Role of the biofilm matrix in resistance of *Candida* biofilms to antifungal agents

By

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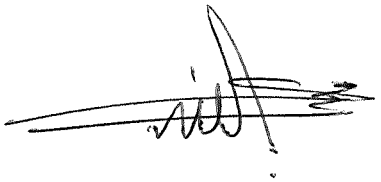
PREFACE

This thesis is the original work of the author.



Mohammed A. A. Al-Fattani

DEDICATION

I should like to dedicate this thesis to my wife "Mrs. Fawziah A. Al-Ghamdi" for her appropriate enthusiastic encouragement and participation through my academic career, my wonderful children Doaa, Emad, Awatif, Amnah and lovely new baby born called Ayia, my family and to the memory of my mother, father, mother in law and my first wife Mrs. Awatif A. Rajab.

I should also like to dedicate this thesis to my mother in law Nafisa Nezar for her continual patience and for looking after my two wonderful children Doaa and Emad throughout my period of studying.

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ABBREVIATIONS

A_{492}	Absorbance at 492nm
ABC	ATP-binding cassette
AIDS	Acquired Immune Deficiency Syndrome
ATCC	American tissue culture collection
ATP	Adenosine triphosphate
C	The drug concentration that penetrated the biofilm
CF	Cystic fibrosis
Cfu	Colony forming units
CLSM	Confocal laser scanning microscopy
Cm	Centimetre
C_o	The drug concentration determined for the control
Conc.	concentration (s)
dH_2O	Distilled water
d	Day (s)
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DnaseI	Deoxyribonuclease
ECTA	Extracellular teichoic acid
EDNA	Extracellular deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
EORTC	European organisation for research and treatment of cancer
EPS	Extracellular polymeric substance
Fe^{2+}	Ferrous, iron (II) compounds
G	Gram
GDH	Glasgow Dental Hospital, Scotland UK
Glc	Glucose
GRI	Glasgow Royal Infirmary, Scotland UK
H	Hour (s)
HIV	Human Immuno-deficiency virus
Hwp1	Hyphal wall protein 1

Ica	Intracellular adhesion
IFIG	Invasive fungal infection group
L	Litre
M	Molar
M7	A slime-negative mutant
MAPK	Mitogen activated protein kinase
MAR	Multiple antibiotic resistances
MDR	Multiple drug resistance
MIC	Minimum inhibitory concentration
min	Minute (s)
ml	Mililitre (s)
ml^{-1}	per millilitre
mm	Millimetre
Mm	Millimolar
MOPS	3-(N-morpholino) propanesulfonic acid
MRD	Modified Robbins device
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ND	Not determined
Ng	Nanogram
°C	Degrees Celsius
OD	Optical density
P	Probability value
PBS	Phosphate-buffered saline
pH	Hydrogen ion concentration
PIA	Polysaccharide intercellular adhesin
PL	Phospholipase
PS/A	Extracellular capsular polysaccharide adhesin
PVC	Polyvinyl chloride
PVE	Prosthetic valve endocarditis
r.p.m	Revolutions per minute
RNA	Ribonucleic acid

RP62A	A slime-producing wild type
RT-PCR	Reverse Transcription Polymerase Chain Reaction
Sap	Secreted aspartic proteinase
SCSP	Synthetic competence signalling peptide
SDA	Sabouraud dextrose agar
sec	Second
SEM	Scanning electron microscopy
spp	Species
TEM	Transmission electron microscopy
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV	Ultraviolet
v/v	Volume/volume ratio
vit K	Menadione
vol.	Volume
w/v	Weight/volume ratio
wt.	Weight
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5 [(phenylamino)carbonyl]-2H tetrazolium hydroxide
YNB	Yeast nitrogen base medium

SUMMARY

Candida albicans and related *Candida* species are frequently found in the normal microbiota of humans; this facilitates their encounter with most implanted biomaterials and host surfaces. The major complication associated with the use of medical implants such as catheters or prostheses is infection. It is now clear that this type of infection is usually due to the development of drug-resistant biofilms on the surface of implanted devices. The aim of this project was to investigate the possible role of the biofilm matrix as a barrier to drug diffusion in *Candida* biofilms and in mixed species fungal-bacterial biofilms.

The penetration of antifungal agents through single- and mixed-species biofilms containing *Candida* was investigated using a novel filter disk bioassay. Fluconazole permeated all single-species *Candida* biofilms more rapidly than flucytosine. Drug penetration was more extensive with *C. albicans* than with the other species and the rates of diffusion of either drug through biofilms of three strains of *C. albicans* were similar. However, the rates of drug diffusion through biofilms of *C. glabrata* or *C. krusei* were faster than those through biofilms of *C. parapsilosis* or *C. tropicalis*. In all cases, after 3 to 6h the drug concentration at the distal edge of the biofilm was very high (many times the MIC). Nevertheless, drug penetration failed to produce complete killing of biofilm cells. These results indicate that poor antifungal penetration is not a major drug resistance mechanism for *Candida* biofilms under these conditions. The abilities of flucytosine, fluconazole, amphotericin B, and voriconazole to penetrate mixed-species biofilms containing *C. albicans* and *Staphylococcus epidermidis* (a slime-producing wild-type strain, RP62A, and a slime-negative mutant, M7) were also

investigated. All four antifungal agents diffused very slowly through the mixed-species biofilms. In most cases, diffusion was slower with biofilms containing *S. epidermidis* RP62A, but amphotericin B penetrated biofilms containing the M7 mutant more slowly. However, the drug concentrations reaching the distal edges of the biofilms always substantially exceeded the MIC. Thus, although the presence of bacteria and bacterial matrix material undoubtedly retarded the diffusion of the antifungal agents, poor penetration does not wholly account for the drug resistance of *Candida* biofilm cells, even in these mixed-species biofilms.

Matrix material was extracted from biofilms of *C. albicans* and *C. tropicalis* and analysed chemically. Both preparations contained carbohydrate, protein, hexosamine, phosphorus and uronic acid. However, the major component in *C. albicans* matrix was glucose (32.2 %), whereas in *C. tropicalis* matrix it was hexosamine (27.4 %). Further characterization of the matrix material was achieved by enzymatic digestion of biofilms. 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.

It has been reported that the production of extracellular matrix by *Candida* biofilms growing under static incubation conditions is relatively minimal, but increases dramatically when developing biofilms are subjected to a liquid flow. In this study, *Candida* biofilms were grown under flow conditions in a modified

Robbins device (MRD). Biofilms of *C. albicans* grown in the MRD produced more matrix material than those grown statically, and were significantly more resistant ($P<0.001$) 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 *S. 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 *S. epidermidis* M7, on the other hand, were completely drug resistant only when grown under flow conditions. Overall, these findings 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.

INTRODUCTION

1 *Candida* and candidosis

1.1 *Candida* species

The genus *Candida* includes around 196 species. Yeasts belonging to this genus are unicellular fungi that reproduce mainly by budding. In the fifth century Hippocrates described thrush and was the first to describe a yeast infection (Ainsworth, 1986). Since then, the primary etiologic agent of thrush, *C. albicans*, has been demonstrated to cause many forms of disease, some of which are life threatening. By 1963, approximately five medically important species of *Candida* had been described. The species were *C. albicans*, *C. stellatoidea* (which is now considered synonymous with *C. albicans*), *C. parapsilosis*, *C. tropicalis*, and *C. guilliermondii* (Emmons *et al.*, 1963). However, the appearance in the 1960s of new modalities to treat cancer, increasing use of central venous catheters, increases in average life expectancy, and other developments in medicine resulted in a parallel increase in the number of serious *Candida* infections. There are now at least 17 species of *Candida* that have been shown to cause disease in humans (Rinaldi, 1993).

C. albicans is a fungus which may develop into a number of different morphological forms, including yeasts, hyphae and pseudohyphae. This change depends on environmental conditions. The ability to form hyphae is generally considered to be an important virulence factor since hyphae are able to penetrate tissue more easily. Recent studies identified a widely distributed yeast species that is closely related to *C. albicans* (Coleman *et al.*, 1997) This newly described species was isolated from oropharyngeal lesions in HIV-positive patients in Dublin. In fact, *C. dubliniensis* was initially difficult to distinguish from *C. albicans* and

was often misidentified as such in standard clinical laboratory examinations because both species are closely related and share phenotypic and genotypic characteristics (Odds *et al.*, 1998; Sullivan and Coleman, 1998; McCullough *et al.*, 1999).

C. glabrata was considered a relatively non-pathogenic saprophyte of the normal flora of healthy persons, rarely causing serious infection in humans (Haley, 1961; Stenderup and Pedersen, 1962). However, following the widespread and increased use of immunosuppressive drugs, together with broad-spectrum antimycotic therapy, the frequency of infections caused by *C. glabrata* has increased significantly (Komshian *et al.*, 1989; Hitchcock *et al.*, 1993; Knoke *et al.*, 1997). *C. glabrata* is now the second or third most common cause of candidosis after *C. albicans* (Wingard, 1995). It is a non-dimorphic yeast that exists as small blastoconidia (yeasts) under all environmental conditions. In fact, *C. glabrata* is the only *Candida* species that does not form pseudohyphae at temperatures above 37°C.

C. krusei forms pseudohyphae with elongated blastoconidia, creating a crossed matchsticks or treelike appearance. *C. krusei* is an emerging non-*albicans* *Candida* species, with a particular predilection for neutropenic adult cancer patients (Wingard, 1995).

C. parapsilosis forms blastoconidia, singly or in small clusters, which are seen under a microscope along the pseudohyphae. This organism is identifiable by the crooked or curved appearance of relatively short pseudohyphae and the occasional presence of large hyphal elements called giant cells (Davise, 1987). The available data suggest that the role of *C. parapsilosis* as an exogenous pathogen

results from its introduction via contaminated intravenous fluids or biomaterials. The numbers of infections due to this organism are accordingly on the increase (Weems, 1992; Branchini *et al.*, 1994; Price *et al.*, 1994).

C. tropicalis forms blastoconidia singly, or in very small groups, all along the pseudohyphae. True hyphae may also be present. Also, a few teardrop-shaped chlamydospores maybe produced irregularly (Davise, 1987). *C. tropicalis* is one of the three most commonly isolated non-*albicans Candida* species. It accounts for 4 to 25% of all *Candida* species isolated and a higher proportion (approximately 20-45%) of non-*albicans Candida* species isolated from patients with candidemia (Fraser *et al.*, 1992; Viscoli *et al.*, 1999).

1.2 *Candida* Infections

The pathogenic *Candida* species are all opportunistic pathogens capable of inflicting disease only when most defences are in some way impaired, locally or systemically. The principal pathogen of the genus, *C. albicans*, can grow either as oval, budding yeasts or as continous septate hyphae, and both morphological forms are usually seen in infected tissues. Disease states range from common, superficial infections, especially those of the mouth and vaginal, to rarer, but frequently fatal, deep-seated infections.

1.2.1 Superficial candidosis

Candida species are capable of initiating a variety of recurring superficial diseases (Odds, 1988). Thrush is a common form of oral candidosis and occurs especially with those patients receiving chemotherapy and radiation. It is characterized by soft, cream-coloured, elevated plaques that can easily be wiped off the buccal mucosa, tongue dorsum, or palate. This infection most frequently

affects newborns, the elderly, and diabetics. In the absence of other known causes of immunosuppression, oral thrush in an adult is highly suggestive of human immuno-deficiency virus (HIV) infection (Dronda *et al.*, 1996).

Oral candidosis is not a single clinical entity, but a spectrum of very different clinical entities such as pseudomembrane candidosis, erythematous candidosis, denture stomatitis and angular cheilitis.

Denture stomatitis is a term used to describe certain pathological changes of the mucosa under complete or partial, removable dentures. The characteristic clinical feature is a uniform bright erythema, usually found in both jaws, less frequently in the mandible, and it is commonly associated with angular cheilitis (Webb *et al.*, 1998). The prevalence of the condition among denture wearers has been variously reported to be 10-75% depending on the population studied (Espinoza *et al.*, 2003; Shulman *et al.*, 2005). *C. albicans* is the most commonly isolated species associated with denture stomatitis, but other less pathogenic species such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* are also recovered (Barbeau *et al.*, 2003; Dar-Odeh and Shehabi, 2003). In the oral cavity *Candida* species usually co-exist with commensal bacteria within a biofilm matrix. Studies by Budtz-Jorgensen (1999) for instance, showed that, in denture plaque of patients with *Candida*-induced denture stomatitis, 93% of the plaque biofilm is composed of yeasts and the remainder comprises oral bacteria.

Vulvovaginal candidosis is one of the most common female genital tract infections encountered in medical practice. Up to 75% of all women will experience fungal vulvovaginitis at some point in their lives, and approximately 40-50% will experience a second episode of this syndrome (Sobel *et al.*, 1998;

Ferrer, 2000). The clinical symptoms most often described in cases of vulvovaginal candidosis are external dysuria, vulval pruritus, swelling, or redness (Ferrer, 2000; White and Vanthuyne, 2006). Recently, Moreira and Paula (2006) reported a study of 130 symptomatic patients examined during the period from 2002 to 2004. Among patients with positive laboratory cultures, *C. albicans* was the most frequently isolated species, with 90% of isolations, followed by *C. glabrata* with 6%, and *C. parapsilosis* and *C. tropicalis* with 2% (Moreira and Paula, 2006).

1.2.2 Systemic candidosis (Deep seated candidosis)

The incidence of systemic fungal infections has increased dramatically over the past twenty years (Beck-Sague and Jarvis, 1993). Most systemic *Candida* infections are considered to arise from the patient's endogenous flora (Fridkin and Jarvis, 1996). Alternatively, they may be transmitted from contaminated equipment or solutions and from hospital personnel. For many years *C. albicans* was the species most commonly causing systemic infections, but recently other species have emerged. In a review of 36 studies reporting 1479 patients with cancer disease, *C. albicans* was indicated for 54 % of infections and *C. tropicalis* for 25 % (Wingard, 1995). *C. tropicalis* has an apparently greater capacity than *C. albicans* to invade the deep tissues of the immunocompromised host; several studies have shown positive surveillance cultures for *C. tropicalis* to be highly predictive of subsequent systemic infection (Komshian *et al.*, 1989). Among children with leukaemia, Marina and colleagues (1991) found a higher incidence of colonization with *C. albicans* than with *C. tropicalis*; however, a higher proportion of those colonized by *C. tropicalis* developed fungaemia (Marina *et al.*, 1991). This pattern

has also been seen in adult oncology and bone marrow transplant patients (Wingard, 1995).

Several other *Candida* species can cause serious infections in hospitals; *C. glabrata* causes about 8 %, *C. parapsilosis* 7-11 % while *C. krusei* accounts for 4 % of infections (Wingard *et al.*, 1991; Girmenia *et al.*, 1996; Nguyen *et al.*, 1996). Systemic infection with *Candida albicans* or related species presents clinically different challenges depending on the site of invasion, the effectiveness of the host's immune responses and the presence of underlying disease (Myerowitz *et al.*, 1977).

*Candida*emia has increased in frequency primarily due to the widespread use of intravascular catheters, repeated injection by drug addicts, and long-term use of broad-spectrum antimicrobial or steroid therapy (Giunchi, 1958; Seelig, 1966; Hay, 1986; Crump and Collignon, 2000). Early studies by Hay (1986) identified drug addicts using contaminated lots of heroin or needles with *Candida*l endocarditis syndrome. Disseminated candidosis occurs mostly in immunocompromised patients, especially organ transplant recipients and neutropenic patients (Bodey and Luna, 1974). Thaler *et al.* (1988) found a distinct syndrome known as chronic disseminated candidosis almost exclusively in patients with acute leukemia. Currently, *Candida* infections are a major cause of septicemia in neonatal intensive care units, and are associated with high morbidity and mortality. The neonatal infections most commonly observed are those caused by *C. albicans* and *C. parapsilosis*, but in the last few years there has been an increase in cases caused by *C. tropicalis* (Leibovitz, 2002). Recently, Dixon *et al.* (2004)

described an adult patient with chronic mucocutaneous candidiasis who developed a disseminated infection with *C. tropicalis*.

1.3 Factors predisposing to candidosis

Although risk factors for invasive candidosis have been clearly identified (such as colonization, length of stay, use of parenteral nutrition, antibiotics, central lines, and abdominal surgery), there are few risk assessment strategies that clearly define or predict a population at high risk of getting this disease. Therefore, the severity and extent of *Candida* infection tends to increase with the number and severity of predisposing factors. There are certain diseases and disorders that have for years been especially associated with candidosis (Odds, 1988). Some of these factors are mentioned briefly below.

1.3.1 Debilitating diseases and disorders

Numerous investigators have reported that high blood and tissue glucose levels favoured the growth of *Candida* in diabetics (Kandhari *et al.*, 1969). Stenderup and Schonheyder (1984) state that oral and oesophageal candidosis are common conditions in AIDS patients, and vaginal candidosis is very common among women with AIDS. The incidence of thrush reaches approximately 15 % in patients with malignant diseases (Boggs *et al.*, 1961). *Candida* carriage is one of the common predisposing factors for cancer patients, especially those with leukaemia (Gentle *et al.*, 1984; Ghannoum *et al.*, 1985).

1.3.2 Digressions from normal physiological status

A number of studies have shown that oral infections in newborn infants within the first few days after birth are due to contamination with *Candida* from the birth canal of the mother (Taschdjian and Kozinn, 1957). Vaginal candidosis has

always been found to be greater in pregnant than in non-pregnant women (Odds, 1988).

1.3.3 Dietary factors

Several studies have demonstrated that nutritional deficiencies, such as vitamin C deficiency, predispose to candidosis. Roger *et al.* (1983) showed in an animal model that vitamin C deficiency reduced the resistance of guinea pigs to renal candidosis. Other studies using two animal models showed that hypovitaminosis A increased, and hypervitaminosis A decreased the susceptibility of rats and mice to infection with *C. albicans* (Odds, 1988). Moreover, iron-deficiency anaemia may be an important predisposing factor in some types of candidosis (Higgs and Wells, 1972).

1.3.4 Mechanical factors

Many case reports show that severely burned patients are particularly susceptible to colonization and deep-seated infection with *Candida* (Zanini *et al.*, 1983). However, Holzheimer and Dralle (2002) showed that the mortality of burns patients with fungal infections dropped from 80-100 % to approximately 10 % through good burns management. Oral yeast infections are higher among denture wearers of various types than among non-wearers (Berdicevsky *et al.*, 1980; Mitchell *et al.*, 1982).

1.3.5 Treatment with drugs

Most studies indicate that treatment with antibacterial antibiotics is an important factor in candidosis, especially treatment with tetracycline (Odds, 1988), penicillin and erythromycin (Meads *et al.*, 1951; Heimdahl and Nord, 1982) and ampicillin (Fitzpatr and Topley, 1966). In addition, steroid therapy is known to be

a major factor predisposing to superficial candidosis (Gale, 1982) and systemic candidosis (Date *et al.*, 1983). Wied *et al.* (1966) noted a significant increase in vaginal *Candida* infections among users of hormonal contraceptives.

1.3.6 Surgical procedures

Invasive *Candida* infections are a significant cause of mortality and morbidity for patients undergoing solid organ transplantation, including liver and lung transplantation (Alexander, 2002; Zaas and Alexander, 2004). Bone marrow transplant recipients are at increased risk of a variety of infections from endogenous and exogenous sources (Kusne and Krystofiak, 2001) and a case of necrotizing fasciitis due to *C. albicans* following renal transplantation has been reported (Wai *et al.*, 2001).

1.3.7 Implanted medical devices

A variety of implanted devices are now known to be important risk factors for *Candida* infections (Table 1). They include indwelling catheters (central venous catheters and urinary catheters), joint prostheses, dialysis access devices (permanent haemodialysis and peritoneal dialysis), cardiovascular devices (heart valves, pacemakers, implantable cardioverter defibrillators and left ventricular assist devices), central nervous devices (ventriculoperitoneal shunts), penile implants and endotracheal tubes (Jones *et al.*, 2001; Kojic and Darouiche, 2004).

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. Infection can arise at any time during the use of this type of catheter. According to the National Nosocomial Infections Surveillance data, 87% of primary bloodstream infections occur in patients with a central line (Mermel *et*

al., 2001). 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 (Goldmann and Pier, 1993; Sherertz, 2000). However, if *Candida* species 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 (Goldmann and Pier, 1993). This could be a common entry mechanism for cancer patients receiving chemotherapeutic regimens that cause serious damage to the intestinal mucosa (Kullberg and Filler, 2002).

1.4 Chemotherapy of candidosis

Fungi, like their human hosts, are eukaryotic organisms, and thus the number of potential targets for drug action is very limited. Although it is known that the most common pathogen among *Candida* species is *C. albicans*, in recent years there has been a shift in the spectrum of identifiable *Candida* infections (Nguyen *et al.*, 1996). In view of the significant morbidity and mortality associated with invasive fungal infections, it is particularly important to make the diagnosis as early as possible and to select the best available antifungal drug for treatment. Fortunately, the prevention and treatment of invasive fungal infections have been improved over the last two decades by the development of new antifungal agents. To date, four classes of antifungal agents have been approved for treating invasive fungal infections: (1) Polyenes (amphotericin B and amphotercin B liposomal formulations) which destabilize fungal cell membranes; (2) Nucleoside analogues (5-fluorocytosine) which interfere with DNA and RNA synthesis.

Table 1. Implantable devices in which *Candida* biofilms develop most frequently

Device	Infection risk (%)	Main *Candida* species
Central and peripheral venous catheters	3-8	*albicans*, *glabrata* *parapsilosis*
Haemodialysis and peritoneal dialysis catheters	1-20	*albicans*, *parapsilosis*
Urinary catheters	10-30	*albicans*, *glabrata*
Endotracheal tubes	10-25	*albicans*
Intracardiac prosthetic devices	1-3	*albicans*, *glabrata* *parapsilosis*, *tropicalis*
Breast implants	1-2	*albicans*
Prosthetic joints	1-3	*albicans* *parapsilosis*, *glabrata*
Neurosurgical shunts	6-15	*albicans*
Voice prostheses	50-100	*albicans*, *tropicalis*
Dentures	5-10	*albicans*, *glabrata*

(Ramage *et al.*, 2006)

(3) Azoles (ketoconazole, fluconzole, itraconazole, and newer azoles such as voriconazole, posaconazole, and ravuconazole) which interfere with sterol synthesis and compromise fungal cell membrane integrity; and (4) Echinocandins (caspofungin) which inhibit glucan synthesis leading to increased cell wall permeability and lysis (Abuhammour and Habte-Gaber, 2004).

1.4.1 Polyenes

The first clinically useful antifungal drugs were the polyenes (Fig. 1a), amphotericin B and nystatin, which became available in the 1950s (Hazen and Brown, 1950). They are amphoteric molecules with a hydrophobic polyene face and a hydrophilic surface containing multiple hydroxyl groups. Polyenes bind to ergosterol in the fungal membrane forming a channel in the membrane with the hydrophilic surface of the drug facing the interior of the channel. This mechanism permits leakage of intracellular components, leading to fungal death (Abuhammour and Habte-Gaber, 2004). These fungicidal drugs have the broadest spectrum of activity in the clinical field (Cowen *et al.*, 2000).

1.4.2 Nucleoside analogues

The antifungal activity of flucytosine (5-fluorocytosine; Fig. 1b) was discovered later and was reported in 1963 (Grunberg *et al.*, 1963) during the development of antineoplastic drugs. It is a fluorinated analogue of cytosine. Among the pyrimidine class of antifungal drugs, only 5-fluorocytosine is approved for the chemotherapy of systemic candidosis. It is a fungicidal drug, with a limited activity spectrum (Dismukes, 2000). It works as an antifungal agent through conversion to 5-fluorouracil within the target cells. This compound becomes incorporated into RNA, and inhibits DNA synthesis through effects on thymidylate

synthesis (Odds *et al.*, 2003). 5-Fluorocytosine is thought to enhance the antifungal activity of amphotericin B, especially in anatomical sites where amphotericin B penetration is poor (Denning and Stevens, 1990) and it is generally used in combination with this drug since the prevalence of primary resistance to 5-fluorocytosine among *Candida* isolates is high.

1.4.3 Azoles

The continued search for new and less toxic antifungals led to the discovery of the azoles. The azole class was discovered in the late 1960s and clotrimazole and miconazole were introduced for clinical use in the early 1970s. Ketoconazole was released in the early 1980s, and was selected as the first available compound for the oral treatment of systemic fungal infections. Indeed, azole antifungals are divided into two categories, the imidazole group, which include clotrimazole, miconazole and ketoconazole, and the triazole group, which includes itraconazole, fluconazole, voriconazole and posaconazole. The main difference between them is that compounds in the latter group have three instead of two nitrogen atoms in the azole ring (Maertens, 2004). The azoles inhibit fungi by blocking ergosterol sythesis through inhibition of the enzyme lanosterol 14α-demethylase. Ergosterol depletion coupled with the accumulation of methylated sterol precursors results in inhibition of fungal cell growth, fungal cell death, or both (Torres *et al.*, 2005).

Fluconazole (Fig. 1c) is well tolerated with very low incidence of side effects and is the most effective agent for the treatment of oropharyngeal and vaginal candidiasis, as well as prophylaxis for fungal infections in neutropenic patients undergoing bone marrow transplantation and for oropharyngeal candidiasis in HIV-infected persons (Andriole, 1999).

Recently, voriconazole (Fig. 1d), a novel triazole antifungal, has been approved for treatment of serious fungal infections caused by *Aspergillus*, *Fusarium*, *Scedosporium*, and resistant *Candida* species. Voriconazole has *in vitro* activity against yeasts and yeast-like fungi similar, or superior to, fluconazole, itraconazole and amphotericin B. Additionally, the drug possesses potent fungicidal activity against moulds including *Aspergillus*, *Scedosporium*, and *Fusarium.* Fungicidal activity is likely due to the high affinity of voriconazole for fungal 14-α-demethylase.

Posaconazole is a new second-generation triazole drug for the treatment and prevention of invasive fungal infections. It is structurally closely related to the old broad-spectrum itraconazole. *In vitro*, posaconazole is very active against *Candida* species and is more potent than itraconazole and fluconazole against virtually all of the *Candida* species and *C. neoformans* isolates tested (Pfaller *et al.*, 2001; Pfaller *et al.*, 2004).

1.4.4 Echinocandins

Most recently, a new antifungal drug was licensed which represented a new class of antifungal agents, the echinocandins. Echinocandins are fungal secondary metabolites comprising a cyclic hexapeptide core with a lipid side-chain that is responsible for antifungal activity. In the late 1990s, three echinocandin-class compounds were released, (the three-dimentional structure of all three molecules being similar): anidulafungin, caspofungin and micafungin (Odds *et al.*, 2003) and all entered clinical development (Vanden Bossche, 2002).

Caspofungin (Cancidas) is the first licensed compound. It was isolated from the fungus *Glarea lozoyensis* (Maschmeyer and Glasmacher, 2005). The target for

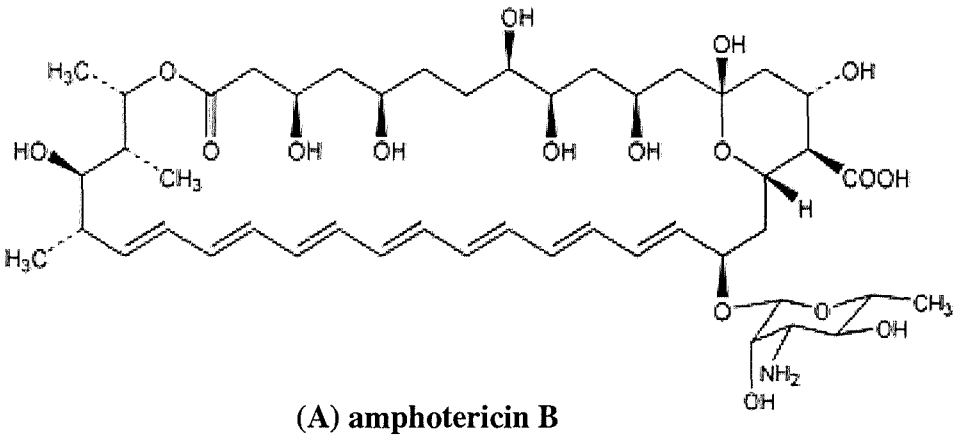
this drug is a complex of proteins responsible for synthesis of cell-wall β-1,3 glucan, which is not present in mammalian cells. The disruption of the cell wall results in osmotic stress, lysis, and then death of the microorganism. Caspofungin may be an effective alternative drug that is better tolerated than amphotericin B (Walsh *et al.*, 2004). It was selected as the most successful treatment for a patient with oropharyngeal candidiasis caused by strains of *C. albicans* and *C. glabrata* resistant to azoles (Nevado *et al.*, 2005).

2 Virulence factors

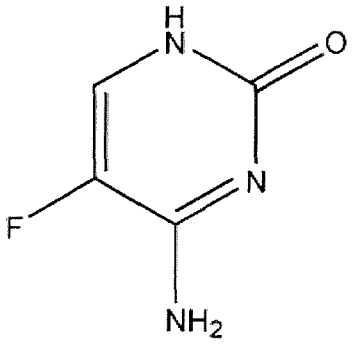
Generally, the organism most often responsible for *Candida* infections is *C. albicans*, and this is due to a number of virulence factors that contribute to pathogenesis. These factors include morphogenesis (the reversible transition between unicellular yeast cells and filamentous growth forms), adhesion (the host recognition biomolecules), and secreted aspartyl proteases and phospholipases (Calderone and Fonzi, 2001). Exactly and simply defined, a virulence factor is any attribute that a fungus possesses which increases its virulence in the host or escalates binding of the organism to host cells.

2.1 Morphogenesis

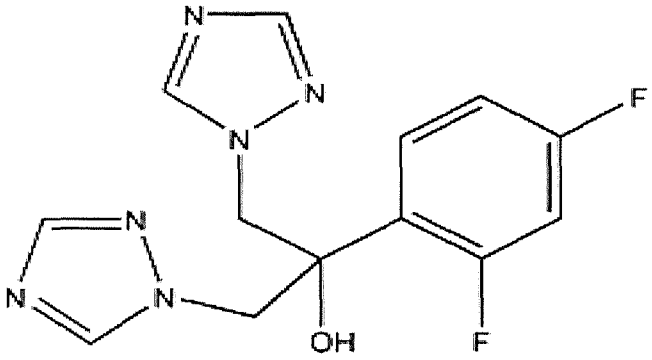
The yeast-to-hyphal transition has been shown to be an important virulence attribute that enables *C. albicans* to invade human tissues (Brown and Gow, 1999). Many fungal pathogens of humans are dimorphic and undergo reversible morphogenetic transitions between budding, pseudohyphal and hyphal growth forms.



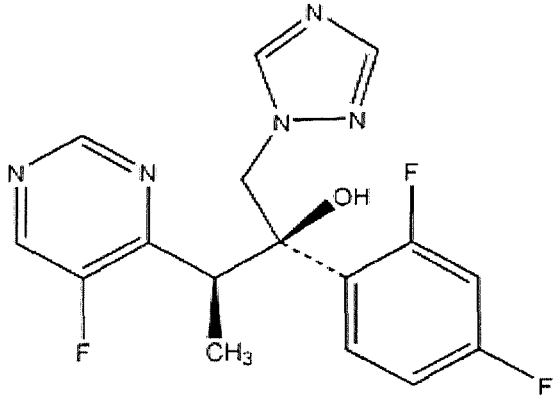
(A) amphotericin B



(B) flucytosine



(C) fluconazole



(D) voriconazole

Figure 1. Antifungal agents

Candida pseudohyphae range from relatively short to extended cells, and are microscopically distinguishable from true hyphae only by conspicuous constrictions of the pseudohyphae at septal junctions (Merson-Davies and Odds, 1989). Morphogenesis can be activated by a wide range of factors and signals, many of which connect signalling pathways within fungal cells. (Gow *et al.*, 2002). For example, hyphal growth is blocked by inactivation of the transcription factors Cph1p and Efg1p which belong to the mitogen-activated protein kinase (MAPK) cascade and Ras-cAMP pathways (Lo *et al.*, 1997). More recently, another protein kinase named CRK1 (in the Cdc2 subfamily of kinases) has also been shown to be essential for conversion of yeast to filamentous growth on serum-containing agar media (Chen *et al.*, 2000).

2.2 Adhesion

Microbial adherence to biomaterial surfaces or to host cells is seen as an essential early step and one of the most important determinants of pathogenesis. *Candida* species can adhere to the surfaces of medical devices, particularly catheters, and form biofilms which results in increased resistance to antifungal drugs compared with planktonic *Candida* cells, and an increase in *Candida*emia related to catheter insertion (Hawser and Douglas, 1994b; Chandra *et al.*, 2001a). There is a correlation between the virulence of different *Candida* species and the ability to form biofilms (Hawser and Douglas, 1994b).

Many studies have shown that several factors influence adhesion *in vitro*. For example, the composition of the growth medium can affect adhesion; yeasts grown in medium containing 500mM galactose or glucose are significantly more adherent to denture acrylic or to buccal epithelial cells than organisms grown in a

low concentration (50mM) of glucose (McCourtie and Douglas, 1981; McCourtie and Douglas, 1984). Increased adhesion appears to be due to enhanced production of a surface mannoprotein that binds to fucose-containing glycolipids in epithelial cell membranes (Cameron and Douglas, 1996).

Recent adhesion studies have focused on the identification of genes that encode a host-recognition protein. The agglutinin-like sequence (ALS) gene family of *C. albicans* encodes large cell-surface glycoproteins that are implicated in the process of adhesion. ALS genes are also found in other *Candida* species that are isolated from cases of clinical disease. Als1p and Als5p participate in adhesion to human buccal epithelial cells and fibronectin (Hoyer, 2001). The role of Als1p is important for the adherence of the organism to the oral mucosa during the early stages of the infection (Kamai *et al.*, 2002).

Another protein called hyphal wall protein 1 (Hwp1), is found on the surface of germ tubes and true hyphae, but not yeasts or pseudohyphae of *C. albicans* (Sundstrom *et al.*, 2002). Hwp1 encodes an outer surface mannoprotein that is supposed to be oriented with its amino-terminal domain surface-exposed and the carboxyl terminus most probably covalently incorporated with cell wall β-glucan (Staab *et al.*, 1996). It is well known that oroesophageal candidiasis is caused by the combined action of fungal virulence factors and host inflammatory responses when protective immunity is absent. Hwp1 is a substrate for mammalian transglutaminase and becomes cross-linked to transglutaminase-expressing surface squames of the oral mucosa. Therefore, Hwp1 is a promising target for development of antifungal drugs for treatment of oroesophageal candidiasis (Sundstrom *et al.*, 2002).

It well known that *C. albicans* binds to several extracellular matrix ligands, including fibronectin, laminin, fibrinogen and collagen (Hostetter, 1994). These binding activities are characteristic of the integrin family of mammalian cell receptors. Hostetter (1994) showed that monoclonal antibodies to the α-chain of integrin proteins bind to proteins of *C. albicans*; some of these reactive proteins were partially characterized. The author suggested that *C. albicans* expresses an integrin-like protein (Int1p). A putative integrin gene *INT1* from *C. albicans* has also been identified (Gale *et al.*, 1996) and it seems likely that *INT1* plays important roles in adherence and filmentation of *C. albicans* (Gale *et al.*, 1998).

2.3 Hydrolytic enzymes

To assist in the invasion of host tissues, many pathogenic microbes possess constitutive and inducible hydrolytic enzymes that destroy, alter, or damage membrane integrity, leading to dysfunction or disruption of host cells. Pathogenic species of *Candida* produce a large variety of secreted hydrolases, among which the secreted aspartic proteinases (Sap) and phospholipases (PL) have been intensively investigated (Calderone and Fonzi, 2001; Haynes, 2001).

2.3.1 Aspartic proteinases

Medically important yeasts of the genus *Candida* have the ability to secrete extracellular aspartic proteinases (Saps), which are of particular interest as virulence factors. This characteristic is shared by *C. albicans*, *C. dubliniesis*, *C. tropicalis* and *C. parapsilosis*. The enzymes produced by these yeasts are all carboxyl proteinases capable of degrading human proteins such as albumin, hemoglobin, keratin, and secretory immunoglobulin A (Hube *et al.*, 1998).

There are at least ten proteins that comprise the Sap family.

In vivo studies show that *SAP* genes 1, 2 and 3 are expressed by yeast cells only, whereas *SAP* 4, 5 and 6 expression has been detected in *C. albicans* undergoing a transition from yeast to hyphae at neutral pH (Schaller *et al.*, 1999). The expression of *SAP* 7 has never been detected under any growth conditions. *SAP* 8 transcripts have been detected in yeast cells grown at 25°C in a defined medium and *SAP* 9 is preferentially expressed in later growth phases (Monod *et al.*, 1998). Like *C. albicans*, *C. tropicalis* secretes *in vitro* Sap activity in a medium containing bovine serum albumin as the only source of nitrogen (Symersky *et al.*, 1997).

2.3.2 Phospholipases and lipases

The role of extracellular phospholipase (PL) as a potential virulence factor has been investigated in several pathogenic fungi, including *C. albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* (Ghannoum, 2000). According to the different and specific ester bond cleaved, these enzymes have been classified into four PLs so far (A, B, C and D). Only PLB activity has been demonstrated in *C. albicans*. This is an 84-kDa glycoprotein that has both hydrolase (fatty acid release) and lysophospholipase transacylase activities (Mukherjee and Ghannoum, 2002). PL activity has also been observed with *C. glabrata*, *C. parasilosis*, *C. tropicalis*, *C. krusei* and *C. lusitaniae* (Ghannoum, 2000). Studies by Dagdeviren *et al.* (2005) demonstrated a correlation between adherence-phospholipase and adherence-aspartic proteinase properties of *C. parasilosis* strains; PL production appeared to be an important virulence factor in bloodstream infections caused by *C. parasilosis*. In a more recent study, 61 isolates of *Candida* recovered from HIV and cancer patients were investigated for PL and

Sap activity. This was more obvious in *C. albicans* isolates, with 100% PL and 94.1% Sap activity. In contrast, non-*C. albicans* species showed only 29.6% PL and 70.3% Sap activity, indicating a relationship between other virulence determinants in these yeasts with colonization and disease (Kumar *et al.*, 2006).

Secreted hydrolytic lipases and esterases are also thought to be important as virulence factors in bacteria and fungi. These enzymes are characterized by their ability to catalyse both the hydrolysis and synthesis of ester bonds of mono-, di- and triacylglycerols or even phospholipids (Schaller *et al.*, 2005). Extracellular lipase activity of pathogenic *Candida* species was first detected by Werner (1966) who noted that the fungi could grow with different Tween detergents as sole sources of carbon. A secreted esterase was later characterized by Tsuboi *et al.* (1996). Subsequently, Fu *et al.* (1997) discovered by chance the first lipase gene of *C. albicans*, *LIP*1, while the authors were searching for phospholipases. Another nine new members of this lipase gene family (*LIP*2-*LIP*10) were later cloned and characterized (Hube *et al.*, 2000). All ten lipase genes encode highly similar proteins with up to 80% identical amino acid sequences. Sequences similar to *LIP*1-*LIP*10 were also detected in other pathogenic *Candida* species such as *C. parasilosis*, *C. krusei* and *C. tropicalis*, but not in *C. glabrata* or *S. cerevisiae* (Fu *et al.*, 1997).

3 Microbial biofilms

Over the past century, microorganisms have primarily been characterised as planktonic, freely suspended cells and described on the basis of their growth properties in nutritionally rich culture media. Rediscovery of a microbiological phenomenon first described by Antonie Van Leeuwenhoek, that microorganisms

attach to and grow on exposed surfaces, led to studies which revealed that surface-associated microorganisms (biofilms) exhibit a distinct phenotype with respect to gene transcription and growth rate. Biofilm formation involves specific mechanisms for initial attachment to a surface, development of a community structure and ecosystem, and detachment (Donlan, 2002). Earlier studies by Heukelekian and Heller (1940) demonstrated that bacterial growth and activity were substantially enhanced by the incorporation of a surface to which these organisms could attach. Also, Zobell (1943) found that the number of bacteria on surfaces was dramatically higher than in the surrounding medium (seawater). Moreover, Characklis (1973) studied microbial slimes in industrial water systems and showed that these microorganisms were not only very tenacious but also highly resistant to disinfectants such as chlorine.

Costerton *et al.* (1978), based on observations of dental plaque and sessile communities in mountain streams, formulated a biofilm hypothesis that explained the mechanisms whereby microorganisms adhere to either biotic (living materials such as tissues and cells) and abiotic (nonliving materials) and the benefits derived from this ecologic niche. Later on, Carpentier and Cerf (1993) simplified the concept of a biofilm as a community of microbes embedded in an organic polymer matrix, adhering to a surface. Furthermore, Elder *et al.* (1995) and Mah and O'Toole (2001) described a biofilm in more co-operative terms as a functional consortium of microorganisms that is difficult to eradicate by conventional antimicrobial therapy and can cause indolent infections. It has become clear that biofilm-grown cells express properties distinct from planktonic cells, one of which is an increased resistance to antimicrobial agents.

Over the last two decades several definitions of a biofilm have been proposed by researchers. One which is widely quoted (Donlan and Costerton, 2002) states that a biofilm is 'a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription'. Underlying this definition are the three basic components of a biofilm: microbes, matrix, and surface. If one of these essentials is removed from the environment, the biofilm will not form. Clearly, this is an over simplification of a fairly complex process that does not take into account the type of microorganisms, the composition of the surface, or the influences of environmental factors (Micheal, 2000).

3.1 Stages of biofilm development

Palmer and White (1997) outlined the steps for the early stages of biofilm formation that included cell-surface and cell-cell interactions, followed by the development of the mature biofilm. Briefly, when microorganisms are transported to the surface by sedimentation, liquid flow, or active swimming, they first make weak and transient attachments. The next phase, irreversible attachment, depends on the properties of both biomaterial surface and the microbial cell surface. During irreversible attachment, a monolayer of single cells firmly adhered to a surface is produced. Next, microcolonies develop, finally forming the mature biofilm. Eventually, if the experimental conditions are no longer favourable, cells can detach from the biofilm and revert to a planktonic lifestyle (Fig. 2) (Wolfaardt *et al.*, 1994; Kierek-Pearscon and Karatan, 2005).

3.2 Biofilm architecture and structure

During the transition from initial attachment to formation of a mature biofilm, the microbial cells undergo significant changes. This process of biofilm development results in the formation of a complex, three-dimensional architecture that usually includes mushroom-like structures, water channels, and pores (Lewandowski, 2000; Reisner *et al.*, 2003). Biofilm architecture can be visualized using a variety of microscopy methods such as transmission electron microscopy (TEM), fluorescence microscopy, scanning electron microscopy (SEM), and confocal laser scanning microscopy (CLSM). In particular, CLSM which allows the visualization of fully hydrated samples, has revealed the complex three-dimensional structure of biofilms (Costerton *et al.*, 1995). For instance, Lawrence *et al.* (1991) used this method to study biofilms formed by *P. aeruginosa*, *P. fluorescens*, and *Vibrio parahaemolyticus* in flow chambers. They found that while each biofilm varied in depth, structure, and ratio of cellular to noncellular material, all of the biofilms developed water channels (Lawrence *et al.*, 1991). Many conditions such as surface and interface properties, the composition of the microbial community, and hydrodynamics, can affect biofilm structure and architecture, especially nutrient availability in the environment (Bowden and Li, 1997; Stoodley *et al.*, 1999).

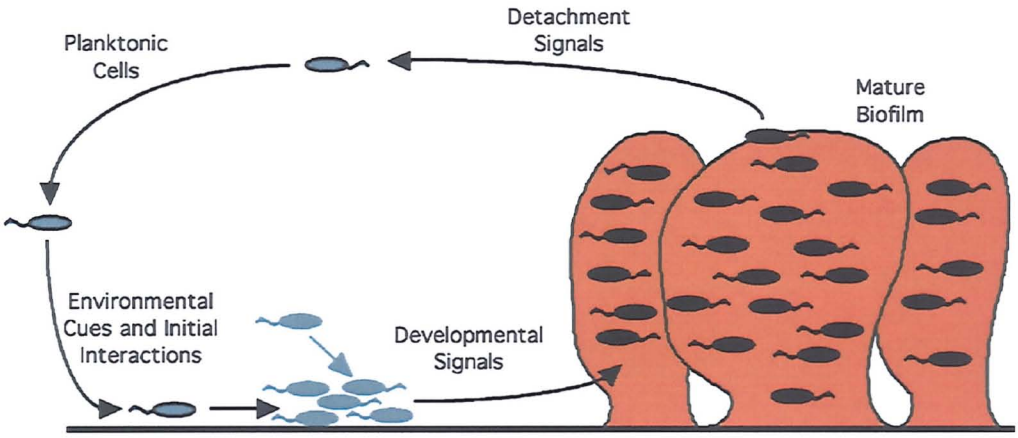


Figure 2. Model of biofilm development

Individual planktonic cells can form cell-to-surface and cell-to-cell contacts resulting in the formation of microcolonies. Cells in the biofilm can return to a planktonic lifestyle to complete the cycle of biofilm development (O'Toole *et al.*, 2000).

3.3 Biofilm matrix and its composition

A characteristic feature of biofilms is the production of an extracellular matrix that envelops the attached cells. This is generally composed of water and microbial macromolecules and provides a complex group of microenvironments surrounding the microorganisms (Sutherland, 2001a). In addition, the matrix contains a range of enzymic and regulatory activities. The matrix architecture is based upon a combination of intrinsic factors such as the genotype of the attached cells and a number of extrinsic factors, including fluctuations in nutrient and gaseous levels and fluid shear. Together, these intrinsic and extrinsic factors combine to produce a dynamic, heterogeneous microenvironment for the attached and enveloped cells (Allison, 2003).

Much research during last two decades has focused on characterizing the matrix material of both Gram-negative and Gram-positive bacterial biofilms, such as *P. aeruginosa* biofilms (Harrison *et al.*, 2005) and *S. epidermidis* biofilms (Sadovskaya *et al.*, 2005). Microbial polymers such as exopolysaccharides, proteins, nucleic acids and various other components are invariably present. However, water is by far the main component of the biofilm matrix, accounting for up to 97% of the mass. The water may be bound within the capsules of the bacterial cells or can exist as a solvent whose physical properties are determined by the solutes dissolved in it (Sutherland, 2001a). In Gram-negative bacteria, some exopolysaccharides are neutral, whereas others are polyanionic because of the presence of uronic acids such as D-glucuronic, D-galacturonic, and D-mannuronic acids, or phosphate or sulphate residues (Sutherland, 2001b). In contrast to Gram-negative bacteria, Gram-positive bacteria often produce polycationic extracellular

polymeric substances (Hussain *et al.*, 1993; Mack *et al.*, 1996). However, some polysaccharides carry hydrophobic groups which may be involved in hydrophobic interactions when adhering to hydrophobic interfaces (Neu *et al.*, 1992). Additionally, many matrix materials possess backbone structures that contain sequences of 1,3- or 1,4-β-linked hexose residues that are likely to be more rigid in structure, and in some cases less soluble or even insoluble (Sutherland, 2001b). Moreover, in the presence of ions, the extracellular polymeric material shows increased viscosity or gelation (Loaec *et al.*, 1997).

Generally, matrix polymer synthesis is dependent on the availability of nutrients such as carbon, nitrogen, potassium, or phosphate. In addition, slow bacterial growth will enhance extracellular polymeric substance production (Sutherland, 2001b).

3.4 Human infections involving biofilms

Several of the common diseases associated with biofilm colonisation will be discussed in this and the next (3.5) section. These will include biofilms which attach to tissues in different organs of the human body and device-associated infections where the biofilm is attached to some sort of prosthesis placed within the body.

3.4.1 Native valve endocarditis

Native valve endocarditis is a condition that results from the interaction between the vascular endothelium, generally of the mitral, aortic, tricuspid, and pulmonic valves of the heart, and bacteria or fungi circulating in the bloodstream (Livornese and Korzeniowski, 1992). An early study by Tunkel and Mandell (1992) noted that of 2,300 cases of infective endocarditis, 56% were caused by

streptococci (including viridans streptococci, enterococci, pneumococci, and *S. bovis*), 25% by staphylococci, and the balance by Gram-negative bacteria and fungi including *Candida* and *Aspergillus* species. These organisms gain access to the bloodstream, primarily via the oropharynx, gastrointestinal tract, and genitourinary tract. Fungal endocarditis is a serious condition that affects particular groups of patients and is associated with considerable morbidity and mortality. The incidence of this disease has increased during the past 2 decades, and fungi now account for 1%–10% of organisms isolated in patients with infective endocarditis (Giamarellou, 2002).

3.4.2 Cystic fibrosis

Cystic fibrosis (CF), a chronic disease of the lower respiratory system and an autosomal inherited disease of Caucasians, is caused by dysfunction of the CF transmembrane conductance regulator protein. This disease is characterized by the production of copious amount of hyperviscous mucus, which covers the surface of the epithelial cells and impairs mechanical clearance mechanisms (Gibson *et al.*, 2003). Chronic infections of biofilm-forming bacteria such *P. aeruginosa* are frequently found in the lungs of CF patients. These biofilm infections cause chronic endobronchiolitis in the lungs and they have been studied extensively. Characteristic features of these infections are their resistance to the innate and immune defence mechanisms and their resistance to antimicrobial agents (Hoiby *et al.*, 2001).

3.4.3 Periodontitis

Periodontal disease, infections involving the supporting tissues of teeth, range from mild inflammation of the gums (gingiva) to chronic destruction of

periodontal tissues. The channel between the tooth root and the gingiva, termed the subgingival crevice, is the primary site of periodontal infection (Lamont and Jenkinson, 1998). Moore *et al.* (1983) characterized the organisms isolated from patients with periodontal disease and found that *Lactobacillus* species, *Actinomyces naeslundii*, *Fusobacterium nucleatum*, *Peptostreptococcus micros*, and *Haemophilus aphrophilus* were all positively correlated with gingivitis. As the organisms develop biofilms in the subgingival crevice, they produce proteolytic enzymes that can damage tissue directly or interfere with the host defences. Collagenase and hyaluronidase are also present and are capable of degrading collagen (Marsh, 1995).

3.5 Implant-associated infections

Medical devices are responsible for a large proportion of nosocomial infections. Device-associated infections can cause major medical and economic sequelae. Moreover, the pathogenesis of device-related infection results from the complicated interaction of microorganism, device, and host factors. Microbial factors are probably the most important in the pathogenesis of infection, whereas device factors are the most amenable to modification with the objective of preventing infection (Burns, 2000; Darouiche, 2001; Donlan, 2001). Biofilms formed by bacteria on various medical devices have been studied extensively over the last 20 years. Until recently, less attention has been focused on the formation of fungal biofilms. However, *Candida* species are now recognised as important nosocomial pathogens, and an implanted device with a detectable biofilm is frequently associated with these infections (Table 1) (Douglas, 2003).

3.5.1 Central venous catheters

Maki (1994) noted that central venous catheters pose a greater risk of device-related infection than does any other indwelling medical device, with infection rates of 3 to 5%. Colonization and biofilm formation may occur within 3 days of catheterization (Anaissie *et al.*, 1995). Analysis of National Nosocomial Infections Surveillance data shows that 87% of primary bloodstream infections occur in patients with a central line (Mermel *et al.*, 2001). A separate study by Richards *et al.* (2000) found that a total of 72 to 87% of bloodstream infections, including *Candida*emia, are catheter related in intensive care unit patients. *C. albicans* accounts for up to 63% of all cases of *Candida*emias, followed by *C. glabrata* or, in some hospitals, *C. tropicalis* (Fraser *et al.*, 1992). A recent study (Yapar *et al.*, 2006) has demonstrated a progressive increase in the frequency of nosocomial *Candida*emia, especially among critically ill or immunocompromised patients. The results showed that *C. albicans* was the most common species (57.7%) and non-*albicans* species accounted for (42.3%) of all episodes. The most common non-*albicans Candida* species isolated was *C. tropicalis* (20.2%) followed by *C. parapsilosis* (12.5%). The most frequent risk factors associated with the *Candida*emia were previous antibiotic treatment (76.9%), presence of a central venous catheter (71.2%) and total parenteral nutrition (55.8%).

3.5.2 Joint prostheses

In modern medicine, the most commonly implanted joint prostheses are hip and knee prostheses. The incidence of infection is low: 1% in primary cases and up to 3% in secondary procedures. However, *Candida* accounts for less than 1% of all cases of infections of joint prostheses (Stocks and Janssen, 2000).

3.5.3 Dialysis access

The number of persons with a dialysis access continues to rise. Infection is the second most common cause of death in patients with end-stage renal disease. Additionally, infection is the most frequent cause of hospitalization and is the leading cause of morbidity and mortality in patients requiring dialysis (Cheung and Wong, 2001). Fungal infections of hemodialysis access sites are rare. However, current reports indicate that *Candida* accounts for 2.6 to 7% of peritoneal dialysis-related infections (Lew and Kaveh, 2000; Vas and Oreopoulos, 2001). Moreover, non-*albicans Candida* species account for up to two-thirds of *Candida* isolates. *C. parapsilosis* seems to be the most prevalent non-*C. albicans* species. This infectious complication is associated with a high mortality and morbidity, including prolonged hospital stay and recourse to hemodialysis (Asim *et al.*, 1999; Prasad and Gupta, 2005).

3.5.4 Central nervous system devices

Most recently used ventriculoperitoneal shunts are made of silicone polymers. Obstruction and infection are the two most common complications occurring in patients with these devices. *Candida* is the causative agent in 1% of such infections and the mortality rate is estimated to be 9% (Sanchezportocarrero *et al.*, 1994).

3.5.5 Cardiovascular devices

Two major groups of prosthetic heart valves are currently used: mechanical valves and bioprostheses (tissue valves). The rates of prosthetic valve endocarditis (PVE), or microbial infection of the valve and surrounding tissues of the heart, are similar for both types of valve (Braunwald, 1997). Fungi are responsible for 2 to 10% of all cases of PVE, and *Candida* species account for up to 90 % of these

fungal infections (Melgar *et al.*, 1997). Furthermore, fungal PVE is associated with a higher mortility rate than bacterial PVE (Kojic and Darouiche, 2004).

Infectious complications involving pacemakers have decreased in frequency as a result of improvements in surgical methods and device technology, but they remain in the range of 0.5 to 7 %. *Candida* species account for up to 4.5 % of these infections (Joly *et al.*, 1997; Wilhelm *et al.*, 1997). Pacemaker-related endocarditis represents about 10 % of pacemaker infections due to infection of the subcutaneous portion adjacent to the intravascular section, which seeds the intracardiac electrode (Joly *et al.*, 1997). Pacemaker endocarditis due to *C. albicans*, *C. glabrata* and *C. tropicalis* have all been reported (Cacoub *et al.*, 1998; Kurup *et al.*, 2000; Roger *et al.*, 2000).

3.5.6 Urinary catheters

Indwelling urinary catheters are used frequently in older patient populations. For either short- or long-term catheters, the infection rate is about 5%. *E. coli* remains the most common infecting organism, but a wide variety of other organisms may be isolated, including *Candida* species as the second most common group. Urinary catheter biofilms are unique in that certain of the component organisms may alter the local pH through the production of urease, which hydrolyzes the urea of the urine to form free ammonia (Tunney *et al.*, 1999). *Candida* infections of the urinary tract are strongly associated with the presence of a urinary catheter. According to a recent National Nosocomial Infections Surveillance system report, *C. albicans* caused 21% of catheter-associated urinary tract infections, in contrast to 13% of non-catheter-associated infections (Richards *et al.*, 1999).

