candida albicans* biofilms. (a) Thin biofilm formed under flow conditions on a cellulose acetate filter in a perfused biofilm fermenter. Extensive matrix material, indicated by an arrow, is present. (b) Biofilm formed on a PVC catheter disc under static conditions showing a bilayer structure at low magnification. The thin, basal, yeast layer and upper, mainly hyphal layer are indicated by arrows. Matrix material is not visible. (c) Biofilm formed on a PVC catheter disc showing a bilayer structure at higher magnification. Matrix material is not visible. (d) Mixed-species biofilm of *C. albicans* and *Staphylococcus epidermidis* (slime-negative mutant) formed on a PVC catheter disc. Clumps of smaller bacterial cells (arrow) can be seen attached to yeasts and hyphae. Matrix material is not visible. Scale bars = 10 μm.

by the microorganisms, and in which they are embedded [6]. The matrix is usually difficult to preserve for scanning electron microscopy and special drying procedures are required [21]. However, the amount of *Candida* matrix visible depends not only on preparative techniques but also on the incubation conditions during biofilm development. Substantially increased amounts of matrix are formed when biofilms of *C. albicans* are incubated with gentle shaking, instead of statically, to produce a flow of liquid over the surface of the cells. Under these conditions the microorganisms can be almost hidden by the enveloping matrix [27]. Matrix production is similarly increased when conventional flow systems such as the perfused biofilm fermenter [28] (Fig. 1) are used.

Biofilm ultrastructure

One distinguishing feature of *C. albicans* biofilms is the mixture of morphological forms usually present. Biofilm development on catheter discs was first examined by scanning electron microscopy, which showed that initial attachment of yeast cells was followed, after 3–6 hours, by germ-tube formation. Fully mature biofilms, produced after incubation for up to 48 hours, consisted of a dense network of yeasts, hyphae and pseudohyphae [22]. Hyphal forms were not seen when the organism was grown in liquid culture or on an agar surface of the same medium (yeast nitrogen base with glucose), suggesting that morphogenesis is triggered by contact with the plastic PVC surface. In this context, it is interesting that a mutant of *C. albicans*, defective in filamentous growth and lacking the transcription factors Efg1p and Cph1p, which are involved in morphogenetic signalling pathways, colonized polyurethane catheters very poorly [33]. Similarly, a recent study with five defined filamentation mutants [34] demonstrated that strains lacking the *EFG1* gene formed only sparse monolayers of elongated yeast cells on plastic coverslips *in vitro*. These results appear to implicate the Efg1p signalling pathway in biofilm development.

The role of morphogenesis in overall biofilm structure was also investigated using scanning electron microscopy. Biofilms produced by wild-type strains of *C. albicans* were compared with those formed by two morphological mutants incapable of yeast and hyphal growth, respectively [29]. Wild-type biofilms on catheter discs consisted of two distinct layers: a thin, basal region of densely packed yeast cells and an overlying thicker, but more open, hyphal layer (Fig. 1). The hypha-negative mutant produced only the basal yeast layer, whereas the yeast-negative mutant formed a thicker, hyphal biofilm resembling the outer zone of wild-type structures. The finding that both mutants were able to generate biofilms, albeit of different thickness, shows that dimorphism is not an absolute prerequisite for biofilm formation but might be necessary for the development of a spatially organized structure. Biofilms of the yeast-negative mutant were more easily detached from catheter discs than the other strains, indicating that the basal yeast layer has an important function in anchoring the biofilm to the surface. On a different type of surface (cellulose fibres), biofilms of wild-type *C. albicans* consisted exclusively of yeast cells and the bilayer structure was absent [28,29], suggesting that biofilm architecture is dependent on highly specific contact-induced gene expression.

Despite its excellent resolution properties, scanning electron microscopy has the disadvantage that all samples examined must be fully dehydrated. Confocal laser scanning microscopy (CLSM), however, allows the examination of fully hydrated, living biofilms if fluorescence is introduced to visualize the cells. Using this technique, bacterial biofilms have been characterized as complex 3-D structures consisting of matrix-enclosed microcolonies, often described as 'towers' or 'mushroom-shaped stacks'. Importantly, the microcolonies are separated by water channels, which provide a mechanism for nutrient circulation within the biofilm [6]. Recent CLSM studies (Fig. 2) suggest that biofilms of both *C. albicans* and *Candida dubliniensis* have similar 3-D structures consisting of microcolonies surrounded by water channels [35–37]. Such studies have also confirmed the yeast–hyphal bilayer structure of *C. albicans* microcolonies when biofilms are grown on plastic surfaces [31] (Fig. 3). On the other hand, investigation of *C. albicans* biofilms using spectroscopic techniques such as Raman microscopy [38] failed to reveal complex architectural features. Instead, the biofilms, which were grown on a germanium surface, appeared to take the form of relatively homogeneous, planar sheets. These observations lend further support to