4 *Candida* biofilms

4.1 *Candida* infections and biofilms

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, *S. aureus* and enterococci (Calderone, 2002). *Candida* species 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 (Crump and Collignon, 2000; Maki and Tambyah, 2001). 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 *Candida*l infection, usually at the time of surgical placement.

Superficial *Candida* infections associated with implanted devices are much less serious, but are encountered frequently and can be difficult to manage. The commonest is probably denture stomatitis, which is a *Candida* infection of the oral mucosa that is promoted by a close-fitting upper denture (Budtz-Jorgensen, 1999). Silicone rubber voice prostheses which are fitted in laryngectomized patients can also become contaminated by polymicrobial biofilms containing *Candida* species

(Van der Mei *et al.*, 2000). Non-device-related infections, too, can involve biofilms; these include *Candida* endocarditis and *Candida* vaginitis (Donlan and Costerton, 2002).

4.2 Model biofilm systems

Various model systems have been used to characterise *Candida* biofilms. They include catheter disks, acrylic strips, microtitre plates, cylindrical cellulose filters, and the perfused biofilm fermenter (Baillie and Douglas, 1999b; Douglas, 2003). Almost all of these model systems have been adapted from methods reported previously for bacteria. The simplest method, and the first to be described, involves growing adherent populations on the surfaces of small disks cut from catheters (Hawser and Douglas, 1994b; Hawser and Douglas, 1995b; Baillie and Douglas, 1999b). A similar model system has been used to study the formation of biofilms on strips of denture acrylic (Nikawa *et al.*, 1996; Chandra *et al.*, 2001b). For rapid processing of large numbers of samples, biofilms can be grown in 96-well microtitre plates (Ramage *et al.*, 2001b). 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. Several flow systems have been described, including the cylindrical cellulose filter (Baillie and Douglas, 1998b; Baillie and Douglas, 1999a; Baillie and Douglas, 1999b) and the perfused biofilm fermenter (Baillie and Douglas, 1998a; Baillie and Douglas, 1999b). In the former system, biofilms are formed on filters which consist of cylindrical paper sleeves with a packed cellulose filling. With the perfused biofilm fermenter, a biofilm is established on the underside of a cellulose

membrane (0.22μm) in the base of a fermenter, and the membrane is perfused with medium from the sterile side. 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.

Recently, two different animal models of catheter-associated *Candida* infections have been described. Visualization of the resulting *in vivo*-formed biofilms indicated structural features similar to those of biofilms formed *in vitro*. Schinabeck *et al.* (2004) described a rabbit model of catheter-associated infection with *C. albicans*, and also showed that antifungal lock therapy with liposomal amphotericin B was an effective treatment for biofilm infections using this model. The second *in vivo* system, developed by Andes *et al.* (2004) , used a rat central venous catheter model for *C. albicans* biofilm formation.

4.3 Biofilm ultrastructure

Visualisation of *Candida* biofilm structure has been achieved using different microscopic techniques, including fluorescence microscopy, scanning electron microscopy, and confocal laser scanning microscopy. One distinguishing feature of *C. albicans* biofilms is the mixture of morphological forms usually present. Biofilm development on catheter disks was first examined by SEM, 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, pseudohyphae, and extracellular polymeric material was visible on the surfaces of some of these morphological forms (Hawser and Douglas, 1994b).

The role of morphogenesis in overall biofilm structure was also investigated using SEM. 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 (Baillie and Douglas, 1999a). Wild-type biofilms on catheter disks consisted two distinct layers: a thin, basal region of densely packed yeast cells and an overlying thicker, but more open, hyphal layer. In contrast, 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. Baillie and Douglas (1999a) observed that biofilms of the yeast-negative mutant were more easily detached from catheter disks than the other strains, indicating that the basal yeast layer has an important function in anchoring the biofilm to the surface.

Despite its excellent resolution properties, scanning electron microscopy has the disadvantage that all samples examined must be totally dehydrated (Douglas, 2003), since water of hydration is not compatible with the vacuum used with the electron beam. This dehydration process results in significant sample distortion and artifacts; the extracellular polymeric substances, which are approximately 95% water (Characklis and Marshall, 1990) will appear more as fibres than as a thick gelatinous matrix surrounding the cells. The development of the confocal laser scanning microscope in the 1980s provided researchers with the ability to examine biofilms *in situ* without the limitations associated with the scanning electron microscope, albeit at lower magnification (Donlan and Costerton, 2002). CLSM allows the examination of fully hydrated, living biofilms if fluorescence is introduced to visualise the cells (Douglas, 2003).

Recent CLSM studies suggest that biofilms of both *C. albicans* and *C. dubliniensis* have similar three-dimensional structures consisting of microcolonies surrounded by water channels (Chandra *et al.*, 2001a; Ramage *et al.*, 2001c; Ramage *et al.*, 2001a). Such studies have also confirmed the yeast–hyphal bilayer structure of *C. albicans* microcolonies when biofilms are grown on plastic surfaces (Fig. 3) (Kuhn *et al.*, 2002a). However, investigation of *C. albicans* biofilms using spectroscopic techniques such as Raman microscopy (Suci *et al.*, 2001) failed to reveal complex architectural features.

4.3.1 Composition of matrix material

The matrix material of *Candida* biofilms has received relatively little attention. Baillie and Douglas (2000) isolated the matrix of *C. albicans* biofilms and compared its composition with that of extracellular polymeric material obtained from culture supernatants of planktonically grown organisms. They found that both preparations contained carbohydrate, protein, phosphorus and hexosamine; however, 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 (Baillie and Douglas, 2000).

4.4 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 (Donlan and Costerton, 2002). Corresponding resistance of *Candida* biofilms to antifungal agents was first studied by Hawser and

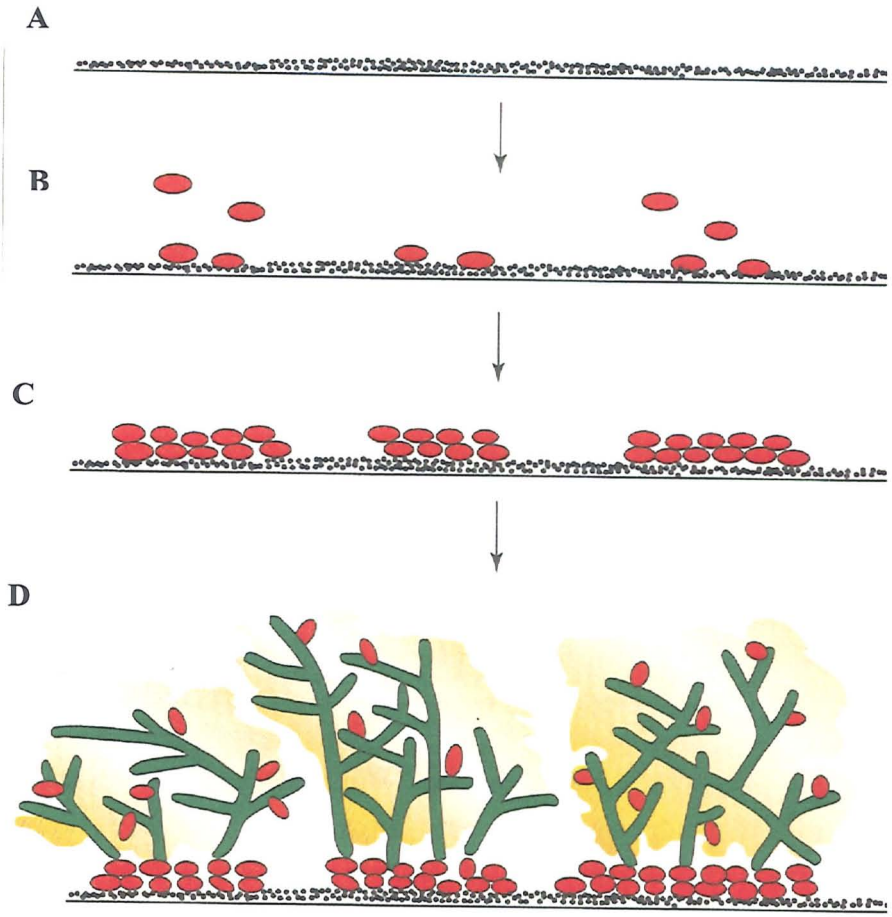


Figure 3. Stages in the formation of *C. albicans* biofilm

Stages in the formation of a *C. 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. (D) Completion of microcolony formation by addition of the upper, mainly hyphal layer and matrix material (yellow) that surrounds both yeast (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 (Douglas, 2003).

Douglas (1995b). They used a catheter disk assay to determine drug concentrations that caused a 50% inhibition of metabolic activity.

The biofilms were tested with five clinically important antifungal agents, namely amphotericin B, fluconazole, flucytosine, itraconazole, and ketoconazole. All of these agents were much less active against *C. albicans* biofilms on PVC disks than against planktonic cells. Subsequent studies indicated that *C. albicans* cells growing in biofilms were 100-fold more resistant to fluconazole and 20 to 30-fold more resistant to amphotericin B compared to planktonic cells (Baillie and Douglas, 1998a; Chandra *et al.*, 2001a; Ramage *et al.*, 2001b). Biofilms of non-*C. albicans* species, such as *C. tropicalis*, *C. parasilosis*, *C. glabrata*, and *C. krusei* were also drug resistant (Hawser and Douglas, 1995b; Zhang *et al.*, 2006).

Separate studies have demonstrated drug resistance for *Candida* biofilms grown on different surfaces such as cellulose (Baillie and Douglas, 1998b; Baillie and Douglas, 1999a), polystyrene (Ramage *et al.*, 2001b; Ramage *et al.*, 2001c), silicone elastomer (Chandra *et al.*, 2001a), polyurethane (Lewis *et al.*, 2002), and denture acrylic (Chandra *et al.*, 2001b). 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) (Kuhn *et al.*, 2002b). The efficacy of caspofungin against *C. albicans* biofilms *in vitro* has now been confirmed by other workers (Bachmann *et al.*, 2002; Ramage *et al.*, 2002a). These intriguing recent

findings could lead to important developments in the treatment of fungal infections of implants.

5 Mechanisms of biofilm drug resistance

The mechanisms of biofilm resistance to antimicrobial agents are not fully understood. At least four possible explanations for increased resistance of biofilms have been proposed. These include: (1) phenotypic changes resulting from a decreased growth rate or nutrient limitation; (2) differential gene expression (induction of biofilm phenotype) including drug efflux pumps; (3) delayed or restricted penetration of drug through the biofilm matrix; and (4) the existence of a small number of 'persisters' cells. It is now thought likely that a combination of these mechanisms operate in both bacterial and fungal biofilms.

5.1 Slow growth rate or nutrient limitation

Phenotypic changes conferring drug resistance could be induced by a slow growth rate or by nutrient limitation within the biofilm. Both growth rate and nutrient limitation are known to affect the cell surface composition of microorganisms and hence perhaps their susceptibility to antimicrobial agents. Growth rate could therefore be an important modulator of drug activity in biofilms. Using a method of cell culture designed to study the effect of growth rate separately from other biofilm processes, Evans *et al.* (1990a) found that the slowest growing *E. coli* cells in biofilm culture were the most resistant to the drug, cetrimide. However, biofilm and planktonic cells were equally susceptible to the drug when exposed at growth rates higher than 0.3 generation per hour (Evans *et al.*, 1990a). Another study by the same group showed that drug susceptibility increased for both *S. epidermidis* biofilm and planktonic cultures with increases in

growth rate. The dependence of susceptibility upon growth rate was greatest for slow growing cells and it was concluded that the faster the rate of cell growth, the more rapid the rate of inactivation by ciprofloxacin (Duguid *et al.*, 1992).

The effect of growth rate on *Candida* biofilm resistance to amphotericin B has been investigated using the approach taken by Evans *et al.* (1990b), in which biofilms, resuspended biofilm cells, and daughter cells were separately tested for drug susceptibility after growth at different rates in a perfused biofilm fermentor. The results were then compared with those obtained for planktonic cells grown at identical 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 (Baillie and Douglas, 1998a). Interestingly, very similar results have been reported for biofilm and planktonic cells of a mucoid strain of *P. aeruginosa* tested by the same protocol for susceptibility to the quinolone ciprofloxacin (Evans *et al.*, 1991).

A separate study using the cylindrical cellulose filter model system (Baillie and Douglas, 1998b) demonstrated that glucose-limited and iron-limited biofilms of *C. albicans* grown at the same low rate were equally resistant to amphotericin B. However, daughter cells from iron-limited biofilms were significantly more susceptible to the drug than those from glucose-limited biofilms. 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 would be unaffected.

5.2 Differential gene expression (induction of biofilm phenotype)

A second possible explanation for biofilm drug resistance is that when microorganisms attach to a surface and form a biofilm there is an upregulation of genes which affect antimicrobial susceptibility. Altered gene expression by organisms within the biofilm can result in a phenotype with reduced susceptibility to an antimicrobial agent.

The multiple antibiotic resistance (*mar*) operon is a global regulator controlling the expression of various genes in *E. coli* which constitute the *mar* regulon. Upregulation of *mar* leads to a multi-drug resistant phenotype, which includes resistance towards structurally unrelated antibiotics, organic solvents and the disinfectant pine oil. Maira-Litran *et al.* (2000a) used *E. coli mar O(II)-lacZ* fusion strains to monitor *mar* expression under various growth conditions including batch, continuous and biofilm culture. From this study they found that *LacZ* expression by the *mar O(II)-lacZ* fusion was generally low within the total biofilm population and equivalent to that of stationary phase cultures grown in batch culture, suggesting that its induction within biofilms cannot explain the elevated levels of antibiotic resistance observed (Maira-Litran *et al.*, 2000a). The same researchers (Maira-Litran *et al.*, 2000b) subsequently used *mar*- and *acrAB*-deleted strains to determine if the resistance of *E. coli* biofilms to ciprofloxacin was affected by loss of these loci. They demonstrated no differences between the wild-type and *mar*-deleted isolates, and showed that isolates constitutive for *mar* displayed reduced susceptibility to ciprofloxacin at low concentration (0.004mg/l) but not at a higher concentration (0.1mg/l). From these results the authors

concluded that antimicrobial agent resistance in *E. coli* biofilms is not mediated by the upregulation of the *mar* or *acrAB* operons (Maira-Litran *et al.*, 2000b).

Work has now began to focus on the identification of genes that are activated or repressed in *Candida* biofilms compared with planktonic cells, and which might contribute to a multidrug-resistant phenotype. For example, upregulation of genes coding for multidrug efflux pumps would result in a multidrug-resistant phenotype. *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 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 (Ramage *et al.*, 2002b).

Subsequent studies by Mukherjee *et al.* (2003) determined the antifungal susceptibilities of biofilms developed by *C. albicans* mutants carrying single, double, or triple deletion mutations of the CDR and MDR1 genes. These results showed that at an early phase of biofilm development these mutants were more susceptible to fluconazole than the wild-type strain. Interestingly, at later time points (12 and 48 h), all the strains became resistant to fluconazole. These observations indicate a lack of involvement of efflux pumps in resistance at late stages of biofilm formation and suggest that multicomponent, phase-specific mechanisms are effective in antifungal resistance of *Candida* biofilms (Mukherjee *et al.*, 2003).

Andes *et al.* (2004) investigated differential gene expression in planktonic cells of *C. albicans* and *in vivo* biofilm-associated cells grown in a rat model system by using quantitative RT-PCR. The authors found that there was no difference in expression of the azole target enzyme gene *ERG11*, or *MDR1*, between planktonic and biofilm-associated cells. However, mRNAs from both of the ATP-binding cassette pumps, *CDR1* and *CDR2* were significantly increased in the biofilm state. Interestingly, *CDR2* was more noticeably affected, with a nearly 10-fold increase in expression (Andes *et al.*, 2004).

5.3 Restricted or delayed penetration of drug through the biofilm matrix

The production of matrix material, or glycocalyx, is one of the distinguishing characteristics of biofilms. Undoubtedly, one of the most important functions of the matrix is to provide the structural complexity and mechanical stability of the biofilm. A number of other functions have been ascribed to the biofilm matrix. Among these, protection of the enveloped cells from antimicrobial treatments is one of the most important (Allison, 2003). Since the matrix structure and integrity is heavily influenced by changes in the surrounding environment, it might exclude or limit the access of antimicrobial agents to microorganisms deep in biofilm (Sutherland, 2001a). However, most studies with bacterial biofilms indicate that the matrix material does not form a major barrier to drug diffusion, although for certain compounds penetration can be delayed (Mah and O'Toole, 2001).

5.3.1 Studies with bacteria

A number of studies have shown an apparent failure of certain antimicrobial agents to penetrate the biofilm. For example, chlorine, a commonly used

disinfectant, did not reach more than 20% of the bulk media's concentration within a mixed *K. pneumoniae* and *P. aeruginosa* biofilm, as measured by a chlorine-detecting microelectrode (De Beer *et al.*, 1994). In fact, the penetration profile was suggestive of a substrate being consumed within the matrix material. Similarly, Suci and co-workers (1994) used infrared spectroscopy to show that the rate of transport of the antibiotic ciprofloxacin to a colonized surface was reduced compared with transport to a sterile surface; what normally required 40s for penetration to a sterile surface required 21 min for a biofilm-containing surface. The authors suggested that the ciprofloxacin was binding and reacting with the biofilm components (Suci *et al.*, 1994).

Studies by other researchers have taken different approaches. Hoyle *et al.* (1992) examined the penetration of the antibiotic piperacillin through a *P. aeruginosa* biofilm grown on one side of a dialysis membrane. Their results were consistent with a role for *P. aeruginosa* biofilm matrix as a physical rather than an enzymatic diffusion barrier to this antibiotic. Ca^{2+} treatment of biofilms, which alters the structure of the mucoid exopolysaccharide, eliminated the diffusion of piperacillin, consistent with the behaviour of the exopolysaccharide as a molecular sieve. In a separate investigation, *S. epidermidis* biofilms were established on a dialysis membrane in a model similar to that of Hoyle and colleagues (Dunne *et al.*, 1993). It was found that the antibiotics rifampicin and vancomycin diffused across the membrane, indicating that these drugs could efficiently penetrate *S. epidermidis* biofilms. These results suggested that inhibition of diffusion cannot always explain resistance to antimicrobial compounds (Dunne *et al.*, 1993). Moreover, these

observations also provided evidence that bactericidal levels of both rifampicin and vancomycin can be attained at the surface of an infected implant.

Anderl and colleagues (2000) investigated the penetration of two antibiotics, ampicillin and ciprofloxacin, through biofilms of *Klebsiella pneumoniae* developed on microporous membranes resting on agar plates with or without antibiotics. The study showed that ampicillin was unable to penetrate the wild-type biofilm, and that the production of the ampicillin-degrading enzyme β-lactamase was responsible for this phenomenon. However, β-lactamase-deficient *K. pneumoniae* biofilms in which ampicillin was shown to penetrate completely were still resistant to ampicillin treatment. Moreover, ciprofloxacin was able to penetrate the wild-type *K. pneumoniae* biofilms, but, as was the case with ampicillin, was unable to kill the biofilm bacteria (Anderl *et al.*, 2000). These results indicate that other mechanisms contribute to the resistance of biofilm cells.

It well known that cells of mucoid and non-mucoid *P. aeruginosa* in biofilms are at least one-thousandfold less sensitive to the antibiotics tobramycin and cefsulodin than are cells of the same bacteria in planktonic suspension. Nichols and co-workers (1989) constructed a mathematical model in order to estimate time-courses of penetration of tobramycin and cefsulodin into biofilms and microcolonies of mucoid and non-mucoid *P. aeruginosa.* They demonstrated that the longest time-period for tobramycin concentration at the base of a biofilm 100μm deep to rise to 90 % of the concentration outside the biofilm would be 2.4 hour. However, the calculations predicted that the cefsulodin concentration at the base of a biofilm 100μm deep would rise to 90% of the external concentration in

only 29s when β-lactamase was synthesized at the basal level (Nichols *et al.*, 1989).

Hatch and Schiller (1998) demonstrated that a 2% suspension of *P. aeruginosa* alginate prepared from two clinical *P. aeruginosa* isolates can completely block the diffusion of gentamicin, tobramycin, and polymyxin B, showing how alginate production can help protect *P. aeruginosa* growing within alginate microcolonies in patients with cystic fibrosis from the effects of aminoglycoside. This aminoglycoside diffusion barrier was degraded with a semipurified preparation of alginate lyase (Hatch and Schiller, 1998). The authors suggested that the blocking activity of the alginate might be due to its ability to form viscous gels as well as to its ionic charge, and that when the mucoid polymer is degraded, the aminoglycoside-blocking activity is effectively reduced. This supports the theory that degradation of mucoid polymers within the lungs of cystic fibrosis patients may make the infecting pathogen more susceptible to aminoglycoside chemotherapy (Hatch and Schiller, 1998).

In summary, many researchers have proposed that biofilm drug resistance is due to the glycocalyx which creates a diffusion barrier to antimicrobial agents (Brown *et al.*, 1995; Chen and Stewart, 1996; Liu *et al.*, 1998). Early studies showed that diffusion through a biofilm may be affected by charge (ionic) interactions between the matrix material and the antimicrobial agent, by an increase in the distance the agent must diffuse, by molecular sieving (size exclusion), and by the viscosity of the matrix material. Moreover, some researchers suggest that the polyanionic nature of the glycocalyx creates a barrier (charge interactions) to the diffusion of cationic antimicrobial agents (Chester *et al.*, 1972; Costerton *et al.*,

1981). For example, the negatively charged biofilm matrix delays some drugs such as aminoglycosides, which carry a positive charge. Walters *et al.* (2003) investigated the penetration of tobramycin through *P. aeruginosa* biofilms and found that it was completely retarded for the first 12 hours of a 36-hour assay, whereas ciprofloxacin, a fluoroquinolone, diffused across completely within the first 12 hours. However, at 36 hours approximately 40% of the antibiotic tobramycin had diffused across the biofilm. They concluded that once the binding capability of the matrix is exceeded, free diffusion could ensue (Walters *et al.*, 2003).

5.3.2 Studies with *Candida*

To investigate whether the matrix plays a role in the antifungal resistance of *Candida* biofilms, Baillie and Douglas (2000) compared the susceptibility of *C. albicans* biofilms grown statically (which have minimal matrix material) with those of biofilms incubated with gentle shaking (which produce much more matrix material). The results of the study showed that the extent of matrix formation in *Candida* biofilms grown with or without shaking did not significantly affect biofilm susceptibility to any of the clinically important antifungal agents tested, including amphotericin B, flucytosine, and fluconazole (Baillie and Douglas, 2000). However, other investigations with biofilms developed under flow conditions suggested that matrix material might play a minor role in biofilm resistance, since resuspended cells (which presumably had lost most of their matrix material) were 20% less resistant to amphotericin B than intact *C. albicans* biofilms (Baillie and Douglas, 1998a; Baillie and Douglas, 1998b). These findings with

resuspended biofilm cells were subsequently confirmed elsewhere (Ramage *et al.*, 2002b).

Mixed species fungal-bacterial biofilms consisting of *C. albicans* and *S. epidermidis* have been investigated by Adam and collaborators (2002). Drug susceptibility studies suggested that fungal cells can modulate the action of antibiotics, and that bacteria can affect antifungal activity in these mixed fungal-bacterial biofilms, possibily as a result of increased viscosity of the matrix. Overall, these results seem to indicate that the matrix material plays a partial role in fungal biofilm resistance, but other factors are also likely to be involved.

5.4 'Persister' cells

One the newest hypotheses for the reduced susceptibility of biofilms to antimicrobials is the formation of a special class of protected subpopulation cells termed 'persisters' (Spoering and Lewis, 2001). Little is known about persisters, no doubt because of the technical difficulties of working with a small fraction of cells (usually 10^{-6}-10^{-4} of the population) expressing a temporary phenotype of uncertain functional significance (Lewis, 2001).

Brooun *et al.* (2000) studied a dose-response killing of *P. aeruginosa* biofilms by the quinolones, ofloxacin and ciprofloxacin. The results proved that the majority of cells were effectively killed by low, clinically achievable concentrations of drug akin to the concentrations which are lethal to planktonic cultures. These experiments indicated that the presence of a small fraction of persister cells was primarily responsible for the very high level of resistance of the biofilms (Brooun *et al.*, 2000). It has been suggested that persisters, and biofilms that contain them, display tolerance, which means that cells do not grow in the

presence of antimicrobial agents; however, they do not die either. This capability to avoid being killed is one of the defining features of persisters (Keren *et al.*, 2004; Cogan, 2006).

Because the study of persister cells in bacterial biofilms is in its infancy, it is not known whether the same phenomenon occurs in *Candida* biofilms. A recent publication by LaFleur *et al.* (2006) reported that biofilms formed by *C. albicans* exhibited a strikingly biphasic killing pattern in response to two antifungal agents. The results indicated that a subpopulation of highly tolerant cells existed. Interestingly, surviving *C. albicans* persisters were only observed in biofilms and not in planktonic exponentially growing, or stationary, populations. The authors concluded that *C. albicans* persisters are not mutants, but phenotypic variants of the wild type which switch on "persister" genes in response to the biofilm mode of growth (LaFleur *et al.*, 2006). Another recent study by Khot and co-workers (2006) demonstrated that *C. albicans* biofilms cultured in a tubular flow cell system harboured a subpopulation of yeast cells that were significantly more resistant to amphotericin B than planktonic populations. This subpopulation of yeast cells formed a basal layer that was tenaciously attached to the surface of the tubing (Khot *et al.*, 2006).

6 Mixed species 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*, several model systems have been used to investigate mixed species fungal-bacterial biofilms, including catheter disks, polycarbonate membrane filters, and denture acrylic disks. Adam *et al.* (2002) used a simple

catheter disk model system to study the development *in vitro* of mixed-species biofilms 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. Interestingly, in mixed fungal-bacterial biofilms, both staphylococcal strains showed extensive interactions with both yeasts and hyphae under scanning electron microscopy. Similar observations have been made with biofilms consisting of *C. albicans* and oral streptococci (*Streptococcus gordonii* and *Streptococcus salivarius*) on denture acrylic (Jenkinson and Douglas, 2002).

Recently O'May and co-workers (2005) designed a complex *in vitro* fermenter system to replicate the biofilm that forms within gastric feeding tubes. This chemostat fermenter was inoculated with the most common strains found in clinical gastric feeding tube samples such as *Lactobacillus sharpeae*, *Lactobacillus paracasei*, *E. coli*, *K. pneumoniae*, *S. aureus*, *C. albicans* and *C. famata.* In this model, the mixed-species biofilms formed on the chemostat tubing, and were composed of both yeast and bacterial cells. Surprisingly, acid suppression therapy was found to change community structure in favour of *Candida* (O'May *et al.*, 2005).

Over the last decade, two opportunistic pathogens, *P. aeruginosa* and *C. albicans*, have been shown to be significantly involved in many device-associated nosocomial infections. Recent *in-vitro* studies have shown antagonistic interactions between these organisms (Hogan and Kolter, 2002; Hogan *et al.*, 2004). *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 beginning 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 (Hogan and Kolter, 2002). The authors suggested that many microbial virulence factors, normally considered in the context of human infection, might also be involved in bacterial–fungal interactions. Subsequently, Hogan *et al.* (2004) identified *P. aeruginosa* mutants incapable of inhibiting *C. albicans* filamentation. During these studies they found that 3-oxo-C_{12} homoserine lactone, a cell-cell signalling molecule produced by *P. aeruginosa*, was sufficient to inhibit *C. albicans* filamentation without affecting fungal growth rates (Hogan *et al.*, 2004).

In the oral cavity, *Candida* or other yeast species always co-exist with commensal bacteria within a biofilm matrix. *In vitro*, Lamfon *et al.* (2005) used denture acrylic (polymethylmethacrylate) disks to co-culture fungal-bacterial biofilms derived from denture plaque from denture stomatitis patients. They found that *Actinomyces*, *Lactobacillus*, *Streptococcus* and *Candida* species grew readily in the biofilm mode *in vitro* and suggested that the system could be used to assess the susceptibility of *Candida* species within these biofilms to antifungal agents (Lamfon *et al.*, 2005). Similar studies have been performed by Shapiro *et al.* (2002) to examine the efficacy of different mouth-rinses containing chlorhexidine, hexetidine, octenidine, Triclosan and aminefluoride against mixed-species fungal-bacterial biofilms.

In a recent investigation, Thein and colleagues (2006) evaluated the effect of varying concentrations of eight different aerobic and anaerobic oral commensal bacterial species such as *Actinomyces israelii*, *Lactobacillus acidophilus*, *Prevotella nigrescens*, *Porphyromonas gingivalis*, *P. aeruginosa*, *E. coli*, *Streptococcus mutans*, and *Streptococcus intermedius* on *in vitro C. albicans* biofilm formation. Polystyrene plastic surfaces were used as a substrate for biofilm development. They found that co-culture with different concentrations of bacteria had variable effects on *Candida* biofilm formation. Moreover, co-culture with the highest concentrations of bacteria resulted in a consistent reduction in the yeast count in *Candida*l biofilms. The data demonstrated the quantitative and qualitative nature of the bacteria modulating *C. albicans* biofilm formation in mixed-species environments such as the oral cavity (Thein *et al.*, 2006).

7 Aims and objectives of research

In recent years, the use of medical implants such as catheters, prosthetic heart valves and joint replacements has increased dramatically. These devices can be colonised by microorganisms that form an adherent biofilm consisting of a mono- or multilayer of cells embedded within a matrix of extracellular polymric material. They are phenotypically different from planktonic or suspended cells; notably, they resist host defences and display a significantly decreased susceptibility to antimicrobial agents. Fungal implant infections are less common than bacterial ones but tend to be more serious and are an increasing problem. They are most often caused by the pathogenic *Candida* species, particularly *C. albicans*. Some of these infections involve mixed species biofilms of *C. albicans* with, for example *S. epidermidis*. *C. albicans* biofilms, like their bacterial counterparts, are

inherently less susceptible to antimicrobial agents than planktonic cells but the mechanism of this drug resistance is unknown. The aim of this project was to explore the possible role of the extracellular matrix material as a barrier to drug penetration in both single species *Candida* biofilms and in mixed species bacterial-fungal biofilms.

Specific objectives included the following:

1) Determination of the penetration of antifungal agents through both single species *Candida* biofilms and mixed species bacterial-fungal biofilms using a novel filter disk assay.
2) Comparison of the drug susceptibility of biofilms grown statically and under conditions of continuous flow using the modified Robbins device.
3) Isolation and analysis of matrix material extracted from biofilms formed by different *Candida* species.
4) Enzymic detachment of biofilms formed by different *Candida* species using a range of commercially available enzymes of known specificity.

MATERIALS AND METHODS

1 Origin and maintenance of organisms

Three strains of *C. albicans* were used in this study. Strains GDH 2346 (NCYC 1467) and GDH 2023 (NCYC 1468) were originally isolated from patients with denture stomatitis at Glasgow Dental Hospital. Strain GRI 682 (NCYC 1473) was obtained from a routine cervical smear taken from an asymptomatic woman at Glasgow Royal Infirmary.

C. glabrata AAHB 12, *C. parapsilosis* AAHB 4479 and *C. tropicalis* AAHB 73 were isolated from patients with line infections at Crosshouse Hospital, Kilmarnock, Scotland. *C. krusei* was obtained from a clinical specimen and came from the Regional Mycology Reference Laboratory, Glasgow, Scotland.

Two strains of *S. epidermidis* (RP62A and M7) were used in this study. Both were kindly supplied by Professor C. G. Gemmell, Glasgow Royal Infirmary. Strain RP62A (ATCC 35984) is known to be a 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).

Candida isolates were maintained on slopes of Sabouraud dextrose agar (SDA; Difco; Appendix 1.2) and *S. epidermidis* strains on Tryptic soy agar (TSB; Difco) at 4°C, and subcultured monthly. Every two months, the cultures were replaced by new ones freshly grown from freeze-dried stocks. Both freeze-dried fungi and bacteria were stored in small, evacuated glass ampoules kept at -20°C.

2 Growth media and culture conditions

Two media were used in this study. Yeast nitrogen base (YNB) was used for the growth of *Candida* species while Tryptic soy broth (TSB) was employed for *S. epidermidis*. In some experiments, mixed species biofilms containing both *Candida* and *S. epidermidis* were also grown in TSB.

2.1 Yeast nitrogen base medium (YNB; Difco; Appendix 1.1)

Yeast nitrogen base (6.7g per litre) was supplemented with 9.0g of glucose to give a final concentration of 50 mM glucose as the carbon source (McCourtie and Douglas, 1981). This medium had a final pH of 5.4 and was autoclaved at 10 p.s.i. for 10min. Batches of medium (50ml, in 250ml Erlenmeyer flasks) were inoculated with *Candida* isolates from fresh culture slopes and incubated at 37°C for 24h in an orbital shaker operating between 60-90 rpm. All *Candida* strains and species, with the exception of the *C. tropicalis* isolate, grew exclusively in the budding-yeast phase under these conditions. Overnight cultures were harvested by centrifugation at 3000 rpm for 5 min and washed twice with sterile 0.15 M phosphate-buffered saline (PBS; pH 7.2, Ca^{2+} and Mg^{2+} free) then resuspended in PBS before use in biofilm experiments. All washed cell suspensions were adjusted to an optical density of 0.8 at 520 nm or 0.2 at 600 nm (for penetration assays).

2.2 Tryptic soy broth (TSB; Difco; Appendix 1.3)

As a result of preliminary experiments with a range of media, Tryptic soy broth (TSB) was selected as the liquid medium best able to support the growth of both fungi and bacteria. Isolates of *C. albicans* GDH 2346 and *S. epidermidis* (RP62A and M7) grew at similar rates in this medium (Adam *et al.*, 2002). The

medium was autoclaved at 121°C for 15 min. Batches of medium (50 ml, in 250-ml Erlenmeyer flasks) were inoculated from fresh culture slopes and incubated at 37°C for 24h on a shaking platform operating at 60-90 rpm. Cells were harvested, washed twice in sterile PBS and finally resuspended in PBS to an optical density of 0.8 at 520 nm or 0.2 at 600 nm (for penetration assays). For development of mixed species biofilms, equal volumes from the standardized suspensions of each organism were mixed immediately before use.

3 Penetration of biofilms by antifungal agents

Several model systems have been developed to study the biofilm mode of growth. In this study, the penetration of antifungal drugs through *Candida*, *S. epidermidis* and mixed fungal-bacterial biofilms was investigated by adapting a novel filter disk assay described by Anderl *et al.* (2000) for bacterial biofilms.

3.1 Antifungal agents

Four clinically important antifungal agents were used in this study. Flucytosine (5-fluorocytosine) and amphotericin B were obtained from Sigma. Fluconazole and voriconazole were kindly donated by Pfizer Ltd. All drug solutions were prepared immediately before use. Flucytosine and fluconazole were dissolved in sterile distilled water and then added to molten culture medium at 50°C via a minisart sterile filtration unit (Sartorius AG, Goettingen, Germany) to create antifungal-supplemented agar for biofilm experiments. Voriconazole and amphotericin B were dissolved in dimethyl sulfoxide (DMSO) and filtered into the warm growth medium. The medium was buffered to pH 7 with 0.165 M morpholinopropanesulfonic acid (MOPS) buffer (Sigma). High drug

concentrations were used in the antifungal-supplemented agar. They were selected on the basis of their ability to give large zones (Fig. 4) of growth inhibition in control assays for drug penetration outlined below. The concentrations used were as follows: flucytosine, 6 µg/ml (30 times the MIC for planktonic *C. albicans* GDH 2346); fluconazole, 24 µg/ml (60 times the MIC); voriconazole, 10 µg/ml (10 times the MIC); and amphotericin B, 78 µg/ml (60 times the MIC).

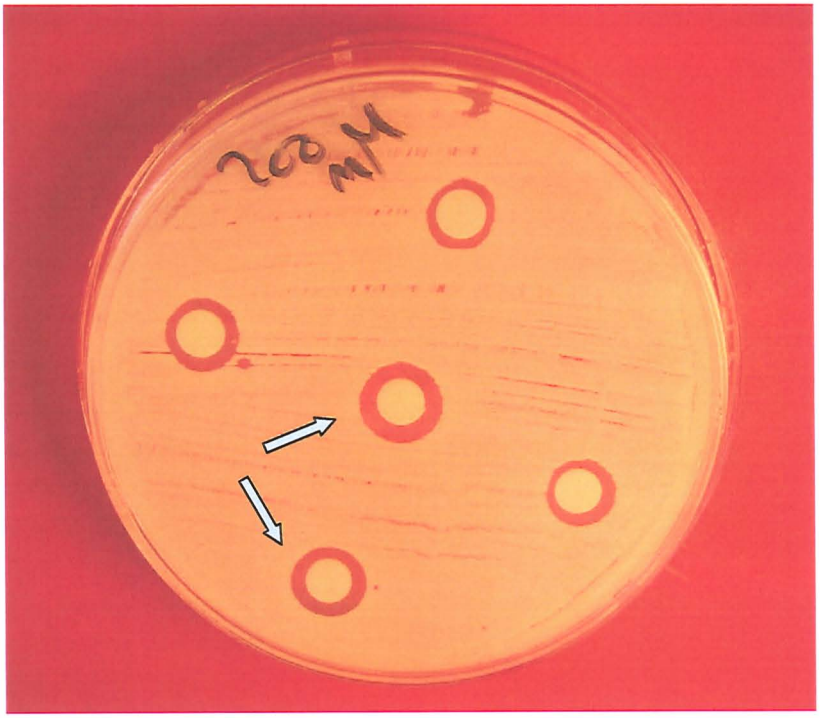


Figure 4. Measurement of drug penetration by zone-of-inhibition bioassay. YNB agar containing 200mM glucose was seeded with 150µl of a standardized suspension of *C. albicans* GDH 2346 to determine zone of growth inhibition (arrows) around disks containing antifungal agents.

3.2 Biofilm formation on membrane filters

Biofilms were grown on membrane filters resting on agar culture medium in petri dishes. Polycarbonate membrane filters (diameter, 25 mm; pore size, 0.2μm; Whatman) were sterilized by exposure to ultraviolet radiation for 15min on both sides prior to inoculation, and placed on the surface of YNB agar containing 50 mM glucose for *Candida* species or TSB agar for *S. epidermidis* and mixed species biofilms. A standardized cell suspension (50 μl) was applied to the surface of each sterile membrane. All plates were incubated at 37°C for 24 h. The membrane-supported biofilms were then transferred to fresh agar (YNB agar with 50 mM glucose or TSB agar) for a further 24 h, giving a total incubation time of 48 h for biofilm formation. Smaller, polycarbonate membrane filters (diameter, 13 mm; pore size, 0.2 μm; Whatman) were sterilized by exposure to ultraviolet radiation for 15 min on both sides, and were then carefully placed on top of the 48-h biofilms. Paper concentration disks (diameter, 6 mm; Becton Dickinson) were also sterilized by exposure to ultraviolet radiation for 15 min per side, and then moistened with growth medium (normally 29 μl) prior to placement on top of the 13-mm-diameter membranes. Because of an occasional variation in disk thickness, a slightly higher or lower volume of medium was sometimes required to saturate the disks. Wetting the disks helped prevent the capillary action of the antifungal medium through the biofilms. Biofilms sandwiched between the membranes and moistened disks were transferred to antifungal agent–containing agar culture medium (Fig. 5). All plates were incubated for specified exposure times, namely, 60, 90, 120, 180, 240, or 360 min.

The amount of antifungal agent which had penetrated each biofilm and which had reached the concentration disk was determined by using the disk in a standard drug diffusion assay. Plates of YNB agar supplemented with 200 mM glucose were seeded with 150 µl of a standardized suspension of planktonic *C. albicans* GDH 2346 (used here as an indicator organism, and adjusted to an optical density of 1.0 at 520nm). After the appropriate exposure time, concentration disks were removed from the biofilm 'sandwiches' and placed on the seeded plates, which were then incubated at 37°C for 24 h. The zones of growth inhibition (Fig. 4) were measured (in mm) at four points around each disk and the mean value calculated. This value was then used to determine the concentration of active antifungal agent in the disk by reference to a standard curve prepared using drug solutions of different concentrations but fixed volumes.

All drug penetration assays were carried out in duplicate on at least two separate occasions. In control assays, concentration disks were placed on the two-membrane system to which no cells had been added, i.e., the unit without the biofilm. The drug concentration that penetrated the biofilms (C) was divided by the drug concentration determined for the controls (C_o) to provide a normalized penetration curve (Anderl *et al.*, 2000).

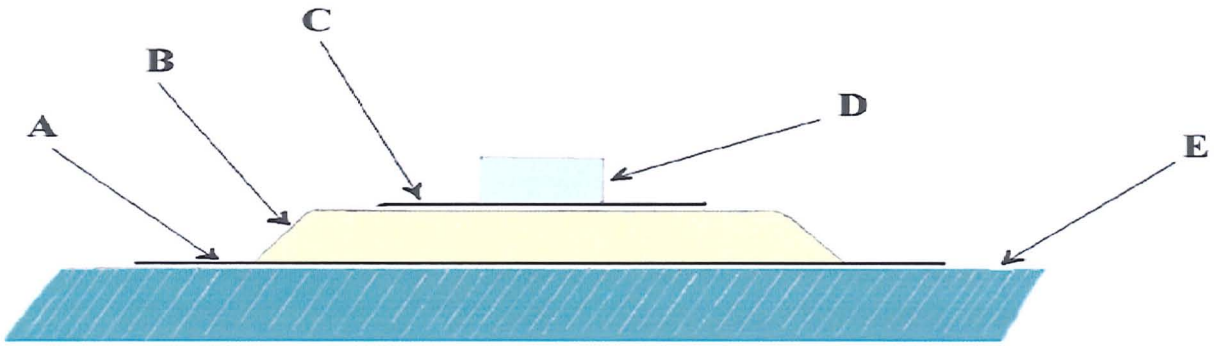


Figure 5. The experimental system used to determine the penetration of antifungal agents through biofilm.

(A) A 25mm-diameter microporous polycarbonate membrane

(B) Biofilm

(C) A 13mm-diameter microporous polycarbonate membrane

(D) A concentration disk

(E) Antifungal agent-containing agar

3.3 Preparation of standard curves for drug penetration assays

3.3.1 Flucytosine (5-fluorocytosine)

A standard curve of flucytosine concentration plotted against diameter of zone of growth inhibition was prepared using flucytosine solutions (in water) of 2, 4, 6, 8, 10, and 20 µg/ml (Table 2). Overnight cultures of *C. albicans* GDH 2346 were harvested by centrifugation at 3000 rpm for 5 min, washed twice in sterile 0.15 M PBS (pH 7.2) and then resuspended in PBS to an optical density of 1.0 at 520 nm. YNB agar supplemented with 200 mM glucose was seeded with 150 µl of this standardized suspension (used here as an indicator organism) to measure the zones of inhibited growth. Blank paper concentration disks were moistened with a fixed volume (24 µl) of drug solution before being transferred to the pre-seeded plates. All plates were incubated at 37°C for 24 h. A total of 9 disks was used for each drug concentration (Table 2). Mean values for the diameter of the zone of growth inhibition were calculated, and plotted against the flucytosine concentration used, to produce a standard curve. Thereafter, test zones of growth inhibition were compared to the standard curve to determine the concentration of flucytosine in experimental disks (Fig. 6).

3.3.2 Fluconazole

A standard curve of fluconazole concentration against diameter of zone of growth inhibition was prepared as described for flucytosine except that fluconazole solutions (in water) of 8, 10, 12, 14, 18, 20, and 25 µg/ml were used (Table 3; Fig. 7). A total of 5 disks was used for each drug concentration (Table 3).

Table 2. Zone of growth inhibition due to flucytosine on plates seeded with *C. albicans* GDH 2346 [a]

	Diameter of zone (mm) of growth inhibition produced by flucytosine at a concentration (µg/ml) of					
Disk [b]	**2**	**4**	**6**	**8**	**10**	**20 [c]**
1	2.0	4.0	6.0	6.50	7.0	11.0
2	2.0	4.50	6.0	6.50	7.50	9.50
3	2.0	4.50	7.0	7.0	7.0	10.50
4	3.0	4.50	7.0	6.0	8.0	9.50
5	2.0	4.0	6.0	7.0	7.50	9.50
6	3.0	4.50	6.0	6.50	7.0	9.0
7	3.0	4.50	7.0	6.0	7.0	9.0
8	2.0	4.50	7.0	6.50	7.0	9.50
9	2.50	4.0	8.0	6.50	7.50	9.0
Total	21.50	39.0	60.0	58.50	65.50	86.50
Mean[d]	2.39	4.33	6.67	6.50	7.28	9.61

[a] Growth medium (YNB supplemented with 200 mM glucose) was seeded with 150 µl of a standardized suspension of *C. albicans*.

[b] Blank paper concentration disks (9 for each drug concentration) were moistened with a fixed volume (24µl) of drug solution.

[c] Different concentrations (µg/ml) of flucytosine were used to draw the standard curve.

[d] Data are means from three independent experiments done in duplicate.

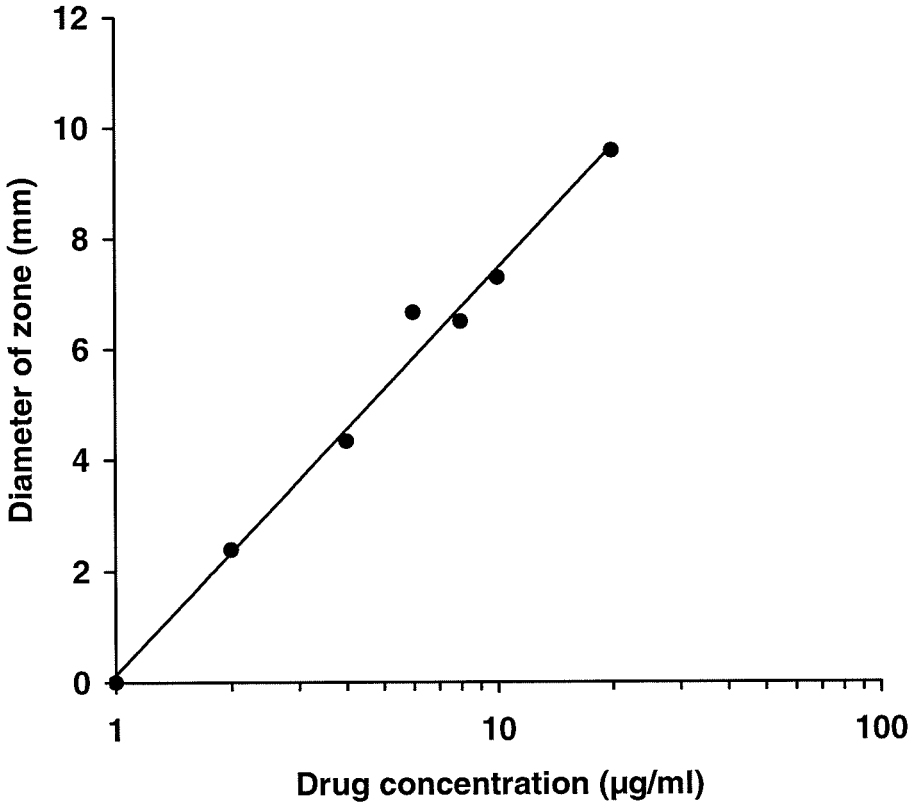


Figure 6. Standard curve for flucytosine in drug penetration assay

Table 3. Zone of growth inhibition due to fluconazole on plates seeded with *C. albicans* GDH 2346 [a]

	Diameter of zone (mm) of growth inhibition produced by fluconazole at a concentration (µg/ml) of						
Disk [b]	**8**	**10**	**12**	**14**	**18**	**20**	**25 [c]**
1	0.0	0.0	0.0	2.50	3.75	5.25	7.50
2	0.0	0.0	0.0	2.50	3.25	5.50	7.50
3	0.0	0.0	0.0	2.50	3.75	5.50	7.25
4	0.0	0.0	0.0	2.50	3.25	5.75	7.75
5	0.0	0.0	0.0	2.50	4.00	5.50	7.25
Total	0.0	0.0	0.0	12.50	18.00	27.50	37.25
Mean [d]	0.0	0.0	0.0	2.50	3.60	5.50	7.45

[a] Growth medium (YNB supplemented with 200mM glucose) was seeded with 150µl of a standardized suspension of *C. albicans*.

[b] Blank paper concentration disks (5 for each drug concentration) were moistened with a fixed volume (27µl) of drug solution.

[c] Different concentrations (µg/ml) of fluconazole were used to draw the standard curve.

[d] Data are means from two independent experiments done in duplicate.

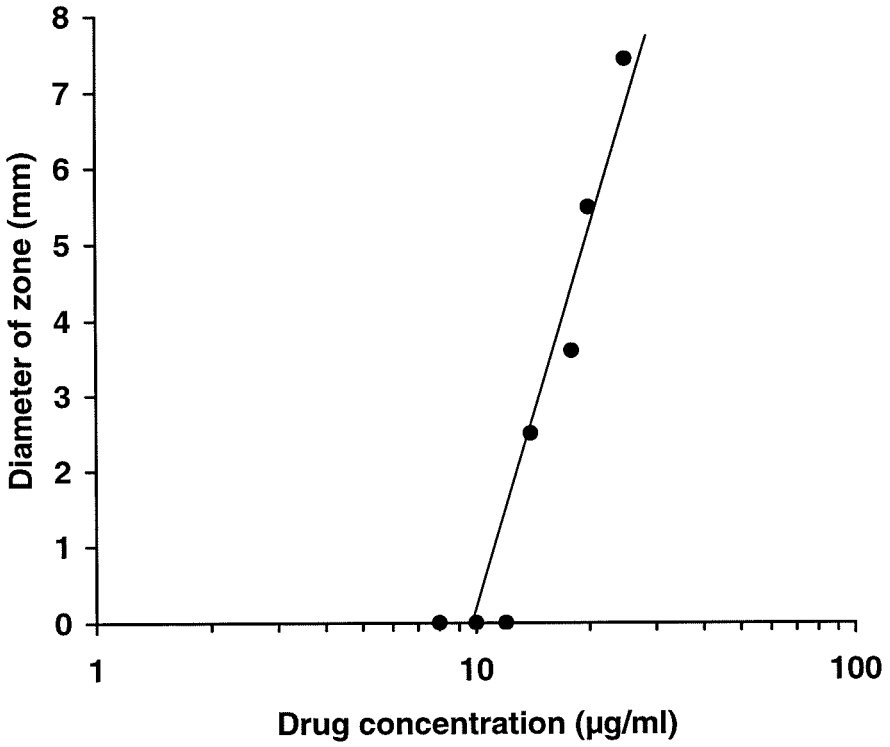


Figure 7. Standard curve for fluconazole in drug penetration assay

3.3.3 Amphotericin B

A standard curve of amphotericin B concentration against diameter of zone of growth inhibition was prepared as described for flucytosine except that amphotericin B solutions (in dimethyl sulfoxide) of 20, 30, 40, 50, 60, 70, and 80 μg/ml were used (Table 4; Fig. 8).

3.3.4 Voriconazole

A standard curve of voriconazole concentration against diameter of zone of growth inhibition was prepared as described for flucytosine except that voriconazole solutions (in dimethyl sulfoxide) of 2, 5, 10, 15, and 20 μg/ml were used (Table 5; Fig. 9).

3.4 Viable counts of biofilm cells exposed to antifungal agents

After biofilm formation on 25-mm-diameter membrane filters, biofilms were capped with sterile, 13-mm-diameter filters, transferred to antifungal agent-containing agar, and incubated at 37°C for 6 h (the maximum exposure period in drug penetration assays) or 24 h. The longer time period was chosen to check whether prolonged drug exposure might increase biofilm susceptibility. After incubation, biofilm cells were scraped gently from the membranes using a sterile scalpel and resuspended in 10 ml of PBS. Serial dilutions (10^{-1} to 10^{-6}) of each biofilm cell suspension were then prepared. Triplicate samples (0.1 ml) of the 10^{-4}, 10^{-5} and 10^{-6} dilutions were spread on YNB agar containing 200 mM glucose, and the plates were incubated at 37°C for 24 h. In control assays, the membranes were transferred to growth medium containing no antifungal agent.

Table 4. Zone of growth inhibition due to amphotericin B on plates seeded with *C. albicans* GDH 2346 [a]

	Diameter of zone (mm) of growth inhibition produced by amphotericin B at a concentration (µg/ml) of						
Disk [b]	**20**	**30**	**40**	**50**	**60**	**70**	**80 [c]**
1	1.75	2.50	3.0	3.87	4.0	5.0	5.0
2	2.0	2.75	3.0	4.0	4.13	4.88	5.0
3	1.13	2.63	3.13	3.38	4.0	4.75	4.88
Total	4.88	7.88	9.13	11.25	12.13	14.63	14.88
Mean [d]	1.63	2.63	3.04	3.75	4.04	4.88	4.96

[a] Growth medium (YNB supplemented with 200mM glucose) was seeded with 150µl of a standardized suspension of *C. albicans*.

[b] Blank paper concentration disks (3 for each drug concentration) were moistened with a fixed volume (33µl) of drug solution.

[c] Different concentrations (µg/ml) of amphotericin B were used to draw the standard curve.

[d] Data are means from two independent experiments done in duplicate.

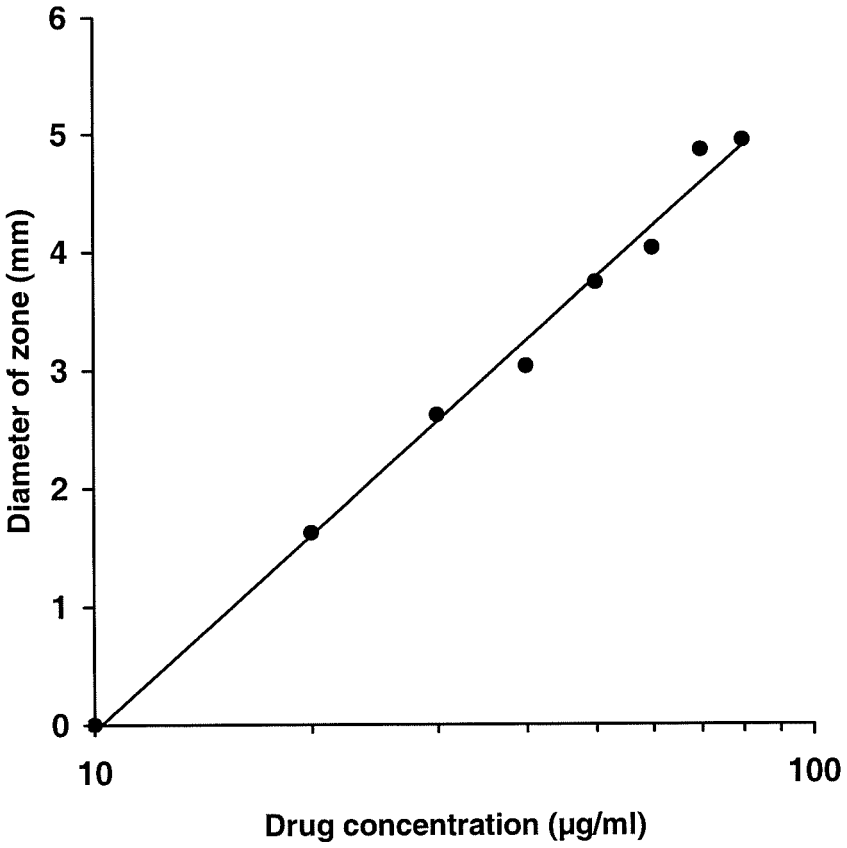


Figure 8. Standard curve for amphotericin B in drug penetration assay

Table 5. Zone of growth inhibition due to voriconazole on plates seeded with *C. albicans* GDH 2346 [a]

	Diameter of zone (mm) of growth inhibition produced by voriconazole at a concentration (µg/ml) of				
Disk [b]	**2**	**5**	**10**	**15**	**20 [c]**
1	5.50	9.50	12.25	13.00	15.00
2	5.50	9.25	12.25	13.00	15.00
3	5.25	9.00	12.25	13.75	14.75
Total	16.25	27.75	36.75	39.75	44.75
Mean [d]	5.42	9.25	12.25	13.25	14.92

[a] Growth medium (YNB supplemented with 200mM glucose) was seeded with 150µl of a standardized suspension of *C. albicans*.

[b] Blank paper concentration disks (3 for each drug concentration) were moistened with a fixed volume (30µl) of drug solution.

[c] Different concentrations (µg/ml) of voriconazole were used to draw standard curve.

[d] Data are means from two independent experiments done in duplicate.

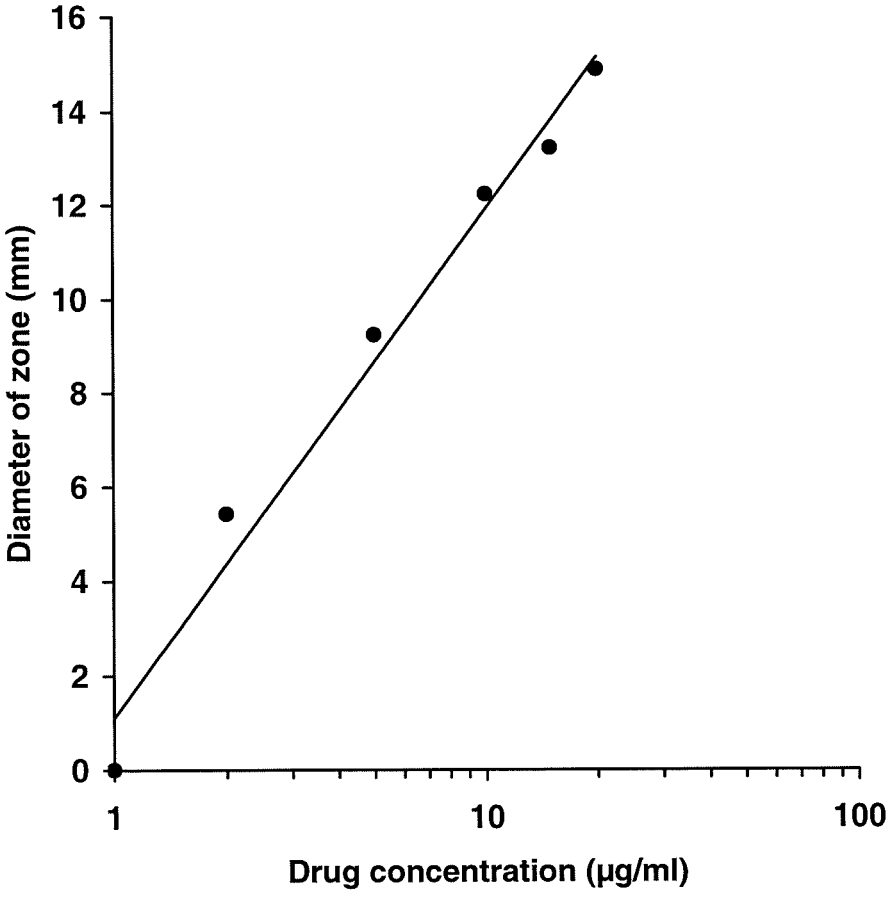


Figure 9. Standard curve for voriconazole in drug penetration assay

4 Drug susceptibility of biofilms formed under static and flow conditions

4.1 Biofilm formation under static conditions on catheter disks

Biofilms grown statically were formed on the surfaces of small disks (diameter, 0.8cm) cut from polyvinyl chloride (PVC) Faucher tubes (French gauge 36; Vygon, Cirencester, UK) using a punch (Fig. 10). Disks were sterilized by exposure to ultraviolet radiation for 30 min on both sides and then placed in the wells of 24-well Nunclon tissue culture plates (Corning Incorporated 3524, USA). Standardized cell suspensions (80 µl) were applied to the surface of each sterile disk. Initially, incubation lasted for 1 h at 37°C (adhesion period). Thereafter, each disk was gently dipped into a well containing 3ml of sterile PBS to remove any non-adherent cells. The disks were transferred to wells of new tissue culture plates each containing 1 ml of YNB supplemented with 50 mM glucose, or TSB medium. They were then incubated statically at 37°C for 48 h, submerged in growth medium, for biofilm formation.

4.2 Biofilm formation under flow conditions using the modified Robbins device (MRD)

Several model systems have been developed to study the biofilm mode of growth under conditions of continuous flow. The modified Robbins device (MRD, Fig. 11 a and b) is one of the most widely used and well-known systems (Lappin-Scott *et al.*, 1993). It provides quantifiable samples of biofilms growing on submerged surfaces in aqueous systems.

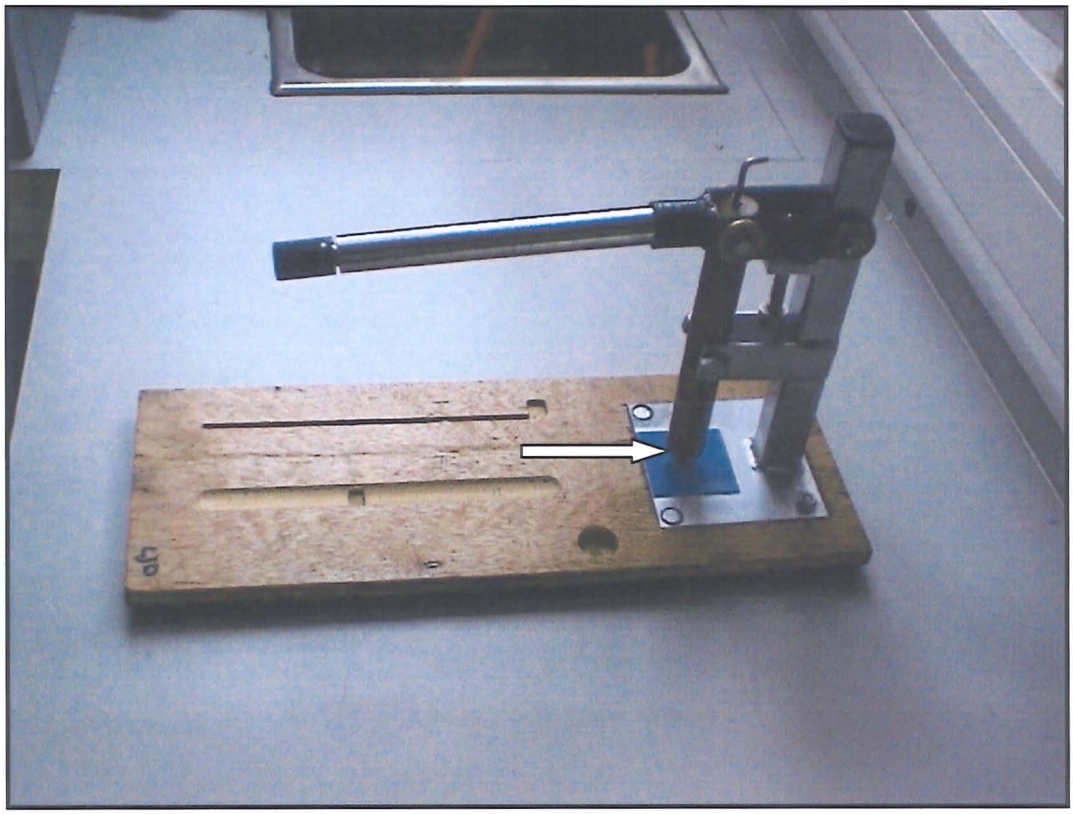
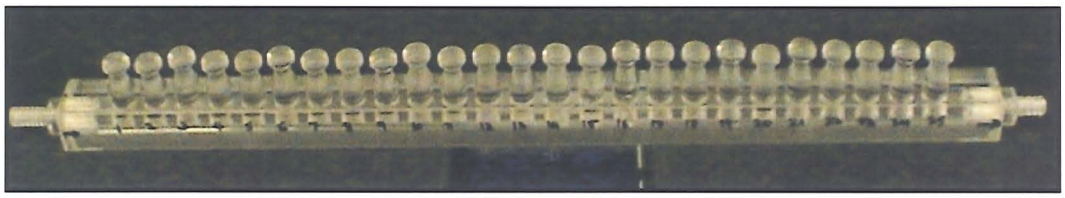
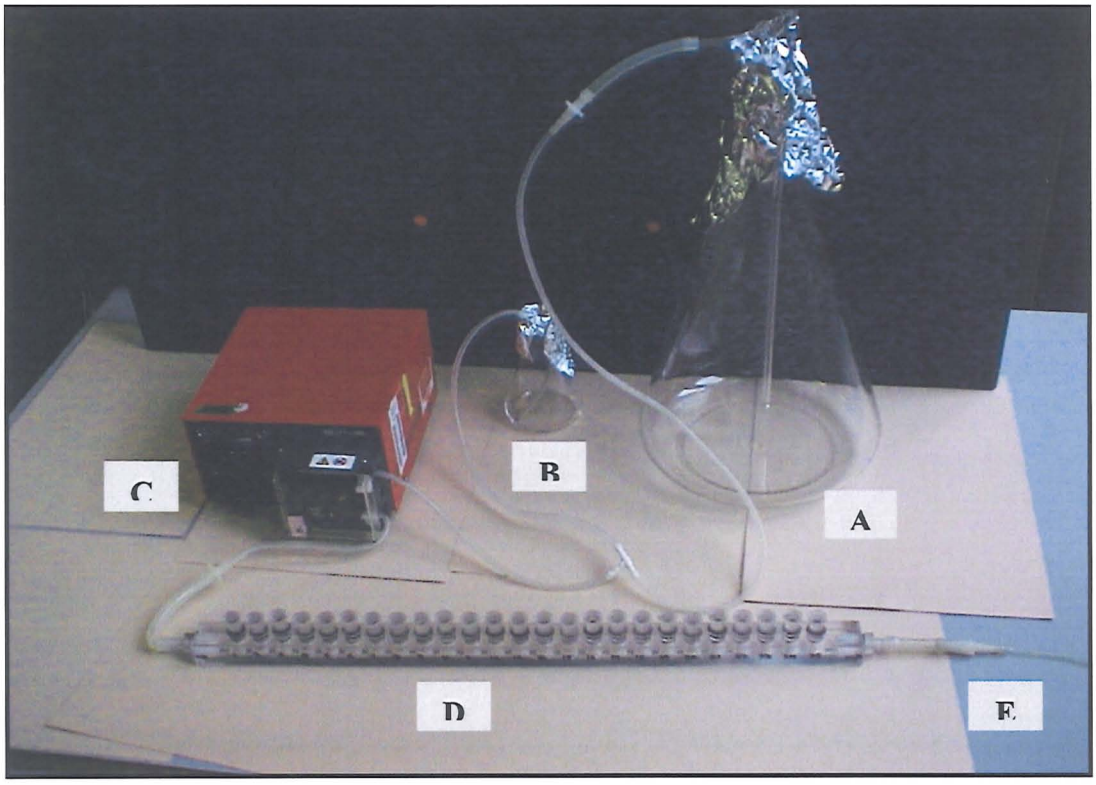


Figure 10. Punch device used to prepare small disks (diameter, 0.8cm) from PVC Faucher tubes for biofilm formation under static and flow conditions. Arrow cutting edge of punch.

Figure 11



(a) The modified Robbins device (MRD)



(b) The experimental system used in this study to provide a continuous flow of cell suspension and culture medium for biofilm formation

(A) 5-litre Erlenmeyer flask with YNB or TSB culture medium.

(B) 250-ml Erlenmeyer flask containing cell suspension.

(C) Peristaltic pump to adjust flow rate.

(D) Modified Robbins device (MRD).

(E) Translucent silicone tubing connected to waste flask.

4.2.1 MRD model system

The MRD is an artificial multiport sampling catheter, constructed of an acrylic block (41.5 cm long, 20 mm high and 26 mm across) with a rectangular lumen (2 mm deep by 10 mm wide). There are 25 evenly spaced sampling ports, 11 mm in diameter, with polypropylene connectors at either end for attaching the tubing from the culture vessel (Lappin-Scott *et al.*, 1993). The sample studs, also made of acrylic, are 26 mm in length and are inserted into the sample ports; this is assisted by rubber O-rings which effectively seal the ports to prevent leakage. Each O-ring is positioned in a narrow groove, 21 mm from the top of the stud. The bottom surface of the sample stud has a 1cm rim designed to hold a sterile disk of the material being studied (Fig. 12). The sampling studs can be removed and replaced aseptically. To establish a biofilm on the surface of disks in the MRD, 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 (Fig. 11 a and b).

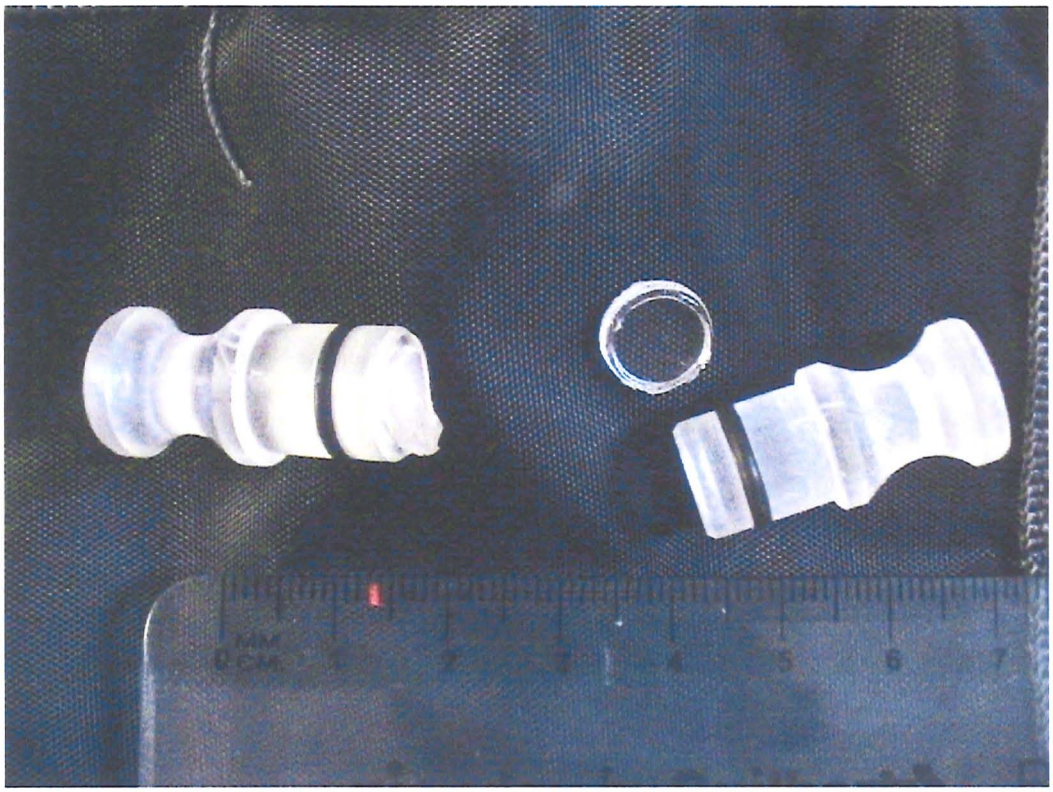


Figure 12. Sampling studs containing sterile catheter disks are inserted into MRD model system for biofilm formation under continuous flow.

4.2.2 Cleaning and maintenance of the MRD

The MRD was completely dismantled and thoroughly cleaned between each assay. After the completion of each experiment, the MRD was sterilized with 0.05 % hibitane, which was pumped through the MRD for 1 h at a flow rate of 60 ml/h. This was followed by a 1-h wash with sterile distilled water, pumped through at a rate of 200 ml/h to remove any traces of hibitane. The device was occasionally soaked in bleach (Virkon) for a short time to remove any build-up of attached biofilm along the lumen. Afterwards, it was washed thoroughly by running tap water through it for an extended period of time. The MRD was stored at ambient temperature on a flat, clean surface, away from direct sun light.

4.3 Susceptibility of biofilms to antifungal agents

The susceptibilities of biofilms grown statically or in the MRD to amphotericin B and fluconazole were tested by treatment with YNB or TSB growth medium containing these antifungal agents. Drug solutions were prepared immediately prior to use. Stock solutions of the drug in DMSO (amphotericin B; 1.95 mg / 50ml) or water (fluconazole; 0.6 mg/50ml) were sterilized by filtration (filter pore size, 0.2µm; Sartorius minisart), and then diluted in warm growth medium buffered to pH 7 with 0.165 M morpholinepropanesulphonic acid (MOPS) buffer (Sigma). After incubation of biofilms on catheter disks for 48 h at 37°C, the disks were transferred to fresh 24-well tissue culture plates. Buffered growth medium (1ml) containing amphotericin B (6.5 or 39 µg/ml) or fluconazole (12 µg/ml) was added to each well and the plates were incubated at 37°C for 5 h or 24 h. Following exposure to antifungal agents, the biofilms were washed gently in 5 ml of PBS and transferred to fresh tissue culture plates. Thereafter, biofilm

activity was assessed quantitatively by tetrazolium salt (XTT; Sigma) reduction assays. The effect of an antimicrobial agent was measured in terms of XTT reduction by biofilms as compared with values obtained for control biofilms incubated under identical conditions in the absence of the drug.

4.4 Quantitative analysis of biofilm growth by the XTT assay

XTT is converted to a coloured formazan product (brown colour) in the presence of metabolic activity (Fig. 13). The primary mechanisms of XTT-to-formazan conversion are the mitochondrial succinoxidase and cytochrome P450 systems, as well as flavoprotein oxidase (Kuhn *et al.*, 2003). However, addition of the electron coupling agent menadione (Vit.K_3; 2-Methyl-1,4-naphthoquinone; Sigma) is essential in the XTT assay.

A stock solution of XTT (2.5 mg/ml) was prepared in prewarmed (37°C) PBS containing 1 % of glucose, 1 ml of XTT solution was added to each well of a new tissue culture plates which containing disks with 48-h mature biofilms grown on the surface of PVC catheter. Menadione solution (1mM in acetone; 4 µl; Sigma) an electron-coupling agent was also added to each well. All plates were incubated at 37°C for 5 h, in dark conditions. After incubation, the XTT solution in each well was transferred to a microfuge tube and clarified at 13,000 rpm for 3 min. The supernatant was then transferred to a spectrophotometer cuvette and formazan production measured at 492 nm (Mosmann, 1983; Tellier *et al.*, 1992; Meshulam *et al.*, 1995; Hawser, 1996; Baillie and Douglas, 1999b).

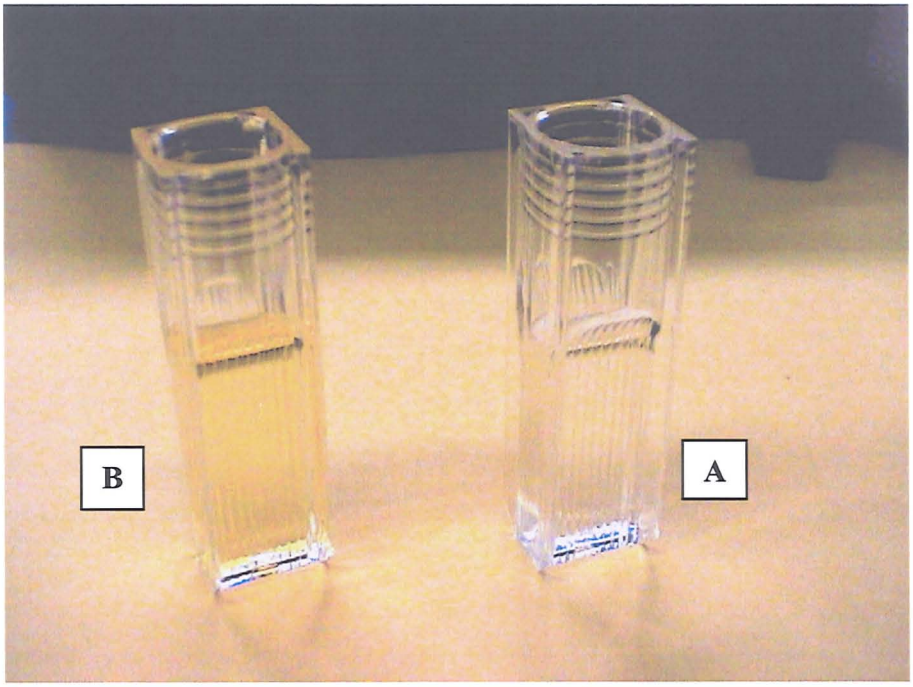


Figure 13. Colorimetric tetrazolium assays (XTT) were used to measure biofilm growth and for drug susceptibility testing. XTT is converted to a coloured formazan product (brown colour) after incubation for 5h at 37°C (B). The blank is colourless (A).

5 Isolation of extracellular polymeric substances (EPS)

5.1 Biofilm formation

Biofilms were formed on sections (4 cm long) of PVC Faucher tubes (French gauge 36; Vygon, Cirencester, UK) that had been cut into three equal concave strips. These were sterilized by exposure to ultraviolet radiation for 15 min on each side. Standardized cell suspension (400 µl) was applied to the surface of each one and the strips were incubated for 1 h at 37°C. Non-adherent cells were removed by gently dipping each strip into a wide-neck conical flask containing 10 ml of sterile PBS. The strips were then transferred to 250-ml, wide-neck Erlenmeyer flasks, (six strips per flask) containing yeast nitrogen base (50 ml) supplemented with 50mM glucose. Flasks were incubated at 37°C for 48 h on an orbital shaker operating at low shaking speeds between 20-50 rpm. This allowed biofilm formation with increased synthesis of extracellular matrix material (Hawser *et al.*, 1998a).

5.2 Isolation of biofilm EPS

Extracellular polymeric material from *Candida* biofilms was prepared by a modification of the method of Baillie and Douglas (2000). Biofilm EPS was physically extracted, without adding any chemical extractants. Catheter strips containing mature biofilms were transferred to universal bottles (six strips per bottle), each containing 10 ml of distilled water. The bottles were sonicated in a water bath at room temperature for 5 min and vortexed vigorously for 1 min to disrupt the biofilms. Cell suspensions were then pooled and the extracellular material was removed from yeast cells by centrifugation at 4,000g for 15 min at room temperature. The supernatants were concentrated to one-tenth of the original

volume using an Amicon DC2 hollow-fibre concentration system with a 3.0 K filter (Millipore Ltd, Watford, UK). The concentrated solutions which contained low molecular-weight metabolites were dialysed through a dialysis membrane (3.5 kDa cut-off; Pierce, Cheshire, U.K) at 4°C for 3 days against five changes (5 litres each) of distilled water and then retentates (crude EPS) were freeze-dried.

6 Chemical analysis of EPS

After lyophilization, the total weight of extracted EPS was determined. EPS preparations were then analysed quantitatively for total carbohydrate, protein, phosphorus, uronic acid, hexosamine and glucose using procedures described below. Full details of these procedures, together with standard curves, are given in Appendix 4 and 5.

6.1 Estimation of total carbohydrate

Total carbohydrate, including both neutral and charged polysaccharides, was determined using the phenol-sulphuric acid method. This utilizes phenol as the specific organic colour-developing agent. Glucose was used as a standard (Dubois *et al.*, 1956).

6.2 Estimation of protein

The Lowry method, with a protein standard of bovine serum albumin (BSA, fraction V; Sigma) was used. It is based on the reaction of peptide bonds with copper in alkaline solution (Biuret reaction) followed by reduction of phosphomolybdic and phosphotungstic acids by the Folin-Ciocalteau phenol reagent (Lowry *et al.*, 1951).

6.3 Estimation of phosphorus

All methods currently used to determine phosphorus in biological material on a micro scale are photometric and based on the same principle. Organically combined phosphorus in the sample is first converted to inorganic phosphate by digestion with acid. The phosphate formed then reacts with ammonium molybdate to give phosphomolybdate. The concentrations of this compound were measured spectrophotometrically, according to Chen *et al.*, (1956) with potassium phosphate (KH_2PO_4) solution as a standard.

6.4 Estimation of uronic acid

The reaction of uronic acids with carbazole is the most satisfactory method of estimating uronic acids. The procedure established by Bitter and Muir (1962) using borate in concentrated sulphuric acid was adopted here. There is an approximately two-fold increase in sensitivity as compared with the original method described by Dische (1947) for glucuronolactone. In addition, the absorbance is a linear function of concentration between 4 – 40 µg/ml, maximum colour develops immediately, and there is greater reproducibility and reduction of interference by chloride ion and oxidants. Glucuronic acid (Sigma) was used as a standard.

6.5 Estimation of total hexosamine

Total hexosamine was determined by using Ehrlich's reagent, according to the method of Blumenkrantz and Asboehansen (1976), with glucosamine as a standard. This procedure yields a chromogen of equal intensity with equal concentrations of glucosamine and galactosamine.

6.6 Glucose determination

Prior to glucose analysis, EPS samples from *C. albicans* GDH 2346 (0.5 ml of 2 mg/ml solution of EPS) and from *C. tropicalis* AAHB 73 (0.25ml of 5.9 mg/ml solution of EPS) were hydrolysed in 1.0 M HCl for 5 h in sealed ampoules at 100°C, and then neutralized with 0.5 M NaOH. Glucose was determined by an enzymatic method, using a glucose assay kit (Sigma-Aldrich, USA) with a standard of D-glucose.

Enzymatic methods are based on the oxidation of glucose to gluconic acid and hydrogen peroxide by glucose oxidase. The hydrogen peroxide formed reacts with O-dianisidine in the presence of peroxidase to form a coloured product. Oxidized O-dianisidine then reacts with sulphuric acid to form a more stable coloured product.

7 Enzymatic treatment of *Candida* biofilms

Eight enzymes were tested for their ability to cause the detachment of *Candida* biofilms from plastic surfaces. All enzyme solutions were prepared and buffered immediately before use. The following enzymes were used (i) proteinase K (in 0.2 M disodium hydrogen phosphate – 0.2 M sodium dihydrogen phosphate buffer, pH 7.5) extracted from *Tritirachium album*; (ii) protease type XIV (in 0.2 M disodium hydrogen phosphate – 0.2 M sodium dihydrogen phosphate buffer, pH 7.5) from *Streptomyces griseus*; (iii) deoxyribonuclease (DNase I) type IV (in 0.1 M citric acid – 0.2 M disodium hydrogen phosphate buffer, pH 5.0) from bovine pancreas; (iv) β-*N*-acetylglucosaminidase (in 0.1 M citric acid – 0.2 M disodium hydrogen phosphate buffer, pH 5.0) from *Canavalia ensiformis* (Jack

bean); (v) chitinase (in 0.1 M citric acid – 0.2 M disodium hydrogen phosphate buffer, pH 6.0) from *Streptomyces griseus*; (vi) lipase type VII (in 0.2 M disodium hydrogen phosphate – 0.2 M sodium dihydrogen phosphate buffer, pH 7.2) from *Candida rugosa* ; (vii) phospholipase A_2 (in 0.2 M Tris maleate – 0.2 M sodium hydroxide, pH 8.0) from bovine pancreas; and (viii) lyticase (in 0.2 M disodium hydrogen phosphate – 0.2 M sodium dihydrogen phosphate buffer, pH 7.5) from *Arthrobacter luteus.* All enzymes were purchased from Sigma.

7.1 Detachment assay

To investigate whether these enzymes could cause the removal of *Candida* biofilms from plastic surfaces, a detachment assay based on that reported by Kaplan *et al.* (2004) for *S. epidermidis* biofilms was used. For detachment experiments, *Candida* species were grown in YNB containing 50mM glucose at 37°C in an orbital shaker at 60 rmp. Cells were harvested after 24 h and washed twice in 0.15 M PBS, pH 7.2. All washed cell suspensions were standardized in growth medium to an optical density of 0.8 at 520nm. *Candida* biofilms were formed in 96-well polystyrene microtitre plates (model 3596; Corning). The wells of 96-well polystyrene microtitre plates were filled with standardized cell suspensions (100 µl), and the plates were incubated statically for 48 h at 37°C for biofilm formation.

To assay biofilm detachment, mature biofilm cells were exposed to different test enzymes. Following incubation of biofilms in 96-well polystyrene microtitre plates for 48 h at 37°C, the growth medium was removed from each well and replaced by an equal volume (100µl) of test enzyme. Enzyme treatments were carried out for 2 h at 25°C (for chitinase, deoxyribonuclease, lyticase and β-

N-acetylglucosaminidase) or 37°C (for lipase type VII, phospholipase A_2, protease type XIV and proteinase K) according to the temperature optimum for the enzyme and at a final enzyme concentration of 50 µg/ml. Following incubation, biofilm cells were stained with Gram-staining reagent (2 g of crystal violet, 0.8 g of ammonium oxalate, and 20 ml of ethanol per 100 ml), then washed twice gently with 200µl of distilled water and left to dry. Thereafter, the amount of biofilm was quantitated by measuring the optical densities of the wells using a Bio-Rad Benchmark microplate reader set to 570 nm.

7.2 Studies with lyticase

Further experiments were carried out to investigate whether lyticase was acting solely on biofilm matrix material, or whether some cell lysis also occurred. The wells of a 24-well polystyrene microtitre plate (model 3524; Corning) were filled with 400 µl of standardized cell suspension, and the plates were incubated for 48 h at 37°C for biofilm formation, the same conditions as those used in the detachment assay. Following incubation, the growth medium was removed from each well and replaced by an equal volume (400 µl) of lyticase in 50 mM tris-HCl buffer (pH 7.2), at a final enzyme concentration of 50 µg/ml. In control experiments, growth medium was removed from each well and replaced by an equal volume of 50 mM tris-HCl buffer without lyticase. Both test and control samples were incubated for 2 h at 25°C, the same conditions as those used in the detachment assay. After incubation with lyticase, the 50 mM tris-HCl buffer was discarded from control samples, and the cells were resuspended in 50 mM tris-HCl buffer (pH 7.2) containing 10 mM $MgCl_2$ and 1 M sorbitol. The optical densities of suspensions were adjusted to 0.8 – 1.0 at 520 nm and 600 nm. The

volume of buffer used was noted. For the test samples, the lyticase solution was discarded from the wells, and the cells were resuspended in 50 mM tris-HCl-$MgCl_2$-sorbitol buffer using the same volume as that used for the control suspensions. At this stage, the optical densities were recorded at 520 and 600 nm for both control and test samples (zero time). Thereafter, further readings for control and test samples were taken at 15 and 30 min during incubation at room temperature. All biofilm and control assays were carried out in triplicate.

8 Scanning electron microscopy (SEM) of biofilms

Biofilm development in all the model systems utilised in this study was observed using scanning electron microscopy.

8.1 Air-drying procedure

Biofilms formed on polycarbonate membranes and catheter disks were fixed with 2.5% (v/v) glutaraldehyde in PBS (pH 7.2) for 1 h at room temperature. They were then treated with 1% (w/v) osmium tetroxide for 1 h, washed three times in 3 ml of distilled water, treated with 1% (w/v) uranyl acetate for 1 h, and finally washed twice in 3 ml of distilled water. Dehydration of the samples was accomplished by immersion in a series of ethanol solutions (30%, 50%, 70%, 90% absolute ethanol, and dried absolute ethanol) for 10 minutes each. All samples were air dried in a desiccator for 48 h then mounted on aluminium stubs before being gold coated with a polaron coater, and viewed under a Philips 500 scanning electron microscope (Hawser and Douglas, 1994a).

8.2 Freeze-drying procedure

The drying procedure selected markedly affects the extent to which the biofilm structure, particularly the matrix of extracellular polymeric material, is retained. Freeze-drying is an effective technique for matrix preservation. Samples were fixed with 2.5% (v/v) glutaraldehyde in 0.1M cacodylate buffer (pH 7.0), washed gently in distilled water three times and then plunged into a liquid propane-isopentane mixture (2:1, v/v) at –196°C before freeze-drying under vacuum (10^{-6} Torr). Disks were mounted on aluminium stubs and coated with gold before being viewed using a Philips 500 scanning electron microscope (Steinbrecht and Muller, 1981; Hawser *et al.*, 1998a).

RESULTS

1 Penetration of antifungal agents through biofilms using a novel filter disk assay

In this part of the project a novel, filter disk assay devised by (Anderl *et al.*, 2000) was adapted to investigate the penetration of antifungal agents through single and mixed species biofilms containing *Candida*. The technique involves the formation of a 48-h–old colony biofilm on a polycarbonate membrane filter and the capping of this biofilm with a second, smaller membrane filter and then a wetted paper disk of the type used in zone-of-inhibition bioassays. The assembly, which represents a primitive diffusion cell, is transferred to agar medium containing the antifungal agent. During subsequent incubation, the drug diffuses out of the agar and through the biofilm sandwich to the moistened paper disk. The drug concentration in the disk can finally be determined by measuring the zone of growth inhibition that it produces on medium seeded with an indicator strain of *C. albicans* in standard bioassays (Anderl *et al.*, 2000).

1.1 Penetration of flucytosine through biofilms of different strains of C. albicans

1.1.1 Susceptibility of *C. albicans* to flucytosine

The minimum inhibitory concentration (MIC) of flucytosine for planktonic *C. albicans* GDH 2346 was 0.2µg/ml (Hawser and Douglas, 1995a). In the experiments presented here, using biofilms of *C. albicans*, 30 times the MIC of flucytosine (6µg/ml) was used. Planktonic cells were rapidly killed by the drug at this concentration.

1.1.2 Flucytosine penetration through *C. albicans* GDH 2346 biofilms

The rate of penetration of flucytosine through *C. albicans* GDH 2346 biofilms was initially low (Fig. 14). The drug concentration (C) at the distal edge of the biofilm (i.e., distal with respect to the agar) was 55 % of the control value (C_o) after 180 min. Within 240 min, the flucytosine concentration had reached 70% of the control value.

1.1.3 Flucytosine penetration through *C. albicans* GDH 2023 biofilms

Drug penetration after 60 min was 29 % of the control value. After 120 min the rate of drug penetration increased slightly and had reached approximately 45% of the control value after 180 min. Within 240 min, the flucytosine concentration had reached 65 % of the control value (Fig. 15).

1.1.4 Flucytosine penetration through *C. albicans* GRI 682 biofilms

The rate of diffusion of flucytosine through *C. albicans* GRI 682 biofilms was relatively rapid overall. Within 60 min, the drug concentration at the distal edge of biofilm was 25 % of the control value. After 180 min it had reached 50%, and after 240 min, it was 80 % of the control value (Fig. 16).

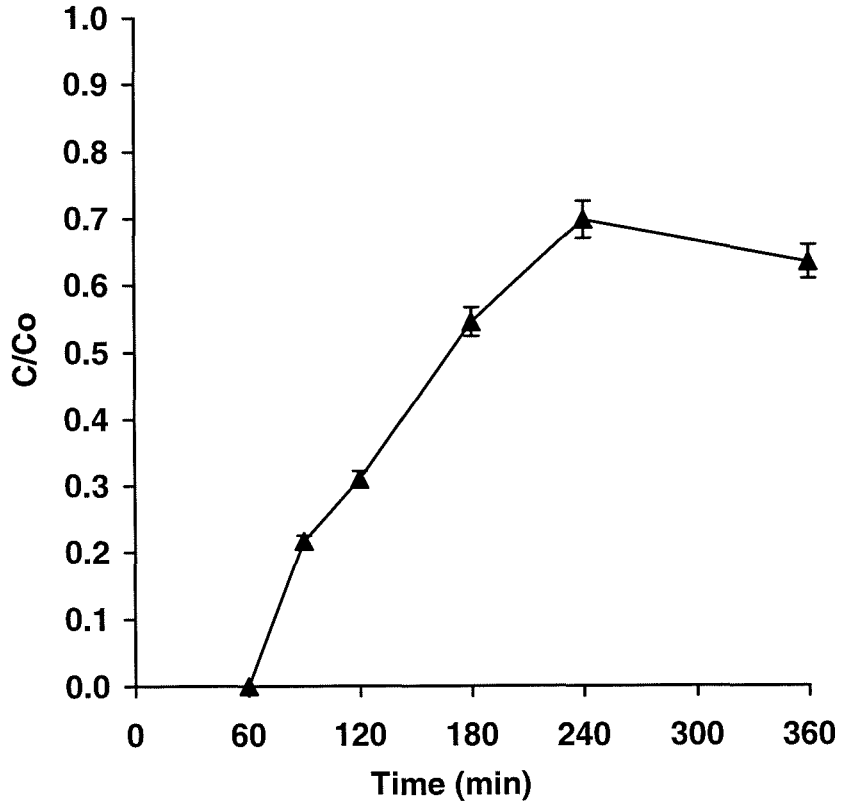


Figure 14. Penetration of flucytosine through biofilms of *C. albicans* GDH 2346, grown on polycarbonate membranes on YNB agar containing 50mM glucose. Error bars indicate the standard errors of the means.

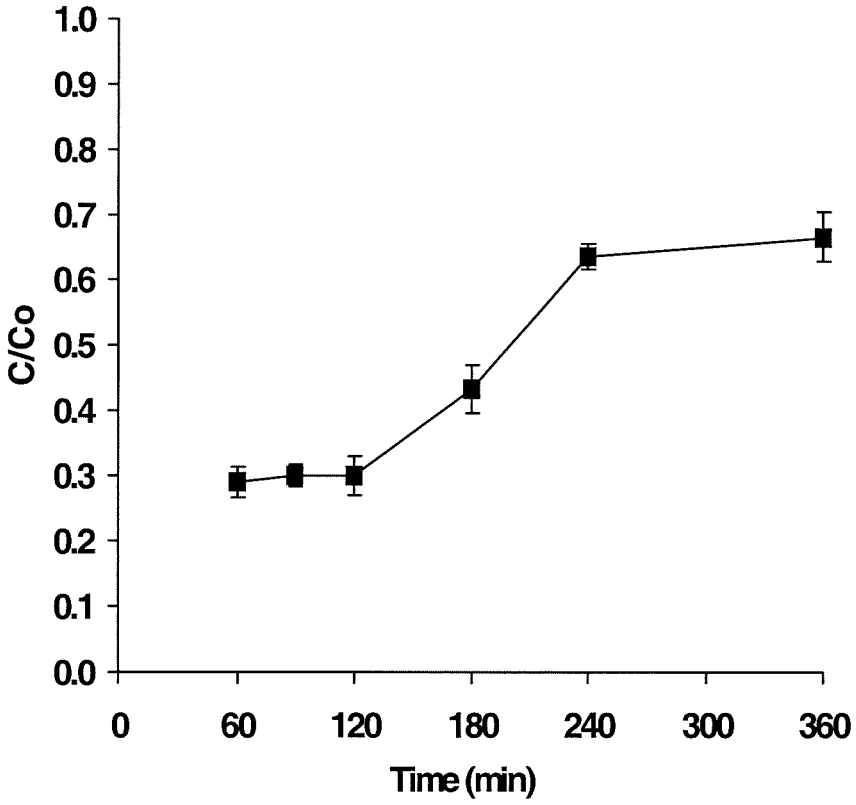


Figure 15. Penetration of flucytosine through biofilms of *C. albicans* GDH 2023, grown on polycarbonate membranes on YNB agar containing 50mM glucose. Error bars indicate the standard errors of the means.

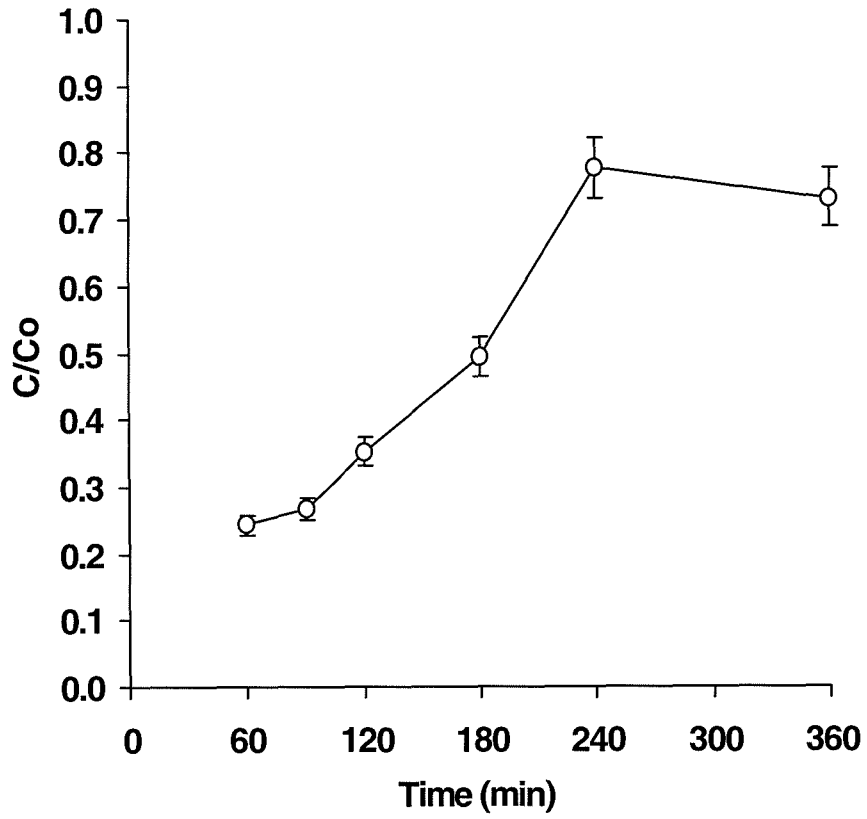


Figure 16. Penetration of flucytosine through biofilms of *C. albicans* GRI 682, grown on polycarbonate membranes on YNB agar containing 50mM glucose. Error bars indicate the standard errors of the means.

1.1.5 Comparison of flucytosine penetration through biofilms of different *C. albicans* strains

Flucytosine penetration of biofilms of three *C. albicans* strains was similar, but initially was lower with strain GDH 2346. The drug concentration at the distal edge of the biofilms was approximately 50 % of the control value after 180 min. After 240 min it had reached 63 to 78 % of the control value (Fig. 17). Drug penetration rates levelled off or fell slightly between 240 min and 360 min.

1.2 Penetration of flucytosine through biofilms of other *Candida* species

1.2.1 Flucytosine penetration through *C. glabrata* AAHB 12 biofilms

The rate of penetration of flucytosine through *C. glabrata* AAHB 12 biofilms was initially rapid, reaching 50 % of the control value after 60 min. A decrease to a stable level of 30 % (Fig. 18) followed the initial speedy penetration.

1.2.2 Flucytosine penetration through *C. krusei* (Glasgow) biofilms

The drug rapidly penetrated through *C. krusei* biofilms. Within 60 min, the flucytosine concentration at the distal edge was 52 % of the control concentration. A decrease to a stable level of 32 to 35 % after 120 to 360 min (Fig. 19) followed.

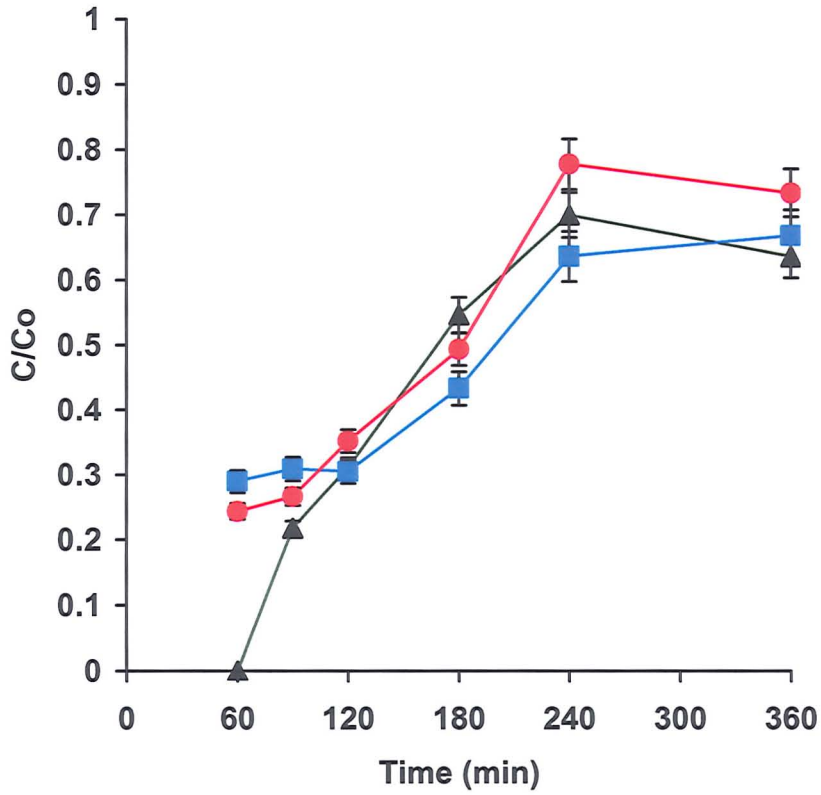


Figure 17. Penetration of flucytosine through biofilms of *C. albicans* GDH 2346 (▲), *C. albicans* GDH 2023 (■), and *C. albicans* GRI 682 (●) grown on polycarbonate membranes on YNB agar containing 50mM glucose. Error bars indicate the standard errors of the means. The mean C_o after 6h was 22.7µg of flucytosine/ml.

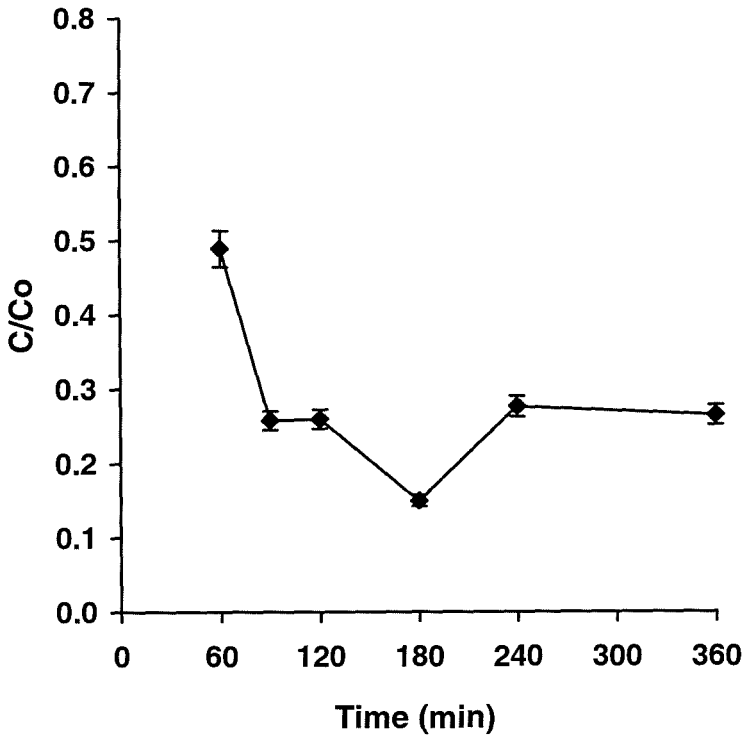


Figure 18. Penetration of flucytosine through biofilms of *C. glabrata* AAHB 12, grown on polycarbonate membranes on YNB agar containing 50mM glucose. Error bars indicate the standard errors of the means.

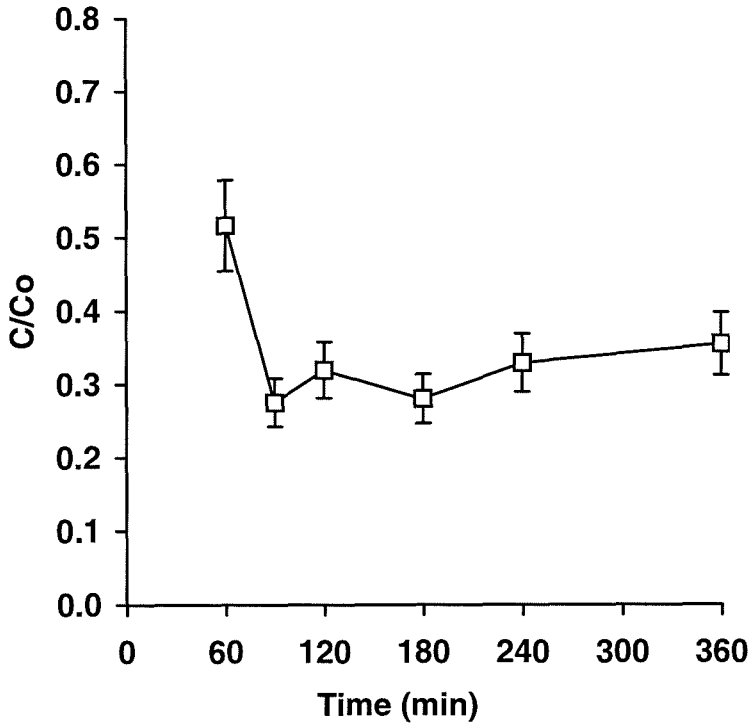


Figure 19. Penetration of flucytosine through biofilms of *C. krusei* grown on polycarbonate membranes on YNB agar containing 50mM glucose. Error bars indicate the standard errors of the means.

1.2.3 Flucytosine penetration through *C. parapsilosis* AAHB 479 biofilms

Flucytosine diffusion through *C. parapsilosis* biofilms was 0% after 60 and 90 min. Slow penetration then ensued and the drug concentration at the distal edge of the biofilm was 25% of the control by 360 min (Fig. 20).

1.2.4 Flucytosine penetration through *C. tropicalis* AAHB 73 biofilms

The rate of penetration of flucytosine through biofilms of *C. tropicalis* was very low (10.4 % of the control value after 180 min). The drug concentration reached a maximum (15 % of the control) after 360 min (Fig. 21).

1.2.5 Comparison of flucytosine penetration through non-*C. albicans Candida* biofilms

The penetration of flucytosine through biofilms of different *Candida* species is compared in Fig. 22. Initially, there was rapid diffusion of the drug (approximately 50 % of the control value after 60 min) through both *C. glabrata* and *C. krusei* biofilms, followed by a decrease to a stable level of around 30%. In contrast, flucytosine penetrated slowly through *C. parapsilosis* biofilms, reaching a maximum of 25 % by 360 min. However, the slowest drug diffusion was detected with *C. tropicalis* biofilms (15 % after 360 min; Fig. 22).

When compared with flucytosine penetration through *C. albicans* biofilms (Fig. 17), penetration through *C. krusei* and *C. glabrata* biofilms was initially faster, while that through *C. parapsilosis* and *C. tropicalis* biofilms was much slower. However, after 360 min, drug penetration through all of these non-*C. albicans Candida* biofilms was lower than that through any of the *C. albicans* strains tested.

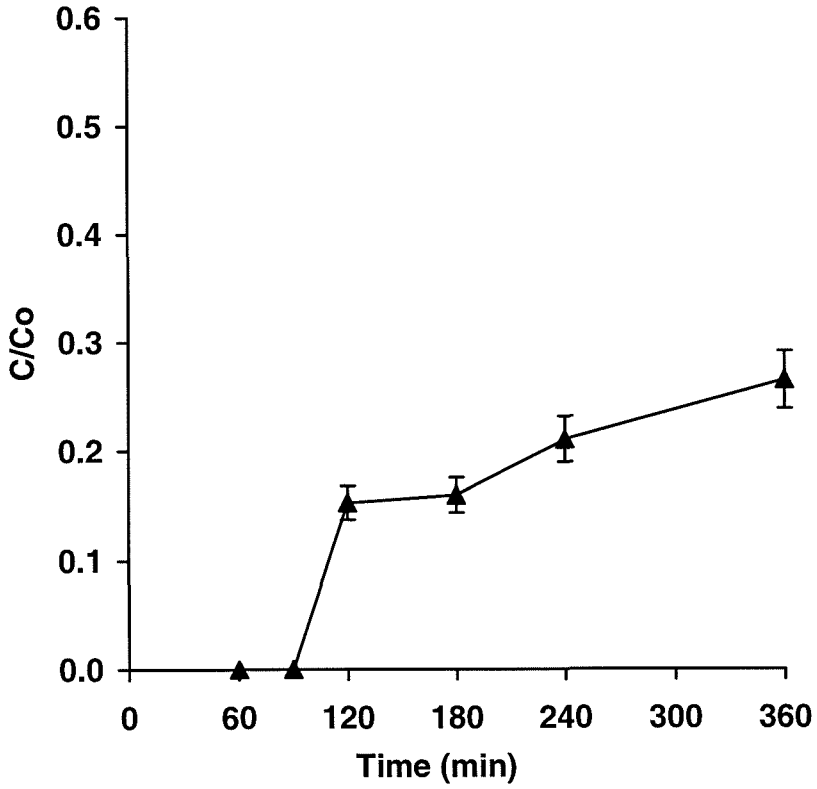


Figure 20. Penetration of flucytosine through biofilms of *C. parapsilosis* AAHB 479, grown on polycarbonate membranes on YNB agar containing 50mM glucose. Error bars indicate the standard errors of the means.

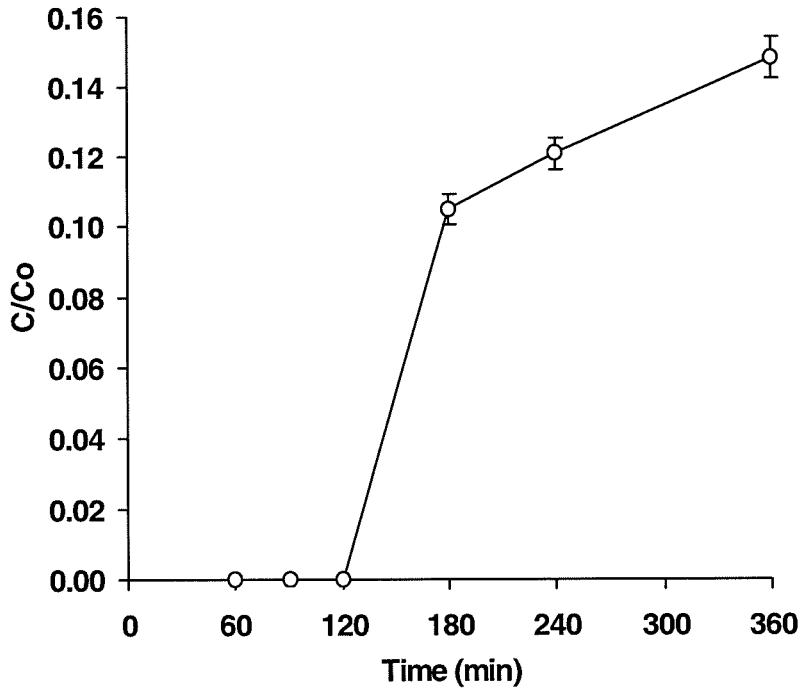


Figure 21. Penetration of flucytosine through biofilms of *C. tropicalis* AAHB 73, grown on polycarbonate membranes on YNB agar containing 50mM glucose. Error bars indicate the standard errors of the means.