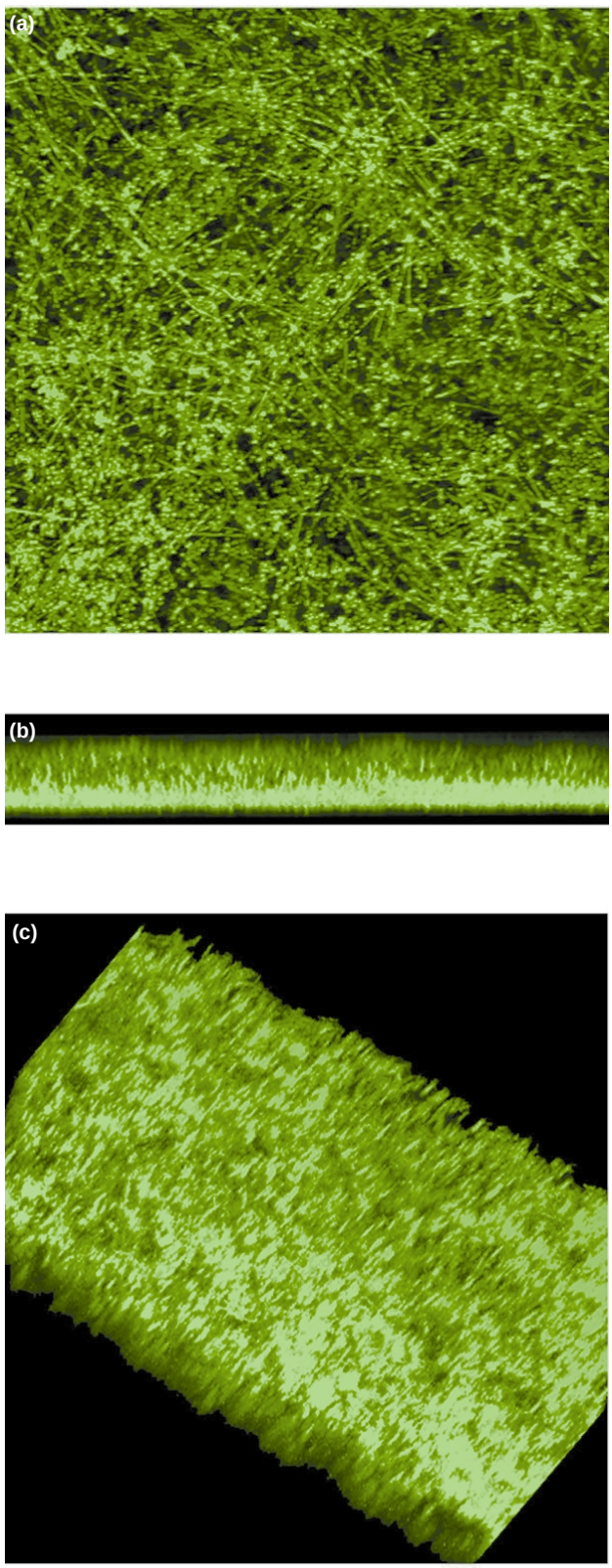

Fig. 2. 3-D reconstruction of a *Candida albicans* biofilm using confocal microscopy and the associated software for the compilation of *xy* optical sections taken across the *z* axis. (a) View from the top. (b) Lateral view to show biofilm thickness (70 μm). (c) Rotated view to provide a global perspective of the biofilm. Reproduced with permission from [36].

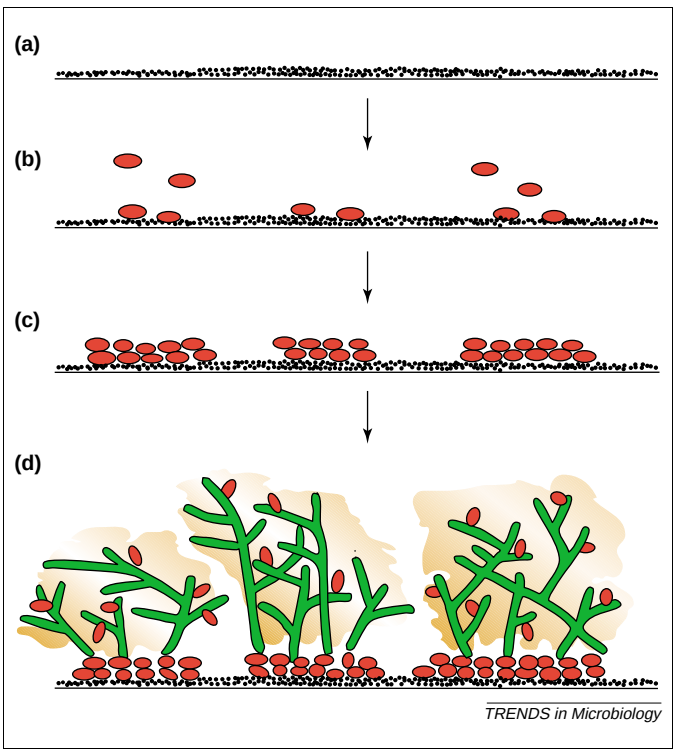

Fig. 3. Stages in the formation of a *Candida albicans* biofilm on a polyvinylchloride (PVC) catheter surface. (a) Catheter surface with an adsorbed conditioning film of host proteins (black dots). (b) Initial yeast (red) adhesion to the surface. (c) Formation of the basal layers of yeast microcolonies. These anchor each microcolony to the surface. (d) Completion of microcolony formation by addition of the upper, mainly hyphal layer and matrix material (yellow) that surrounds both yeasts (red) and hyphae (green). Mature biofilms contain numerous microcolonies with interspersed water channels to allow circulation of nutrients. On other surfaces (e.g. cellulose fibres) microcolonies consisting entirely of yeast cells are produced.

the conclusion that biofilm structure might depend on highly specific, surface-induced gene expression.

Matrix polymers of bacterial biofilms are primarily exopolysaccharides and many are negatively charged. Smaller amounts of proteins, nucleic acids and various other components can also be present. However, much of the biofilm matrix (up to 97%) is water [39]. The matrix of *C. albicans* biofilms has been isolated and its composition compared with that of extracellular polymeric material obtained from culture supernatants of planktonically grown organisms [40]. 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, and contained galactose, suggesting that it might possess components unique to biofilms [40].

Drug resistance of biofilms

Microbial biofilms are notoriously resistant to a variety of antimicrobial agents, including antibiotics, antiseptics and industrial biocides. For example, when bacteria exist in the biofilm form they are 10–1000 times more resistant to antibiotics than are planktonic cells [6]. Corresponding resistance of *Candida* biofilms to antifungal agents was first demonstrated in 1995 [23]. A catheter disc assay was used to determine drug concentrations that caused a 50% inhibition of metabolic activity (as measured by tetrazolium salt reduction or [^{3}H]leucine

incorporation). Clinically important antifungal agents – amphotericin B, fluconazole, flucytosine, itraconazole and ketoconazole – were tested. All of these agents were much less active against *C. albicans* biofilms on PVC discs than against planktonic cells. Drug concentrations required to reduce metabolic activity by 50% were five to eight times higher for biofilms than for planktonic cells, and 30–2000 times higher than the corresponding minimum inhibitory concentrations (MICs). Scanning electron microscopy revealed that the biofilm structure remained intact at an amphotericin B concentration of 11 times the MIC. Biofilms of non-*C. albicans* species, such as *C. tropicalis* and *C. parapsilosis*, were also drug resistant [23].

Subsequent studies have demonstrated drug resistance for *Candida* biofilms grown on cellulose [28,29], polystyrene [26,35], silicone elastomer [37], polyurethane [41] and denture acrylic [25]. Recently, however, it has been reported that some of the newer antifungal agents are active against *Candida* biofilms. Although biofilms of *C. albicans* and *C. parapsilosis* were clearly resistant to two new triazoles (voriconazole and ravuconazole), there appeared to be some antibiofilm activity with lipid formulations of amphotericin B and two echinocandins (caspofungin and micafungin) [42]. The efficacy of caspofungin against *C. albicans* biofilms *in vitro* has now been confirmed by other workers [43,44]. Caspofungin is the first antifungal agent to be licensed that inhibits the synthesis of β 1,3-glucan, the major structural component of *Candida* cell walls; glucan synthesis might prove to be a particularly effective target for biofilms if, as seems possible from analytical data [40], the biofilm matrix also contains this polysaccharide. These intriguing recent findings could lead to important developments in the treatment of fungal infections of implants. For bacterial biofilms, it has been suggested that effective antibiotic regimes could still be possible with some conventional drugs acting either singly or in combination, and encouraging results have been obtained with fluoroquinolones [45].