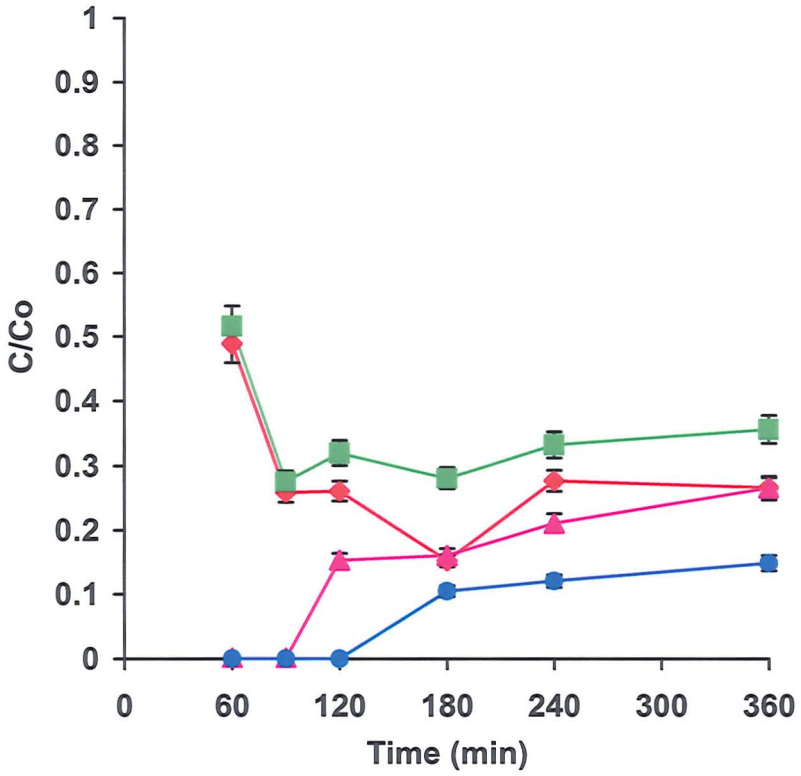


Figure 22. Penetration of flucytosine through biofilms of *C. parapsilosis* (▲), *C. krusei* (■), *C. glabrata* (♦), *C. tropicalis* (●) grown on polycarbonate membranes on YNB agar containing 50mM glucose. Error bars indicate the standard errors of the means. The mean C_o after 6h was 22.7µg of flucytosine/ml.

1.2.6 Flucytosine degradation

Enzymatic degradation of flucytosine by *C. glabrata* and *C. krusei* was investigated qualitatively and did not occur. This was demonstrated by removing membrane-supported biofilms from antifungal agent-containing plates and spreading a sensitive strain (strain GDH 2346) of *C. albicans* on to the plates. These indicator organisms were unable to grow on any part of the plate, including the area that had been under the biofilm (results not shown).

1.3 Penetration of fluconazole through biofilms of different strains of *C. albicans*

1.3.1 Susceptibility of *C. albicans* to fluconazole

The MIC of fluconazole for *C. albicans* GDH 2346 is 0.4µg/ml (Hawser and Douglas, 1995a). In the work presented here, biofilms were challenged with 60 times the MIC of fluconazole (24µg/ml). The drug rapidly killed planktonic cells of *C. albicans* at this concentration.

1.3.2 Fluconazole penetration through *C. albicans* GDH 2346 biofilms

Fluconazole diffused through biofilms of *C. albicans* GDH 2346 very quickly (Fig. 23). The drug concentration at the distal edge of the biofilm was 85-90 % of the control value after 60-90 min. There was then a slight decrease and a levelling off at 80 % of the control value after 360 min (Fig. 23).

1.3.3 Fluconazole penetration through *C. albicans* GDH 2023 biofilms

Penetration of *C. albicans* GDH 2023 biofilms by fluconazole was also very rapid. The fluconazole concentration at the distal edge of the biofilm was 93% of the control value after 60 min. Later, there was a slight decrease and levelling off at 70 % of the value of the control after 360 min (Fig. 24).

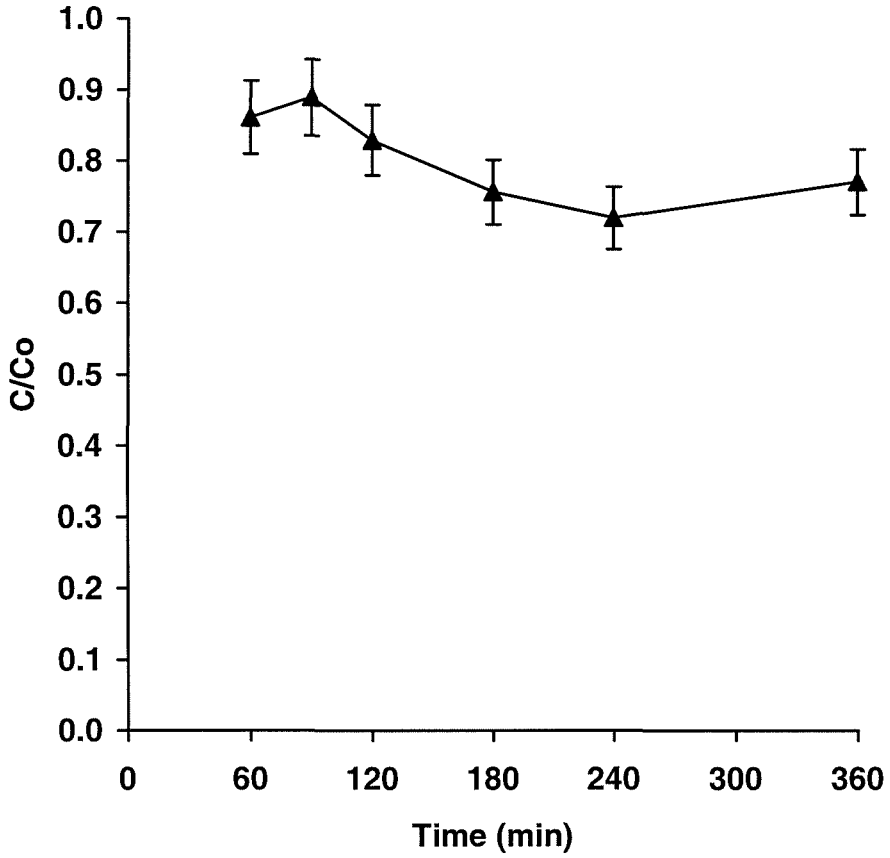


Figure 23. Penetration of fluconazole through biofilms of *C. albicans* GDH 2346, grown on polycarbonate membranes on YNB agar containing 50mM glucose. Error bars indicate the standard errors of the means.

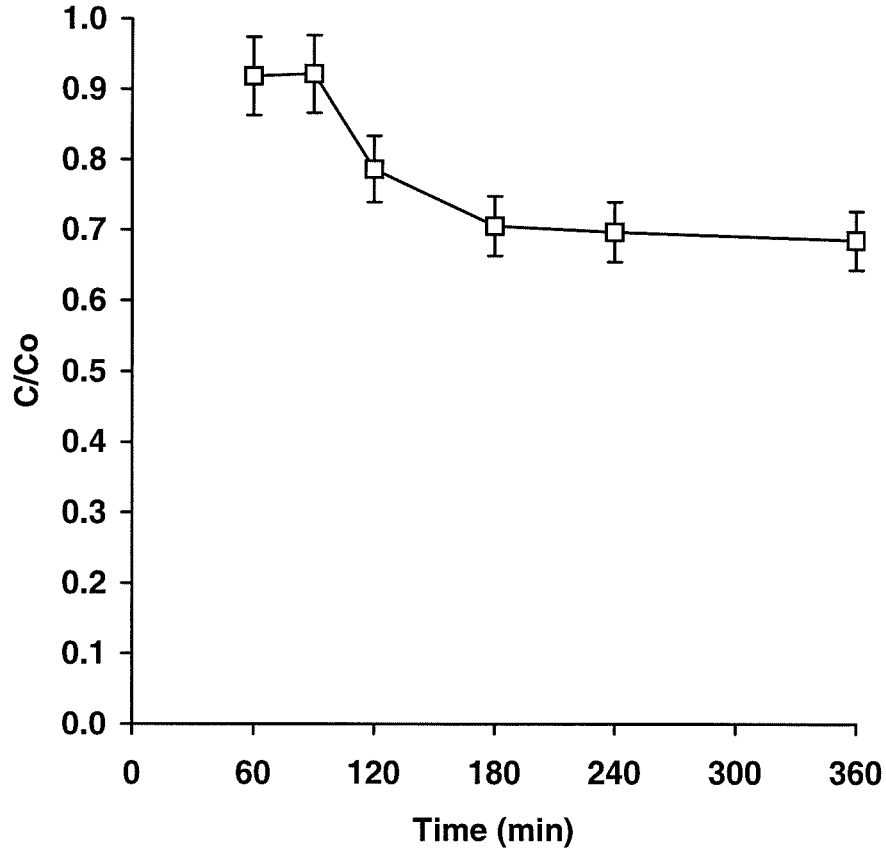


Figure 24. Penetration of fluconazole through biofilm of *C. albicans* GDH 2023, grown on polycarbonate membranes on YNB agar containing 50mM glucose. Error bars indicate the standard errors of the means.

1.3.4 Fluconazole penetration through *C. albicans* GRI 682 biofilms

Fluconazole penetration of biofilms of this third strain of *C. albicans* was also extremely rapid. Within 60 min, the drug concentration at the distal edge of biofilm was 95 % of the control. Subsequently, the rate of penetration of fluconazole decreased and levelled off at a C/Co value of 0.77 (Fig. 25).

1.3.5 Comparison of fluconazole penetration through biofilms of different *C. albicans* strains

Fluconazole penetration was similar and rapid for three *C. albicans* strains reaching approximately 90 % of the control value after 60 min, then decreasing and stabilising at 70 % of the control value after 360 min (Fig. 26). Interestingly, diffusion was much more rapid with fluconazole than with flucytosine, but the final levels of drug penetration were similar after 360 min (Figs. 17 and 26). However, this represents a higher drug concentration with fluconazole.

1.4 Penetration of fluconazole through biofilms of other *Candida* species

1.4.1 Fluconazole penetration through *C. glabrata* AAHB 12 biofilms

The rate of penetration of fluconazole through *C. glabrata* AAHB 12 biofilms was rapid (80-85 % of that for the control after 60-90 min). There was then a slight decrease to a stable level of 75 % after 360 min (Fig. 27).

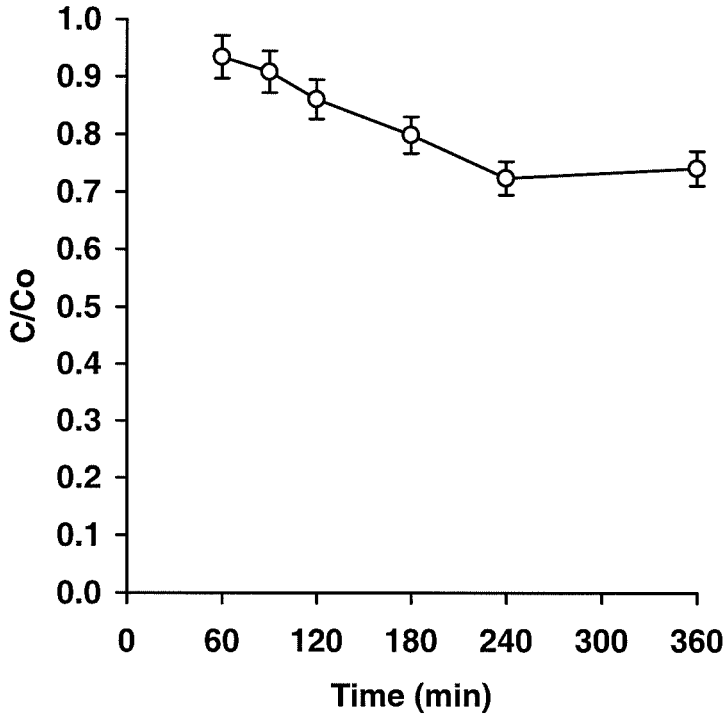


Figure 25. Penetration of fluconazole through biofilms of *C. albicans* GRI 682, grown on polycarbonate membranes on YNB agar containing 50mM glucose. Error bars indicate the standard errors of the means.

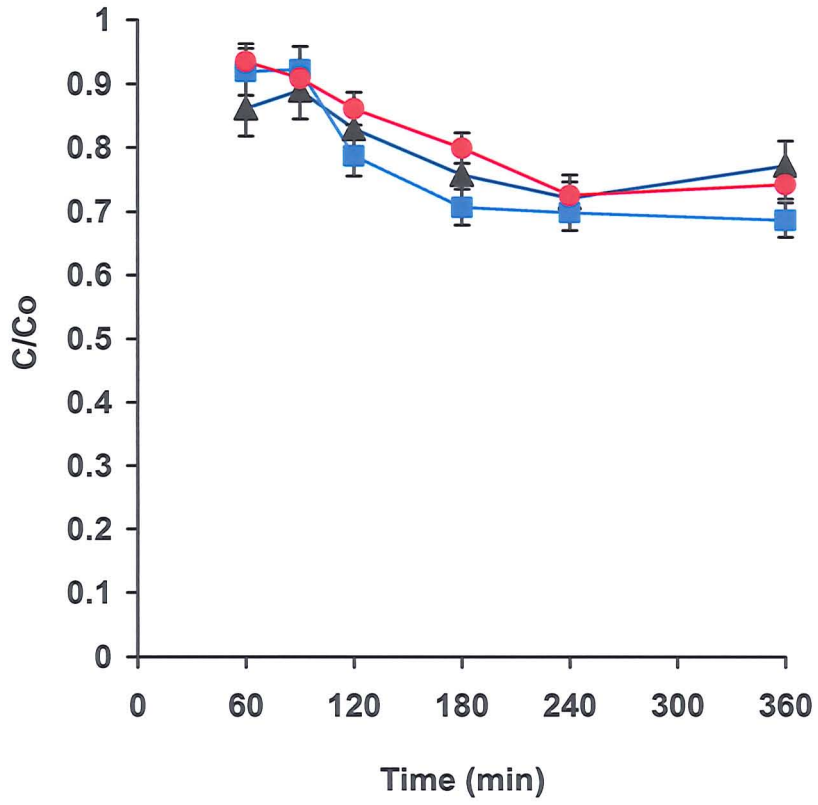


Figure 26. Penetration of fluconazole through biofilms of *C. albicans* GDH 2346 (▲), *C. albicans* GDH 2023 (■), and *C. albicans* GRI 682 (●) grown on polycarbonate membranes on YNB agar containing 50mM glucose. Error bars indicate the standard errors of the means. The mean C_o after 6h was 26.6µg of fluconazole/ml.

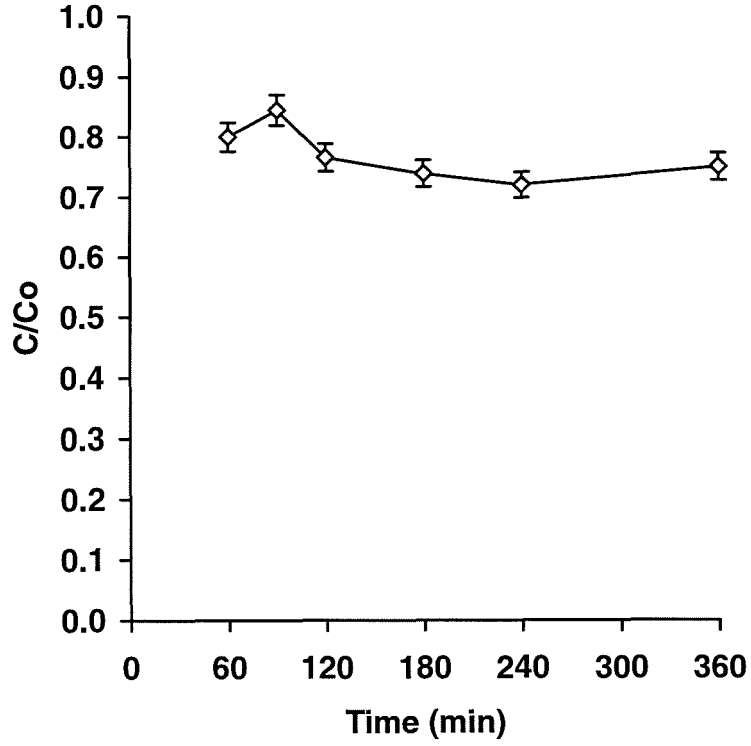


Figure 27. Penetration of fluconazole through biofilms of *C. glabrata* AAHB 12 grown on polycarbonate membranes on YNB agar containing 50mM glucose. Error bars indicate the standard errors of the means.

1.4.2 Fluconazole penetration through *C. krusei* (Glasgow) 12 biofilms

The drug also diffused through *C. krusei* biofilms rapidly. Within 60 min, the fluconazole concentration at the distal edge of biofilm was approximately 80% of the control concentration. This was followed by a decrease to a stable level of 70 % of the control after 360 min (Fig. 28).

1.4.3 Fluconazole penetration through *C. parapsilosis* AAHB 479 biofilms

Fluconazole diffusion through biofilms of *C. parapsilosis* was zero after 60 min. However, there was a sharp rise to 75 % of the control value after 90 min. This was followed by a levelling off to 65 % after 360 min (Fig. 29).

1.4.4 Fluconazole penetration through *C. tropicalis* AAHB 73 biofilms

The diffusion of fluconazole through biofilms of *C. tropicalis* was very slow, with 0 % penetration after 90 min. After 120 min, the drug concentration at the distal edge of biofilms was 65 % of the control value. A stable level of 58 % of the control was attained after 180 min (Fig. 30).

1.4.5 Comparison of fluconazole penetration through biofilms of other *Candida* species

When non-*C. albicans Candida* species were used, there was rapid fluconazole penetration through biofilms of either *C. glabrata* or *C. krusei*, followed by a decrease to approximately 70 % of the control value (Fig. 31). Drug penetration through *C. parapsilosis* biofilms was zero after 60 min but rose rapidly to 75 % of the control value and then levelled off at 65 %. As noted for flucytosine the slowest penetration was with *C. tropicalis* biofilms.

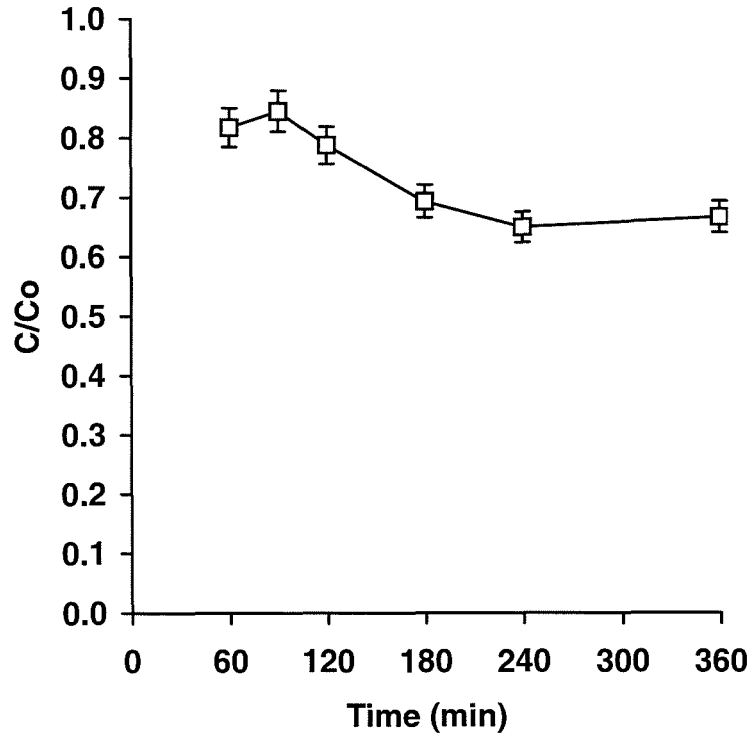


Figure 28. Penetration of fluconazole through biofilms of *C. krusei* grown on polycarbonate membranes on YNB agar containing 50mM glucose. Error bars indicate the standard errors of the means.

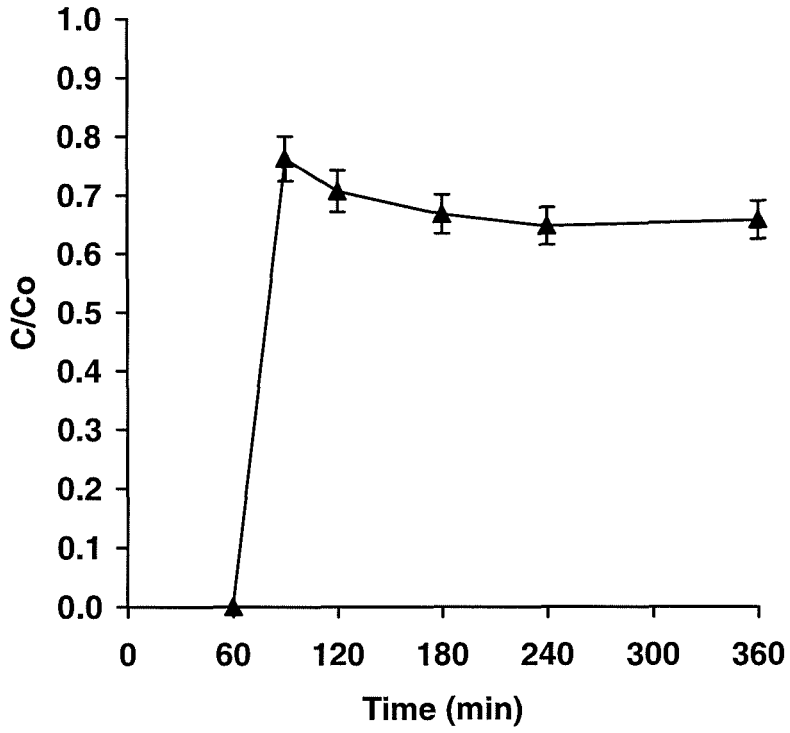


Figure 29. Penetration of fluconazole through biofilms of *C. parapsilosis* AAHB 479, grown on polycarbonate membranes on YNB agar containing 50mM glucose. Error bars indicate the standard errors of the means.

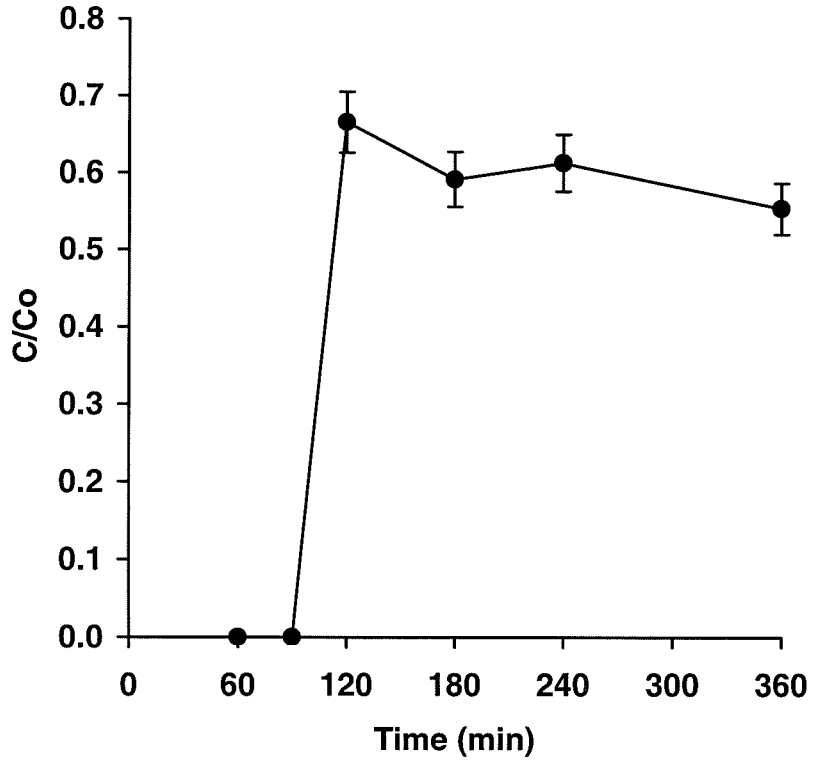


Figure 30. Penetration of fluconazole through biofilms of *C. tropicalis* AAHB 73, grown on polycarbonate membranes on YNB agar containing 50mM glucose. Error bars indicate the standard errors of the means.

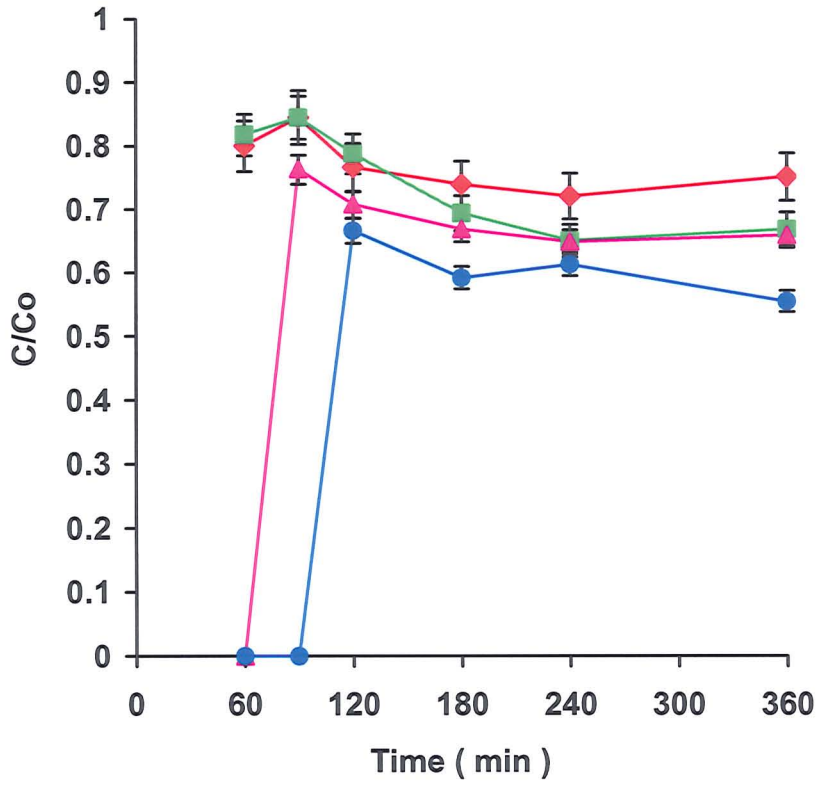


Figure 31. Penetration of fluconazole through biofilms of *C. glabrata* (♦), *C. krusei* (■), *C. parapsilosis* (▲), and *C. tropicalis* (●) grown on polycarbonate membranes on YNB agar containing 50mM glucose. Error bars indicate the standard errors of the means. The mean C_o after 6h was 26.6 µg of fluconazole/ml.

For all non-*C. albicans* species, the overall level of penetration of fluconazole (55% to 85 %; Fig. 31) was higher than that of flucytosine (15 % to 50 %; Fig. 22).

When compared with fluconazole penetration through *C. albicans* biofilms (Fig. 26), penetration through *C. krusei* and *C. glabrata* biofilms was slightly slower, which that through *C. parapsilosis* and *C. tropicalis* biofilms was considerably slower. However, after 360 min, drug penetration through all of these non-*C. albicans Candida* biofilms was only slightly lower than that through any of the *C. albicans* strains tested.

1.5 Penetration of flucytosine through single- and mixed-species biofilms of *C. albicans* and *S. epidermidis*

1.5.1 Flucytosine penetration through single-species biofilms of *C. albicans* and *S. epidermidis*

Flucytosine diffused through biofilms of *C. albicans* GDH 2346 fairly slowly, as described above (Fig. 14). In these experiments, however, the *C. albicans* biofilms, like the mixed-species biofilms, were grown on tryptic soy agar (TSA) rather than YNB agar. The drug concentration at the distal edge of biofilm was 40 % of the control value after 240 min. Within 360 min, the flucytosine concentration had reached a maximum of 45-50 % of the control value (Fig. 32). These results are slightly different from those obtained when YNB was used (Fig. 14).

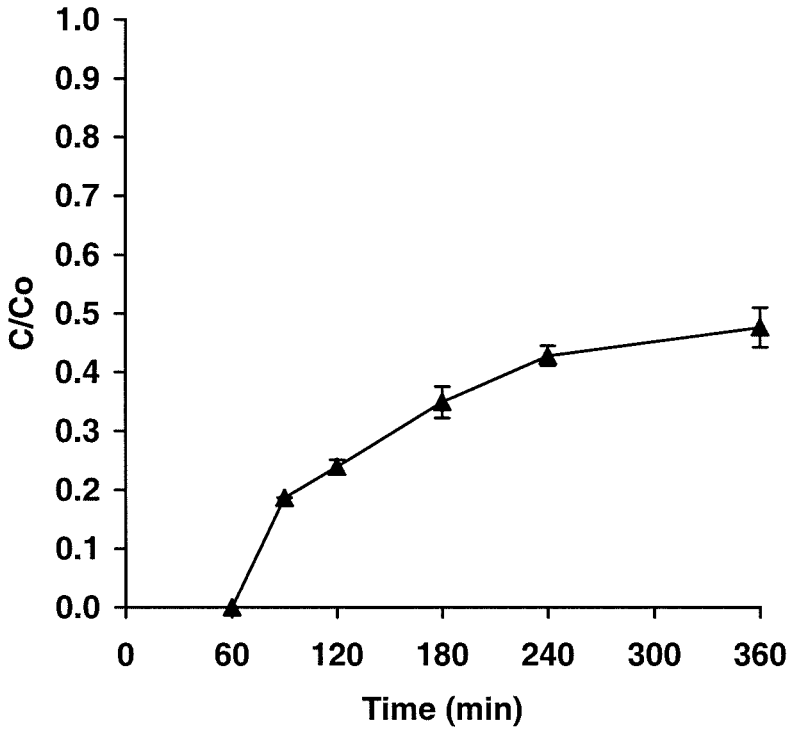


Figure 32. Penetration of flucytosine through biofilms of *C. albicans* GDH 2346, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

By contrast, flucytosine penetration through *S. epidermidis* RP62A biofilms was very slow and poor. There was zero penetration after 180 min and only 12 % after 240 min. Subsequently, there was a levelling off to 10 % of the control value (Fig. 33) after 360 min.

The diffusion of flucytosine through *S. epidermidis* M7 biofilms was also slow, but slightly faster than with wild–type biofilms. Drug diffusion started after 120 min and the concentration at the distal edge of the biofilms was 14 % of the control value by 180 min. Within 360 min, the drug concentration decreased to a stable level of approximately 12 % (Fig. 34).

1.5.2 Flucytosine penetration through mixed-species biofilms of *C. albicans* and *S. epidermidis*

The concentration of flucytosine at the distal edge of mixed-species *Candida*-RP62A biofilms was only 11 % of the control value after 240 min. There was then a slight decrease and a levelling off at 9 % of the control value after 360 min (Fig. 35). However, diffusion through *Candida*-M7 biofilms by flucytosine was slightly more rapid. The drug concentration at the distal edge of the biofilm was approximately 13 % of the control value after 180 min. Later, there was a slight decrease and levelling off at 11% of the control value after 360 min (Fig. 36). A composite graph, with results for all single and mixed-species biofilms shows the poor penetration by flucytosine overall; it also illustrates the finding that diffusion was more rapid with *Candida*-M7 biofilms than with *Candida*-RP62A biofilms (Fig. 37).

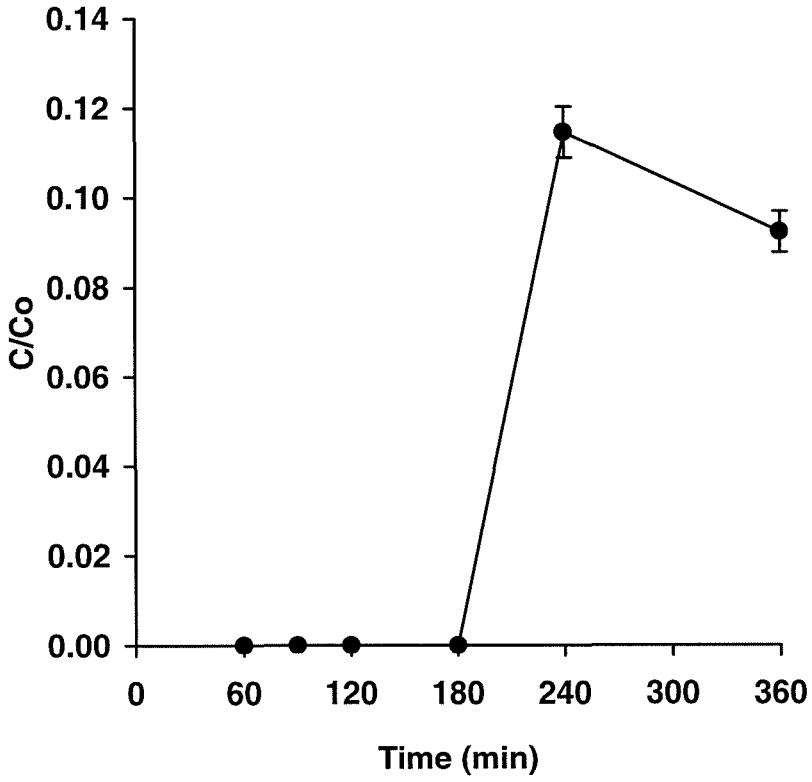


Figure 33. Penetration of flucytosine through biofilms of *S. epidermidis* RP62A, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

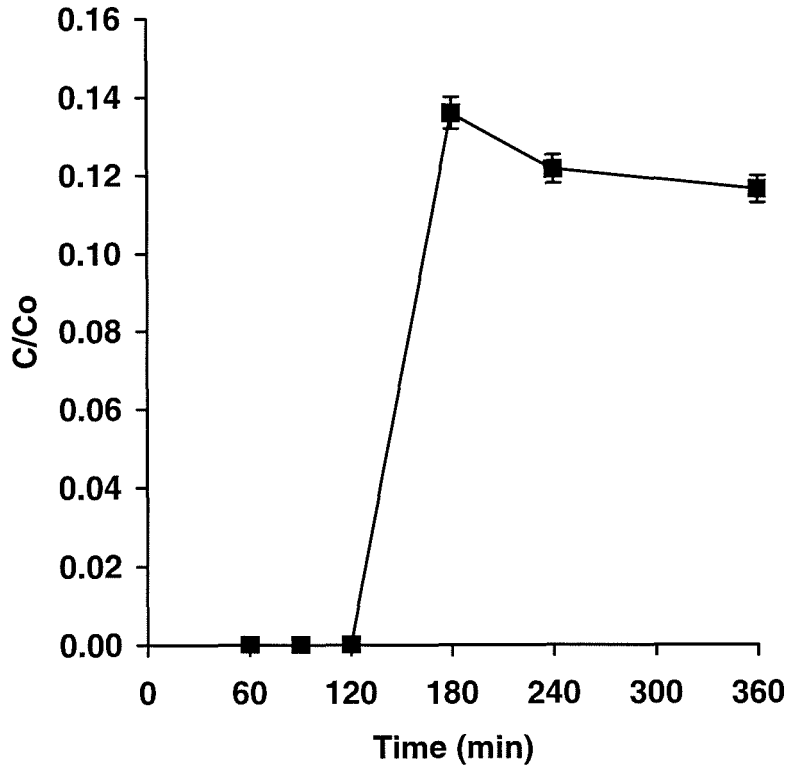


Figure 34. Penetration of flucytosine through biofilms of *S. epidermidis* M7, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

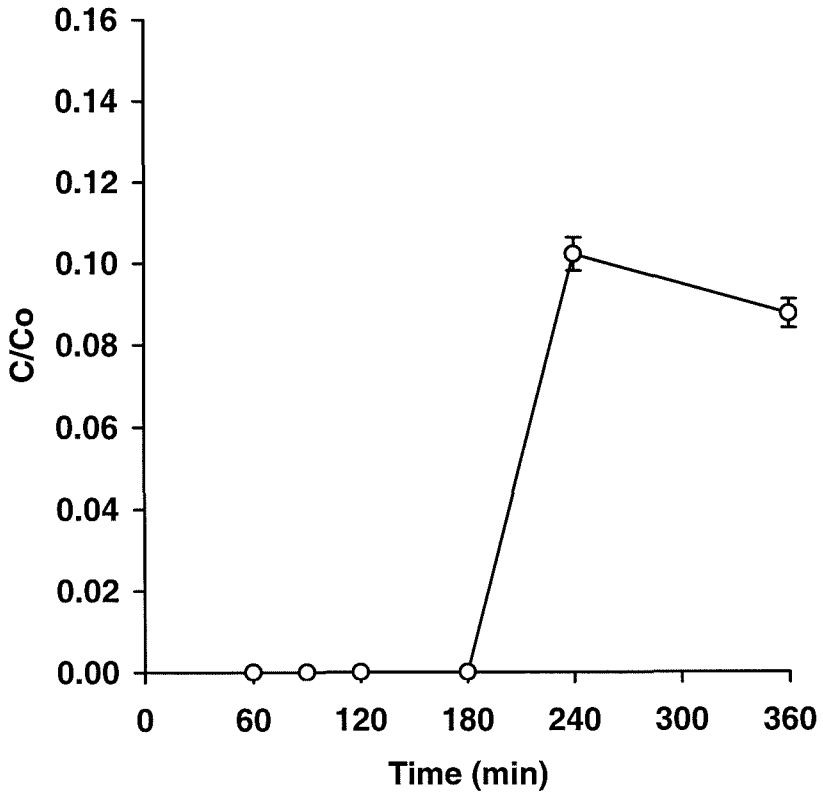


Figure 35. Penetration of flucytosine through mixed-species biofilms of *C. albicans* GDH 2346 and *S. epidermidis* RP62A, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

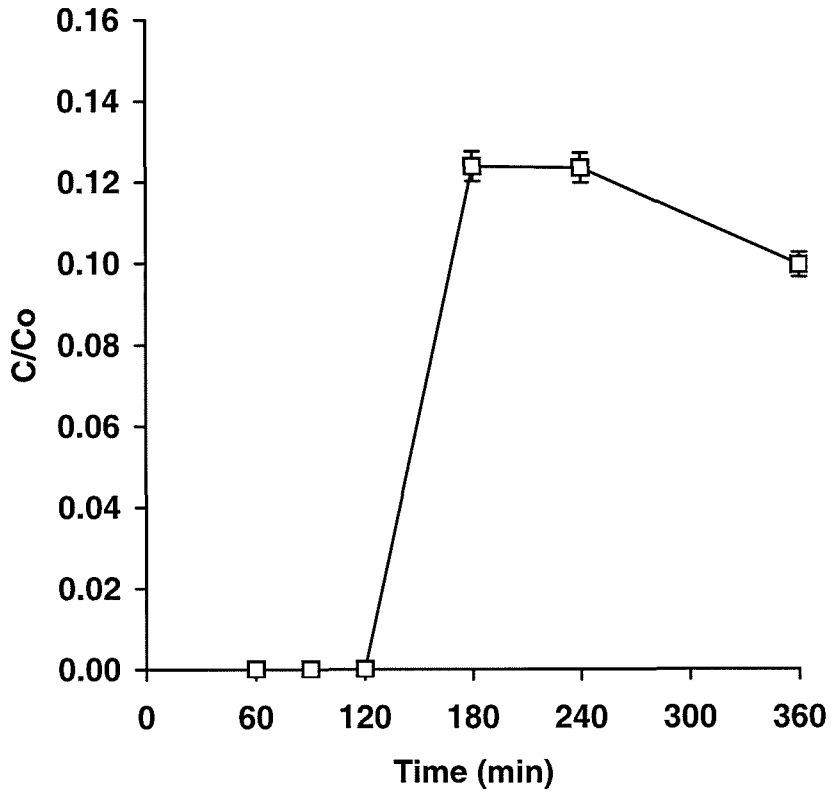


Figure 36. Penetration of flucytosine through mixed-species biofilms of *C. albicans* GDH 2346 and *S. epidermidis* M7, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

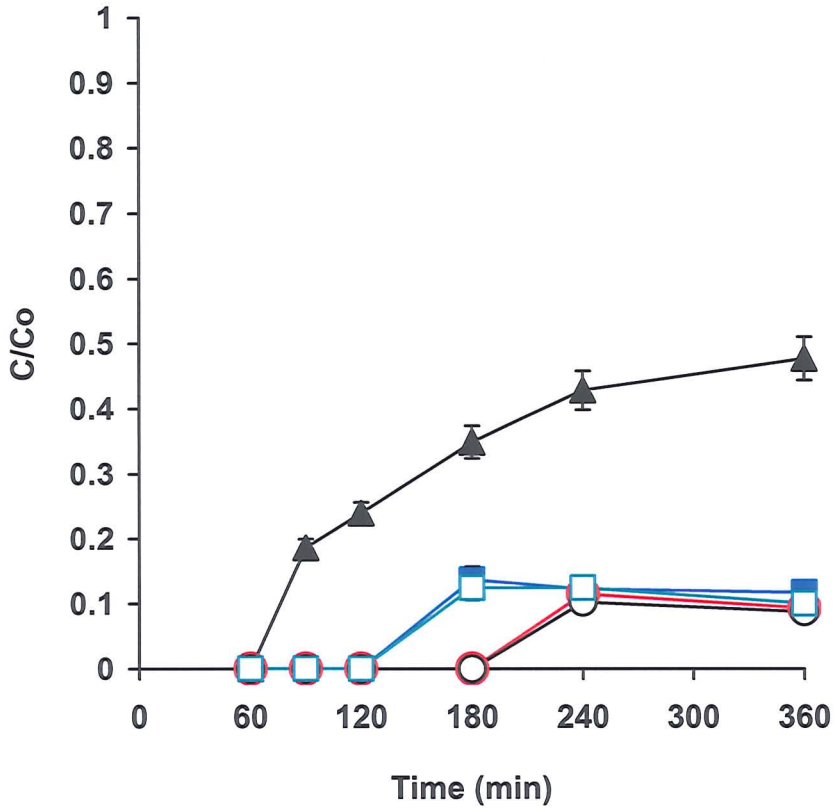


Figure 37. Penetration of flucytosine through single and mixed species biofilms of *C. albicans* GDH 2346 (▲), *S. epidermidis* RP62A (●), *S. epidermidis* M7 (■), *C. albicans* GDH 2346 and *S. epidermidis* RP62A (O), and *C. albicans* GDH 2346 and *S. epidermidis* M7 (□) grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means. The mean C_o after 6h was 24.2 µg of flucytosine/ml.

1.6 Penetration of fluconazole through single- and mixed-species biofilms of *C. albicans* and *S. epidermidis*

1.6.1 Fluconazole penetration through single-species biofilms

Penetration of fluconazole through biofilms of *C. albicans* was rapid and extensive, as before. In these experiments, however, the *C. albicans* biofilms, like the mixed-species biofilms, were grown on TSA rather than YNB agar. The drug concentration at the distal edge of the biofilm was 90 % of the control value after 90 min. After 360 min, the fluconazole concentration had dropped slightly to 75 % of that of the control (Fig. 38).

There was much slower and poorer penetration of fluconazole through biofilms of *S. epidermidis* RP62A (wild-type strain). The fluconazole concentration reached a maximum of 55 % of the control value after 180 min. It then decreased slightly to a stable level of approximately 50 % after 240-360 min (Fig. 39).

The rate of drug penetration through biofilms of the slime-negative mutant *S. epidermidis* M7 was also slower and poorer than that through *C. albicans* biofilms. The fluconazole concentration reached a maximum of 55 % of the control value after 180 min, then levelled off to 50 %, in a manner almost identical to that observed with biofilms of the wild-type strain (Fig. 40).

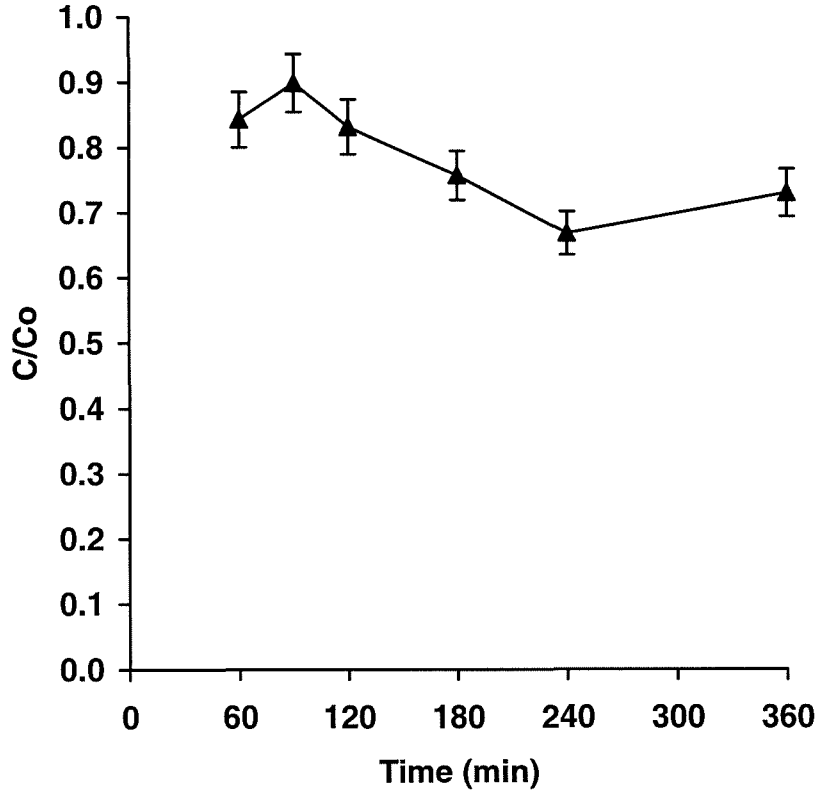


Figure 38. Penetration of fluconazole through biofilms of *C. albicans* GDH 2346, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

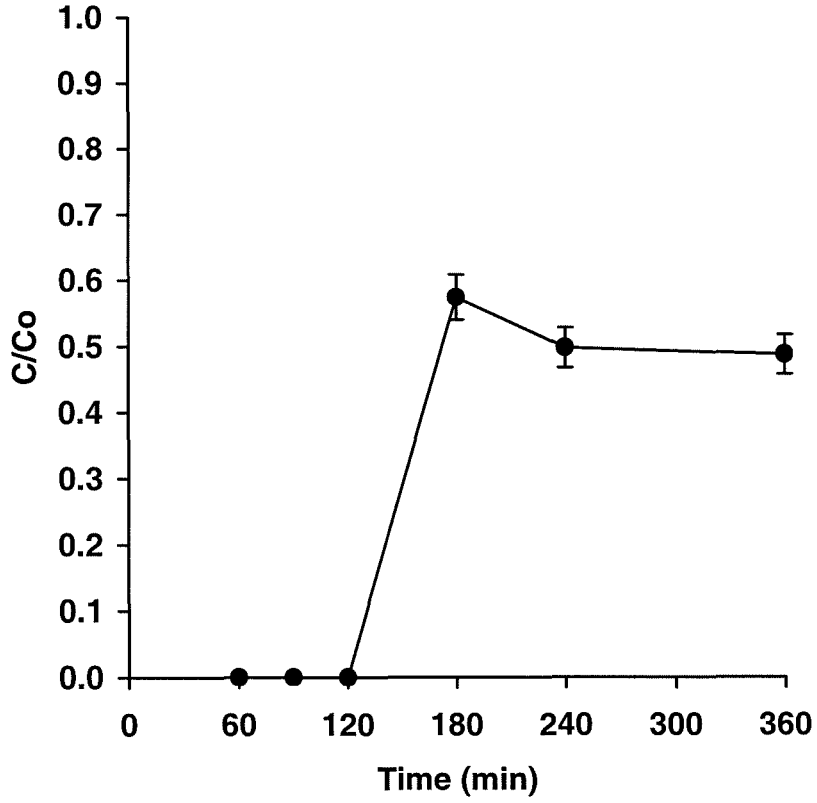


Figure 39. Penetration of fluconazole through biofilms of *S. epidermidis* RP62A, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

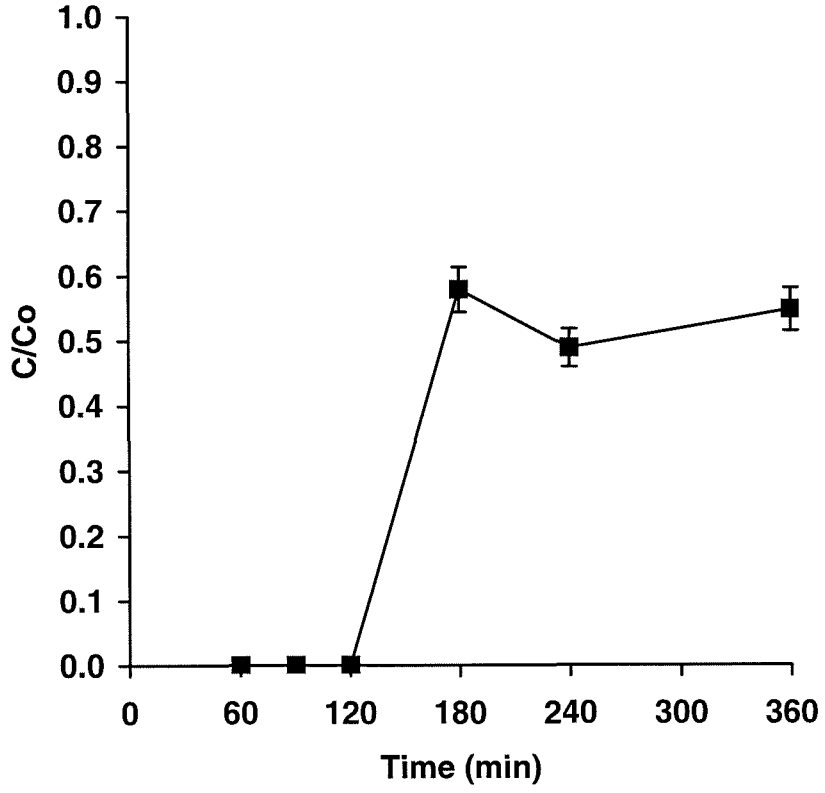


Figure 40. Penetration of fluconazole through biofilms of *S. epidermidis* M7, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

1.6.2 Fluconazole penetration through mixed-species biofilms of *C. albicans* and *S. epidermidis*

With mixed fungal-bacterial biofilms, there was also relatively slow and poor penetration of fluconazole. The drug concentration at the distal edge of the biofilm reached 49 % of the control value after 360 min for *Candida*-RP62A biofilms (Fig. 41). However, with biofilms containing the slim-negative mutant, *S. epidermidis* M7, diffusion was more rapid and the drug concentration reached 50 % after 240 min (Fig. 42). A composite graph, with results for all single- and mixed-species biofilms is presented in Fig. 43. Fluconazole penetrated mixed-species biofilms to a greater extent than any other antifungal agent tested.

1.7 Penetration of amphotericin B through single- and mixed-species biofilms of *C. albicans* and *S. epidermidis*

1.7.1 Susceptibility of *C. albicans* to amphotericin B

The MIC of amphotericin B for planktonic *C. albicans* GDH 2346 is 1.3 μg/ml (Hawser and Douglas, 1995a). In the work presented here, biofilms of *C. albicans* were challenged with 60 times the MIC of amphotericin B (78 μg/ml). The drug rapidly kills planktonic cells at this concentration.

1.7.2 Amphotericin B penetration through single-species biofilms of *C. albicans* and *S. epidermidis*

Despite its low solubility in water, amphotericin B diffused rapidly through biofilms of *C. albicans* GDH 2346. In these experiments the *C. albicans* biofilms, like the mixed-species biofilms, were grown on tryptic soy agar (TSA) rather than YNB agar. The drug concentration at the distal edge of the biofilm was 65 % of the control value after 60 min. There was then a slight decrease and a levelling off at 50 % of the control value after 360 min (Fig. 44).

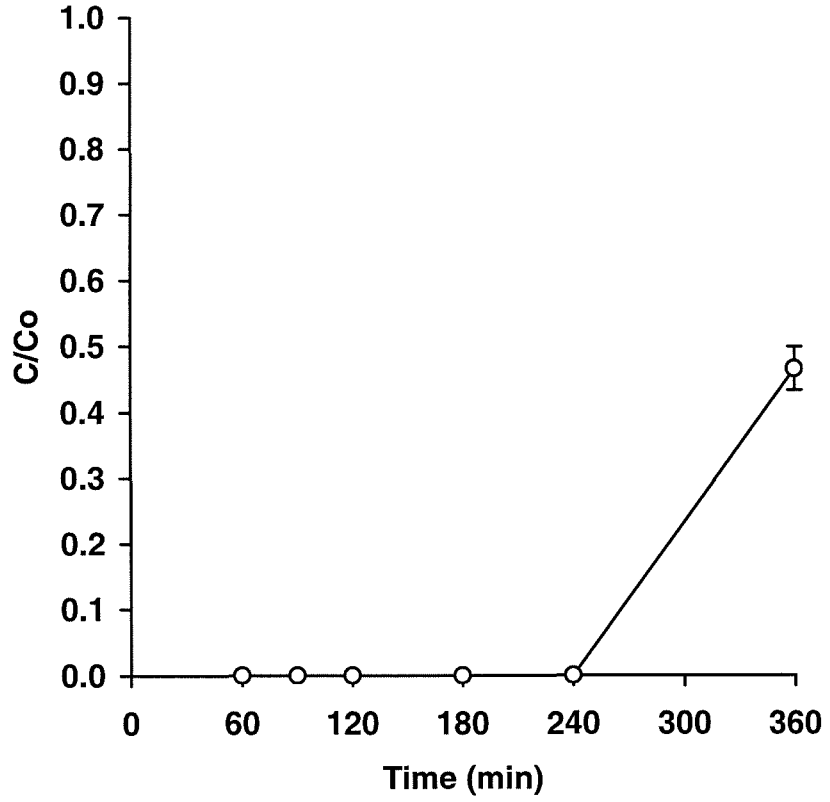


Figure 41. Penetration of fluconazole through mixed-species biofilms of *C. albicans* GDH 2346 and *S. epidermidis* RP62A, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

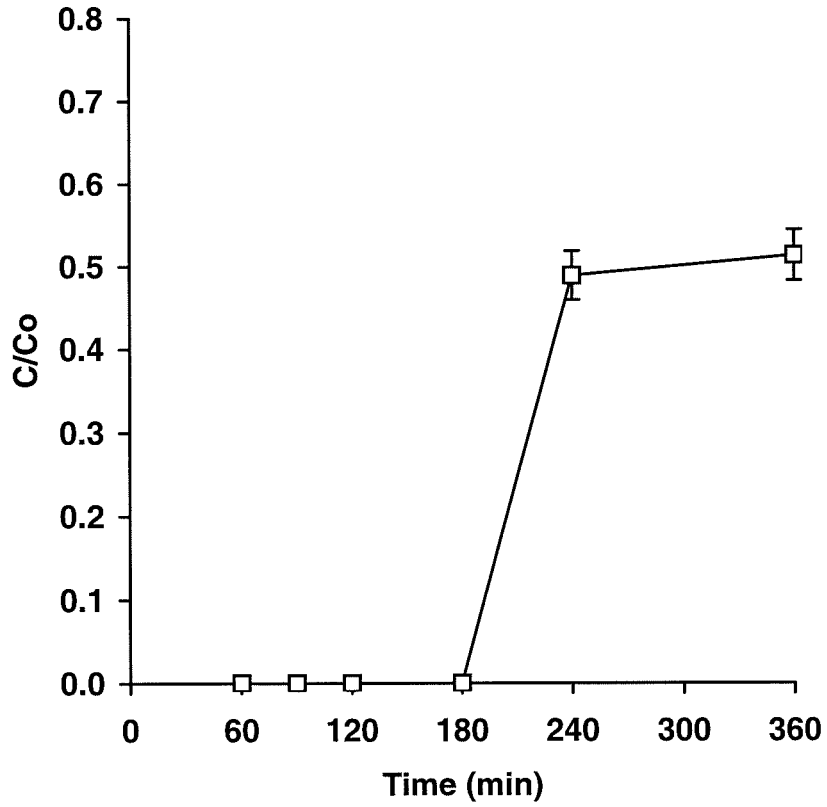


Figure 42. Penetration of fluconazole through mixed-species biofilms of *C. albicans* GDH 2346 and *S. epidermidis* M7, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

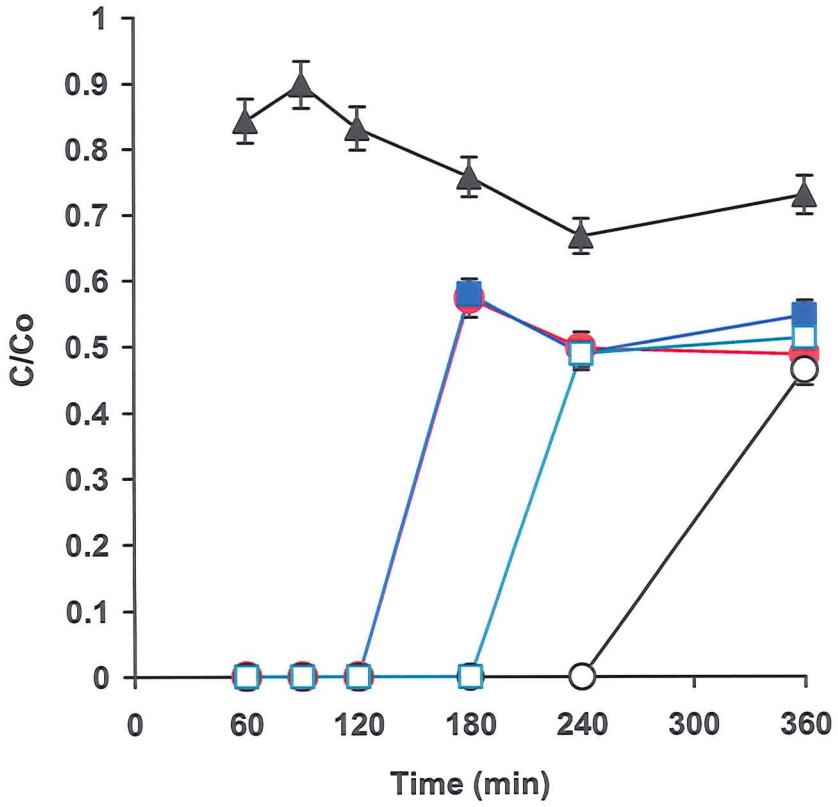


Figure 43. Penetration of fluconazole through single and mixed-species biofilms of *C. albicans* GDH 2346 (▲), *S. epidermidis* RP62A (●), *S. epidermidis* M7 (■), *C. albicans* GDH 2346 and *S. epidermidis* RP62A (○), and *C. albicans* GDH 2346 and *S. epidermidis* M7 (□) grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means. The mean C_o after 6 h was 27.0 µg of fluconazole/ml.

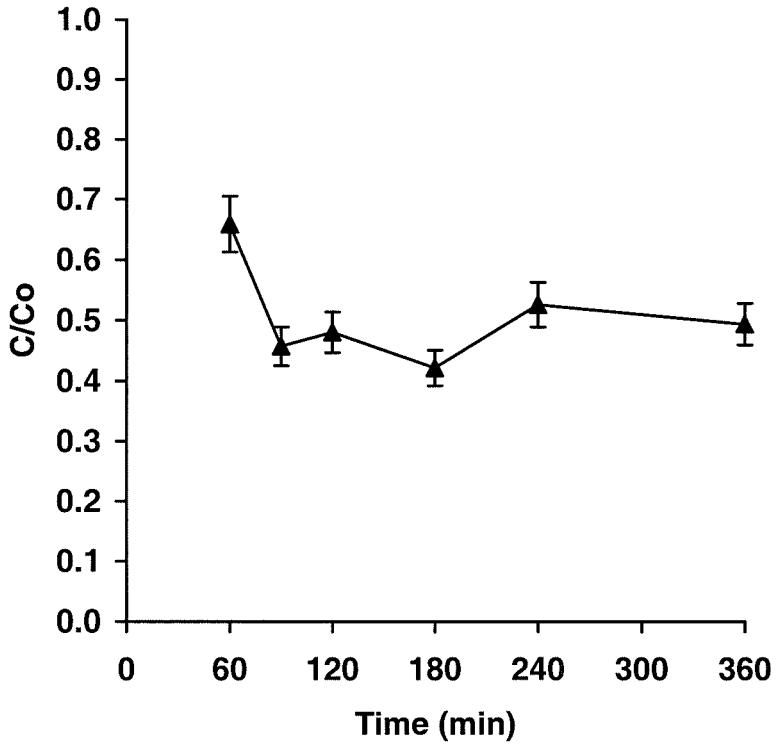


Figure 44. Penetration of amphotericin B through biofilms of *C. albicans* GDH 2346, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

The diffusion of amphotericin B through *S. epidermidis* biofilms was slower and less extensive. In this instance, penetration was faster through biofilms containing the wild-type strain RP62A than through those containing the slime-negative mutant M7. Within 120 min, the drug concentration at the distal edge of *S. epidermidis* RP62A (wild-type strain) biofilms was 32 % of the control value. This was followed by a decrease to a stable level of roughly 27 % of the control value after 360 min (Fig. 45).

By contrast, the rate of drug diffusion through biofilms of the slime-negative mutant was slower than that through wild-type biofilms. The drug concentration at the distal edge of the biofilm was 21 % of the control value after 180 min. The amphotericin B concentration reached a maximum of 31 % of the control value after 360 min (Fig. 46).

1.7.3 Amphotericin B penetration through mixed-species biofilms of *C. albicans* and *S. epidermidis*

With mixed fungal-bacterial biofilms, amphotericin B penetration was also slow and poor, although in this instance diffusion was faster through biofilms containing *S. epidermidis* RP62A than through those containing *S. epidermidis* M7. The drug concentration at the distal edge for *Candida*-RP62A biofilms was 23 % of the control value after 180 min; it then increased to 30 % after 360 min (Fig. 47). However, with biofilms containing the slime-negative mutant, *S. epidermidis* M7, penetration was slower and the drug concentration reached 16 % after 240 min. After 360 min, the rate of concentration reached a maximum of 18 % of the control value (Fig. 48).

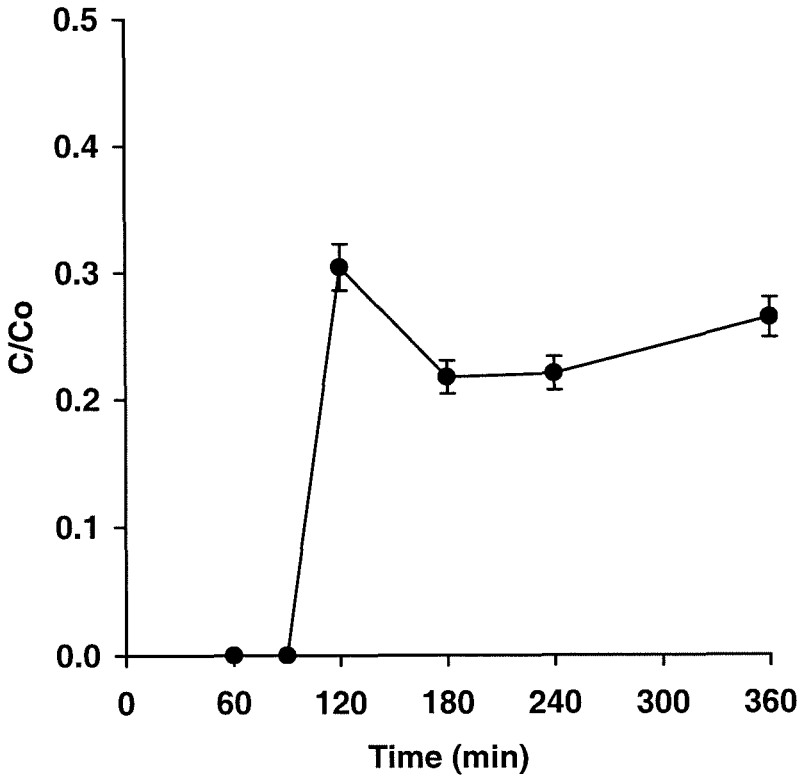


Figure 45. Penetration of amphotericin B through biofilms of *S. epidermidis* RP62A, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

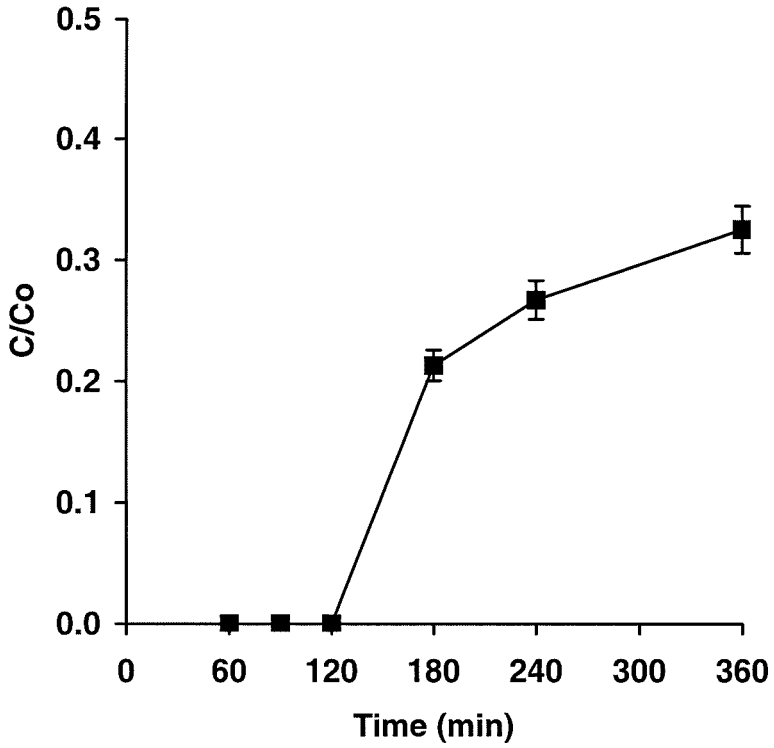


Figure 46. Penetration of amphotericin B through biofilms of *S. epidermidis* M7, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

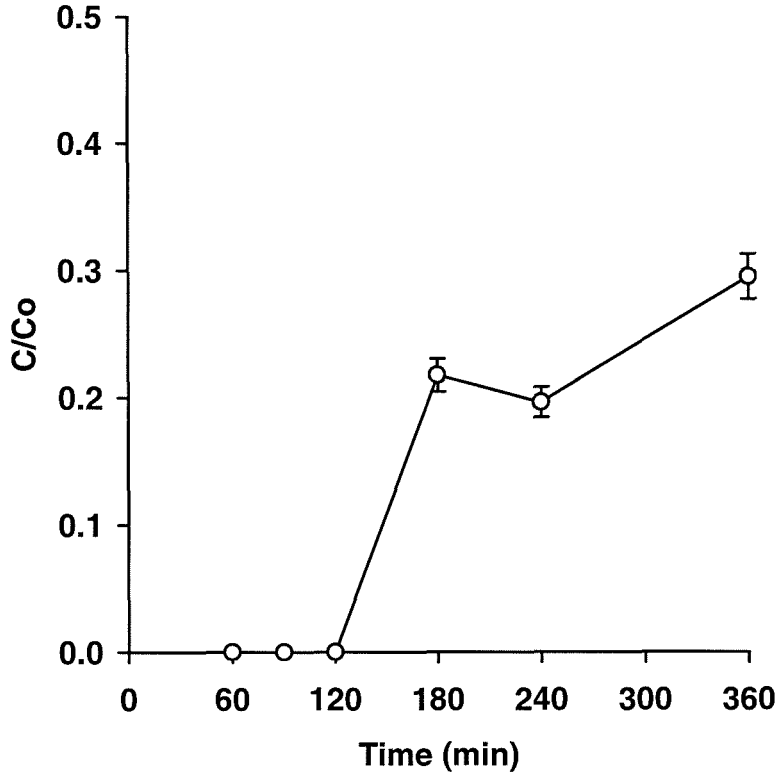


Figure 47. Penetration of amphotericin B through mixed-species biofilms of *C. albicans* GDH 2346 and *S. epidermidis* RP62A, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

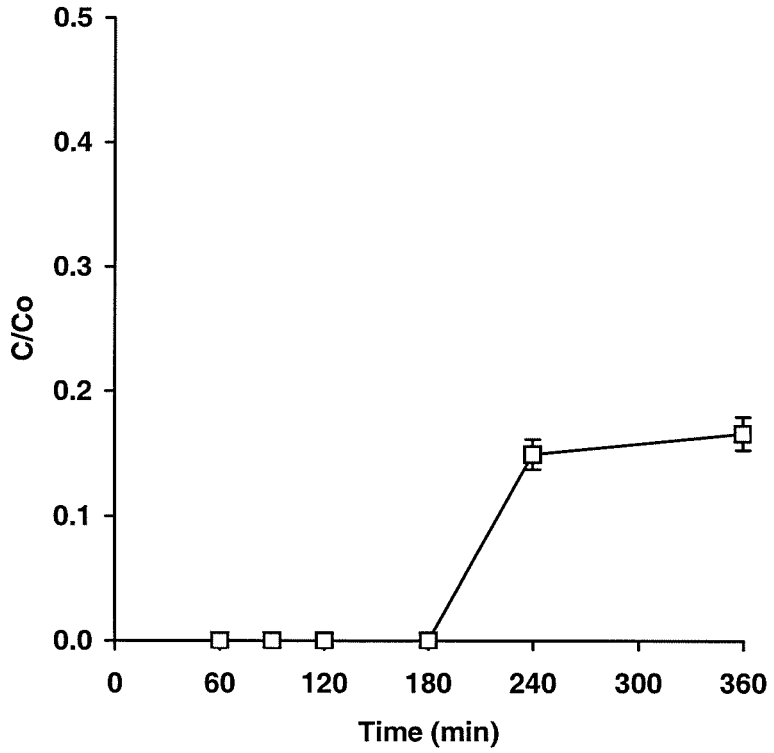


Figure 48. Penetration of amphotericin B through mixed-species biofilms of *C. albicans* GDH 2346 and *S. epidermidis* M7, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

A composite graph, with results for all single and mixed-species biofilms is presented in Fig. 49. Surprisingly, the C_o of amphotericin B (Fig. 49), like that of flucytosine (Fig. 22), was higher than the drug concentration in the agar. The reason for this is not clear. However, it is conceivable that during the drug penetration assay some drugs bind to the disk cellulose (thus effectively reducing their concentration in solution) but then are released during the zone-of-inhibition assay.

1.8 Penetration of voriconazole through single- and mixed-species biofilms of *C. albicans* and *S. epidermidis*

1.8.1 Susceptibility of *C. albicans* to voriconazole

The MIC of the newer azole, voriconazole, for planktonic *C. albicans* is 1.0 µg/ml (Lozano-Chiu *et al.*, 1999). In the experiments presented here biofilms of *C. albicans* were challenged with 10 times the MIC (10µg/ml) of the drug. Voriconazole rapidly killed planktonic cells at this concentration.

1.8.2 Voriconazole penetration through single–species biofilms of *C. albicans* and *S. epidermidis*

In these experiments, the *C. albicans* biofilms, like the mixed-species biofilms, were grown on TSA rather than YNB agar. Voriconazole penetrated rapidly through biofilms of *C. albicans* GDH 2346. The drug concentration at the distal edge of the biofilm was 56 % of that of the control value after 60 min. The voriconazole concentration reached a maximum of 59 % of the control value after 90 min. It then decreased and levelled off at 23 % of the control value after 360 min (Fig. 50).

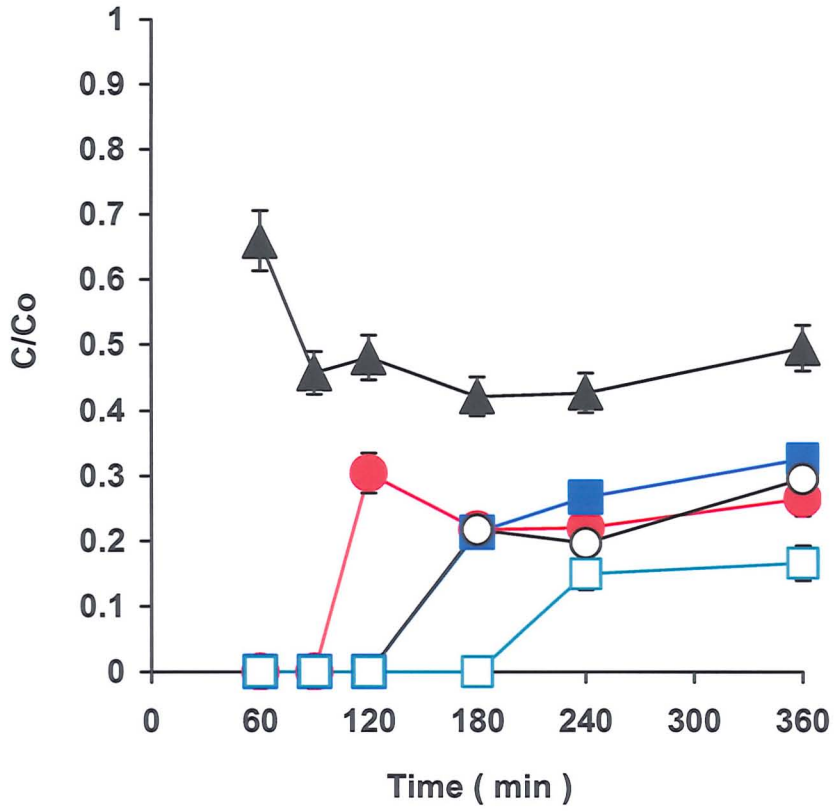


Figure 49. Penetration of amphotericin B through single- and mixed-species biofilms of *C. albicans* GDH 2346 (▲), *S. epidermidis* RP62A (●), *S. epidermidis* M7 (■), *C. albicans* GDH 2346 and *S. epidermidis* RP62A (O), and *C. albicans* GDH 2346 and *S. epidermidis* M7 (□) grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means. The mean C_o after 6 h was 120.6 µg of amphotericin B/ml.

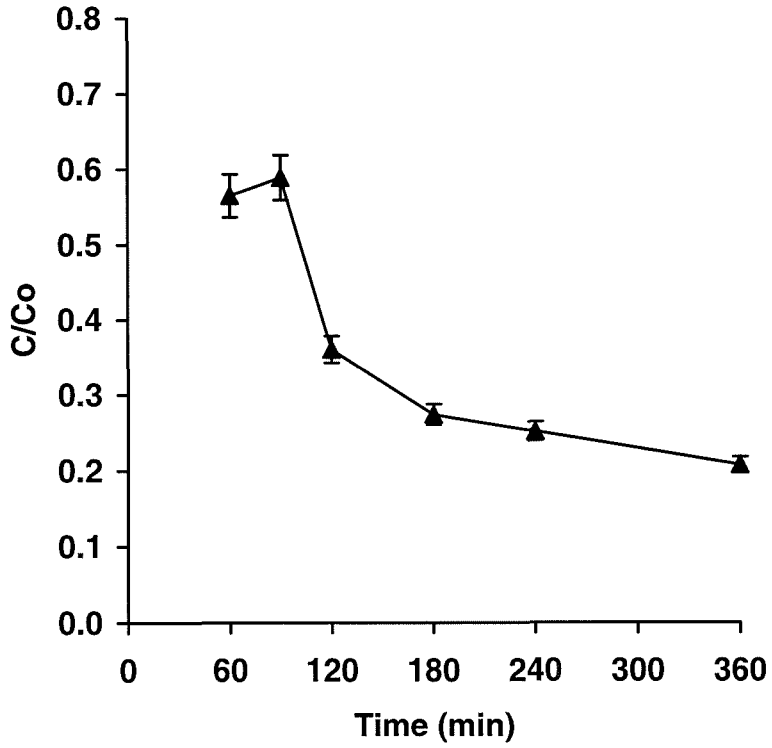


Figure 50. Penetration of voriconazole through biofilms of *C. albicans* GDH 2346, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

There was much slower and poorer penetration of voriconazole through *S. epidermidis* biofilms. Drug diffusion through biofilms of the wild-type strain was approximately 18 % of the control value after 180 min. Drug penetration rates fell slightly between 180 min and 360 min (Fig. 51). Similarly, with biofilms containing the slime-negative mutant, *S. epidermidis* M7, diffusion was 17 % of the control value after 180 min. The concentration of drug at the distal edge of the biofilm was 15 % of the control by 360 min (Fig. 52).

1.8.3 Voriconazole penetration through mixed-species biofilms of *C.albicans* and *S. epidermidis*

With mixed fungal-bacterial biofilms there was also slower and poorer penetration of voriconazole. Drug diffusion to the distal edge of the biofilm reached 17 % of the control value after 180 min, then decreased slightly to level off at 12 % of the control value after 360 min for *Candida*-RP62A biofilms (Fig. 53). However, with biofilms containing the slime-negative mutant, *S. epidermidis* M7, diffusion was faster and the drug concentration reached 25 % of the control value after 120 min. After 360 min, the drug concentration had fallen to 12 % of the control value (Fig. 54).

A composite graph, with the results for all single and mixed-species biofilms, shows that the diffusion of voriconazole, like that of flucytosine, was more rapid with mixed-species biofilms containing *S. epidermidis* M7 than with those containing wild-type strain RP62A (Fig. 55).

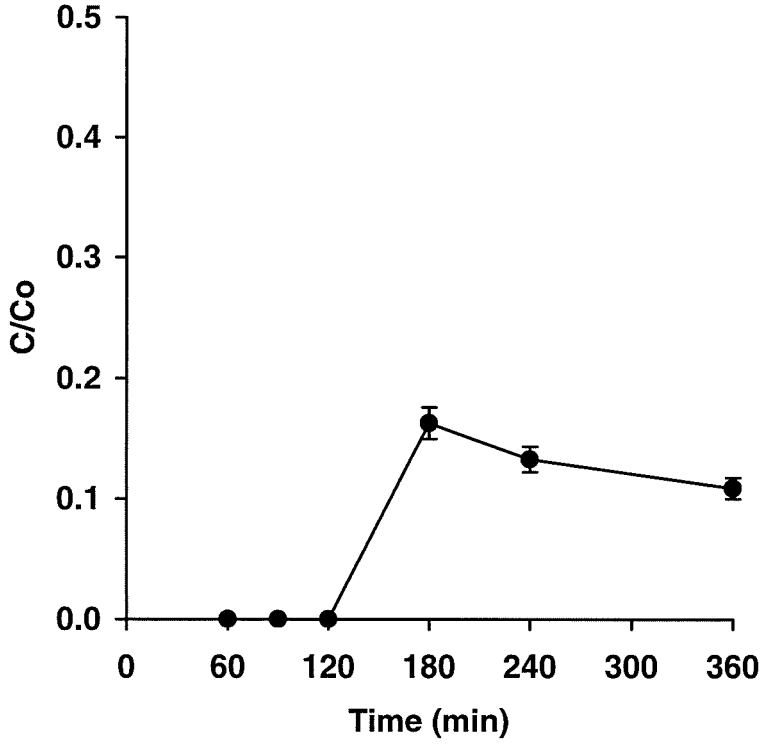


Figure 51. Penetration of voriconazole through biofilms of *S. epidermidis* RP62A, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

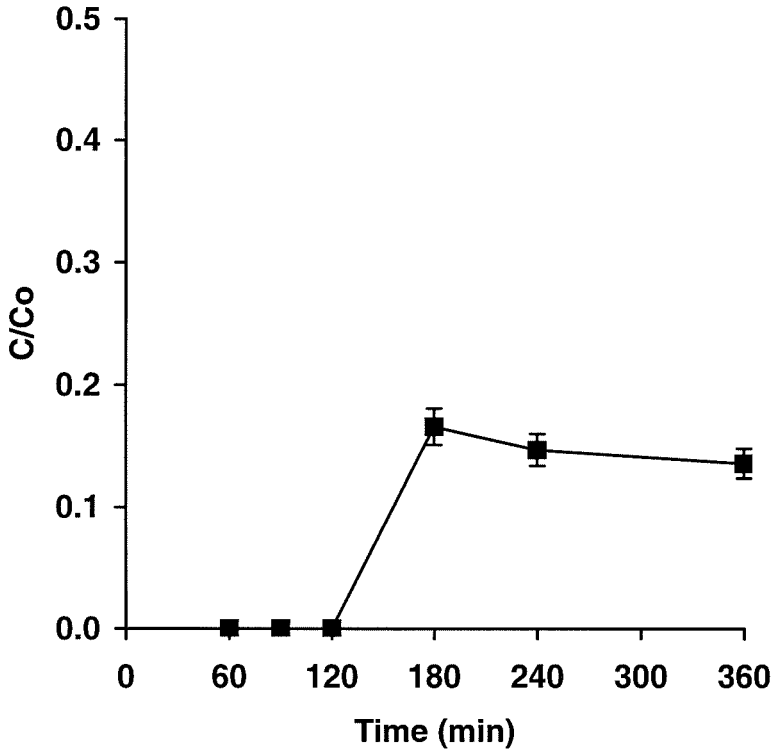


Figure 52. Penetration of voriconazole through biofilms of *S. epidermidis* M7, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

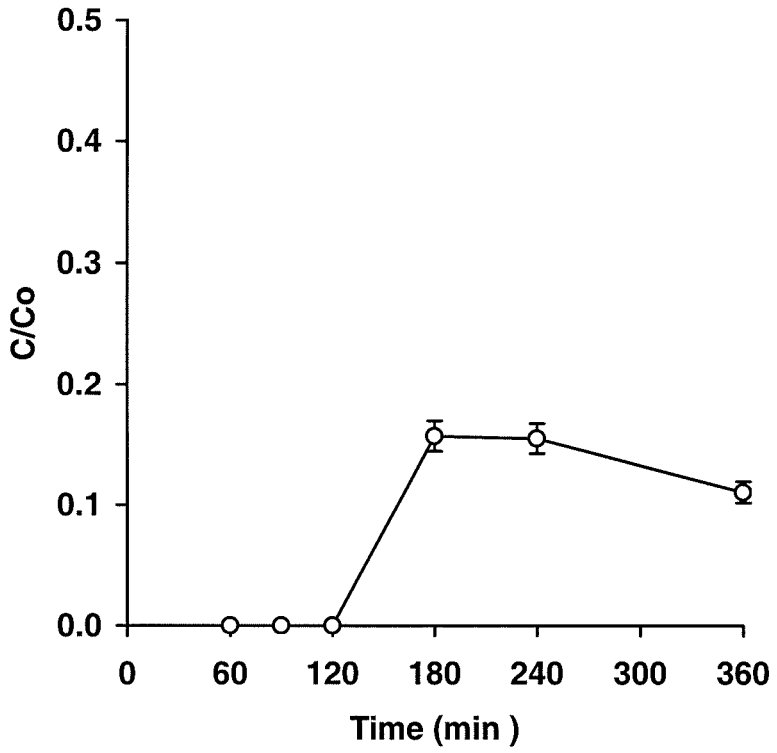


Figure 53. Penetration of voriconazole through mixed-species biofilms of *C. albicans* GDH 2346 and *S. epidermidis* RP62A, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

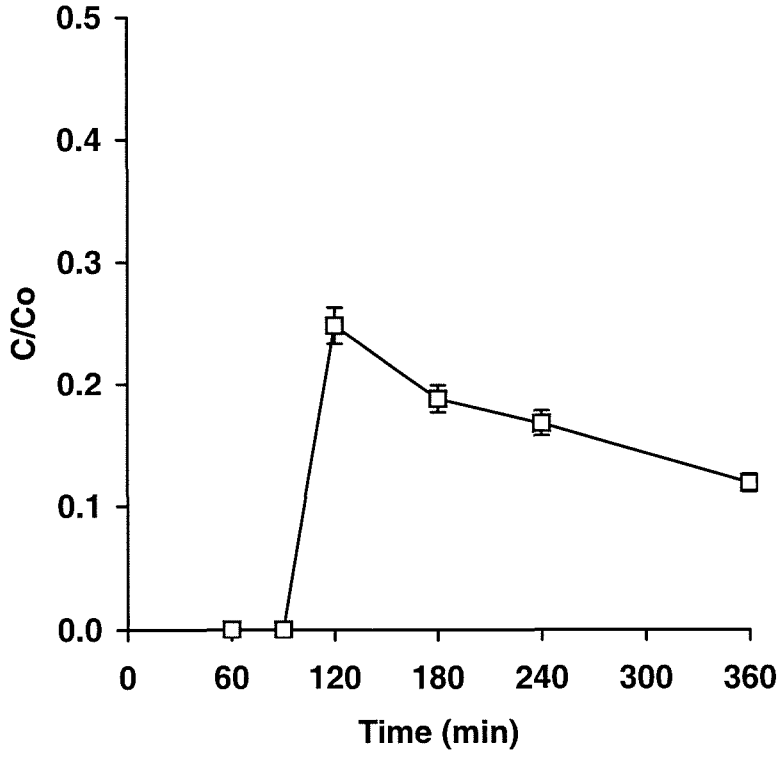


Figure 54. Penetration of voriconazole through mixed-species biofilms of *C. albicans* GDH 2346 and *S. epidermidis* M7, grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means.

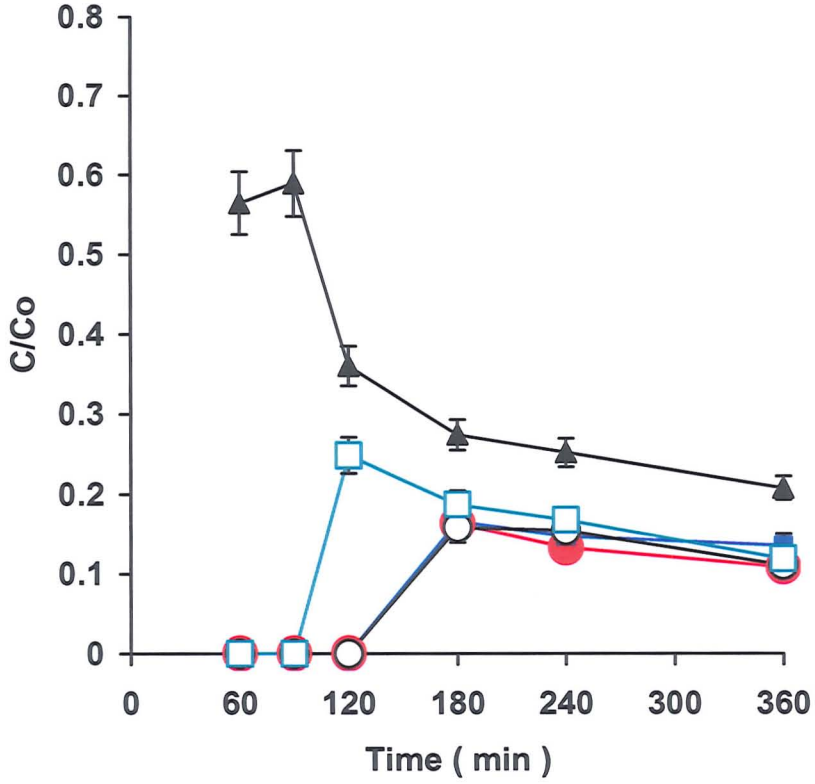


Figure 55. Penetration of voriconazole through single- and mixed-species biofilms of *C. albicans* GDH 2346 (▲), *S. epidermidis* RP62A (●), *S. epidermidis* M7 (■), *C. albicans* GDH 2346 and *S. epidermidis* RP62A (O), and *C. albicans* GDH 2346 and *S. epidermidis* M7 (□) grown on polycarbonate membranes on tryptic soy agar. Error bars indicate the standard errors of the means. The mean C_o after 6 h was 7.9 µg of voriconazole/ml.

1.9 Effect of antifungal agents on the viability of biofilms cells

To assess the effects of antifungal agents on biofilm cells, biofilms sandwiched between the two membranes, as in the drug penetration assay, were exposed to antifungal-containing agar at 37°C for 6 h (the time period during which drug penetration was determined) or 24 h. Antifungal agents (flucytosine or fluconazole) were present at concentrations identical to those used in the drug penetration assay. After incubation, the numbers of viable biofilm cells were determined by a standard procedure of serial dilution followed by plating. In no case did drug penetration result in complete killing of biofilm cells (Table 6).

Biofilms of *C. glabrata* AAHB 12 were wholly unaffected by fluconazole after 6h. However, many strains of this species are known to be resistant to fluconazole even when grown in planktonic culture (Cox and Perfect, 1993). The results with fluconazole overall were not unexpected, despite the high concentration used, since this drug is generally considered to be fungistatic only. However, a recent study (Moosa *et al.*, 2004) has demonstrated that fluconazole can be fungicidal under certain conditions.

Table 6. Viability of 48-h biofilm cells of *Candida* species after exposure to flucytosine or fluconazole for 6 or 24 h [a]

Organism	Viability (%)			
	Flucytosine (6 µg/ml)		Fluconazole (24 µg/ml)	
	6 h	24 h	6 h	24 h
C. albicans GDH 2346	34.0 ± 2.3	28.9 ± 0.5	64.1 ± 1.7	81.0 ± 1.5
C. albicans GDH 2023	45.6 ± 1.5	29.9 ± 1.6	73.0 ± 2.0	62.1 ± 1.4
C. albicans GRI 682	38.2 ± 2.2	29.4 ± 0.9	67.6 ± 1.6	78.7 ± 2.9
C. glabrata AAHB 12	15.8 ± 1.2	24.2 ± 0.6	101.7 ± 1.1	81.1 ± 1.5
C. krusei (Glasgow)	71.3 ± 1.3	66.9 ± 0.5	74.4 ± 0.6	47.8 ± 1.2
C. parapsilosis AAHB 479	74.9 ± 0.7	62.1 ± 1.5	67.4 ± 3.0	51.1 ± 1.2
C. tropicalis AAHB 73	65.0 ± 1.8	40.5 ± 1.4	49.6 ± 0.9	37.7 ± 2.1

[a] Viability is expressed as a percentage of that of control cells incubated under identical conditions in the absence of antifungal agent. The results are means ± standard errors of the means of triplicate determinations. Biofilms were grown on membrane filters on YNB agar containing 50mM glucose.

1.10 Scanning electron microscopy (SEM) of biofilms grown on polycarbonate filter disks

Biofilms formed by different *Candida* species on filter disks were fixed and prepared for SEM by a dehydration procedure that involved air drying (Baillie and Douglas, 1999b); biofilm matrix material is not preserved using this protocol. Examination of biofilms by SEM showed that, after 48 h, those formed by *C. albicans* GDH 2346 consisted of a dense network of yeasts, germ tubes, pseudohyphae and hyphae (Fig. 56). However, *C. glabrata* AAHB 12 biofilm contained mainly small round-to-oval, budding yeast cells. Occasionally, pseudohyphae were present (Fig. 57). Biofilms of *C. krusei*, on the other hand, contained large numbers of pseudohyphae with fewer yeast cells, creating a cross-matchsticks appearance (Fig. 58). Biofilms of *C. parapsilosis* consisted of yeast cells, many of which were elongated, together with relatively short pseudohyphae (Fig. 59).

Biofilms of *C. tropicalis* AAHB 73 had a very slimy appearance, suggestive of an extensive matrix, and were poorly penetrated by both flucytosine and fluconazole. Examination of these biofilms by SEM showed that they consisted of a dense network of yeasts and filaments (pseudohyphae with possibly some hyphae). Many of the filaments appeared to lie parallel to each other in the form of bundles (Fig. 60). The procedure used for sample preparation allows clear visualization of biofilm cells but normally fails to preserve the biofilm matrix. However, fairly extensive matrix material could still be seen adhering to and linking some of the cells, a finding consistent with the slimy appearance of *C. tropicalis* biofilms.

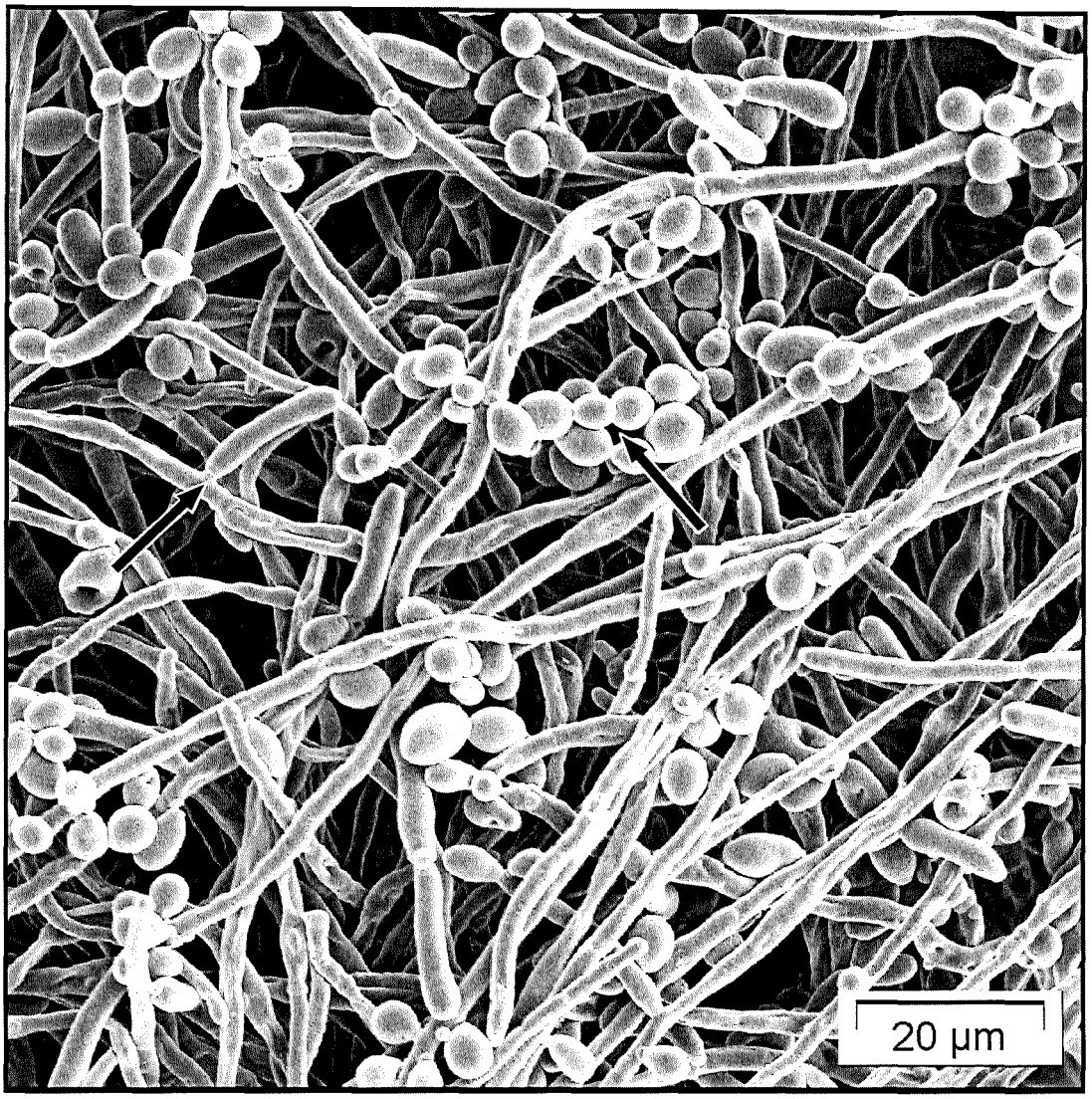


Figure 56. Scanning electron micrograph of biofilm formation by *C. albicans* GDH 2346 on polycarbonate membrane filters after air-drying of samples. Biofilms consisted of a dense network of yeasts, germ tubes, pseudohyphae and hyphae (arrows). Scale bar, 20µm.

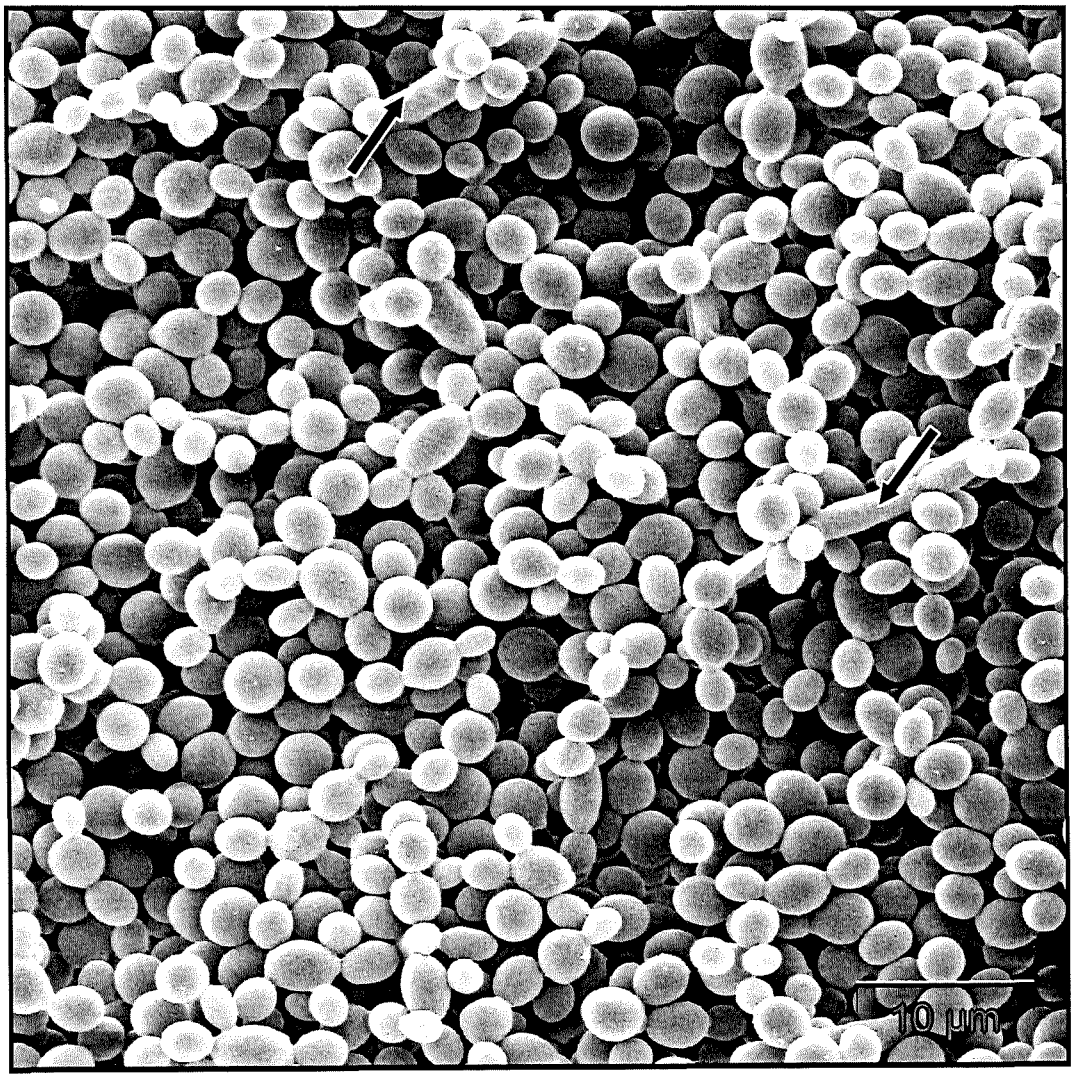


Figure 57. Scanning electron micrograph of biofilm formation by *C. glabrata* AAHB 12 on polycarbonate membrane filters after air-drying of samples. Biofilms consisted of round to oval, budding yeast cells with occasional pseudohyphae (arrows). Scale bar, 10µm.

Figure 58. Scanning electron micrograph of biofilm formation by *C. krusei* on polycarbonate membrane filters after air-drying of samples. Biofilms consisted of large numbers of pseudohyphae (arrows) with fewer yeast cells. Bar, 10µm.

Figure 59. Scanning electron micrograph of biofilm formation by *C. parapsilosis* AAHB 479 on polycarbonate membrane filters after air-drying of samples. Biofilms consisted of yeast cells, many of which were elongated, and pseudohyphae (arrows). Bar, 10 µm.

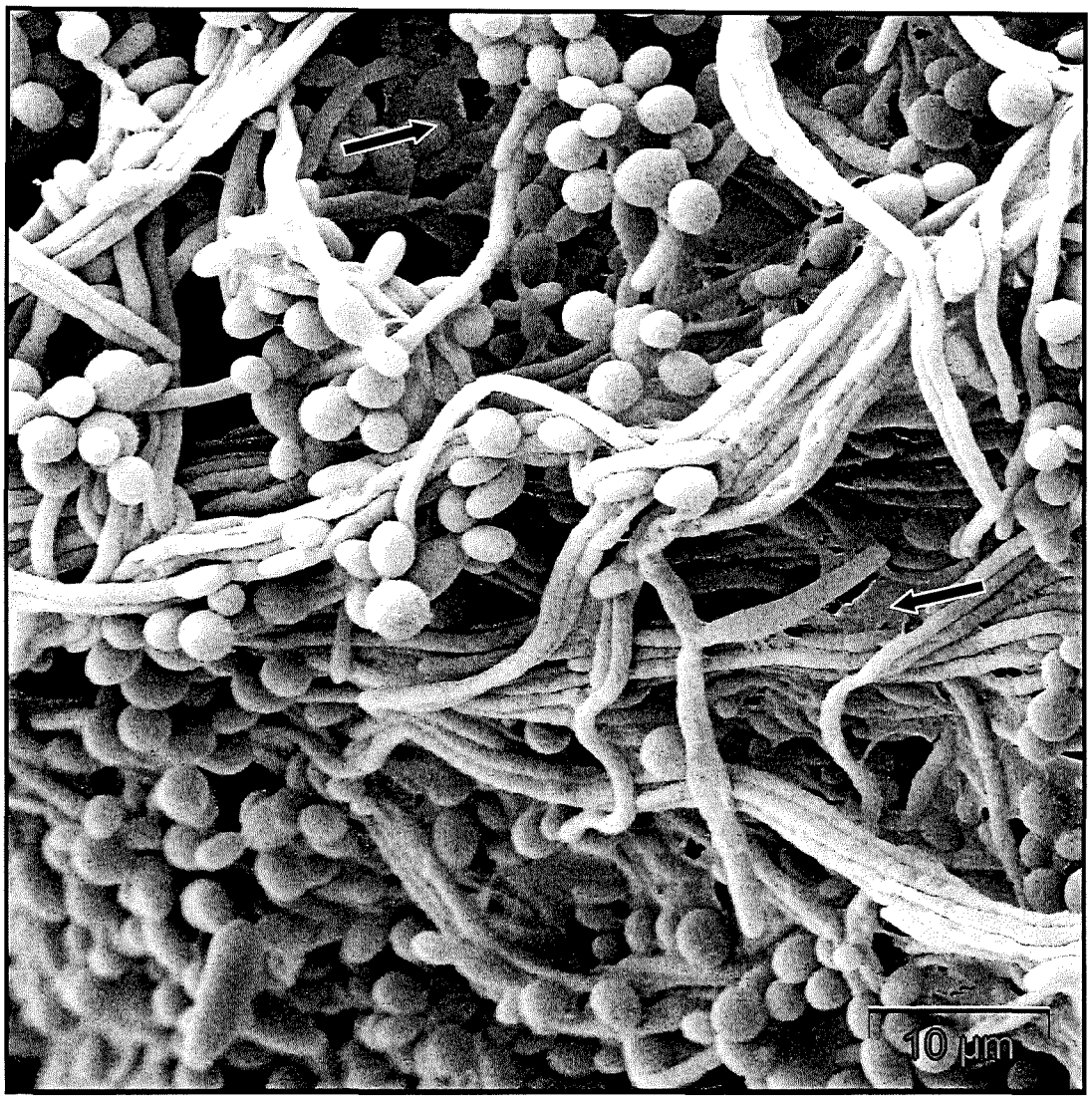


Figure 60. Scanning electron micrograph of biofilm formation by *C. tropicalis* AAHB 73 on polycarbonate membrane filters after air-drying of samples. Biofilms consisted of a dense network of yeast and filaments. Many of the filaments appeared to lie parallel to each other in the form of bundles. Matrix material is visible, indicated by arrows. Bar, 10 µm.

Previous work (Adam *et al.*, 2002) demonstrated that both strains of *S. epidermidis* used in this study formed thick biofilms on catheter disks. However, the wild-type strain RP62A, unlike the M7 mutant, produced abundant matrix material, or slime. This was not readily visible with the preparative techniques used here. SEM revealed that biofilms of mixed fungal-bacterial biofilms of *C. albicans* and *S. epidermidis* RP62A formed on filter disks contained clumps and clusters of smaller bacterial cells, some of which were attached to yeasts (Fig. 61). A similar examination of mixed fungal-bacterial biofilms of *C. albicans* GDH 2346 and *S. epidermidis* M7 grown on polycarbonate membranes also revealed multiple interactions between bacterial and fungal cells. Staphylococci were clearly adherent to both morphological forms of the fungus (Fig. 62).

Although some hyphae were produced in mixed species biofilms with *S. epidermidis* M7 (Fig. 62) they appeared to be absent from mixed species biofilms with *S. epidermidis* RP62A (Fig. 61). It is therefore possible that the wild-type strain of *S. epidermidis* produces a farnesol-like compound which inhibits hyphal production by *C. albicans*. A similar finding has been reported recently for mixed species biofilms containing *C. albicans* and *P. aeruginosa* (Hogan and Kolter, 2002; Hogan *et al.*, 2004).

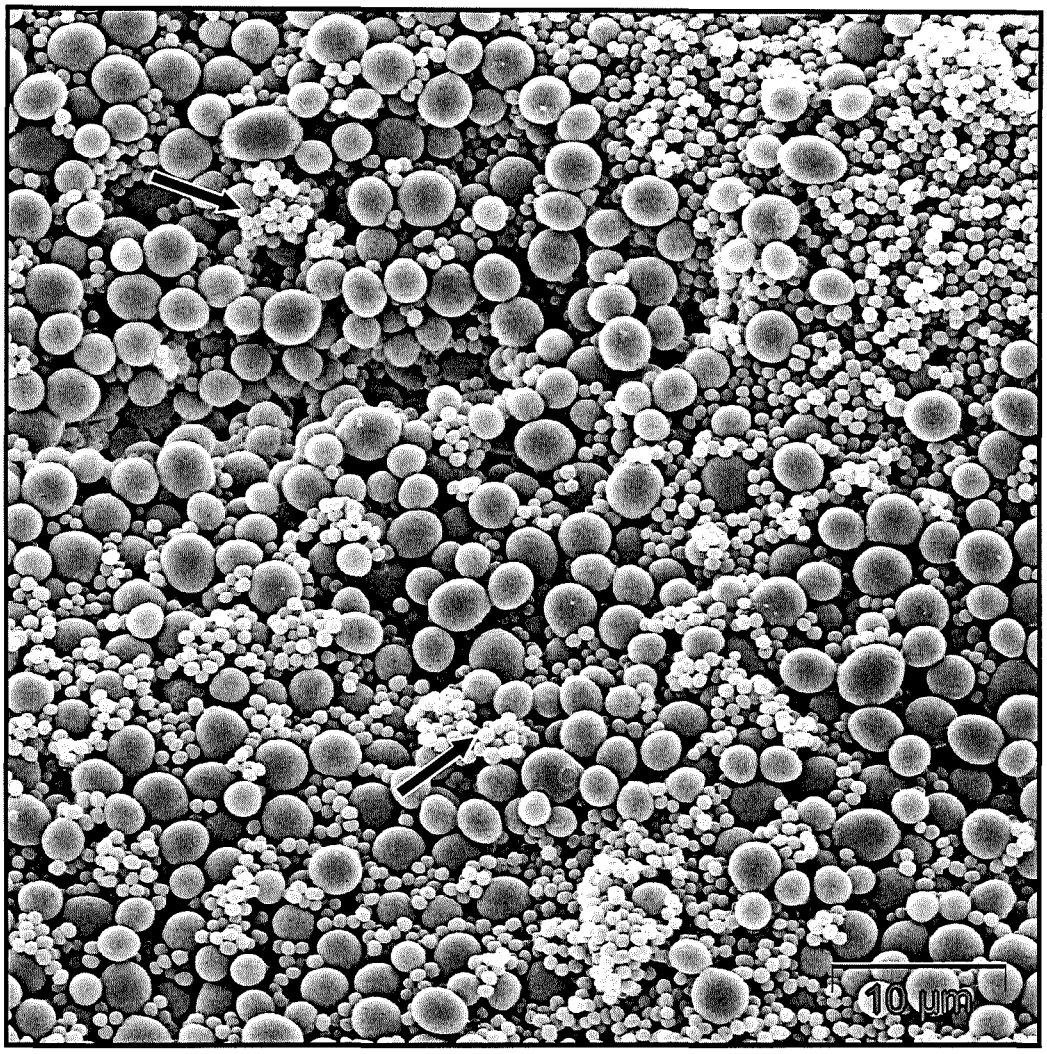


Figure 61. Scanning electron micrograph of mixed-species biofilm formation by *C. albicans* GDH 2346 and *S. epidermidis* RP62A on polycarbonate filters after air-drying of samples. Clumps and clusters of smaller bacterial cells (arrows) can be seen attached to yeasts. Bar, 10 µm.