Possible mechanisms of drug resistance

The mechanisms of biofilm resistance to antimicrobial agents are not fully understood. Possible mechanisms include: (1) restricted penetration of drugs through the biofilm matrix; (2) phenotypic changes resulting from a decreased growth rate or nutrient limitation; and (3) expression of resistance genes induced by contact with a surface [3,6]. Another recent suggestion is that a small number of 'persister' cells are responsible for resistance [46]. Multiple mechanisms appear to operate in bacteria, and these vary with the bacteria present in the biofilm and the nature of the antimicrobial agent being administered [3].

Restricted penetration

It has long been supposed that the matrix of extracellular polymeric material might exclude or limit the access of drugs to organisms deep in a biofilm. Most studies with bacterial biofilms indicate that the matrix does not form a major barrier to drug diffusion, although for certain compounds penetration can be delayed [3]. To investigate whether the matrix plays a role in the resistance of *C. albicans* 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 failed to exhibit significant differences in susceptibility to any of the drugs tested, indicating that drug resistance is unrelated to the extent of matrix formation [40]. However, separate studies with biofilms produced under flow conditions showed that resuspended cells (which presumably had lost most of their matrix) were some 20% less resistant to amphotericin B than intact biofilms, suggesting that the matrix might play a minor role in drug resistance [28,30].

Slow growth rate or nutrient limitation

Biofilm cells are thought to grow slowly because of the limited availability of nutrients, particularly at the base of the biofilm. A slow growth rate is often accompanied by changes in cell surface composition that could, in turn, affect the susceptibility of the microorganisms to antimicrobial agents. Growth rate could therefore be an important modulator of drug activity in biofilms [3,6]. To investigate this possibility with *C. albicans*, a perfused biofilm fermenter was used to generate 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 same growth rate 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 [30]. Biofilm resistance is therefore not only the result of a low growth rate but depends on some other feature of the biofilm mode of growth. A separate study [28] using the cylindrical cellulose filter model system (Table 1) demonstrated that glucose-limited and iron-limited biofilms grown at the same low rate were equally resistant to amphotericin B. Iron-limited biofilms probably resemble more closely biofilms growing *in vivo*, as although there is an abundance of iron in the human body, most of it is located intracellularly or tightly complexed to iron-binding glycoproteins, and so is relatively inaccessible to microorganisms. In the same study, daughter cells from iron-limited biofilms were significantly more susceptible to the drug [28]. An acute disseminated infection produced by the release of such cells from an implant biofilm might therefore respond rapidly to amphotericin B but the biofilm itself would be unaffected.

Surface-induced expression of resistance genes

When microorganisms attach to a surface and form a biofilm they express an altered phenotype. Work has now started to identify genes that are activated or repressed in *Candida* biofilms compared with planktonic cells, and there is particular interest in genes that might contribute to drug resistance. For example, upregulation of genes coding for multidrug efflux pumps would result in a multidrug-resistant phenotype. To date, evidence for this as a resistance mechanism in bacterial biofilms is equivocal [47,48]. *C. albicans* possesses two different types of efflux pump: ATP-binding cassette (ABC) transporters and major facilitators, which are encoded by CDR

and MDR genes, respectively. A recent study has demonstrated that genes encoding both types of efflux pump are upregulated during biofilm formation and development. However, mutants carrying single or double deletion mutations in some of these genes were highly susceptible to fluconazole when growing planktonically but still retained the resistant phenotype during biofilm growth [49]. These results strongly suggest that drug resistance in *C. albicans* biofilms, like that in bacterial biofilms, is a complex process that cannot be explained by a single molecular mechanism.