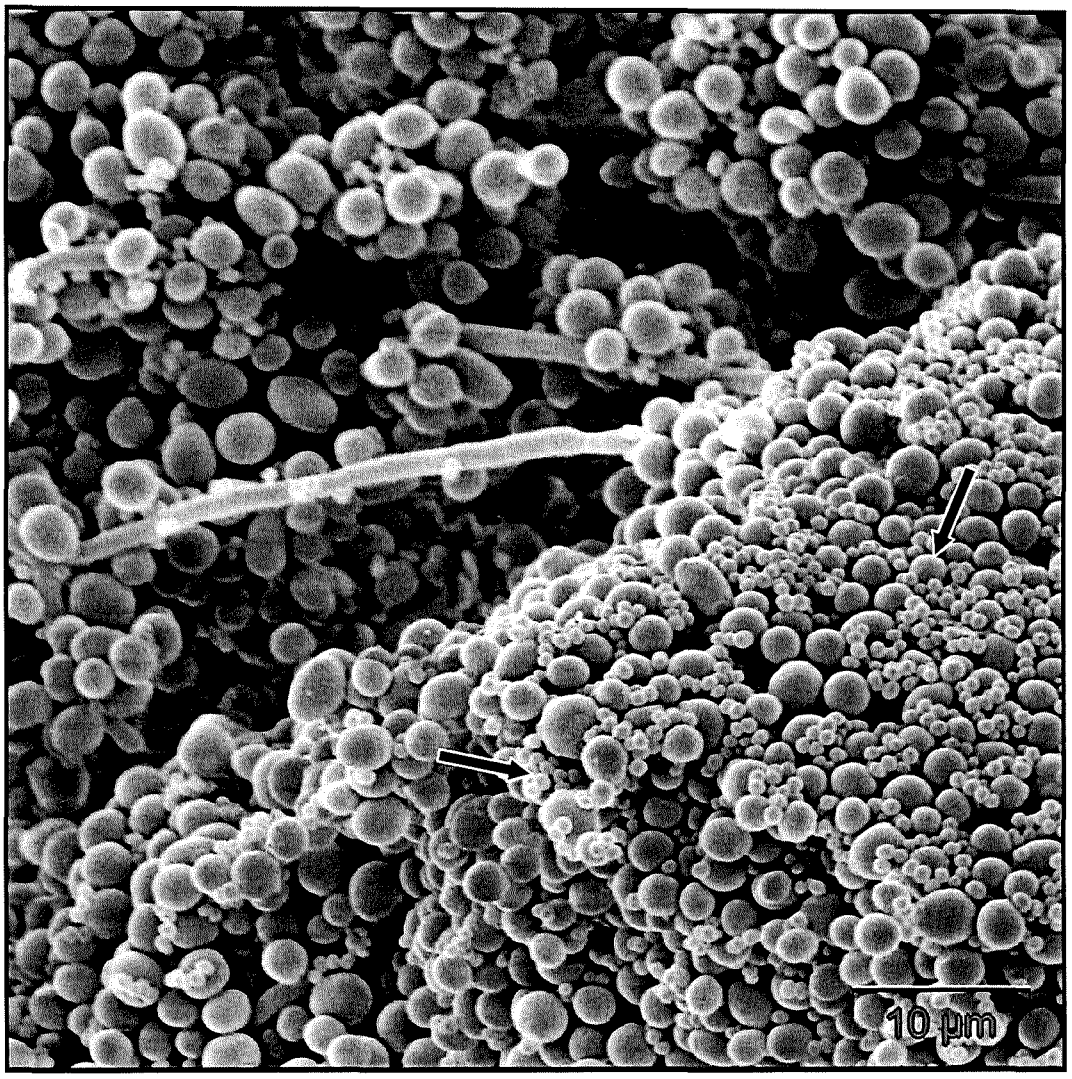


Figure 62. Scanning electron micrograph of mixed-species biofilm formation by *C. albicans* GDH 2346 and *S. epidermidis* M7 on polycarbonate filters after air-drying of samples. Multiple, physical interactions between bacterial and fungal cells are evident (arrows). Bar,10 µm.

2 Drug susceptibility of biofilms grown statically and under conditions of continuous flow

2.1 Comparison of biofilms grown statically and under conditions of continuous flow

Two *Candida* species were assayed for biofilm formation under both static and continuous-flow conditions after incubation for 48 h in YNB medium containing 50 mM glucose. The organisms used were *C. albicans* GDH 2346 and *C. tropicalis* AAHB 73. Both of these isolates came originally from active infections; *C. tropicalis* AAHB 73 was from a line infection (Hawser and Douglas, 1994a).

2.1.1 Biofilms grown statically

The model system used for static biofilm culture involved growing adherent populations on the upper surface of disks cut from catheter material (Hawser and Douglas, 1994a; Hawser and Douglas, 1994c; Baillie and Douglas, 1999b). This model has been well characterised and is known to produce reproducible biofilm populations. In this investigation, biofilms were grown statically on the surfaces of small disks (diameter, 0.8 cm) cut from polyvinyl chloride (PVC) Faucher tubes using a punch (Fig. 10). The disks were placed, concave side up, in wells of 24-well Nunclon tissue culture plates. This simple model system was originally adapted from one used to evaluate the effects of antibiotics on biofilms of *Escherichia coli* and other Gram-negative bacteria (Prosser *et al.*, 1987). Scanning electron micrographs of biofilms formed by *C. albicans* GDH 2346 after incubation for 48 h, revealed a complex mixture of yeasts and hyphae (Fig. 63).

These observations confirmed previous studies undertaken with this system (Hawser and Douglas, 1994a).

2.1.2 Biofilms grown under flow conditions

The Robbins device, which provides quantifiable samples of biofilm growing on submerged surfaces in aqueous systems, is one of the best-known and most widely used systems for biofilm formation under continuous flow (Costerton *et al.*, 1987). The device was later modified by McCoy *et al.* (1981) and was called the modified Robbins device (MRD) (Fig. 11a). There are 25 evenly spaced sampling ports, each of which is fitted with a stud. At the bottom of each stud a disk of catheter material can be fitted. Biofilms are established on the surfaces of the disks (Fig. 12), exposed to a liquid flow in the chamber of the MRD. Initial biofilm inoculation is done by pumping a standardised cell suspension of the test organism through the device at flow rate of 60 ml/h for 1 h at 37°C, followed by sterile YNB or TSB medium at the same flow rate (Fig. 11b). Incubation of catheter disks under conditions of continuous flow for 48 h increased the production of extracellular matrix material associated with the biofilm. The amount of extracellular matrix was monitored by SEM. Samples were prepared by a freeze-drying protocol which permits better preservation of the matrix than air-drying. A flow rate of 60 ml/h increased the synthesis of matrix material, as compared with that found in biofilms incubated statically. Biofilms grown under these conditions in the MRD possessed an extensive coating of matrix material (Fig. 64).

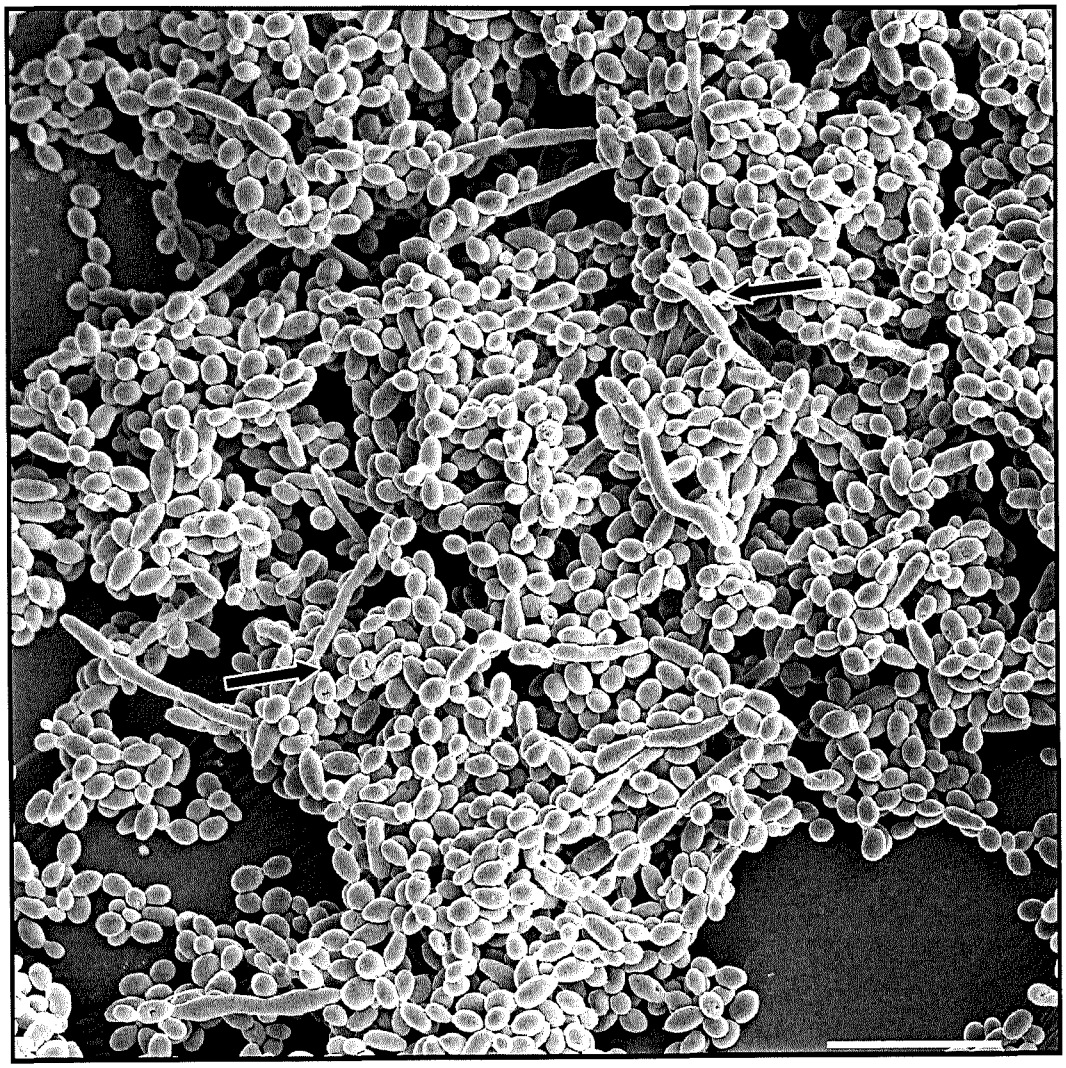


Figure 63. Scanning electron micrograph of a biofilm formed by *C. albicans* GDH 2346 on a PVC catheter disk after incubation for 48h under static conditions in YNB medium containing 50mM glucose. The biofilms consisted of a mixture of yeasts, germ tubes, hyphae and pseudohyphae (arrows). Bar, 20 µm.

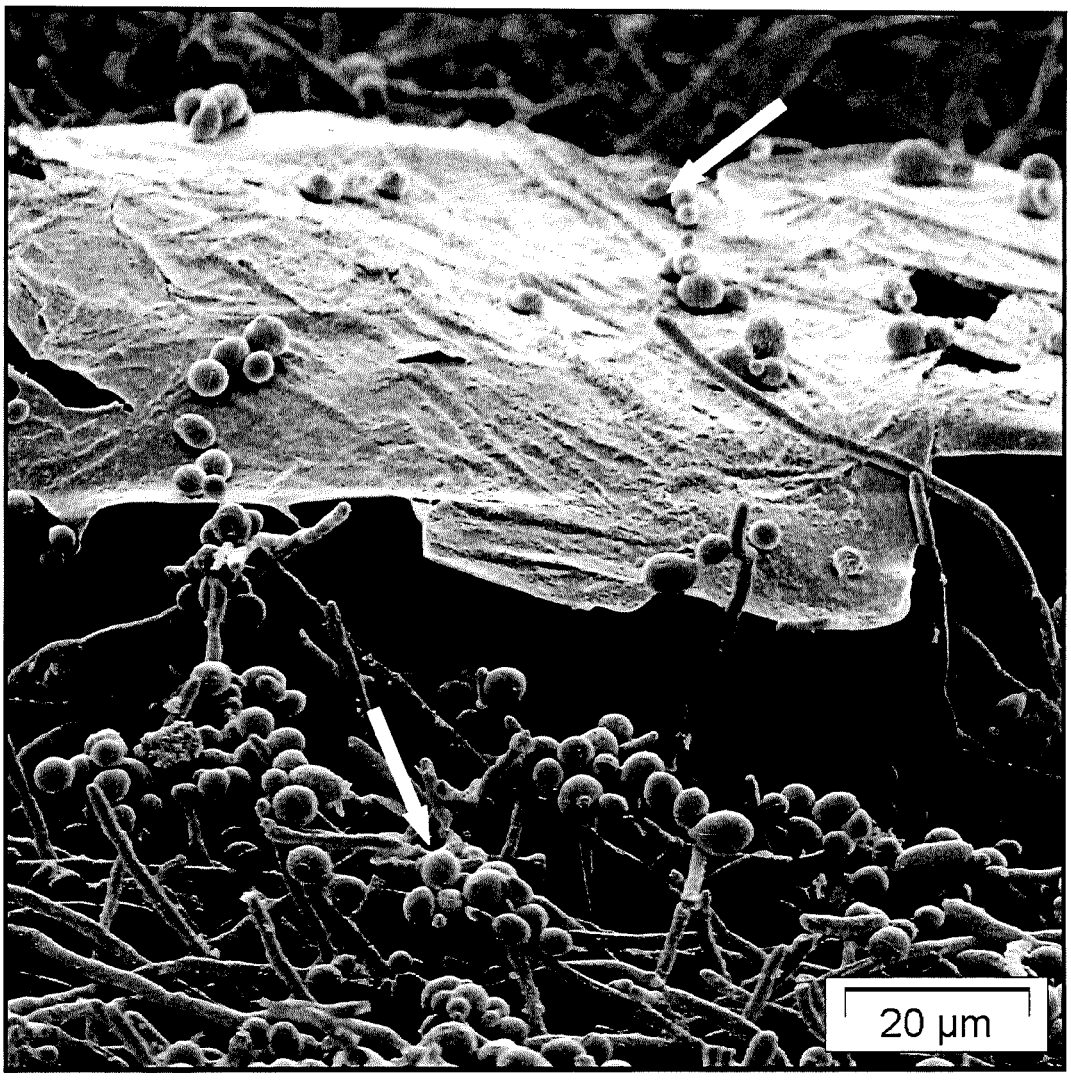


Figure 64. Scanning electron micrograph of biofilm formation by *C. albicans* GDH 2346 on the surface of PVC catheter disks processed by freeze-drying to preserve the biofilm matrix. Biofilms were incubated for 48h under flow conditions (MRD) in medium containing 50mM glucose. The biofilms consisted of a mixture of yeasts and hyphae (arrows) with extensive matrix material. Bar, 20 µm.

2.2 Possible mechanisms of resistance to antifungal agents

Candida biofilms, like those of other microorganisms, are drug resistant. They are resistant to the action of a range of important antifungal agents in current clinical use (Hawser and Douglas, 1995). The mechanisms by which *Candida* biofilms resist the action of antimicrobial agents are poorly understood. However, recent investigations indicate that at least four possible mechanisms of drug resistance are possible. Firstly, resistance could be due to the drug exclusion by the biofilm matrix material. Secondly, phenotypic changes conferring drug resistance could be induced by a slow growth rate, or by nutrient limitation within the biofilm. The third possibility is that attachment to a surface causes the switching on or off of genes which coincidentally affect antimicrobial susceptibility (Brown and Gilbert, 1993). In the present study, the aim was to investigate the first possibility. This was done by increasing the amount of extracellular matrix in biofilm cultures using the MRD continuous-flow system. Drug susceptibility profiles of biofilms incubated under flow conditions were then compared with those of biofilms incubated statically. The effect of an antifungal agent was measured in terms of the percentage inhibition of XTT-formazan formation by biofilms compared with values obtained for control biofilm cells incubated in the absence of the agent.

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

The susceptibility of mature, 48-h *C. albicans* biofilms to antifungal agents was assessed colorimetrically by measuring the ability of biofilm cells to reduce the tetrazolium salt, XTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to a formazan product after exposure to a drug. The assay (Baillie and

Douglas, 1999b) was modified from that described by Mosmann (1983) . Amphotericin B, one of the commonest antifungal agents used clinically, was added to buffered growth medium at different concentrations (5 and 30 times the MIC). Biofilm cells of *C. albicans* grown under static and flow conditions were exposed to this buffered medium containing amphotericin B at 37°C for 5 or 24 h. After incubation, 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 7). *C. albicans* biofilms grown under flow conditions were highly resistant to amphotericin B at a concentration of 5 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 (5h), the biofilms were rather less resistant. However, for both drug treatments, biofilms formed under flow conditions were significantly more resistant that those grown statically (Table 7). Large clumps of biofilm were observed on the surfaces of catheter disks removed from the MRD during these experiments. Scanning electron microscopy showed that more matrix material was present in these biofilms than in those grown statically (Fig. 64). These findings suggest that the more extensive matrix of these biofilms could contribute to their enhanced resistance to amphotericin B.

Table 7. Metabolic activity of *C. albicans* GDH 2346 biofilms after treatment with amphotericin B for 5 h or 24 h

Drug treatment	XTT formazan formation [a]			
	Static conditions [b]		Flow conditions [b]	
	OD_{492}	% of control	OD_{492}	% of control
5 x MIC (24h)	1.194 ± 0.025	60.7±1.3	2.948 ± 0.035	99.4 ± 1.2
30 x MIC (5h)	0.944 ± 0.003	39.6±0.1	1.615 ± 0.218	54.6 ± 7.4

[a] The data are means ± SEM of two independent experiments carried out in quadruplicate.

[b] T-test showed statistically significant differences at $P < 0.001$ for biofilms grown under flow conditions as compared with those grown statically.

2.4 SEM of *C. albicans* GDH 2346 biofilms grown under static or flow conditions on PVC catheter disks

Scanning electron microscopy showed that biofilms formed by *C. albicans* GDH 2346 incubated statically on PVC catheter disks comprised a dense network of yeasts, germ tubes, hyphae, and pseudohyphae (Fig. 63). By contrast, incubation of catheter disks under conditions of continuous flow at 60 ml/h produced biofilms consisting of a mixture of yeasts and hyphae, as before, but the cells were surrounded by extensive matrix material (Fig. 64).

2.5 Effect of amphotericin B and fluconazole on biofilms of *C. tropicalis* AAHB 73 grown under static and flow conditions

The strain of *C. tropicalis* used in this study was a clinical isolate from a line infection and had a very slimy appearance on solid medium. Some of this slime, or matrix material, could be seen when biofilm preparations were observed under the scanning electron microscope (Fig. 65). The effect of antifungal agents on biofilm activity with this organism was assessed only with biofilms grown under static conditions since growth under flow conditions resulted in blockage of the MRD (see below). Following incubation for 48 h at 37°C, biofilms on catheter disks were further incubated with buffered growth medium containing the test antifungal agents at a concentration 30 times the MIC for a further 5 or 24 h at 37°C (Table 8). The metabolic activity of *C. tropicalis* biofilms was then measured using the XTT assay. *C. tropicalis* biofilms were highly resistant to the action of both drugs at both exposure times (5 and 24h), despite the high drug concentration used (30 times the MIC; Table 8). SEM revealed that a large amount of matrix material, which seemed to envelope the cells, was present even under these static

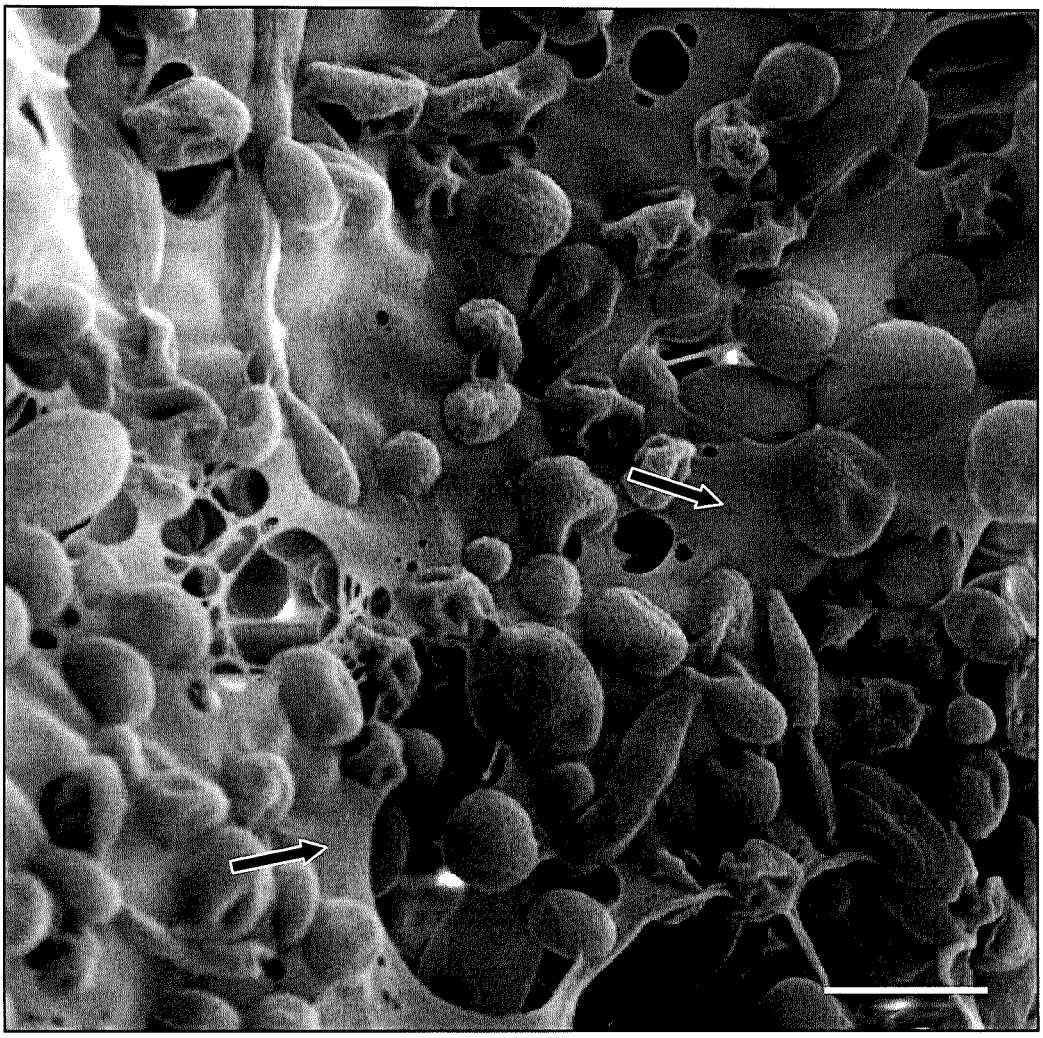


Figure 65. Scanning electron micrograph of biofilm formation by *C. tropicalis* AAHB 73 on the surface of PVC catheter disks processed by freeze-drying to preserve the biofilm matrix. Biofilms were incubated for 48h under static conditions in YNB medium containing 50mM glucose. Extensive matrix material is visible (arrows). Bar, 10 µm.

Table 8. Effect of amphotericin B and fluconazole on biofilms of *C. tropicalis* AAHB 73 after exposure for 5 h or 24 h

Antifungal agent [b]		XTT formazan formation [a]		
		Static conditions [c]		Flow conditions [d]
		OD_{492}	% of control	
Amphotericin B	(5h)	1.981 ± 0.044	109.7 ± 2.4	ND
Amphotericin B	(24h)	1.916 ± 0.063	104.7 ± 3.4	ND
Fluconazole	(5h)	1.923 ± 0.084	106.5 ± 4.7	ND
Fluconazole	(24h)	1.862 ± 0.060	101.8 ± 3.3	ND

[a] The data are means ± SEM of two independent experiments carried out in quadruplicate.

[b] The concentrations of the two drugs used in this assay were equivalent (30 x MIC).

[c] There was no significant difference ($P > 0.05$) between values for drug – treated biofilms and those for the controls.

[d] ND, not determined.

growth conditions with this organism (Fig. 65). These observations again suggest that the drug resistance of *Candida* biofilms may be partly accounted for by drug exclusion by the biofilm matrix.

Attempts to grow biofilms of *C. tropicalis* AAHB 73 for 48h under flow conditions in the MRD were unsuccessful. Although biofilms of the organism appeared to develop quickly, the silicone tubing that connected the medium to the MRD (Fig. 11b) rapidly became blocked by fungal growth. Consequently, it was not possible to produce 48-h biofilms under conditions of continuous flow with this organism, and therefore no assays of biofilm drug susceptibility could be carried out for these conditions.

2.6 SEM of *C. tropicalis* AAHB 73 biofilms grown under static conditions on PVC catheter disks

Scanning electron microscopy showed that *C. tropicalis* biofilms grown statically on PVC catheter disks consisted of yeasts with some pseudohyphae. Extensive matrix material was visible at both lower and higher magnification and many of the microorganisms were almost hidden by the enveloping matrix (Fig. 65). At high magnification, a large amount of extracellular polymeric material was visible on the surface of the filaments (Fig. 66).

2.7 Effect of amphotericin B and fluconazole on mixed fungal-bacterial biofilms grown under static and flow conditions

The catheter disk model system (Hawser and Douglas, 1994c; Baillie and Douglas, 1999b) was used to follow the development of biofilms containing *C. albicans* and *S. epidermidis* statically, while the MRD was used to provide conditions of continuous flow. Two strains of *S. epidermidis* were used in this

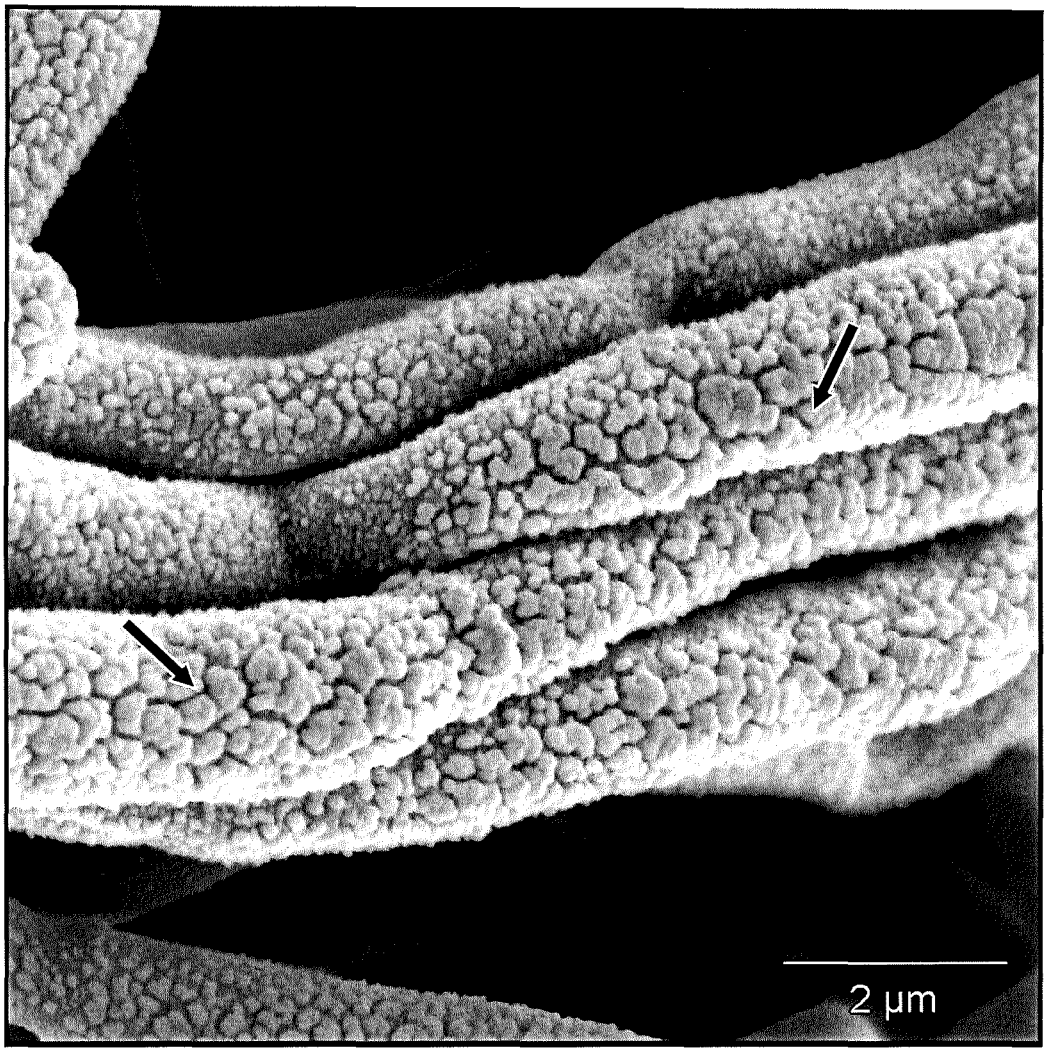


Figure 66. Scanning electron micrograph of biofilm formation by *C. tropicalis* AAHB 73 on the surface of PVC catheter disks processed by freeze-drying to preserve the biofilm matrix. Biofilms were incubated for 48h under static conditions in YNB medium containing 50mM glucose. Arrows indicate substantial amounts of extracellular polymeric material on the surfaces of filaments at high magnification. Bar, 2µm.

study: a slime-producing wild type (RP62A) and a slime-negative mutant (M7) 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). The effects of the antifungal agents amphotericin B and fluconazole on mixed-species biofilms containing *C. albicans* and either the wild-type or mutant strain of *S. epidermidis* were examined.

2.7.1 Effect of amphotericin B and fluconazole on mixed-species biofilms of *C. albicans* GDH 2346 and *S. epidermidis* RP62A

After growth for 48 h, mixed-species biofilms containing *C. albicans* plus the slime-producing *S. epidermidis* RP62A were treated with amphotericin B and fluconazole by the procedure of Adam *et al.* (2002). Briefly, the biofilms were incubated for a further 5 or 24 h at 37°C in buffered growth medium containing fluconazole (12 μg/ml), or amphotericin B (39 μg/ml) at concentrations 30 times the MIC. Biofilm activity was assessed by the XTT reduction assay, after transfer of the disks to new wells. The effect of antifungal agents was measured in terms of XTT reduction by biofilms as compared with values obtained for control biofilms incubated in an identical fashion in the absence of the agents.

C. albicans biofilms were shown from previous studies to be highly resistant to the action of amphotericin B and fluconazole, when compared with planktonic cells of the same strain (Hawser and Douglas, 1994c). Mixed-species biofilms of *C. albicans* and *S. epidermidis* RP62A grown statically or under continuous flow conditions were also highly resistant to the action of both drugs

(Table 9). At exposure times of 5 and 24 h the drugs had no effect on the metabolic activity of mixed-species biofilms, despite the high drug concentration used (30 times the MIC). Moreover, biofilms produced statically were just as resistant as those grown under flow conditions. These results contrast with those obtained for single-species *C. albicans* biofilms treated with amphotericin B where biofilms grown statically were more susceptible to the drug (Table 7). 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.

2.7.2 Effect of amphotericin B and fluconazole on mixed-species biofilms of *C. albicans* GDH 2346 and *S. epidermidis* M7

Mixed-species biofilms of *C. albicans* and *S. epidermidis* M7 (the slime-negative mutant) on catheter disks grown statically or under conditions of continuous flow were also exposed to buffered medium containing fluconazole (12 µg/ml) or amphotericin B (39 µg/ml) for 5 or 24 h at 37°C. The metabolic activity of mature mixed-species biofilms was then determined colorimetrically by the XTT reduction assay. The metabolic activity of drug-treated biofilms was determined as a percentage of that of control biofilms incubated under identical conditions in the absence of the antimicrobial agents. 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 the MIC; Table 10). They were, however, slightly less resistant than biofilms containing the slime-producing *S. epidermidis* RP62A treated in the same way (Table 9).

Table 9. Metabolic activity of mixed-species biofilms of *C. albicans* GDH 2346 and *S. epidermidis* RP62A after treatment with antifungal agents for 5 h or 24 h

Antifungal agent[b]	XTT formazan formation [a]			
	Static conditions [c]		Flow conditions [c]	
	OD_{492}	% of control	OD_{492}	% of control
Amphotericin B (5h)	2.86 ± 0.03	98.9 ± 1.0	2.31 ± 0.16	105.8 ± 7.3
Amphotericin B (24h)	2.87 ± 0.05	99.8 ± 1.7	2.97 ± 0.06	93.2 ± 1.9
Fluconazole (5h)	2.76 ± 0.06	95.5 ± 2.1	2.18 ± 0.06	100.1 ± 2.8
Fluconazole (24h)	2.95 ± 0.02	102.4 ± 0.7	3.04 ± 0.06	95.3 ± 1.9

[a] The data are means ± SEM of two independent experiments carried out in quadruplicate.

[b] The concentrations of the two drugs used in this assay were equivalent (30 x MIC).

[c] T-test showed no statistically significant differences ($P > 0.05$) between biofilms grown under static and flow conditions after treatment with antifungal drugs.

Table 10. Metabolic activity of mixed-species biofilms of *C. albicans* GDH 2346 and *S. epidermidis* M7 after treatment with antifungal agents for 5 h or 24 h

Antifungal agent [b]	XTT formazan formation [a]			
	Static conditions [c]		Flow conditions [c]	
	OD_{492}	% of control	OD_{492}	% of control
Amphotericin B (5h)	2.05 ± 0.03	88.7 ± 1.3	2.73 ± 0.01	99.0 ± 0.4
Amphotericin B (24h)	1.69 ± 0.09	71.8 ± 3.8	2.94 ± 0.09	91.9 ± 2.8
Fluconazole (5h)	1.53 ± 0.07	66.4 ± 3.0	2.58 ± 0.11	93.5 ± 3.9
Fluconazole (24h)	1.59 ± 0.11	67.2 ± 4.6	2.92 ± 0.04	91.5 ± 1.3

[a] The data are means ± SEM of two independent experiments carried out in quadruplicate.

[b] The concentrations of the two drugs used in this assay were equivalent (30 x MIC)

[c] T-test showed statistically significant differences ($P < 0.01$) between biofilms grown under static and flow conditions after drug treatment.

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 10). 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.

2.8 SEM of mixed-species biofilms grown under static or flow conditions on PVC catheter disks

Examination of mixed-species fungal-bacterial biofilms by SEM showed that, after 48 h, both staphylococcal strains formed extensive interactions with *C. albicans*; these interactions were more easily seen with the slime-negative mutant, using a preparative procedure that does not preserve the biofilm matrix. Examination of mixed-species biofilms containing the wild-type strain RP62A of *S. epidermidis* processed by the air-drying technique revealed clumps of smaller bacterial cells adherent to yeasts and hyphae. The matrix material was not visible (Fig. 67). The freeze-drying method of sample preparation, which does preserve some of the matrix material, allowed similar interactions to be visualised with biofilms containing the mutant M7 (Fig. 68). Clumps of staphylococci attached to either yeasts or hyphae could again be seen. In addition, a similar examination of mixed-species biofilms by SEM at low magnification revealed the presence of open channels between microcolonies in the biofilm structure. However, it is possible that these channels are an artefact of the sample preparation procedure rather than genuine features of the biofilm. Large numbers of hyphae are also visible at this low magnification (Fig. 69).

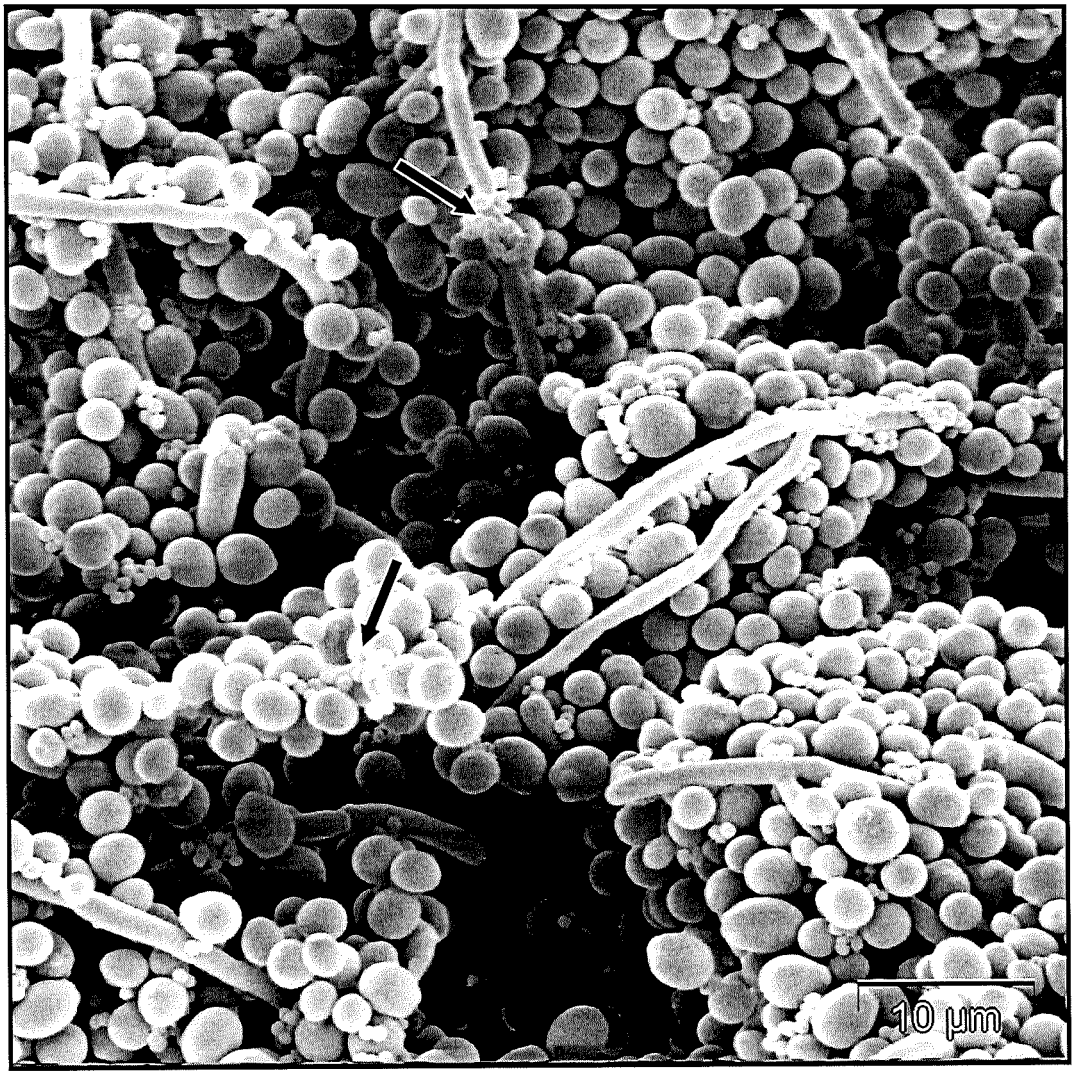


Figure 67. Scanning electron micrograph of mixed-species biofilm formation by *C. albicans* GDH 2346 and *S. epidermidis* RP62A on the surface of PVC catheter disks after air-drying of samples. Biofilms were incubated for 48h under flow conditions (MRD) in tryptic soy broth. Clumps of smaller bacterial cells (arrows) can be seen adherent to yeasts and hyphae. Matrix material is not visible. Bar, 10 µm.

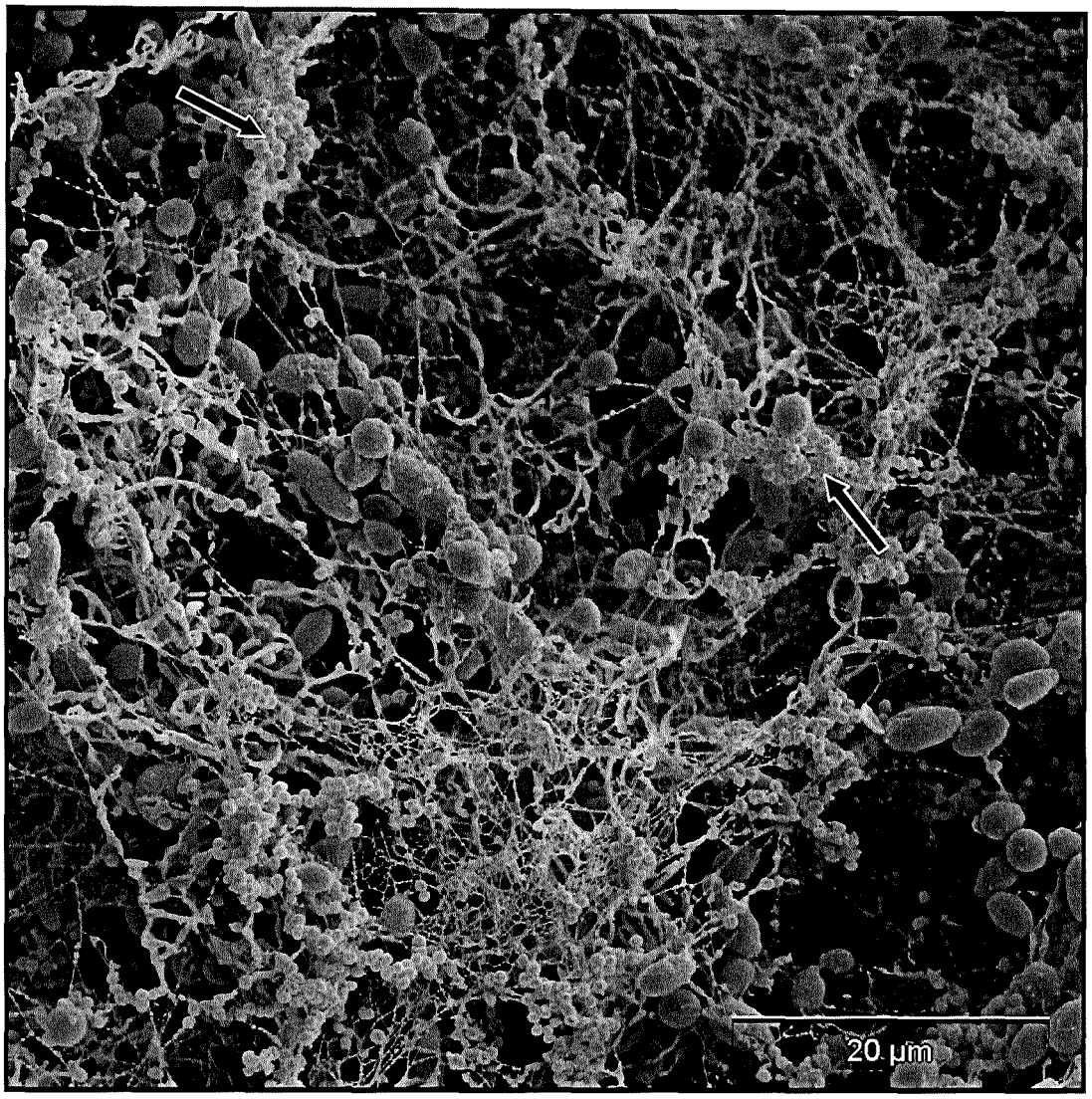


Figure 68. Scanning electron micrograph of mixed-species biofilm formation by *C. albicans* GDH 2346 and *S. epidermidis* M7 on the surface of PVC catheter disks processed by freeze-drying to preserve the biofilm matrix. Biofilms were incubated for 48h in tryptic soy broth under flow conditions in the MRD. The biofilms consisted of clumps and clusters of smaller bacterial cells (arrows) attached to yeasts or hyphae. Fairly extensive matrix material is visible. Bar, 20 µm.

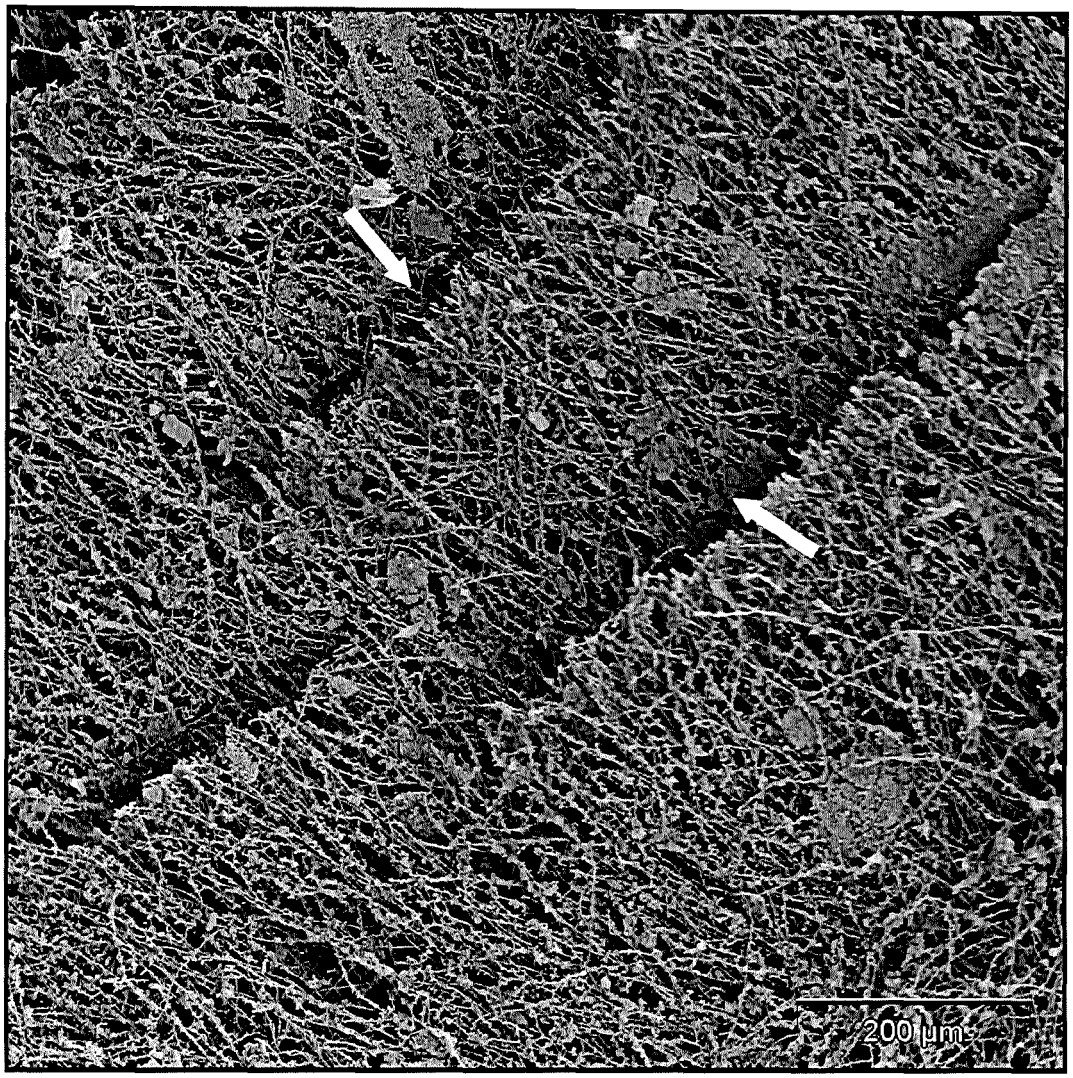


Figure 69. Scanning electron micrograph of mixed-species biofilm formation by *C. albicans* GDH 2346 and *S. epidermidis* M7 on the surface of PVC catheter disks after freeze-drying of samples. Biofilms were incubated for 48h under flow conditions (MRD) in tryptic soy broth. Open channels (arrows) are visible in the biofilm structure at low magnification. Scale bar = 200µm.

3 Isolation and analysis of extracellular polymeric substances (EPS) from biofilms of *C. albicans* and *C. tropicalis*

3.1 Extraction of EPS

To study the composition of EPS, an extraction method is required to separate cells from the matrix material. Various methods have been described for the isolation and analysis of EPS. However, no universal method has yet been adopted because the compromise between EPS yield and minimum cell lysis is difficult to establish (Azeredo *et al.*, 2003). In this study, EPS was extracted from biofilms formed on sections cut from PVC catheter tubing during incubation with gentle shaking at 37°C for 48 h. Quantification of EPS is highly dependent upon the extraction method used. Two main extraction methods have been developed for quantitative analysis, the most common of which is physical extraction. In this work, a physical extraction process modified from the method of Baillie and Douglas (2000) was used. It involved sonication, vortexing and centrifugation of biofilms formed on sections of catheter tubing. EPS was prepared from biofilms of two different *Candida* species, *C. albicans* GDH 2346 and *C. tropicalis* AAHB 73.

3.1.1 Quantitative extraction of EPS from *C. albicans* GDH 2346 biofilms

Following the extraction process, and after lyophilization, the total quantity of extracted EPS was weighed using a sensitive, 5-figure balance. Two separate extraction procedures were carried out with *C. albicans* biofilms to provide sufficient material for chemical analysis. Very similar quantities of EPS were obtained for the two preparations (10.7 and 9.40 mg; Table 11).

Table 11. EPS extracted from different *Candida* biofilms [a]

	Total EPS (mg)	
Preparation number	***C. albicans* GDH 2346**	***C. tropicalis* AAHB 73**
1	10.7	16.5
2	9.40	23.6

[a] EPS was physically extracted, without adding any chemical extractant.

[b] Each preparation was freeze-dried and then dissolved in distilled water (1ml) for biochemical analysis.

3.1.2 Quantitative extraction of EPS from *C. tropicalis* AAHB 73 biofilms

The same extraction procedure was used for *C. tropicalis* biofilms. Again, two extractions were carried out to provide sufficient material for chemical analysis. However, the yield of EPS from biofilms of *C. tropicalis* was much higher than that from biofilms of *C. albicans* (16.5 and 23.6 mg; Table 11). This strain of *C. tropicalis* was isolated from a line infection and had a very slimy appearance on solid medium, suggestive of the production of copious amounts of extracellular polymers.

3.2 Composition of the biofilm matrix material from different *Candida* species

In general, extracellular polymeric substances consist of metabolic products that accumulate on the microorganism's surface (Morgan *et al.*, 1990). They play an important role in protecting the cells against host defences and harsh external environments such as those containing antimicrobial agents. They may also function in nutrition to extend the cells' carbon and energy reserves during starvation. Bacterial EPS is mainly composed of carbohydrate and protein plus smaller amounts of uronic acid, lipid and DNA (Liu and Fang, 2002). Preliminary results with *C. albicans* EPS (Baillie and Douglas, 2000) suggest that it has a similar composition overall.

3.2.1 Composition of EPS extracted from *C. albicans* GDH 2346 biofilms

To analyse the chemical composition of matrix material, biofilms were subjected to a physical extraction process, and two separate EPS preparations were obtained under identical conditions, as described above. The EPS preparations were analysed for carbohydrate, glucose, protein, hexosamine, phosphorus and

uronic acid by colorimetric or enzyme methods. EPS consisted largely 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 12).

These results are similar to those obtained previously by Baillie and Douglas (2000) for the same *C. albicans* strain, except for the glucose content. The glucose content determined in this study (32.2 %) was higher than that reported by Baillie and Douglas (15.9 %). This may reflect the fact that the carbon source used here was glucose, whereas galactose was used in the earlier study.

3.2.2 Composition of EPS extracted from *C. tropicalis* AAHB 73 biofilms

Table 13 shows the chemical composition of EPS extracted from *C. tropicalis* biofilms; no such data for *C. tropicalis* biofilms have been reported elsewhere so far. The EPS consisted largely of hexosamine (27.4 %), together with smaller amounts of carbohydrate (3.3 %, including 0.5 % glucose), protein (3.3 %) and phosphorus (0.2 %; Table 13). The *C. tropicalis* EPS also contained slightly more uronic acid (1.6 %) than that from *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. Interestingly, hexosamine (glucosamine) is also a major component of the matrix material of slime-producing *S. epidermidis* RP62A.

Table 12. Analysis of EPS extracted from *C. albicans* GDH 2346 biofilms

Component	Percentage composition of biofilm EPS [a]
Carbohydrate	39.6 ± 0.3
Glucose	32.2 ± 1.5
Hexosamine	3.3 ± 0.6
Phosphorus	0.5 ± 0.0
Protein	5.0 ± 0.1
Uronic acid	0.1 ± 0.0

[a] The data are means ± SEM for two independent experiments (with two different preparations of EPS) carried out in duplicate or triplicate.

Table 13. Analysis of EPS extracted from *C. tropicalis* AAHB 73 biofilms

Component	Percentage composition of biofilm EPS [a]
Carbohydrate	3.30 ± 0.0
Glucose	0.50 ± 0.0
Hexosamine	27.40 ± 0.2
Phosphorus	0.20 ± 0.0
Protein	3.30 ± 0.0
Uronic acid	1.60 ± 0.0

[a] The data are means ± SEM for two independent experiments (with two different preparations of EPS) carried out in duplicate or triplicate.

4 Enzymatic detachment of *Candida* species biofilms

At present, coagulase-negative staphylococci, mostly *Staphylococcus epidermidis*, are the most prominent organisms responsible for infections associated with implanted biomaterials such as intravascular catheters, prosthetic joints, and prosthetic cardiac valves (Kloos and Bannerman, 1994). *S. epidermidis* grows on medical devices as an adherent biofilm consisting of cells embedded in an amorphous, sticky matrix, which is composed of exopolysaccharides (Mack *et al.*, 1996). Recently, Kaplan *et al.* (2004) identified an enzyme, produced by *Actinobacillus actinomycetemcomitans*, that rapidly and efficiently removed *S. epidermidis* biofilms from plastic surfaces. This enzyme, named dispersin B, is an *N*-acetylglucosaminidase that apparently degrades a polymer of β (1→6)-linked *N*-acetylglucosamine residues, which is a major component of the biofilm matrix of *S. epidermidis*.

In the study described here, the enzymatic detachment assay devised by Kaplan *et al.* (2004) was used to investigate whether *Candida* biofilms could also be detached from plastic surfaces. A range of commercially available enzymes of known specificity was tested in an attempt to further characterise the biofilm matrix material. Both *C. albicans* and *C. tropicalis* were used in these experiments.

4.1 Detachment of *C. albicans* GDH 2346 biofilms by different test enzymes

To assess possible biofilm detachment or degradation of matrix material caused by different test enzymes, biofilms of *C. albicans* GDH 2346 were grown in the wells of a 96-well polystyrene microtitre plate. Following incubation, the wells were washed to remove loosely adherent cells and the remaining fungal cells attached to the bottoms of the wells were stained with crystal violet (Gram-staining

reagent). Adherent biofilm cells were indicated by the presence of dark-staining material on the bottoms of the wells (Fig. 70). The amount of biofilm could be quantified by measuring the OD of the wells at 570 nm with a microtitre plate reader. To assay biofilm detachment or degradation, wells containing mature biofilms were treated with different test enzymes at 37°C or 25°C (according to the temperature optimum) for 2 h at a final enzyme concentration of 50μg/ml. Treatment with lipase type VII, phospholipase A_2, or protease type XIV failed to affect biofilms, which were similar in appearance to untreated biofilms (Table 14). However, treatment with proteinase K, DNase I, chitinase or β-*N*-acetylglucosaminidase resulted in a significant decrease in OD reading suggesting that the enzyme activity removed matrix material and caused some biofilm detachment from the surfaces of the wells. Interestingly, lyticase, which hydrolyses poly (β-1, 3-glucose), such as yeast cell-wall glucan, had by far the greatest effect on biofilms, causing an 85% reduction in optical density ($P < 0.001$). These results suggest that β-1, 3-linked glucan might also be an important component of biofilm matrix material in *C. albicans*.