Mixed fungal–bacterial biofilms

Bacteria are often found with *Candida* species in polymicrobial biofilms *in vivo*, and it is likely that extensive interspecies interactions take place in these adherent populations. *In vitro*, the catheter disc model system has been used to investigate mixed-species biofilms consisting of *C. albicans* and *Staphylococcus epidermidis*, the commonest agent of bacterial catheter-related infection. Two strains of *S. epidermidis* were used: a slime-producing wild-type and a slime-negative mutant. Scanning electron microscopy revealed numerous physical interactions between the staphylococci and both yeasts and hyphae [50] (Fig. 1). Moreover, drug susceptibility studies suggested that fungal cells can modulate the action of antibacterial agents and bacteria can affect the activity of antifungal agents in these biofilms. For example, the presence of *C. albicans* in a biofilm increased the resistance of slime-negative staphylococci to vancomycin. However, *Candida* resistance to fluconazole was enhanced in the presence of slime-producing staphylococci, but was unaffected by the presence of the slime-negative mutant. Similar observations have been made with biofilms consisting of *C. albicans* and oral streptococci (*Streptococcus gordonii* and *Streptococcus salivarius*) on denture acrylic [8]. It will be interesting to determine whether a mixture of bacterial and fungal extracellular polymers produces a more viscous matrix and whether increased matrix viscosity [51] might explain the enhanced antimicrobial resistance of these mixed-species biofilms.

Recently, an antagonistic interaction was described between *C. albicans* and *Pseudomonas aeruginosa*, an opportunistic bacterial pathogen [52]. *P. aeruginosa* formed a dense biofilm on *C. albicans* hyphae, and killed the fungus. By contrast, the bacteria were unable to bind to, or kill, yeast-form *C. albicans*. Hyphal death occurred only after the onset of biofilm formation. To examine the possible role of virulence genes in these interactions, three classes of *P. aeruginosa* mutants were tested for their ability to kill fungal cells. The results indicated that several virulence factors, including pili and secreted molecules such as phospholipase C, were acting in concert to kill *Candida* hyphae [52]. The authors suggest that many microbial virulence factors, normally considered in the context of human infection, might also be involved in bacterial–fungal interactions.

Future perspectives

Although biofilm research on fungi has lagged well behind that on bacteria, the basic structural features and properties of *C. albicans* biofilms have been established. Rather more remains to be determined about biofilms formed by the non-*C. albicans* species. With the sequencing of the *C. albicans* genome and the advent of DNA microarray technology, research in the immediate future is likely to concentrate on defining the biofilm phenotype of *C. albicans* with a view to identifying possible targets for novel, biofilm-specific antifungal agents (Box 1). Further studies *in vivo* with existing new drugs that already show promising activity *in vitro* will also be required. Little is currently known about the chemical composition of the matrix material, and whether it contains any biofilm-specific components essential for biofilm integrity; this might also prove to be a productive area of research in terms of the development of new biofilm-specific drugs.

Finally, the nature of cell-to-cell signalling in *Candida* biofilms has yet to be determined. Quorum sensing, or population-dependent gene expression, is a well known cell-signalling mechanism in bacteria and is now also thought to be significant in biofilm formation [53,54]. In Gram-negative bacteria, acylated homoserine lactones are signal molecules that accumulate in bacterial cultures as a function of cell density. At a threshold population density, described as a bacterial quorum, the accumulated signal molecules interact with cellular receptors that control the expression of a set of specific target genes. Recently, farnesol was identified as a quorum-sensing molecule in planktonic cultures of *C. albicans* and shown to prevent the conversion of yeast cells to mycelia [55]. The possible role of this and other signalling molecules in *Candida* biofilms has not been established. Intercellular signalling in mixed-species biofilms, particularly, is likely to be very complex and probably plays a crucial role in determining the diversity and distribution of different microorganisms within such adherent populations. Moreover, the recent striking demonstration of antagonism by bacteria against fungi within a biofilm [52] indicates that a wide variety of secreted molecules might participate in modulating the composition of bacterial–fungal biofilms *in vivo*.

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Box 1. Questions for future research

- Which genes are differentially expressed in *Candida* biofilms and what is the biofilm phenotype?
- Can any of the genes upregulated in biofilms provide targets for novel biofilm-specific antifungal agents?
- What is the exact chemical nature of the matrix material in *Candida* biofilms and which components are essential for the maintenance of biofilm structure?
- How do fungal and bacterial extracellular polymers interact in mixed-species biofilms and what is the effect on the properties of the biofilm matrix?
- What signalling mechanisms operate in *Candida* biofilms? In particular, what complex signals are exchanged between fungi and bacteria in mixed-species biofilms?

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