4.2 Detachment of *C. tropicalis* AAHB 73 biofilms by different test enzymes

C. tropicalis biofilms were grown in the wells of a 96-well polystyrene microtitre plate by the same procedure as that used for *C. albicans*. The biofilms were then treated with different test enzymes as before (final concentration, 50 μg/ml) for 2 h at 37°C or 25°C according to the temperature optimum for the enzyme. After incubation, biofilm cells remaining attached to the bottom of the wells were stained with crystal violet (Fig. 71).

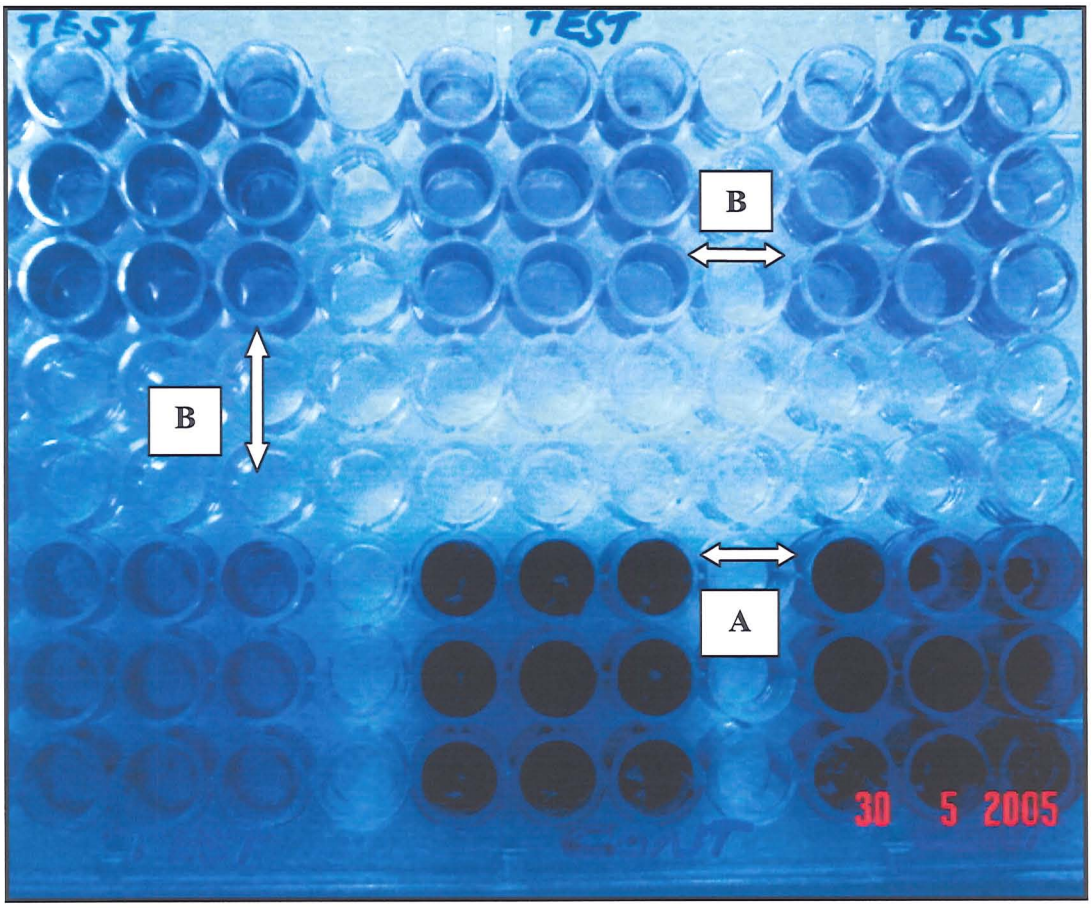


Figure 70. *C. albicans* GDH 2346 biofilm formation in the wells of 96-well polystyrene microtitre plates *C. albicans* GDH 2346 biofilms were grown in triplicate in 3 horizontal rows of wells, and then washed with PBS and stained with crystal violet. Two hours prior to washing, test enzyme was added to horizontal rows of wells labelled B. An equivalent volume of buffer was added to horizontal rows of wells labelled A (control wells).

Table 14. Detachment of *C. albicans* GDH 2346 biofilms after exposure to different test enzymes [a]

Enzyme [b]	OD_{570}	% of control
Lipase type VII	0.144 ± 0.008	>100.0 [c]
Phospholipase A_2	0.161 ± 0.006	>100.0 [c]
Protease type XIV	0.144 ± 0.007	>100.0 [c]
Proteinase K	0.098 ± 0.004	69.2 ± 2.8 [d]
Chitinase	0.131 ± 0.008	77.2 ± 4.7 [d]
DNase I	0.113 ± 0.007	70.8 ± 4.4 [d]
Lyticase	0.025 ± 0.002	15.4 ± 1.2 [e]
β-N–Acetylglucosaminidase	0.120 ± 0.004	79.3 ± 2.6 [d]

[a] The data are means ± SEM of two independent experiments each carried out with 36 replicates for every enzyme tested.

[b] All enzyme treatments were carried out for 2h at 37°C or 25°C with a final enzyme concentration of 50µg/ml.

[c] Value not significantly different ($P > 0.05$) from that for the control.

[d] Value significantly different at $P \leq 0.05$ from that for the control.

[e] Value significantly different at $P < 0.001$ from that for the control.

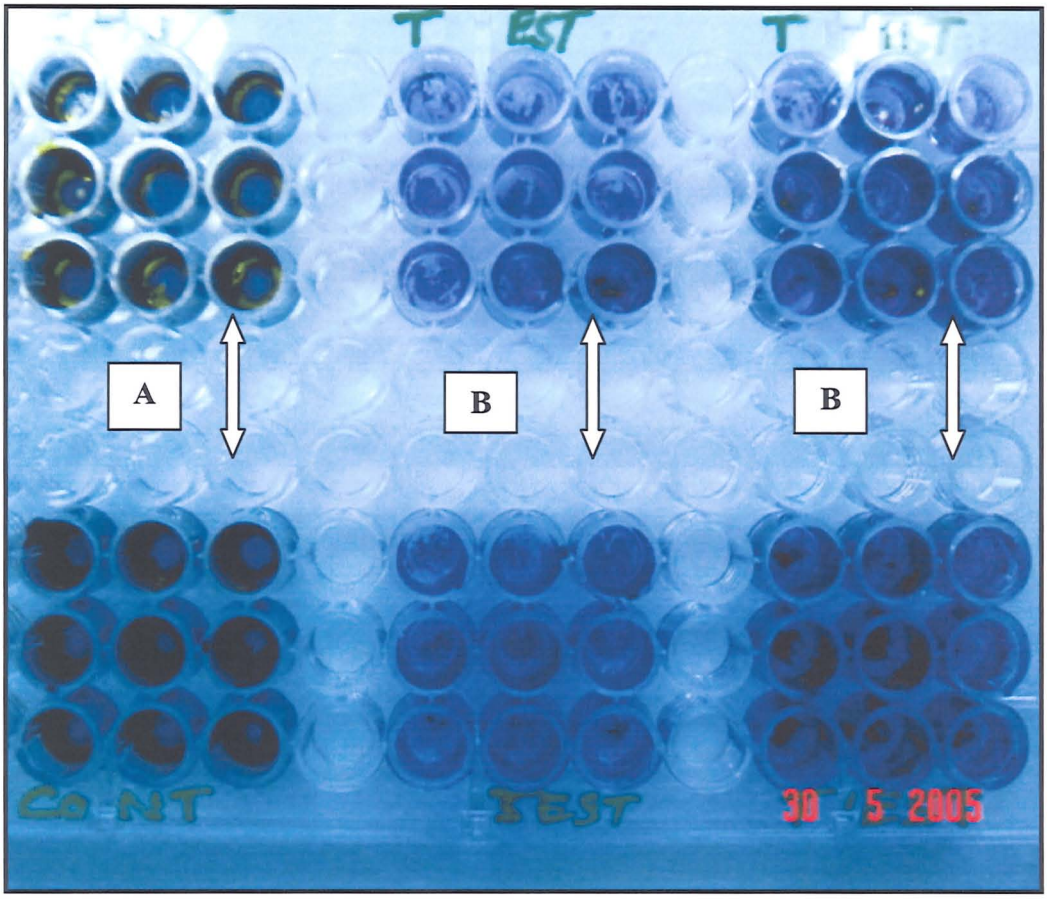


Figure 71. *C. tropicalis* AAHB 73 biofilm formation in the wells of 96-well polystyrene microtitre plates *C. tropicalis* AAHB 73 biofilms were grown in triplicate in 3 horizontal rows of wells, and then washed with PBS and stained with crystal violet. Two hours prior to washing, test enzyme was added to horizontal rows of wells labelled B. An equivalent volume of buffer was added to horizontal rows of wells labelled A (control wells).

Treatment of biofilms with DNase I had no effect (Table 15) and the biofilms had an appearance similar to that of the untreated controls. Phospholipase A_2, protease type XIV, proteinase K and β-*N*-acetylglucosaminidase also failed to produce a statistically significant decrease in OD reading. Treatment with lipase type VII and chitinase, on the other hand, did result in a significant decrease in OD reading ($P \leq 0.05$) suggesting that these enzymes caused some biofilm detachment from the surface of the wells. Again, the greatest effect was with lyticase, which caused a reduction in optical density of over 53% ($P < 0.001$; Table 15). However, lyticase had less effect with *C. tropicalis* biofilms than with *C. albicans* biofilms (Table 14) whose matrix material contains substantially more glucose (Table 12).

5 Further studies on the effect of lyticase on *Candida* biofilms

The cell wall of yeasts and other fungi is a rigid structure essential for the survival of fungal cells. Because of its absence in mammalian cells, the cell wall is an attractive target for antimicrobial agents. Early studies (Yu *et al.*, 1967; Chattawa *et al.*, 1968) on the cell walls of *C. albicans* and *S. cerevisiae* established that the walls are qualitatively similar, being composed mainly of *β*-glucans, mannoproteins (previously called mannans) and a small amount of chitin. In general, *β*-glucans account for 60 % of the cell-wall dry mass, mannoproteins 40 % and chitin approximately 2 % (Klis *et al.*, 2002). Enzymes capable of degrading the yeast cell wall have been isolated from the culture supernatants of several microorganisms. In general, yeast lytic activity can be attributed to a *β*-1,3-glucanase, although lytic *β*-1,6-glucanases have been characterised (Scott and Schekman, 1980). Lyticase is a *β*-1,3-glucanase produced by *Arthrobacter luteus*.

Table 15. Detachment of *C. tropicalis* AAHB 73 biofilms after exposure to different test enzymes [a]

Enzyme [b]	OD_{570}	% of control
Lipase type VII	0.229 ± 0.006	73.9 ± 1.9 [d]
Phospholipase A_2	0.259 ± 0.007	92.2 ± 2.5 [c]
Protease type XIV	0.244 ± 0.015	88.3 ± 5.4 [c]
Proteinase K	0.255 ± 0.016	93.1 ± 5.8 [c]
Chitinase	0.206 ± 0.010	71.3 ± 3.5 [d]
DNase I	0.279 ± 0.006	100.0 ± 2.2 [c]
Lyticase	0.147 ± 0.007	46.2 ± 2.2 [e]
β-N – Acetylglucosaminidase	0.298 ± 0.008	94.9 ± 2.6 [c]

[a] The data are means ± SEM of two independent experiments each carried out with 36 replicates for every enzyme tested.

[b] All enzyme treatments were carried out for 2h at 37°C or 25°C with a final enzyme concentration of 50µg/ml.

[c] Value not significantly different ($P > 0.05$) from that for the control.

[d] Value significantly different at $P \leq 0.05$ from that for the control.

[e] Value significantly different at $P < 0.001$ from that for the control.

It is frequently used in the preparation of yeast spheroplasts because it hydrolyses β-1,3-glucan which is the main structural component of the yeast cell wall.

In this investigation, previous studies (Section 4) showed that lyticase was the enzyme which caused most biofilm detachment from microtitre plates. This suggested that lyticase was capable of degrading one or more components of the biofilm matrix. However, it is also possible that lyticase treatment resulted in some lysis of biofilm cells. The following experiments were designed to investigate this possibility.

5.1 Effect of lyticase on *C. albicans* GDH 2346 biofilm cells

To assess the effect of lyticase enzyme on biofilm cells, biofilms were grown for 48 h in a 24-well microtitre plate and then treated with lyticase (final concentration 50 µg/ml) at 25°C for 2 h. The concentration of the enzyme used in this assay was identical to that used previously in the detachment assay.

After incubation, the enzyme solution was removed and the biofilms were resuspended in 1M sorbitol buffer. Optical density readings at 520nm and 600nm were then taken after 0, 15 and 30 min. There was no decrease in optical density reading during this 30 min period for the lyticase-treated biofilm cells (Table 16). However, the optical density of these cell suspensions was only half that of cell suspensions from control biofilms that had not been exposed to the enzyme. This suggests some degree of cell lysis in the lyticase-treated biofilms although it is also possible that some of the reduction in optical density was due to degradation of matrix material.

Table 16. Effect of lyticase on *C. albicans* GDH 2346 biofilm cells [a, b]

Time after resuspension (min)	OD_{520}	% of control [c]	OD_{600}	% of control [c]
0	0.35 ± 0.02	45.6 ± 2.2	0.32 ± 0.02	48.5 ± 2.4
15	0.37 ± 0.02	49.2 ± 2.2	0.34 ± 0.02	49.1 ± 2.4
30	0.37 ± 0.02	49.8 ± 2.0	0.34 ± 0.01	52.7 ± 2.2

[a] The data are means ± SEM of two independent experiments carried out in triplicate. After lyticase treatment, biofilm cells were resuspended in sorbitol buffer and the optical density of each suspension monitored after 0, 15 and 30 min.

[b] The final concentration of lyticase used was 50 µg/ml.

[c] Optical density readings for lyticase-treated biofilm cells expressed as a percentage of those for control (untreated) cells. The differences between enzyme-treated cells and control cells were statistically significant ($P < 0.001$).

5.2 Effect of lyticase on *C. tropicalis* AAHB 73 biofilm cells

Biofilms were grown for 48 h on polystyrene surfaces in the wells of a 24-well microtitre plate and then treated with the enzyme at a final concentration of 50 µg/ml at 25°C for 2 h. After removal of the enzyme solution the biofilm cells were resuspended in sorbitol buffer and optical density readings taken after 0, 15 and 30 min. As with *C. albicans* biofilms, there was no decrease in optical density over the 30-min period (Table 17). Moreover, with these biofilms, the optical density readings for the cell suspensions treated with lyticase were actually higher than those of the control suspensions.

These results suggest that lysis of *C. tropicalis* cells does not occur during the lyticase treatment, even though *C. tropicalis* is known to be sensitive to the enzyme (Kamiryo *et al.*, 1982).

Table 17. Effect of lyticase on *C. tropicalis* AAHB 73 biofilm cells [a, b]

Time after resuspension (min)	OD_{520}	% of control [c]	OD_{600}	% of control [c]
0	1.05 ± 0.01	> 100.0	0.96 ± 0.02	> 100.0
15	1.05 ± 0.01	> 100.0	0.98 ± 0.02	> 100.0
30	1.04 ± 0.01	> 100.0	0.97 ± 0.01	> 100.0

[a] The data are means ± SEM of two independent experiments carried out in triplicate. After lyticase treatment, biofilm cells were resuspended in sorbitol buffer and the optical density of each suspension monitored after 0, 15 and 30 min.

[b] The final concentration of lyticase used was 50 μg/ml.

[c] Optical density readings for lyticase-treated biofilm cells expressed as a percentage of those for control (untreated) cells.

DISCUSSION

1 Penetration of *Candida* biofilms by antifungal agents

The mechanisms that protect microorganisms in biofilms from antibiotics, biocides and antibodies are still being elucidated. Currently, four mechanisms are under study: (i) slow penetration of the antimicrobial agent into the biofilm, (ii) an altered chemical microenvironment within the biofilm leading to zones of slow or no growth, (iii) adaptive responses to environmental stress, and (iv) the existence of persister cells that are protected from all types of antimicrobial insult (Stewart, 2003). Almost all of this work is being done with bacterial biofilms. At present, no single mechanism seems to account for the exceptional resistance of biofilm cells to a wide variety of antimicrobial agents. Instead, it is likely that two or more mechanisms operate together. Mathematical modelling suggests that multiple mechanisms of biofilm reduced susceptibility are manifested even in biofilms of the same species and that the particular resistance mechanism depends on the biofilm age, antimicrobial agent, and biofilm thickness (Dodds *et al.*, 2000).

All four resistance mechanisms mentioned above appear to depend critically on the multicellular nature of biofilms. For example, when an antimicrobial agent fails to penetrate a biofilm, it is because the drug is reactively neutralized as it diffuses into a cell cluster. This process may involve enzymatic degradation of the drug or drug binding to charged extracellular polymers, but it is effective only when microorganisms are aggregated and exert their collective neutralizing activity (Stewart, 2003). Imipenem induces high levels of β-lactamase production in *Pseudomonas aeruginosa* biofilms. Treatment with piperacillin also induces β-lactamase production in these biofilms but to a lesser

degree. The combination of β-lactamase production with other protective properties of the biofilm mode of growth could be a major reason for the persistence of this sessile bacterium in chronic infections (Giwercman *et al.*, 1991).

In this study, the penetration of antifungal agents through single- and mixed-species biofilms formed by fungal pathogens in the genus *Candida* was investigated. The results demonstrated that fluconazole permeated all single-species *Candida* biofilms more rapidly than flucytosine. The rates of diffusion of either drug through biofilms of three strains of *C. albicans* were similar. On the other hand, the rates of drug diffusion through biofilms of *C. glabrata* and *C. krusei* were faster than those through biofilms of *C. parapsilosis* and *C. tropicalis*. In all cases, the drug concentration reached at the distal edge of the biofilm was very high. However, drug penetration failed to produce complete killing of biofilm cells even when the incubation period was extended from 6 to 24 h. These results indicate that poor drug penetration is not a major resistance mechanism for *Candida* biofilms.

This conclusion is in agreement with previous studies on *P. aerguinosa* and *K. pneumoniae* biofilms by Stewart *et al.* (2001) who also found poor biofilm killing despite effective physical penetration of antimicrobial agents into the biofilm (Stewart *et al.*, 2001). Similarly, Zheng and Stewart (2002) showed that rifampin penetrated uniformly through biofilms formed by *Staphylococcus epidermidis* but failed to effectively kill the bacteria (Zheng and Stewart, 2002). All of these results indicate that biofilms are protected by mechanisms other than simple physical shielding by the matrix material. The results are also consistent

with earlier studies by Darouiche *et al.* (1994) who concluded that the failure of glycopeptide antibiotics to eradicate prosthesis-related infection was not due to poor penetration of drugs into the biofilm but might be due to a diminished antimicrobial effect on bacteria in the biofilm environment.

The biofilms showing the lowest levels of drug penetration, particularly with flucytosine, were those formed by *C. parapsilosis* and *C. tropicalis*. The strain of *C. tropicalis* used in these experiments, a clinical isolate, appeared to produce large amounts of matrix material, some of which could be seen when biofilm preparations were viewed under a scanning electron microscope (Fig. 60). Although the chemical composition of the biofilm matrix of *C. albicans* has been investigated (Baillie and Douglas, 2000), at the outset of this project no comparable studies had been done with *C. tropicalis*. The matrix of *C. albicans* biofilms contains mainly carbohydrate and protein, with a relatively high proportion (16%) of glucose (Baillie and Douglas, 2000). Recent work with *P. aeruginosa* has led to the identification of periplasmic glucans in biofilm cells which appear to sequester antibiotics and slow their diffusion, perhaps preventing them from reaching their sites of action in the cytoplasm (Mah *et al.*, 2003). It has also been postulated that the nature and amounts of extracellular glucans produced by oral streptococci from sucrose in dental plaque are major determinants retarding acid diffusion (Hojo *et al.*, 1976). Unexpectedly, the matrix material of *C. tropicalis* biofilms has hexosamine, not glucose, as its major component (Table 13). These observations suggest that the nature and amount of matrix material could play a minor role in the drug resistance of *C. tropicalis* biofilms by slowing the diffusion of antifungal agents.

Recently, Jefferson and colleagues (2005) analyzed the diffusion of the glycopeptide antibiotic vancomycin into *S. aureus* biofilms using confocal scanning laser microscopy and a fluorescently labelled vancomycin derivative BodipyFL-vancomycin. They found that binding of vancomycin to bacterial cells within the deepest layers of biofilm was not detected for nearly 60 min, whereas the antibiotic bound to planktonic cells within 5 min. These results indicate that the antibiotic diffuses into the depths of the biofilm but that the rate is significantly reduced as compared with diffusion through planktonic cell suspensions. This gradual exposure of microorganisms in the deeper layers of the biofilm might therefore allow them to undergo stress-induced metabolic or transcriptional changes that increase resistance to the antimicrobial agents (Jefferson *et al.*, 2005).

All four antifungal agents tested diffused very slowly through mixed-species biofilms containing *C. albicans* and either the wild-type or M7 mutant strain of *S. epidermidis*. In most cases, diffusion was slower with biofilms containing *S. epidermidis* RP62A, the wild-type, slime-producing strain. Curiously, however, amphotericin B penetrated biofilms containing the M7 mutant more slowly. In all of these experiments with mixed fungal-bacterial biofilms, the drug concentrations reaching the distal edges of the biofilms substantially exceeded the MIC for *C. albicans*. Thus, although the presence of bacteria and bacterial matrix material undoubtedly retarded the diffusion of the antifungal agents, poor penetration does not account for the drug resistance of *Candida* biofilm cells, even in these mixed species biofilms.

The chemical composition of the extracellular matrix of *S. epidermidis* biofilms is not fully established. It appears to contain a polymer of β-1,6-linked *N*-acetylglucosamine residues with some deacetylated amino groups, as well as succinate and phosphate substituents (the intercellular polysaccharide adhesin [PIA]) (Mack *et al.*, 1996). However, early work by Hussain *et al.*(1993) found that the slime is a mixture of about 80% (w/w) teichoic acid and 20% protein. A 140-kDa accumulation-associated protein has also been identified (Hussain *et al.*, 1997). The M7 mutant, which fails to accumulate on glass surfaces (Schumacherperdreau *et al.*, 1994) but which does form biofilms on polyvinyl chloride catheter disks (Adam *et al.*, 2002), has been reported to lack this protein but nevertheless synthesizes PIA (Hussain *et al.*, 1997). Interactions between these polymers and those produced by *C. albicans* in mixed-species biofilms might result in a more viscous matrix. Rheological interactions between matrix polysaccharides from *Pseudomonas cepacia* and *P. aeruginosa* have been shown to decrease the rates of diffusion and antimicrobial activities of antibiotics (Allison and Matthews, 1992). On the other hand, a recent study of oral biofilms containing six microbial species, including *C. albicans*, suggested that retarded diffusion of fluorescent probes through the biofilm was due to tortuosity, i.e., the convoluted paths traversed by macromolecules during biofilm penetration (Thurnheer *et al.*, 2003).

Biofilm cells appear to grow slowly because of the limited availability of nutrients, especially at the base of the biofilm. Growth rate has therefore been considered as an important modulator of drug activity in biofilms (Gilbert *et al.*, 2002; Douglas, 2003). A perfused fermentor 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 same rates in a chemostat. The results indicated that biofilms were resistant to the drug at all growth rates tested, whereas planktonic cells were resistant only at low growth rates (Baillie and Douglas, 1998a). An alternative mechanism of drug resistance might be upregulation of genes coding for multidrug efflux pumps in biofilm cells. *C. albicans* possesses two different types of efflux pump: ATP-binding cassette transporters and major facilitators, which are encoded by *CDR* and *MDR* genes, respectively. Recent work has shown that genes encoding both types of pump are indeed 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 they were growing planktonically but retained the resistant phenotype during biofilm growth (Ramage *et al.*, 2002b) . Overall, it seems probable that drug resistance in *Candida* biofilms, like that in bacterial biofilms, is a complex process involving more than one mechanism.

2 Drug susceptibility of *Candida* biofilms grown statically and under conditions of continuous flow

It is known that the architecture of biofilms can depend on the flow conditions that prevail in the liquid in which they are grown. The present study set out to compare the drug susceptibility of *Candida* biofilms grown in two different models: one was under continuous flow and the other static. The static biofilm model was based on a method described by Prosser *et al.* (1987), previously used for *Escherichia coli* and other gram-negative bacteria. This

involved growing adherent populations of biofilms on the surfaces of small disks cut from polyvinylchloride (PVC) catheter material in static conditions. Biofilm activity was assessed quantitatively by a colorimetric tetrazolium salt reduction assay (XTT) and visualised using scanning electron microscopy (SEM). This simple static model is also inexpensive, rapid, and facilitates accurate testing of the *in vitro* susceptibility of *Candida* biofilms to antifungal agents.

To allow comparison with statically cultured *Candida* biofilms, biofilms were grown under conditions of continuous flow using a modified Robbins device (MRD). Over the years, several systems have been developed *in vitro* to study the biofilm mode of growth under flow conditions but the MRD remains one of the most widely used and well-known. It has been used successfully by many researchers for the study of bacterial adhesion and biofilm formation (Linton *et al.*, 1999; Hoiby *et al.*, 2001). For this reason, their methods were adapted to study the growth of *C. albicans* biofilms under flow conditions. Biofilm formation on PVC catheter sampling studs within the chamber of the MRD was monitored at different time intervals. The MRD is recognised as a good model which mimics intravenous delivery of planktonic microorganisms that eventually adhere to and colonise available plastic surfaces such as catheters within patients. One of the drawbacks of this system is that it is difficult to sterilize before use. It is recommended that the MRD is sterilized using ethylene oxide (Jass and Lappin-Scott, 1992; Kharazmi *et al.*, 1999) although in the present study sterilization was achieved using 0.05 % hibitane. There is also an increased risk of contamination, especially when removing sampling studs. In addition, the MRD requires the use of much larger quantities of material such as

standardized cell suspension and growth medium, and takes longer to set up than static models. However, on the plus side, the MRD has the advantage of allowing a large number of samples to be assessed at any one time by arranging several MRDs in parallel. It has been recommended that this *in vitro* model should be considered for comparative evaluation of biomaterial candidates for medical device use in the urinary tract (Tunney *et al.*, 1997).

2.1 Drug susceptibility of *C. albicans* biofilms grown under static and flow conditions

Biofilms of *C. albicans* grown under static conditions on small PVC disks (surface area, 0.5 cm^2) were exposed to different concentrations of amphotericin B at 37°C for 5 or 24 h. After incubation, the metabolic activity of the sessile cells was assessed by the tetrazolium reduction assay. The effect of the antifungal agent was calculated in terms of the percentage inhibition of formazan formation by biofilms compared with values obtained for control biofilms incubated in the absence of the drug (Table 7). The results showed that statically grown biofilms treated with amphotericin B at a concentration of 6.5 µg/ml (5 times the MIC) or 39 µg/ml (30 times the MIC) showed partial resistance to the drug. The first investigation of biofilm formation by *Candida* species developed on the surface of catheter material used the same system (Hawser and Douglas, 1994b), and the authors went on to show that the biofilms formed were resistant to the action of five clinically important antifungal agents (Hawser and Douglas, 1995b). These early studies were therefore the first to indicate that, as is the case with bacterial biofilms, the *Candida* biofilm phenotype confers resistance to antifungal drug therapy and they have subsequently been confirmed by other

workers (Chandra *et al.*, 2001b). They were also consistent with the clinical observation that current antifungal therapy has poor activity against *Candida* species embedded in a biofilm matrix (Lewis *et al.*, 1998).

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 7). 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.*, 1998b). Biofilms grown with or without shaking did not exhibit significant differences in susceptibility to flucytosine, fluconazole or amphotericin B (Baillie and 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, 2000). For example, the total number of cells producing matrix material is higher in *Pseudomonas fluorescens* biofilms growing under laminar flow than in biofilms formed under turbulent flow (Pereira *et al.*, 2002). The results presented here for *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.

2.2 Drug susceptibility of *C. tropicalis* biofilms grown under static or flow conditions

Since the late 1970s, several researchers have highlighted the potential of *C. tropicalis* to cause severe invasive disease (Wingard *et al.*, 1979; Flynn *et al.*, 1993). *C. tropicalis* has an apparently higher capacity than *C. albicans* to invade the deep tissues of the immunocompromised host; many reports have shown positive surveillance cultures for *C. tropicalis* to be highly predictive of subsequent systemic infection (Komshian *et al.*, 1989).

In the present, study attempts for the first time 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 8). Rates of drug diffusion through statically grown *Candida* biofilms were determined using a filter disk assay (Section 1). Of several *Candida* species and strains tested, the slowest penetration was observed with *C. tropicalis* AAHB 73. In view of the analytical data on matrix preparations (Table 13), 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 (Table 12). 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).

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

It is now known that the complex architecture of most biofilms is intrinsically stratified into spatially organized populations of mixed species communities with a degree of interspecies interaction (El-Azizi *et al.*, 2004). A number of investigators have studied the phenomenon of coaggregation and coadhesion between *Candida* and different bacteria and the effect of modulating factors including saliva, sugars, and pH (Grimaudo *et al.*, 1996; O'Sullivan *et al.*, 2000). An earlier study by Holmes *et al.* (1995) demonstrated that adhesion of *C. albicans* to *Streptococcus gordonii* immobilized on microtitre plates was increased when either the yeasts were suspended in saliva, or when the bacteria were pretreated with saliva.

Various clinical studies have revealed that fungal oropharynx infections in combination with adhering bacteria are very common in bone marrow transplant patients (Hsu *et al.*, 1990) as well as in patients with autoimmune

deficiency disease (Torssander *et al.*, 1987; Coleman *et al.*, 1993). Silicon rubber voice prostheses in laryngectomized patients have to be frequently replaced because of the adhesion of *Candida* together with oral and skin commensal organisms on the surface of the device (Mahieu *et al.*, 1986). Baena-Monroy and colleagues (2005) identified a significant association between *C. albicans* and *Staphylococcus aureus* in patients suffering from atrophic denture stomatitis. These co-species communities, probably existing as mixed fungal/bacterial biofilms, are difficult to treat with both antibiotics and antifungals (Baena-Monroy *et al.*, 2005). Moreover, many reports on implant-related infections have shown that the frequent incidence of mixed-species biofilms on the surface of catheters can enhance resistance against antifungal agents (Crump and Collignon, 2000; Ramage *et al.*, 2005).

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 strain 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 (Schumacherperdreau *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).

In the present study, 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 9). 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 2). 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 and Giamarellou, 1998). Similar results were obtained when staphylococcal 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 and 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 10). They were, however, slightly less resistant than biofilms containing the slime-producing *S. epidermidis* RP62A treated in the same way

(Table 9). 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 10). 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.

2.4 General conclusions on drug susceptibility of biofilms

Overall, these 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. A possible explanation for this might be the synthesis of a hexosamine-containing matrix polymer similar to *S. epidermidis* PIA. Drug diffusion through *C. albicans* / *S. epidermidis* biofilms (Fig. 37, 43, 49 and 55) grown statically on cellulose filters is slower than that through *C. albicans* biofilms or even *C. tropicalis* biofilms alone, as described in earlier sections of this study. 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 and Matthews, 1992). Undoubtedly, matrix polymers do contribute towards drug resistance in both single-species and mixed-species biofilms containing *Candida,* especially under the flow conditions that 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).

2.5 Scanning electron microscopy (SEM) of biofilms grown statically and under conditions of continuous flow

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.*, 1998b), 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. 65). 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.*, 1998b). In contrast with *C. albicans*, biofilms of *C. tropicalis* synthesized large amounts of extracellular material even during growth under static conditions, and many of the cells were almost hidden by the enveloping matrix (Fig. 66). At high magnification, matrix material was clearly visible on the surface of the cells (Fig. 67).

3 Isolation and analysis of matrix material from *Candida* biofilms

3.1 Isolation of matrix material

To study the chemical composition of the biofilm matrix an extraction procedure which can separate cells from the matrix material without cell destruction is desirable. Although several extraction methods have been developed, there is no standard method universally accepted by researchers and a variety of physical and chemical extractions have been reported (Azeredo *et al.*, 1999; Liu and Fang, 2002; Azeredo *et al.*, 2003). Many of these methods promote leakage of intracellular material (Azeredo *et al.*, 1999). In this investigation, matrix material was prepared from biofilms of two different *Candida* species, *C. albicans* GDH 2346 and *C. tropicalis* AAHB 73. A physical extraction process was used which involved adapting the method described by Baillie and Douglas (2000) in an attempt to minimize leakage. This consisted of 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* was much higher than that from biofilms of *C. albicans* (Table 11).

3.2 Chemical composition of the biofilm matrix

For years, carbohydrate was considered to be the predominant constituent in extracellular material produced by many pure cultures (Sutherland and Kennedy, 1996; Sutherland, 1997; Cescutti *et al.*, 1999). Generally, matrix material secreted by bacteria during growth on a surface is mainly composed of

carbohydrate and protein, plus small quantities of uronic acid and deoxyribonucleic acid (Ross *et al.*, 1991; Marty *et al.*, 1992; Wingender *et al.*, 2001; Liu and Fang, 2002; Tsuneda *et al.*, 2003). In this study, matrix isolated from *C. albicans* biofilms consisted of carbohydrate, together with small amounts of protein, hexosamine, phosphorus and uronic acid (Table 12). 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 and 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, with smaller amounts of carbohydrate including glucose, protein and phosphorus (Table 13). It is also noteworthy that the *C. tropicalis* matrix contained slightly more uronic acid 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 13). 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*. The extracellular antigenic markers of this organism were isolated and studied independently by different research groups

(Tojo *et al.*, 1988; Christensen *et al.*, 1990). Tojo *et al.* (1988) first isolated an extracellular capsular polysaccharide adhesin (PS/A) from culture supernatant of *S. epidermidis* strain RP62A. PS/A has been reported (McKenney *et al.*, 1998) to be the component of the bacterial cell surface and biofilms that mediates cell adherence to biomaterials and protects the bacterial cells from host defences. In subsequent studies, Christensen and co-workers (1990) described a slime-associated antigen (SAA) isolated from the same strain that had a similar function. However, after investigation of the chemical composition of SAA, Baldassarri *et al.*, (1996) suggested that SAA and a hexosamine-containing polysaccharide intercellular adhesin (PIA) of *S. epidermidis* strain RP62A, described by Mack *et al.* (1994), could be the same antigenic molecule. Later, Mack *et al.* (1996) elucidated the chemical structure of PIA and identified it as a polysaccharide of β-1,6-linked *N*-acetylglucosamine residues containing some deacetylated amino groups, and succinate and phosphate substituents. Recently, Sadovskaya and colleagues (2005) found another carbohydrate-containing polymer, extracellular teichoic acid (EC TA), to be an essential component of *S. epidermidis* RP62A biofilms. They also studied the relative amounts of extracellular PIA and EC TA produced by the same strain. Production depended on the growth conditions; static culture or moderate shaking in TSB medium favoured PIA synthesis, while more EC TA was produced in brain heart infusion medium (Sadovskaya *et al.*, 2005). PIA mediates cell–cell interactions within the biofilm (Gotz, 2002) and its production has been related to *S. epidermidis* virulence in catheter-infection models in animals.

P. aeruginosa has become a model organism in biofilm research and biofilms of this organism have been intensively studied during the last decade. *P. aeruginosa* is well characterized with respect to its molecular genetics, biochemistry, and physiology (Costerton *et al.*, 1999). During chronic colonization in cystic fibrosis patients, *P. aeruginosa* undergoes conversion from a nonmucoid to a mucoid phenotype (Hentzer *et al.*, 2001). The most characteristic feature of the mucoid phenotype is the secretion of large amounts of highly viscous exopolysaccharides. Alginate from *P. aeruginosa* is a high molecular-weight O-acetylated α-1,4-linked random polymer of mannuronic and guluronic acids similar in structure to seaweed alginate (Evans and Linker, 1973; Marty *et al.*, 1992; Grobe *et al.*, 1995; Rehm and Valla, 1997). In addition to alginate, nucleic acid represents approximately 20 % of the total weight of the biofilm matrix, and is composed of both DNA and RNA. Proteins and several sugars are also present including glucose, mannose, fucose, galactose, ribose and rhamnose, together with the hexosamines (glucosamine) and galactosamine (O'Toole and Kolter, 1998; Costerton *et al.*, 1999; Hentzer *et al.*, 2001). Recently, it was noted that a nonmucoid wild type, as well as alginate-negative mutants of *P. aeruginosa* are also able to form biofilms. These nonmucoid biofilms showed a different architecture from that of biofilms formed by alginate-overproducing, mucoid *P. aeruginosa* (Nivens *et al.*, 2001).

Another study demonstrated that the matrix material isolated from *Helicobacter pylori* biofilms consisted of fucose, glucose, galactose, and glycero-mannoheptose, *N*-acetylglucosamine and *N*-acetylmuramic acid and the author

concluded that these sugars were by far the major components of the biofilm matrix material (Stark *et al.*, 1999).

4 Enzymatic detachment of *Candida* biofilms

Biofilm accumulation is determined by the balance of attachment, growth, and detachment processes. Of these phenomena, the least understood is detachment. Detachment refers to the release of microbial cells and their associated matrix polymers from the biofilm to the bulk fluid of growth media. Moreover, detachment is of fundamental importance to the dissemination of infection and to contamination in both clinical (Nickel *et al.*, 1994) and public health settings (Walker *et al.*, 1995). However, detachment is the least-studied biofilm process and remains poorly understood (Stewart, 1993).

Some factors such as heat, detergents, sonication, and agitation have been suggested to be important in biofilm detachment *in vitro*. Other detachment promoters include matrix-degrading enzymes (Allison *et al.*, 1998; Vats and Lee, 2000; Kaplan *et al.*, 2003b), microbially generated gas bubbles (Ohashi and Harada, 1994), nutrient levels and microbial growth status (Applegate and Bryers, 1991; Stewart, 1993; Sawyer and Hermanowicz, 1998; Jackson *et al.*, 2002; Hunt *et al.*, 2004), availability of multivalent cross-linking cations (Turakhia *et al.*, 1983; Applegate and Bryers, 1991), fluid shear stress (Picioreanu *et al.*, 2001; Stoodley *et al.*, 2001), contact attrition (Chang *et al.*, 1991), quorum-sensing signals (Hentzer *et al.*, 2002), and the activation of a lytic bacteriophage (Webb *et al.*, 2003). Stewart *et al.* (2000) described several strategies to eradicate unwanted biofilms that may be applied to a particular system, depending on its characteristics. These include (i) mechanical cleaning,

(ii) the use of antimicrobial agents, (iii) stopping biofilm growth by removing essential nutrients, (iv) inhibiting microbial attachment to a surface and (v) promoting biomass detachment which is the least investigated of the possible strategies to remove unwanted biofilms. Several enzymes have been observed to induce detachment by destroying the physical integrity of the biofilm matrix. This would be an attractive procedure for both medical and industrial applications where complete biofilm removal is essential. It might involve acting on the matrix by depolymerizing either polysaccharides (Allison *et al.*, 1998; Chen and Stewart, 2000; Itoh *et al.*, 2005) or extracellular DNA (Whitchurch *et al.*, 2002). A number of detachment studies have been performed with bacterial biofilms (Boyd and Chakrabarty, 1994; Stoodley *et al.*, 2001; Hunt *et al.*, 2004; Kaplan *et al.*, 2004; Marion *et al.*, 2006). Currently, there are no reports of detachment studies with *Candida* biofilms.

The aim of the work presented here was to investigate further the biofilm matrix by determining whether *Candida* biofilms could be enzymically detached from plastic surfaces by degradation of the matrix polymers. An assay devised by Kaplan and co-workers (2004) for *S. epidermidis* biofilms was used together with a collection of commercially available enzymes of known specificity. Both *C. albicans* and *C. tropicalis* biofilms were examined.

The results clearly showed that biofilms of *C. albicans* were unaffected by lipase type VII, phospholipase A2 and protease type XIV. However, treatment with proteinase K, chitinase, DNase I and β-*N*-acetylglucoaminidase resulted in a significant decrease in optical density suggesting that these enzymes partially degraded matrix material and caused some biofilm detachment from the surfaces

of the wells. It is known that the yeast lytic activity can be attributed to a β-1,3-glucanase, although lytic β-1,6-glucanases have been characterized (Rombouts and Phaff, 1976). Interestingly, the lyticase enzyme, which hydrolyses β-1,3 glucan, had by far the greatest effect, causing an 85% reduction in optical density (Table 14). 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 (Table 15). Phospholipase A2, protease type XIV, proteinase K, DNase I and β-*N*-acetylglucosaminidase all had no significant effect. By contrast, treatment with lipase type VII and chitinase did appear to produce some biofilm detachment. Chitinase had a similar effect with biofilms of both *C. tropicalis* and *C. albicans*, indicating that most of the hexosamine present in the *C. tropicalis* matrix was unlikely to be 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 (Mack *et al.*, 1996; Cramton *et al.*, 1999; Stark *et al.*, 1999; Kaplan *et al.*, 2003b).

Related studies by Kaplan *et al.* (2003b) have identified a family 20 glycosyl hydrolase produced by *Actinobacillus actinomycetemcomitans* that causes the detachment of cells from *A. actinomycetemcomitans* biofilms grown and attached to plastic surfaces. This enzyme, called dispersin B, is an *N*-acetylglucosaminidase, which degrades an *N*-acetylglucosamine-containing extracellular polysaccharide that mediates *A. actinomycetemcomitans*

intercellular adhesion. The same enzyme also rapidly and efficiently removed *S. epidermidis* biofilms from plastic surfaces (Kaplan *et al.*, 2003a).

The greatest effect on *C. tropicalis* biofilms was again observed with lyticase, which caused a reduction in optical density of over 53% (Table 15). However, lyticase had less effect on these biofilms than on those of *C. albicans* whose matrix material contains substantially more glucose (Table 14).

Further experiments were carried out to investigate possible lysis of *Candida* biofilm cells during their exposure to lyticase 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. 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 and Meyer, 1991).

It is generally assumed that nucleic acids are localized inside living cells and that their primary function is the storage of information; extracellular DNA (eDNA) is mainly considered to be a remnant of lysed cells. Early studies suggested that *P. aeruginosa* excretes large amounts of eDNA through a mechanism independent of cellular lysis and that appeared to involve the release of small vesicles from the outer membrane (Hara and Ueda, 1981; Muto and Goto, 1986). *P. aeruginosa* produces a large amount of eDNA during alginate

biosynthesis, and this is clearly essential for the initial stages of biofilm formation (Steinberger *et al.*, 2002; Whitchurch *et al.*, 2002). A recent publication by Allesen-Holm *et al.* (2006) demonstrated that eDNA in *P. aeruginosa* biofilms and cultures is generated via lysis of a subpopulation of the bacteria. The authors also found a link between DNA release and quorum sensing in planktonic cultures. Their results indicated that the eDNA is generated via a mechanism which is dependent on acyl homoserine lactone and *Pseudomonas* quinolone signalling, as well as on flagella and type IV pili. Microscopic investigation of flow chamber-grown biofilms revealed that the eDNA was located mainly in the stalks of mushroom-shaped multicellular structures with a high concentration particularly in the outer part of the stalks forming a border between the stalk-forming bacteria and the cap-forming bacteria (Allesen-Holm *et al.*, 2006).

Whitchurch and his colleagues (2002) previously showed that eDNA is essential for biofilm stability during the early stages of biofilm growth. On the other hand, Molin and Tolker-Nielsen (2003) noted that the eDNA may enhance gene transfer between biofilm constituent organisms; it may also provide nutrition during oligotrophic conditions (Finkel and Kolter, 2001). Petersen *et al.* (2005) studied the importance of eDNA in the growth of biofilms of *Streptococcus mutans*. The authors found that the addition of synthetic competence signalling peptide (sCSP) to biofilms caused an increase in the growth of biofilms (biomass); however, the addition of DNase I enzyme reversed the effect of the addition of sCSP, showing the importance of DNA for increased biomass (Petersen *et al.*, 2005). As a result of all these investigations, it has been

concluded that DNA is a major structural component of the extracellular matrix of bacterial biofilms (Starkey *et al.*, 2004; Spoering and Gilmore, 2006).

The results of the present study indicate that 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 12). 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 A_2. In this context, it is interesting to note that *C. tropicalis* is capable of producing a fibrillar layer containing mannoprotein with covalently-linked fatty acids (Kappeli and Fiechter, 1977; Kappeli *et al.*, 1984).

Other studies have shown that not only is DNA continuously present in the matrix material of *P. aeruginosa* biofilms but in fact is the most abundant constituent polymer (Steinberger and Holden, 2004). Interestingly, *P. aeruginosa* biofilm formation was inhibited by addition of DNase I, although DNase I did not inhibit planktonic bacterial growth. This observation implies that DNA provides an important structural component for biofilm formation (Whitchurch *et al.*, 2002). It is possible that, eDNA occurring *in vivo* might derive from neutrophils. Analysis of cystic fibrosis (CF) sputum demonstrated high concentrations of granule proteins, actin, and DNA released from necrotic neutrophils (Goldstein and Doring, 1986; Sheils *et al.*, 1996). Neutrophil actin and DNA have been observed to bind together, forming polymers that increase the viscosity of the sputum (Lethem *et al.*, 1990). A recent study showed that the presence of neutrophils enhances initial *P. aeruginosa* biofilm formation through

the formation of polymers comprised of actin and DNA. The authors reported that the addition of DNase I abolished much of the neutrophil-induced enhancement of biofilm formation without significantly inhibiting bacterial growth or neutrophil survival (Walker *et al.*, 2005). Several researchers (Whitchurch *et al.*, 2002; Gibson *et al.*, 2003) have suggested that DNase I treatment in combination with antibiotics might be beneficial as a early prophylatic measure to prevent the establishment of infection in CF patients by inhibiting biofilm formation.

5 Concluding remarks

This study has shown that a variety of antifungal agents are able to penetrate biofilms of *C. albicans* and other *Candida* species at different rates and reach high concentrations (many times the MIC) at the distal edge of the biofilm. However, durg penetration fails to produce complete killing of biofilm cells suggesting that poor antifungal penetration is not a major drug resistance mechanism for *Candida* biofilms formed under static conditions. On the other hand, the drug resistance of *C. albicans* biofilms can be significantly enhanced by increased production of matrix material under flow conditions in the MRD, and by the presence of one or more matrix polymers of *S. epidermidis* in mixed species biofilms. Moreover, biofilms of *C. tropicalis* whose matrix material contains hexosamine as the major sugar substituent are less susceptible to antifungal agents than *C. albicans* biofilms where hexosamine is replaced by glucose. Overall, the 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. However, the mechanism of biofilm drug resistance is likely to be multifactorial involving, in addition, drug-resistance physiologies such as dormant 'quiescent' cells and expression of efflux pumps.

To extend this study further, it would be interesting to investigate the matrix composition of some of the other non-*C. albicans Candida* species. It could be predicted, for example, that *C. parapsilosis* matrix, like that of *C. tropicalis*, contains mainly hexosamine since biofilms of both these species show poor drug penetration. On the other hand, biofilms of *C. krusei* and *C. glabrata* which, like those of *C. albicans* strains, are penetrated fairly rapidly by antifungal drugs might be expected to contain glucose as a major matrix component.

Investigation by viscometry of possible rheological interactions between matrix polymers produced by *C. albicans* and *S. epidermidis* might also prove informative. Such interactions could produce a more viscous matrix thereby further inhibiting drug penetration. Rheological interactions between matrix polysaccharides from *Pseudomonas* (now *Burkholderia*) *cepacia* and *P. aeruginosa* have previously been shown to decrease the rates of diffusion and antimicrobial activities of antibiotics (Allison and Matthews, 1992). Similar studies with *C. albicans* and *S. epidermidis* matrix polymers should provide further important information on the role of the matrix in biofilm drug resistance.

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APPENDICES

Appendix 1. Medium

1.1 Yeast Nitrogen Base (YNB)

Formulation as given by Difco

Chemical	Weight/litre
Ammonium sulfate	5 g
Monopotassium phosphate	1 g
Magnesium sulfate	0.5 g
Sodium chloride	0.1 g
Calcium chloride	0.1 g
L-Histidine monohydrochloride	10 mg
LD-Methionine	20 mg
LD-Tryptophan	20 mg
Biotin	2 µg
Inositol	2000µg
Boric acid	500µg
Niacin (Nicotinic acid)	400µg
Manganese sulfate	400µg
Pyridoxine HCl	400 µg
Zinc sulfate	400µg
Thiamine HCl	400µg
Calcium pantothenate (D-Pantothenic acid)	400µg
Ferric chloride	200µg
Sodium molybdate	200µg
Riboflavin	200µg
p-Aminobenzoic acid	200µg
Potassium iodide	100µg
Copper sulfate	40µg
Folic acid	2µg

Final pH 5.4 ± 0.1 at 25 °C.

1.2 Sabouraud Dextrose Agar (SDA)

Formulation as given by Oxoid

Chemical	Weight (g/l)
Mycological peptone	10.0
Glucose	40.0
Agar	15.0

Autoclaved for 15 min at 15 lbs pressure (121°C). Final pH 5.6 ± 0.2 at 25°C.

1.3 Tryptic Soy Broth (TSB)

Formulation as given by Difco

Chemical	Weight (g/l)
Tryptone, Difco	17.0
Soytone, Difco	3.0
Sodium chloride	5.0
Dipotassium phosphate	2.5
Agar	15.0

Sterilization was by autoclaving for at 121°C for 15 minutes. Final pH 7.3 ± 0.2 at 25 °C.

Appendix 2. Buffers

2.1 0.15 M Phosphate-buffered saline (PBS)

Phosphate buffered saline tablet

Chemical	Quantity
Phosphate buffered saline tablet	1.0
Distilled water	200 ml

Phosphate buffered saline tablets were used. Each tablet added to 200 ml distilled water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride. The solution was autoclaved at 121°C for 15 min. The pH of the subsequent solution was 7.2, at 25 °C.

2.2 0.1 M citric acid – 0.2 M disodium hydrogen phosphate buffer (pH 5 and pH 6)

Chemical	Quantity
Solution (A) 0.1 M citric acid	
$C_6H_8O_7H_2O$	21.01 g
Distilled water	1000 ml
Solution (B) 0.2 M Na_2HPO_4	
Na_2HPO_4	28.4 g
Distilled water	1000 ml

Solutions were made up and mixed to give the appropriate pH.

- To prepare 0.1 M citric acid – 0.2 M disodium hydrogen phosphate buffer (pH 5), 48.50 ml of solution A was mixed with 51.50 ml of solution B.

- To prepare 0.1 M citric acid – 0.2 M disodium hydrogen phosphate buffer (pH 6), 36.85 ml of solution A was mixed with 63.15 ml of solution B.

2.3 0.2 M disodium hydrogen phosphate – sodium dihydrogen phosphate buffer (pH 7)

Chemical	Quantity
Solution (A) 0.2 M disodium hydrogen phosphate	
$Na_2 HPO_4$	35.61 g
Distilled water	1000 ml
Solution (B) 0.2 M Na H_2PO_4	
Na H_2PO_4	27.6 g
Distilled water	1000 ml

Solutions were made up and mixed to give the appropriate pH.

To prepare 0.2 M disodium hydrogen phosphate – sodium dihydrogen phosphate buffer (pH 7), 30.50 ml of solution A was mixed with 19.50 ml of solution B.

2.4 0.2 M Tris maleate - sodium hydroxide buffer (pH 8)

Chemical	Quantity
Solution (A) 0.2 M tris-maleate	
Tris	2.40 g
Maleic acid	23.2 g
Distilled water	1000 ml
Solution (B) 0.2 M Na OH	
Na OH	8.0 g
Distilled water	1000 ml

To obtain (pH 8), 25 ml of solution A was mixed with 34.50 ml of solution B.

Appendix 3. Antimicrobial disinfectant

3.1 0.05 % Chlorhexidine gluconate

Antimicrobial prepared for cleaning and sterilising MRD

Chemical	Quantity
Chlorhexidine gluconate (5 %, concentrated)	1.0 ml
Distilled water	99.0 ml

Appendix 4. Chemical analysis of extracellular polymeric substances (EPS)

4.1 Estimation of total carbohydrate by the phenol-sulphuric acid method (Dubois *et al.* 1956)

Reagents:

(A) Concentrated sulphuric acid

(B) 80% (w/v) phenol

Procedure:

Carbohydrate samples containing 10-70 μg of sugar in 2 ml were used; 0.05 ml of the phenol reagent was added to each solution, followed by the rapid addition of 5.0 ml of concentrated sulphuric acid. Samples were left at room temperature for at least 30 min and the absorbance at 485 nm recorded.

4.2 Estimation of total protein using the Lowry method (Lowry *et al.* 1951)

Reagents:

(A) 2% Na_2SO_3 in 0.1M NaOH

(B) 0.5% $CuSO_4.5H_2O$ in 1% sodium tartrate (separate double strength solutions mixed 1:1 before use)

(C) Folin-Ciocalteau reagent diluted 1:1 with distilled water

(D) 50 volumes of reagent A mixed with 1.0 volume of reagent (B), renewed daily.

Procedure:

Protein samples containing 25 to 100 μg protein in 1.0 ml were used. Solution D (5ml) was added to each tube and the contents mixed and allowed to stand at room temperature for at least 10 min. Solution C (0.5 ml) was added rapidly with immediate mixing. All solutions were allowed to stand for 30 min at room temperature before their absorbance at 750 nm was recorded.

4.3 Estimation of phosphorus (Chen *et al.* 1956)

Reagents required:

1. Digestion mixture: Conc. H_2SO_4 : 60% $HClO_4$ (3:2 ; v / v)
2. Colour reagent:

    1 volume 6 M sulphuric acid

    2 volumes distilled water

    1 volume 2.5 % (w / v) ammonium molybdate

    1 volume 10 % (w / v) ascorbic acid

- Reagent should be prepared fresh, just before use.

3. Standard KH_2PO_4 solution (10 µg P/ ml). Dissolve 87.8 mg KH_2PO_4 and make up to 100 ml with distilled water. Then dilute 5 ml from the concentrated solution KH_2PO_4 and make up to 100 ml with distilled water; the solution contains 10µg phosphorus/ml.

Digestion procedure:

A few carborundum chips were added to duplicate samples of EPS solutions in pyrex tubes. The samples were evaporated to dryness in an oven set at 140°C for 1 h. Digestion mixture (0.1 ml) was added to each tube and the mixture was heated again for 1 h at 140°C. The tubes were allowed to cool for the estimation of phosphorus in the digestion mixture.

Assay procedure:

To the digestion mixture, 3.9 ml of water was added followed by 4.0 ml colour reagent, and samples were mixed by inversion. Tubes were incubated at 37°C for 1.5-2 h, and the absorbance at 820 nm recorded.

4.4 Estimation of uronic acid (Bitter and Muir, 1962)

Reagents:

(A) 0.025 M sodium tetraborate - $10H_2O$ in concentrated sulphuric acid.

(B) 0.125 % carbazole in absolute ethanol.

Procedure:

Solution A (3 ml) was placed in tubes, cooled to 4°C, and 0.5 ml of sample, containing 4-40 µg uronic acid, carefully layered on top. Tubes were closed with ground glass stoppers and shaken gently at first, then vigorously, with constant cooling. Tubes were heated for 10 min in a boiling water bath and then cooled to room temperature. Carbazole reagent (0.1 ml) was added and the tubes were shaken, and then heated in a boiling water bath for a further 15 min. After cooling, the absorbance of each solution at 530 nm was recorded.

4.5 Estimation of hexosamine (Blumenkrantz and Asboehansen, 1976)

Reagents:

Acetylacetone: 3.5 % (v/v) in sodium phosphate-potassium tetraborate solution (98 ml of 1M trisodium phosphate + 2 ml of 0.5M potassium tetraborate).

Ehrlich's reagent: 3.2 g p-dimethylaminobenzaldehyde dissolved in 30 ml of concentrated HCl and diluted with 210 ml propan-2-ol.

Preparation of sample:

Prior to analysis, samples (50µl) were hydrolysed in sealed ampoules with equal an volume of 4 M HCl for 12–14 h at 100°C. After hydrolysis, the hydrochloric acid was neutralized with 0.5 NaOH for and the samples were dried

overnight (over NaOH pellets) at room temperature. Samples were redissolved in distilled water ready for analysis.

Procedure:

Samples containing 20-80 µg hexosamine in 0.8 ml were added to 0.6 ml of acetylacetone reagent in glass-stoppered tubes and the mixture heated for 30 min in a boiling water bath. Ehrlich's reagent (2.0 ml) was added to each tube and the absorbance at 535 nm measured.

4.6 Enzymic determination of glucose (Glucose (GO) assay kit; Sigma)

Reagents:

1. Glucose oxidase / peroxidase reagent:

Each capsule contains 500 units of glucose oxidase (*Aspergillus niger*), 100 purpurogallin units of peroxidase (horseradish) and buffer salts; the contents of the capsule are dissolved in 39.2 ml of deionized water.

2. o-Dianisidine reagent:

Vial contains 5 mg of o-dianisidine dihydrochloride. Reconstitute the vial with 1.0 ml of deionized water. Invert the vial several times to completely dissolve.

3. Assay reagent:

This is prepared by adding 0.8 ml of the o-dianisidine reagent to the glucose oxidase/peroxidase reagent. Invert bottle several times to mix properly.

4. Glucose standard solution:

D-Glucose, 1.0 mg/ml in 0.1 % benzoic acid. Supplied ready to use.

Sample preparation:

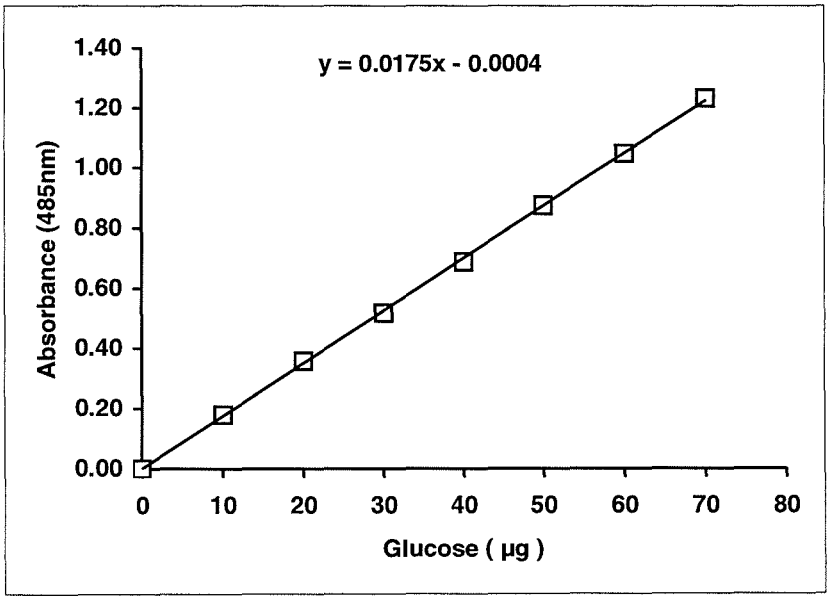
Prior to glucose analysis, EPS samples from *C. albicans* GDH 2346 (0.5 ml of 2 mg/ml solution of EPS) were hydrolysed in 1.0 M HCl (0.5ml) for 5 h in sealed ampoules at 100°C then neutralized with 0.5 M NaOH (1.0ml). For *C. tropicalis* AAHB 73, samples (0.25ml of 5.9 mg/ml solution of EPS) were hydrolysed in 1.0 M HCl (0.25ml) for 5 h in sealed ampoules at 100°C then neutralized with 0.5 M NaOH (0.5ml). Glucose contents of samples were estimated by using glucose (GO) assay kit.

Procedure:

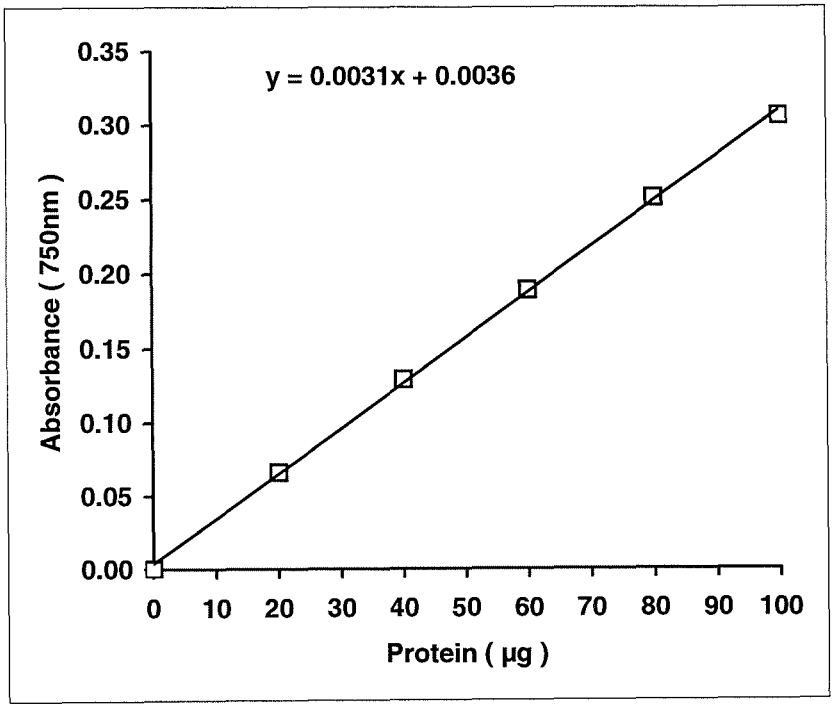
Samples contained 20-80 μg glucose in 1.0 ml of deionized water. At zero time, the reaction was started by adding 2.0 ml of assay reagent to the first tube and mixing. Intervals of 30 seconds were allowed between additions of assay reagent to each subsequent tube. The tubes were incubated for 30 min at 37°C, and then 2.0 ml of 12 M H_2SO_4 was added to each tube to stop the reaction. After careful mixing, the absorbance of each mixture was measured against the reagent blank at 540 nm.

Appendix 5. Standard curves for chemical analysis of EPS

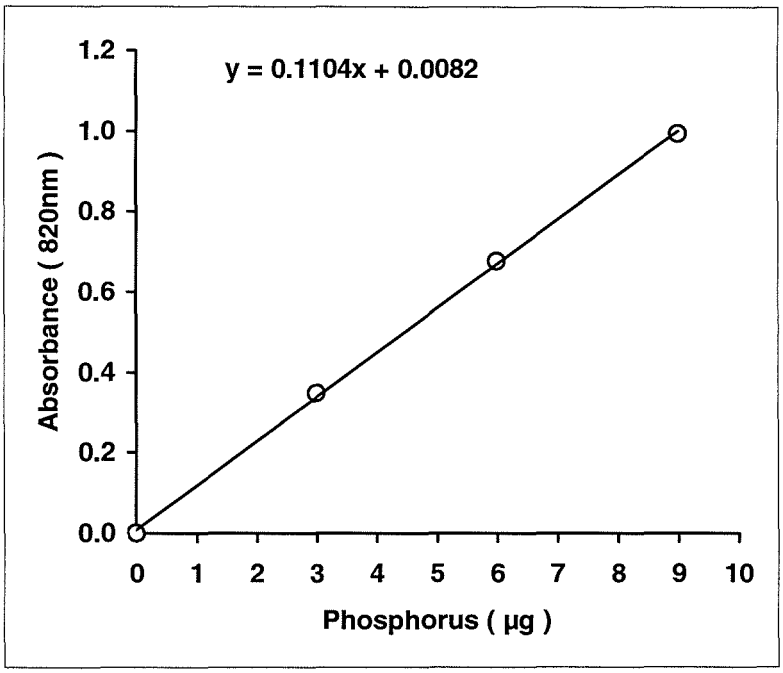
1. Total carbohydrate



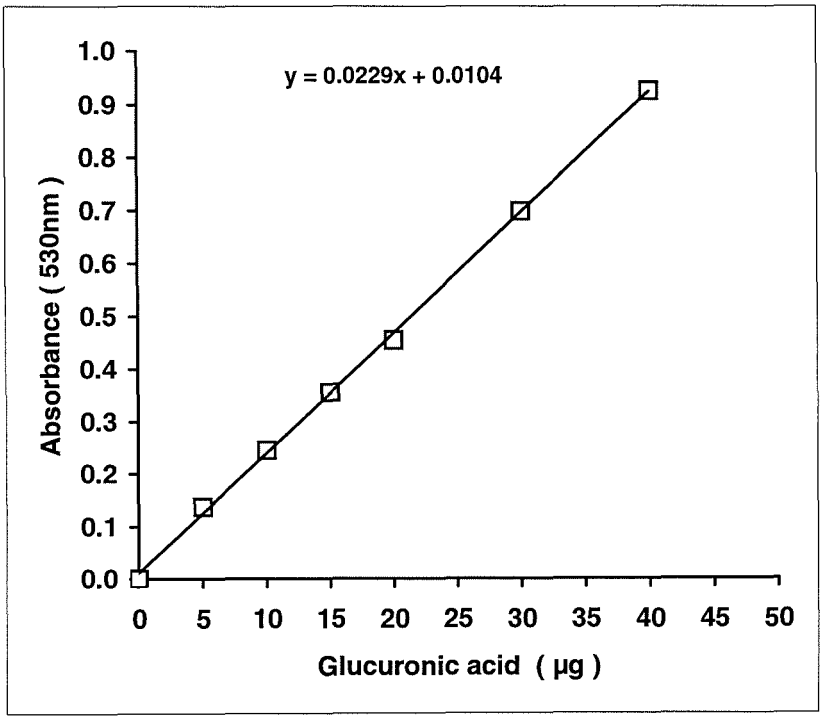
2. Total protein



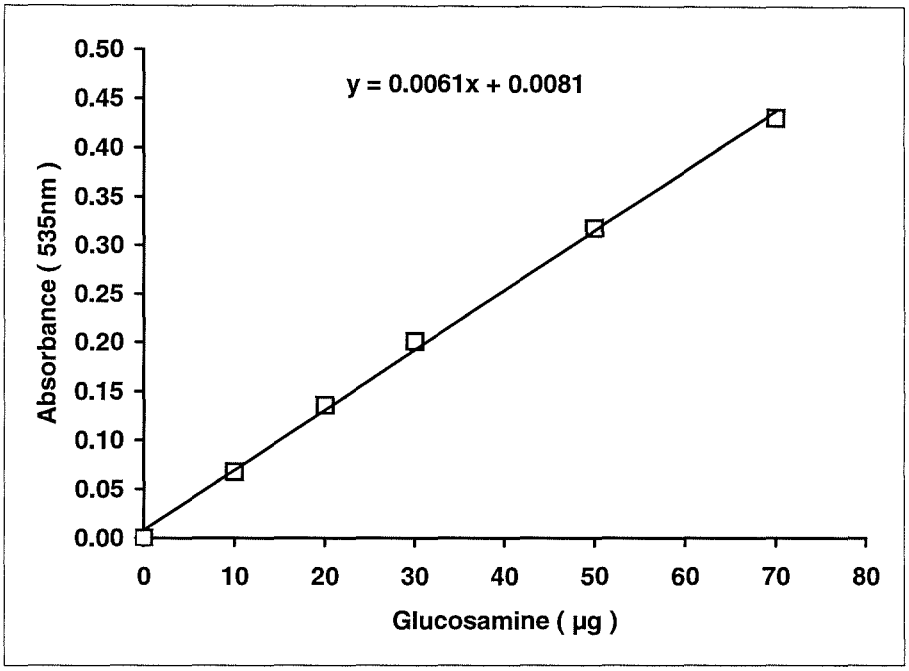
3. Phosphorus



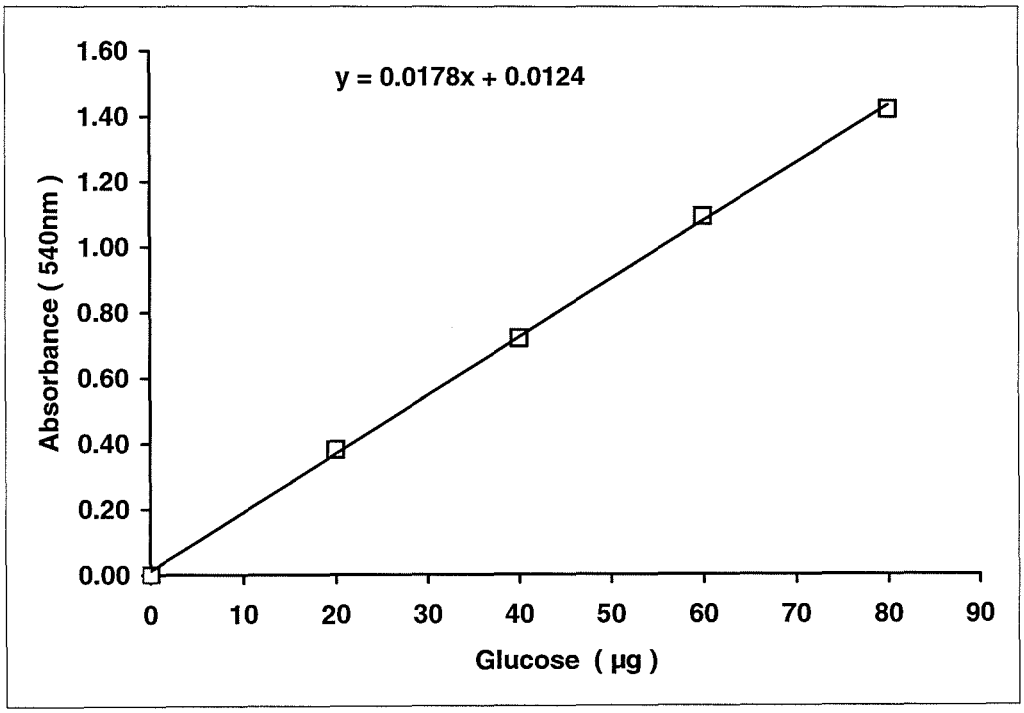
4. Uronic acid



5. Hexosamine



6. Glucose



PUBLICATIONS AND POSTER ABSTRACT

Publications and poster abstract arising from this work

Publications

1. **Al-Fattani, M. A. and Douglas, L. J. (2004).** Penetration of *Candida* biofilms by antifungal agents. *Antimicrobial Agents and Chemotherapy* **48**, 3291-3297.

2. **Al-Fattani, M. A. and Douglas, L. J. (2006).** Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *Journal of Medical Microbiology* **55**, 999-1008.

Poster abstract

THE *CANDIDA* BIOFILM MATRIX: COMPOSITION AND ROLE IN DRUG RESISTANCE *

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One of the most distinctive features of microbial biofilms is that the microorganisms are embedded in an extracellular matrix. Relatively little is known about the composition of the matrix, even in well-studied biofilm systems, although it is generally considered to contain one or more exopolysaccharides as major components. In this study, the chemical composition of matrix material from *Candida albicans* GDH 2346 was compared with that of matrix material from *Candida tropicalis* AAHB 73. A physical extraction process was used which involved sonication, vortexing and centrifugation of biofilms formed on sections of catheter tubing. The yield of matrix material from biofilms of *C. tropicalis* was substantially higher than that from biofilms of *C. albicans*. *C. tropicalis* AAHB 73 was originally isolated from a line infection and had a very slimy appearance on solid medium, suggestive of the production of copious amounts of extracellular polymer. Matrix material isolated from biofilms of both *Candida* species contained carbohydrate, protein, hexosamine, phosphorus and uronic acid. However, in *C. albicans*, glucose was the major sugar component, whereas in *C. tropicalis* this was replaced by hexosamine. Biofilms of *C. albicans* were grown under conditions of continuous flow (60 ml/h) in a modified Robbins device (MRD) which is an *in-vitro* system used for modelling catheter infections. These biofilms produced more matrix material than those grown statically, as determined by scanning electron microscopy, and were significantly more resistant to amphotericin B (at up to 30 times the MIC). Biofilms of *C. tropicalis* could not be grown in the MRD because the copious amounts of extracellular material synthesized by this organism blocked the liquid flow. However, biofilms

of *C. tropicalis* produced large amounts of matrix material even when grown statically, and such biofilms were completely resistant to amphotericin B. Mixed-species biofilms of *C. albicans* and a slimy strain of *Staphylococcus epidermidis* (RP62A), when grown in the MRD, were also completely resistant to amphotericin B. The matrix material of *S. epidermidis*, like that of *C. tropicalis*, contains large amounts of hexosamine present as a polysaccharide of beta 1-6 linked N-acetylglucosamine residues, and production of this polymer has been related to *S. epidermidis* virulence in catheter infection models in animals. Overall, our results demonstrate the importance of matrix production and composition, and its contribution to drug resistance in *Candida* biofilms under conditions similar to those found in catheter infections *in vivo*.

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Penetration of *Candida* Biofilms by Antifungal Agents

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A filter disk assay was used to investigate the penetration of antifungal agents through biofilms containing single and mixed-species biofilms containing *Candida*. Fluconazole permeated all single-species *Candida* biofilms more rapidly than flucytosine. The rates of diffusion of either drug through biofilms of three strains of *Candida albicans* were similar. However, the rates of drug diffusion through biofilms of *C. glabrata* or *C. krusei* were faster than those through biofilms of *C. parapsilosis* or *C. tropicalis*. In all cases, after 3 to 6 h the drug concentration at the distal edge of the biofilm was very high (many times the MIC). Nevertheless, drug penetration failed to produce complete killing of biofilm cells. These results indicate that poor antifungal penetration is not a major drug resistance mechanism for *Candida* biofilms. The abilities of flucytosine, fluconazole, amphotericin B, and voriconazole to penetrate mixed-species biofilms containing *C. albicans* and *Staphylococcus epidermidis* (a slime-producing wild-type strain, RP62A, and a slime-negative mutant, M7) were also investigated. All four antifungal agents diffused very slowly through these mixed-species biofilms. In most cases, diffusion was slower with biofilms containing *S. epidermidis* RP62A, but amphotericin B penetrated biofilms containing the M7 mutant more slowly. However, the drug concentrations reaching the distal edges of the biofilms always substantially exceeded the MIC. Thus, although the presence of bacteria and bacterial matrix material undoubtedly retarded the diffusion of the antifungal agents, poor penetration does not account for the drug resistance of *Candida* biofilm cells, even in these mixed-species biofilms.

Candida albicans is the major fungal pathogen of humans (10). During recent years this organism, together with related *Candida* species, has become one of the commonest agents of hospital-acquired infections (18). Many of these are implant-associated infections, in which adherent microbial populations, or biofilms, are found on the surfaces of devices, including catheters, prosthetic heart valves, endotracheal tubes, and joint replacements (15, 17). Such infections can be caused by a single microbial species or by a mixture of fungal or bacterial species (13, 28). Individual organisms in biofilms are embedded within a matrix of frequently slimy, extracellular polymers and typically display a phenotype that is very different from that of planktonic (free-floating) cells. In particular, biofilm cells are significantly less susceptible to antimicrobial agents (16, 17, 19, 34). As a result, drug therapy for an implant infection may be futile, and often, the only solution is mechanical removal of the implant (13).

Various model systems have been used to investigate the properties of *Candida* biofilms in vitro (17). These range from simple assays with catheter disks to more complex flow systems, such as the perfused biofilm fermentor (7). Biofilms of *C. albicans* usually consist of a mixture of yeasts, hyphae, and pseudohyphae and may have a basal yeast layer that anchors the biofilm to the surface (8). The cells are surrounded by a matrix of extracellular polymeric material, the synthesis of which markedly increases when developing biofilms are exposed to a liquid flow (24). Results from several studies have shown that *Candida* biofilms are resistant to clinically important antifungal agents, including amphotericin B, fluconazole, flucytosine, itraconazole, and ketoconazole (6, 11, 12, 23, 31, 37, 38). Newer azoles (voriconazole and ravuconazole) are also ineffective against biofilms (29), although some antibiofilm activity has been demonstrated with the echinocandin caspofungin in vitro (4, 29, 39). Mixed *Candida-Staphylococcus* biofilms are similarly resistant to fluconazole, and there is evidence that the bacteria can enhance *Candida* resistance (1).

The mechanisms of biofilm resistance to antimicrobial agents are not fully understood. One long-standing hypothesis for the resistance of bacterial biofilms is that the matrix material restricts drug penetration by forming a reaction-diffusion barrier (19) and that only the surface layers of a biofilm are exposed to a lethal dose of antibiotic. The extent to which the matrix acts as a barrier to drug diffusion would depend on the chemical nature of both the antimicrobial agent and the matrix material. Several research groups have investigated antibiotic penetration in *Pseudomonas aeruginosa* biofilms (26, 30, 42, 44). The overall conclusion from that work was that fluoroquinolones penetrate *P. aeruginosa* biofilms readily, whereas the penetration of aminoglycosides is retarded. Further studies suggested that aminoglycosides diffuse more slowly because they bind to matrix polymers such as alginate (20, 21, 36). Analogous investigations with *Candida* species have not been reported. However, the drug susceptibility profiles of *C. albicans* biofilms incubated statically (which have relatively little extracellular matrix material) 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 susceptibilities to amphotericin B, flucytosine, or fluconazole, suggesting that drug resistance is unrelated to the extent of matrix formation (9).

In the study described here, we have investigated the pene-

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tration of antifungal agents through *Candida* biofilms using a filter disk assay adapted from the technique reported by Anderl et al. (3) for bacterial biofilms. The abilities of flucytosine and fluconazole to permeate biofilms of *C. albicans* and related *Candida* species were evaluated in this way. In parallel, the viabilities of drug-treated biofilm cells were determined. The drug penetration of mixed-species biofilms containing *C. albicans* and *Staphylococcus epidermidis* (a slime-producing wild-type strain and a slime-negative mutant) was also assessed. *S. epidermidis* is the organism most frequently isolated from bacterial implant-associated infections and has also been found in polymicrobial infections with *C. albicans* (28). Voriconazole and amphotericin B, as well as flucytosine and fluconazole, were used in these mixed-species experiments.

MATERIALS AND METHODS

Organisms. *C. albicans* GDH 2346 (NCYC 1467) and GDH 2023 were originally obtained from patients with denture stomatitis at Glasgow Dental Hospital; strain GRI 682 was from a vaginal smear at Glasgow Royal Infirmary. *C. glabrata* AAHB 12, *C. tropicalis* AAHB 73, and *C. parapsilosis* AAHB 4479 were isolated from patients with line infections at Crosshouse Hospital, Kilmarnock, Scotland. *C. krusei* was obtained from a clinical specimen and came from the Regional Mycology Reference Laboratory, Glasgow, Scotland. All strains were maintained on slopes of Sabouraud dextrose agar (Difco) and were 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 (41). The growth rate, initial adherence, cell wall composition, surface characteristics, and antimicrobial susceptibility profile of strain M7 are indistinguishable from those of wild-type strain RP62A (41).

Medium and culture conditions. All *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 rpm. Cells were harvested and washed twice in 0.15 M phosphate-buffered saline (PBS; pH 7.2). Before use in biofilm experiments, all washed cell suspensions were adjusted to an optical density at 600 nm of 0.2.

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* (strains RP62A and M7) grew at similar rates in this medium (1). 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 an optical density at 600 nm of 0.2 prior to use in biofilm experiments. For mixed-species biofilms, equal volumes of the standardized suspension of each organism were mixed immediately before use.

Biofilm formation. Biofilms were grown on membrane filters resting on agar culture medium in petri dishes. For experiments with *Candida* species, polycarbonate membrane filters (diameter, 25 mm; pore size 0.2 μm; Whatman) were sterilized by exposure to UV radiation for 15 min on both sides prior to inoculation and were then placed on the surface of YNB agar containing 50 mM glucose. Tryptic soy agar was used for *S. epidermidis* and mixed-species biofilms. A standardized cell suspension (50 μl) was applied to the surface of each sterile membrane. All plates were incubated at 37°C for 24 h. The membrane-supported biofilms were then transferred to fresh agar for a further 24 h, giving a total incubation time of 48 h for biofilm formation.

Penetration of biofilms by antifungal agents. Four clinically important antifungal agents were used in this study. Flucytosine and amphotericin B were obtained from Sigma. Fluconazole and voriconazole were kindly donated by Pfizer Limited. All drug solutions were prepared immediately before use. Flucytosine and fluconazole were dissolved in sterile distilled water and then added to molten culture medium at 50°C by use of a sterile filtration unit (Sartorius) to create antifungal agent-supplemented agar for the biofilm experiments. Voriconazole and amphotericin B were dissolved in dimethyl sulfoxide and filtered into the growth medium. The medium was buffered to pH 7 with 0.165 M morpholinepropanesulfonic acid buffer (Sigma). High drug concentrations were used in the antifungal agent-supplemented agar. They were selected on the basis of their ability to give large zones of growth inhibition in control assays for drug penetration outlined below. The concentrations used were as follows: flucytosine, 6 μg/ml (30 times the MIC for planktonic *C. albicans* GDH 2346); fluconazole, 24 μg/ml (60 times the MIC); voriconazole 10 μg/ml (220 times the MIC); and amphotericin B, 78 μg/ml (60 times the MIC).

Penetration of antifungal agents through biofilms was assessed by a modification of the filter disk technique described previously for bacterial biofilms (3). After biofilm formation on membrane filters, smaller polycarbonate membrane filters (diameter, 13 mm; pore size, 0.2 μm; Whatman) were sterilized by exposure to UV radiation for 15 min on both sides and were then carefully placed on top of the 48-h-old biofilms. Paper concentration disks (diameter, 6 mm; Becton Dickinson) were also sterilized by exposure to UV radiation for 15 min per side and then moistened with growth medium (normally 29 μl) prior to placement on top of the 13-mm-diameter membranes. Because of an occasional variation in disk thickness, a slightly higher or lower volume of medium was sometimes required to saturate the disks. Wetting of the disks helped prevent the capillary action of the antifungal medium through the biofilms. Biofilms sandwiched between the membranes and moistened disks were transferred to antifungal agent-containing agar medium. All plates were incubated for specified exposure times, namely, 60, 90, 120, 180, 240, or 360 min.

The amount of antifungal agent which had penetrated each biofilm and which had reached the concentration disk was determined by using the disk in a standard drug diffusion assay. Plates of YNB agar containing 200 mM glucose were seeded with 150 μl of a standardized suspension of planktonic *C. albicans* GDH 2346 (used here as an indicator organism and adjusted to an optical density at 520 nm of 1.0). After the appropriate exposure time, concentration disks were removed from the biofilm "sandwiches" and placed on the seeded plates, which were then incubated at 37°C for 24 h. The zones of growth inhibition were measured and used to determine the concentration of active antifungal agent in the disks by reference to a standard curve prepared by using drug solutions of different concentrations but fixed volumes. All drug penetration assays were carried out in duplicate on at least two separate occasions. In control assays, concentration disks were placed on the two-membrane system to which no cells had been added, i.e., the unit without the biofilm. The drug concentration that penetrated the biofilms (C) was divided by the drug concentration determined for the controls (C_0) to provide a normalized penetration curve (3).

Viable counts of biofilm cells exposed to antifungal agents. After biofilm formation on 25-mm-diameter membrane filters, biofilms were capped with sterile, 13-mm-diameter filters, transferred to antifungal agent-containing agar, and incubated at 37°C for 6 h (the maximum exposure period in drug penetration assays) or 24 h. After incubation, biofilm cells were gently scraped from the membranes with a sterile scalpel and resuspended in 10 ml of PBS. Serial dilutions (10^{-1} to 10^{-6}) of each biofilm cell suspension were then prepared. Triplicate samples (0.1 ml) of the 10^{-4}, 10^{-5}, and 10^{-6} dilutions were spread on YNB agar containing 200 mM glucose, and the plates were incubated at 37°C for 24 h. In control assays, the membranes were transferred to growth medium containing no antifungal agent.

Scanning electron microscopy (SEM). Biofilms of *C. tropicalis* and mixed-species biofilms of *C. albicans* GDH 2346 and *S. epidermidis* M7 formed on polycarbonate membranes were fixed with glutaraldehyde and then treated with osmium tetroxide and uranyl acetate as described previously (22). After dehydration in a series of ethanol solutions, samples were air dried in a desiccator for 48 h, coated with gold with a Polaron coater, and viewed under a Philips 500 scanning electron microscope.

RESULTS

In this study, we adapted a novel, filter disk assay devised by Anderl et al. (3) to investigate the penetration of antifungal agents through single- and mixed-species biofilms containing *Candida*. The technique involves the formation of a 48-h-old colony biofilm on a polycarbonate membrane filter and the capping of this biofilm with a second, smaller membrane filter and then a wetted paper disk of the type used in zone-of-inhibition bioassays. The assembly, which represents a primitive diffusion cell, is transferred to agar medium containing the antifungal agent. During subsequent incubation, the drug diffuses out of the agar and through the biofilm sandwich to the moistened paper disk. The drug concentration in the disk can finally be determined by measuring the zone of growth inhibi-

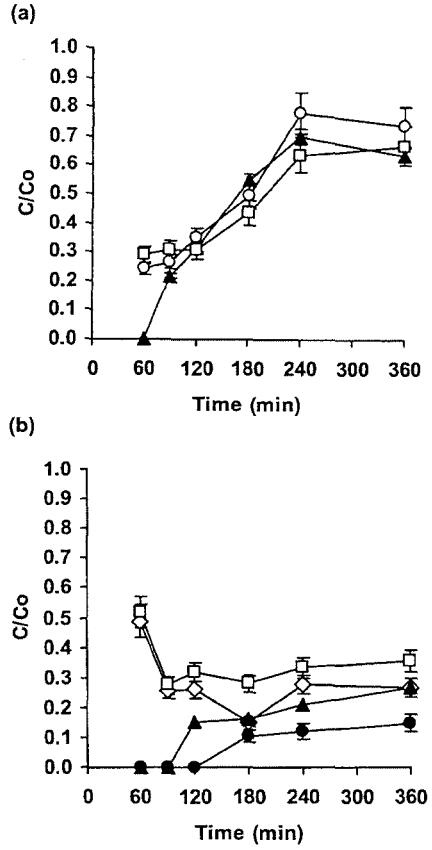

FIG. 1. Penetration of flucytosine through biofilms of *C. albicans* GDH 2346 (closed triangles), *C. albicans* GDH 2023 (open squares), and *C. albicans* GRI 682 (open circles) (a) and through biofilms of *C. krusei* (open squares), *C. glabrata* (open diamonds), *C. parapsilosis* (closed triangles), and *C. tropicalis* (closed circles) (b). Error bars indicate the standard errors of the means. The mean C_0 after 6 h was 22.7 μg of flucytosine/ml.

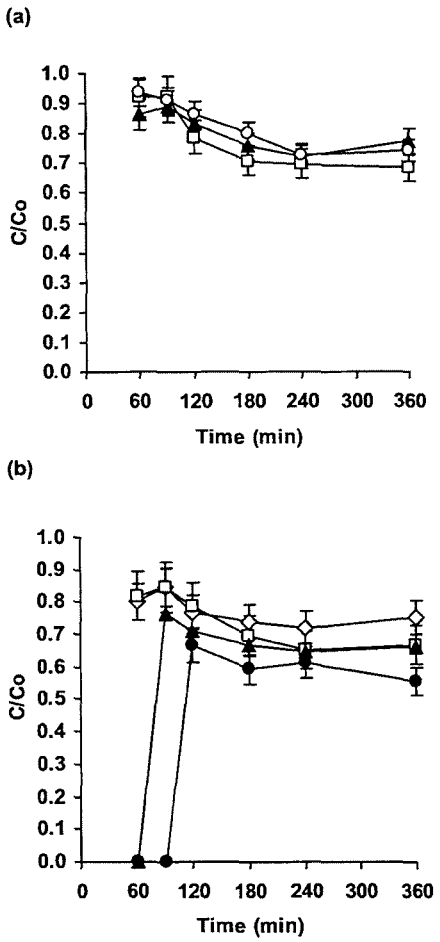

FIG. 2. Penetration of fluconazole through biofilms of *C. albicans* strain GDH 2346 (closed triangles), *C. albicans* GDH 2023 (open squares), and *C. albicans* GRI 682 (open circles) (a) and through biofilms of *C. krusei* (open squares), *C. glabrata* (open diamonds), *C. parapsilosis* (closed triangles), and *C. tropicalis* (closed circles) (b). Error bars indicate the standard errors of the means. The mean C_0 after 6 h was 26.6 μg of fluconazole/ml.

tion that it produces on medium seeded with an indicator strain of *C. albicans* in standard bioassays. The great advantage of this system is that because there is physical access to both sides of the biofilm, the penetration of solutes can be measured directly. Moreover, colony biofilms appear to lack the water channels that typically surround matrix-enclosed microcolonies in many other biofilms (46). The possibility that drugs simply move through water channels without reaching cells deep within the microcolonies is therefore largely eliminated by using this model system.

Flucytosine penetration through *Candida* biofilms. The levels of flucytosine penetration of biofilms of three *C. albicans* strains were similar, but they were initially lower with strain GDH 2346 (Fig. 1a). The drug concentration at the distal edge of the biofilm (i.e., distal with respect to the agar) was approximately 50% of that of the control value after 180 min. After 240 min it had reached 63 to 78% of that of the control value (Fig. 1a).

When biofilms of other *Candida* species were used, there was rapid penetration of the drug (approximately 50% of that for the control after 60 min) through both *C. glabrata* and *C. krusei* biofilms and then a decrease to a stable level of approximately 30% after 120 to 360 min. By contrast, there was slow diffusion through *C. parapsilosis* biofilms (25% by 360 min; Fig. 1b). However, the slowest penetration was observed with *C. tropicalis* biofilms (approximately 15% after 360 min; Fig. 1b).

Enzymatic degradation of flucytosine by *C. glabrata* and *C. krusei* did not occur. This was demonstrated by removing membrane-supported biofilms from antifungal agent-containing plates and spreading a sensitive strain (strain GDH 2346) of *C. albicans* onto the plates. These indicator organisms were unable to grow on any part of the plate, including the location that had been beneath the biofilm (results not shown).

Fluconazole penetration through *Candida* biofilms. Fluconazole penetration was similar and rapid for three *C. albicans* strains (approximately 90% of that for the control after 60 min; Fig. 2a). There was then a slight decrease and a leveling off at 70% of the control value after 360 min. Interestingly, diffusion was much more rapid with fluconazole than with flucytosine, but the final extents of drug penetration were similar after 360

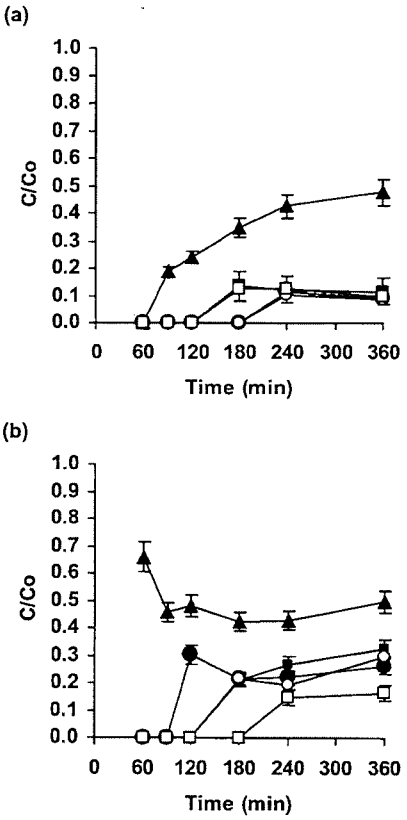

FIG. 3. Penetration of flucytosine (a) and amphotericin B (b) through single- and mixed-species biofilms of *C. albicans* and *S. epidermidis*. Biofilms contained *C. albicans* GDH 2346 (closed triangles), *S. epidermidis* RP62A (closed circles), *S. epidermidis* M7 (closed squares), *C. albicans* GDH 2346 and *S. epidermidis* RP62A (open circles), and *C. albicans* GDH 2346 and *S. epidermidis* M7 (open squares). Error bars indicate the standard errors of the means. Mean C_0s after 6 h were 24.2 μg of flucytosine/ml and 120.6 μg of amphotericin B/ml.

min (Fig. 1a and 2a). However, this represents a higher drug concentration with fluconazole.

When non-*C. albicans Candida* species were used, there was rapid fluconazole penetration through biofilms of either *C. glabrata* or *C. krusei*, followed by a decrease to roughly 70% of the control value (Fig. 2b). The level of drug penetration through *C. parapsilosis* biofilms was zero after 60 min but then rose rapidly and leveled off at 65% of the control value. The slowest penetration was again with *C. tropicalis* biofilms (Fig. 2b). For all non-*C. albicans* species, the overall level of penetration of fluconazole (55 to 85%) was higher than that of flucytosine (15 to 50%; Fig. 1b and 2b).

Penetration of antifungal agents through single- and mixed-species biofilms of *C. albicans* and *S. epidermidis*. Flucytosine diffused through biofilms of *C. albicans* GDH 2346 fairly slowly, as noted above, with penetration of about 50% of the control value after 360 min (Fig. 3a). This value was slightly lower than that shown in Fig. 1a (65% after 360 min). In these experiments, however, the *C. albicans* biofilms, like the mixed-species biofilms, were grown on tryptic soy agar rather than YNB agar. By contrast, the drug penetrated *S. epidermidis* biofilms very poorly (Fig. 3a); there was approximately 12% penetration after 180 min for slime-negative mutant M7 and 10% penetration after 240 min for wild-type strain RP62A. Mixed fungal-bacterial biofilms showed similarly slow and poor drug penetration, although diffusion was more rapid with *Candida*-M7 biofilms than with *Candida*-RP62A biofilms (Fig. 3a).

Despite its low solubility in water, amphotericin B diffused rapidly through biofilms of *C. albicans* GDH 2346 (65% of the control value after 60 min; Fig. 3b). Penetration through *S. epidermidis* biofilms was slower and less extensive. With mixed fungal-bacterial biofilms, drug diffusion was also slow and poor, but in this instance diffusion was faster through biofilms containing wild-type strain RP62A than through those containing slime-negative mutant M7 (Fig. 3b). Surprisingly, the C_0 of amphotericin B (Fig. 3 legend), like that of flucytosine (Fig. 1 legend), was higher than the drug concentration in the agar. The reason for this is not clear. However, it is conceivable that during the drug penetration assay some drugs bind to the disk cellulose (thus effectively reducing their concentration in solution) but then are released during the zone-of-inhibition assay.

Both fluconazole and the newer azole voriconazole diffused rapidly through biofilms of *C. albicans* GDH 2346, although penetration by fluconazole was more extensive (Fig. 4a and b). There was slower and poorer penetration of *S. epidermidis* biofilms. These drugs also diffused through mixed fungal-bacterial biofilms slowly, but fluconazole penetrated mixed biofilms to a greater extent than any other antifungal agent tested (Fig. 4a). The diffusion of both azoles, like that of flucytosine, was more rapid with biofilms containing *S. epidermidis* M7 than with those containing wild-type strain RP62A (Fig. 4a and b).

Effects of antifungal agents on the viability of biofilm cells. To assess the effects of the antifungal agents on biofilm cells, biofilms sandwiched between the two membranes, as in the drug penetration assay, were exposed to antifungal agent-containing agar at 37°C for 6 h (the time period during which drug penetration was determined) or 24 h. Antifungal agents (flucytosine or fluconazole) were present at concentrations identical to those used in the drug penetration assay. After incubation, the numbers of viable biofilm cells were determined by a standard procedure of serial dilution followed by plating. In no case did drug penetration result in the complete killing of biofilm cells (Table 1). *C. glabrata* AAHB 12 was wholly unaffected by fluconazole after 6 h, but many strains of this species are known to be resistant to fluconazole even when they are grown in planktonic culture (14). The results with fluconazole overall were not unexpected, despite the high concentration used, since this drug is generally considered to be fungistatic only. However, a recent study (35) has demonstrated that fluconazole can be fungicidal under certain conditions.

SEM. Biofilms of *C. tropicalis* had a very slimy appearance, suggestive of an extensive matrix, and were poorly penetrated by both flucytosine and fluconazole. *C. tropicalis*, whose ability to produce biofilms has received little attention, is able to grow in the form of yeast cells or filaments. Examination of the biofilms by SEM showed that they consisted of a dense cell network containing both morphological types. Many of the filaments appeared to lie parallel to each other in the form of bundles (Fig. 5). The procedure used for sample preparation allows clear visualization of biofilm cells but normally fails to preserve the biofilm matrix. However, fairly extensive matrix

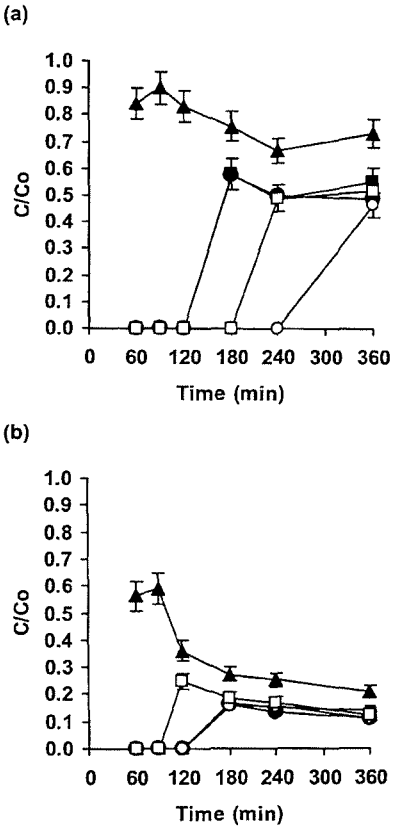

FIG. 4. Penetration of fluconazole (a) and voriconazole (b) through single- and mixed-species biofilms of *C. albicans* and *S. epidermidis*. Biofilms contained *C. albicans* GDH 2346 (closed triangles), *S. epidermidis* RP62A (closed circles), *S. epidermidis* M7 (closed squares), *C. albicans* GDH 2346 and *S. epidermidis* RP62A (open circles), and *C. albicans* GDH 2346 and *S. epidermidis* M7 (open squares). Error bars indicate the standard errors of the means. Mean C_0s after 6 h were 27.0 μg of fluconazole/ml and 7.9 μg of voriconazole/ml.

material could still be seen adhering to and linking some of the cells, a finding consistent with the slimy appearance of *C. tropicalis* biofilms.

Previous work (1) demonstrated that both strains of *S. epidermidis* used here formed thick biofilms on catheter disks. However, wild-type strain RP62A, unlike mutant M7, produced abundant matrix material, or slime. The physical interactions between staphylococci and *C. albicans* were more easily seen with the slime-free mutant (1). A similar examination of mixed-species biofilms of *C. albicans* GDH 2346 and *S. epidermidis* M7 grown on polycarbonate membranes in this study also revealed multiple, adhesive interactions between bacterial and fungal cells. Staphylococci were clearly adherent to both yeasts and hyphae (Fig. 6).

DISCUSSION

The mechanisms that protect microorganisms in biofilms from antibiotics and biocides are still being elucidated. Currently, four mechanisms are under study: (i) slow penetration of the antimicrobial agent into the biofilm, (ii) an altered chemical microenvironment within the biofilm leading to zones of slow or no growth, (iii) adaptive responses to environmental stress, and (iv) the existence of persister cells that are protected from all types of antimicrobial insult (43). Almost all of this work is being done with bacterial biofilms. At present, no single mechanism seems to account for the exceptional resistance of biofilm cells to a wide variety of antimicrobial agents. Instead, it is likely that two or more mechanisms operate together. All four mechanisms appear to depend on the multicellular nature of biofilms. For example, when an antimicrobial agent fails to penetrate a biofilm, it is because the drug is reactively neutralized as it diffuses into a cell cluster. This process may involve enzymatic degradation of the drug or drug

TABLE 1. Viability of biofilm cells of *Candida* species after exposure to flucytosine or fluconazole for 6 or 24 h[a]

Organism	Viability (%)			
	Flucytosine (6 μg/ml)		Fluconazole (24 μg/ml)	
	6 h	24 h	6 h	24 h
C. albicans GDH 2346	34.0 ± 2.3	28.9 ± 0.5	64.1 ± 1.7	81.0 ± 1.5
C. albicans GDH 2023	45.6 ± 1.5	29.9 ± 1.6	73.0 ± 2.0	62.1 ± 1.4
C. albicans GRI 682	38.2 ± 2.2	29.4 ± 0.9	67.6 ± 1.6	78.7 ± 2.9
C. glabrata AAHB 12	15.8 ± 1.2	24.2 ± 0.6	101.7 ± 1.1	81.1 ± 1.5
C. krusei	71.3 ± 1.3	66.9 ± 0.5	74.4 ± 0.6	47.8 ± 1.2
C. parapsilosis AAHB 479	74.9 ± 0.7	62.1 ± 1.5	67.4 ± 3.0	51.1 ± 1.2
C. tropicalis AAHB 73	65.0 ± 1.8	40.5 ± 1.4	49.6 ± 0.9	37.7 ± 2.1

[a] Viability is expressed as a percentage of that of control cells incubated under identical conditions in the absence of antifungal agent. The results are means ± standard errors of the means of triplicate determinations.

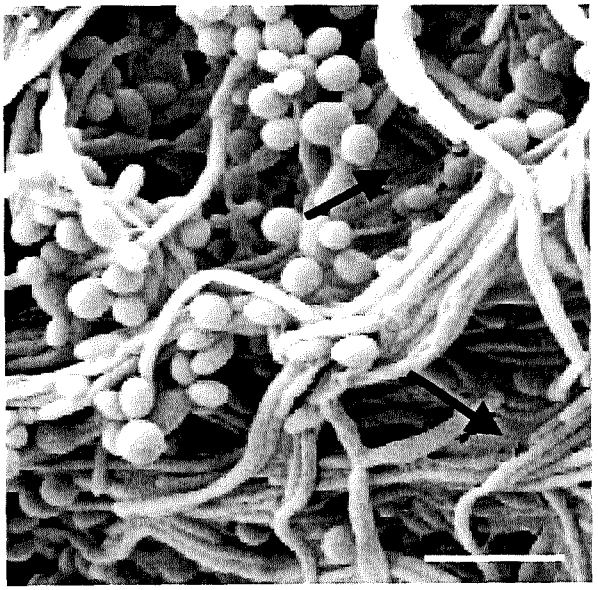

FIG. 5. Scanning electron micrograph of a 48-h-old colony (membrane-supported) biofilm of *C. tropicalis*. Arrows indicate extracellular matrix material. Bar, 10 μm.

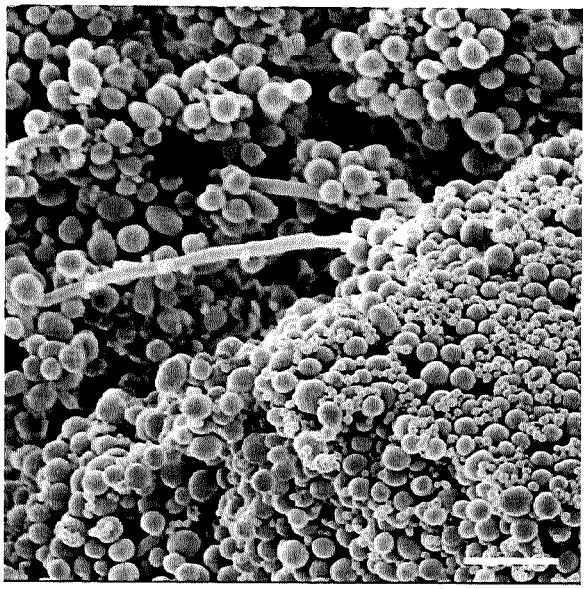

FIG. 6. Scanning electron micrograph of a 48-h-old colony (membrane-supported) biofilm of *C. albicans* GDH 2346 and *S. epidermidis* M7. Bar, 10 μm.

binding to charged extracellular polymers, but it is effective only when microorganisms are aggregated and exert their collective neutralizing activity (43).

In this study, we have investigated the penetration of antifungal agents through single- and mixed-species biofilms formed by fungal pathogens in the genus *Candida*. Our results demonstrated that fluconazole permeated all single-species *Candida* biofilms more rapidly than flucytosine. The rates of diffusion of either drug through biofilms of three strains of *C. albicans* were similar. On the other hand, the rates of drug diffusion through biofilms of *C. glabrata* and *C. krusei* were faster than those through biofilms of *C. parapsilosis* and *C. tropicalis*. In all cases, the drug concentration reached at the distal edge of the biofilm was very high. However, drug penetration failed to produce complete killing of biofilm cells even when the incubation period was extended from 6 to 24 h. These results indicate that poor drug penetration is not a major resistance mechanism for *Candida* biofilms.

The biofilms showing the lowest levels of drug penetration, particularly with flucytosine, were those formed by *C. parapsilosis* and *C. tropicalis*. The strain of *C. tropicalis* used in these experiments, a clinical isolate, had a very slimy appearance on solid medium, and some of this slime, or matrix material, could be seen when biofilm preparations were viewed under a scanning electron microscope. As yet, nothing is known of the chemical composition of this material, but it could play a minor role in the drug resistance of *C. tropicalis* biofilms by slowing the diffusion of antifungal agents. The matrix of *C. albicans* biofilms has been isolated and shown to contain mainly carbohydrate and protein, with a relatively high proportion (16%) of glucose (9). Recent work with *P. aeruginosa* has led to the identification of periplasmic glucans in biofilm cells which appear to sequester antibiotics and slow their diffusion, perhaps preventing them from reaching their sites of action in the cytoplasm (33). It has also been postulated that the nature and amounts of extracellular glucans produced by oral streptococci from sucrose in dental plaque are major determinants retarding acid diffusion (25). It will be interesting to determine whether the matrix material of *C. tropicalis* biofilms is especially rich in glucan polysaccharides.

All four antifungal agents tested diffused very slowly through mixed-species biofilms containing *C. albicans* and either the wild-type or M7 mutant strain of *S. epidermidis*. In most cases, diffusion was slower with biofilms containing *S. epidermidis* RP62A, the wild-type, slime-producing strain. Curiously, however, amphotericin B penetrated biofilms containing the M7 mutant more slowly. In all of these experiments with mixed fungal-bacterial biofilms, the drug concentrations reaching the distal edges of the biofilms substantially exceeded the MIC for *C. albicans*. Thus, although the presence of bacteria and bacterial matrix material undoubtedly retarded the diffusion of the antifungal agents, poor penetration does not account for the drug resistance of *Candida* biofilm cells, even in these mixed-species biofilms.

The nature of the extracellular matrix of *S. epidermidis* biofilms is not fully established. It appears to contain a polymer of β-1,6-linked *N*-acetylglucosamine residues with some deacetylated amino groups, as well as succinate and phosphate substituents (the intercellular polysaccharide adhesin) (32). A 140-kDa accumulation-associated protein has also been identified (27). The M7 mutant, which fails to accumulate on glass surfaces (41) but which does form biofilms on polyvinyl chloride catheter disks (1), has been reported to lack this protein but nevertheless synthesizes intercellular polysaccharide adhesin (27). Interactions between these polymers and those produced by *C. albicans* in mixed-species biofilms might result in a more viscous matrix. Rheological interactions between matrix polysaccharides from *Pseudomonas cepacia* and *P. aeruginosa* have been shown to decrease the rates of diffusion and antimicrobial activities of antibiotics (2). On the other hand, a recent study of oral biofilms containing six microbial species, including *C. albicans*, suggested that retarded diffusion of fluorescent probes through the biofilm was due to tortuosity, i.e., the convoluted paths traversed by macromolecules during biofilm penetration (45).

Biofilm cells appear to grow slowly because of the limited availability of nutrients, especially at the base of the biofilm. Growth rate has therefore been considered as an important modulator of drug activity in biofilms (17, 19). A perfused fermentor 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 same rates in a chemostat. The results indicated that biofilms were resistant to the drug at all growth rates tested, whereas planktonic cells were resistant only at low growth rates (5). An alternative mechanism of drug resistance might be upregulation of genes coding for multidrug efflux pumps in biofilm cells. *C. albicans* possesses two different types of efflux pump: ATP-binding cassette transporters and major facilitators, which are encoded by *CDR* and *MDR* genes, respectively. Recent work has shown that genes encoding both types of pump are indeed 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 they were growing planktonically but retained the resistant phenotype during biofilm growth (40). Overall, it seems probable that drug resistance in *Candida* biofilms, like that in bacterial biofilms, is a complex process involving more than one mechanism.

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ERRATUM

Penetration of *Candida* Biofilms by Antifungal Agents

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

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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.

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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 multi-port 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.

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

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.

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

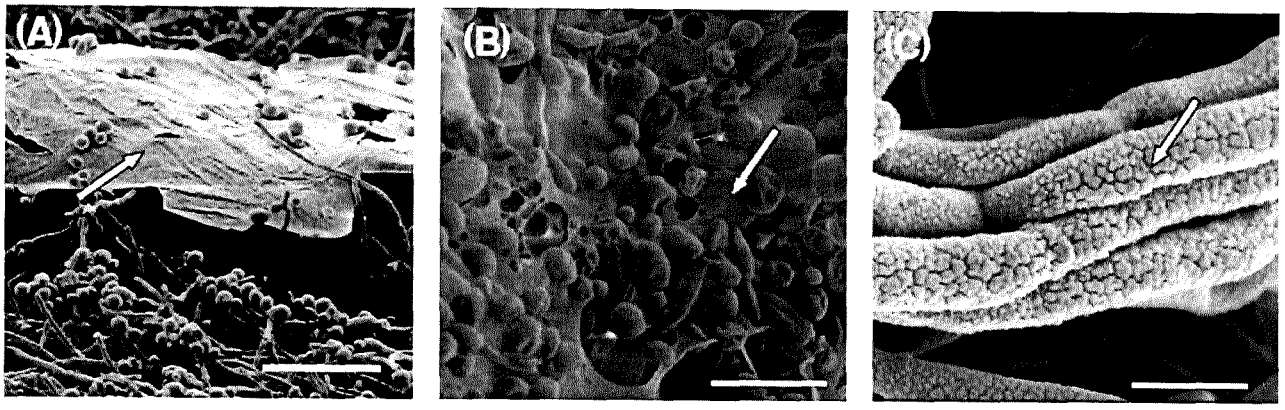

Fig. 1. Scanning electron micrographs of biofilm formation by *C. albicans* (A) and *C. tropicalis* (B, C) on PVC catheter disks. Biofilms were incubated under flow conditions in the MRD (A), or statically (B, C), for 48 h in YNB medium containing 50 mM glucose. Arrows indicate matrix material. Bars,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

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