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Drug Resistance Updates 7 (2004) 301–309
DRUG RESISTANCE Updates
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Candida biofilm resistance

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Received 1 August 2004; received in revised form 1 September 2004; accepted 16 September 2004

Abstract

Device-related infections in most nosocomial diseases can be traced to the formation of biofilms (microbial communities encased within polysaccharide-rich extracellular matrix) by pathogens on surfaces of these devices. *Candida* species are the most common fungi isolated from these infections, and biofilms formed by these fungal organisms are associated with drastically enhanced resistance against most antimicrobial agents. This enhanced resistance contributes to the persistence of this fungus despite antifungal therapy. *Candida* biofilms exhibit enhanced resistance against most antifungal agents, except echinocandins and lipid formulations of AmB. The expression of drug efflux pumps during the early phase of biofilm formation and alterations in membrane sterol composition contribute to resistance of these biofilms against azoles. Metabolic dormancy and ECM do not appear to contribute to resistance, although in a mixed-species biofilm, ECM does retard the diffusion of drugs across biofilm. These multifactorial mechanisms of resistance in fungal biofilms constitute a broad-spectrum defense that is effective against many types of antifungal agents, and represent a common theme present across microbial biofilms.

Keywords: Biofilm; Intensive Care Unit; Antifungal therapy

1. Introduction

A vast majority of nosocomial infections in medical Intensive Care Units (ICUs) in the United States are linked with the use of medical devices. For example, 87% of primary bloodstream infections, 86% of nosocomial pneumonia, and 95% of urinary tract infections are associated with central lines, mechanical ventilation, and urinary catheters, respectively (Richards et al., 1999). Most of the organisms responsible for device-related infections can exist in polysaccharide-rich extensive biofilms (Ghannoum, 2004; Donlan and Costerton, 2002; Costerton et al., 1995). *Candida* species are the most common fungi isolated from these infections (Budtz-Jorgensen, 2000, 1990; Nicastri et al., 2001; Darouiche, 2001; Kojic and Darouiche, 2004). Formation of biofilms by *Candida* species have been demonstrated on a number of devices, including central venous catheters, joint devices, dialysis access devices, cardiovascular devices, urinary catheters, penile implants, voice prostheses, dentures, and ocular implants (Kojic and Darouiche, 2004; Lane and Matthay, 2002). In a recent study, Kuhn et al. (2004) showed that *Candida parapsilosis* isolates from an outbreak had a significantly higher ability to form biofilms than sporadic isolates, and suggested that biofilm formation may play an important role in outbreaks of infection due to this organism. Another example of microbial biofilms involved in diseases is denture plaque, present in denture stomatitis (Budtz-Jorgensen, 2000, 1990, 1978). Ultrastructural studies have shown that denture plaques are complex biofilms composed of bacteria, yeasts (mostly *Candida* spp.) and desquamated epithelial cells (Catalan et al., 1987; Radford and Radford, 1993; Theilade and Budtz-Jorgensen, 1980). Taken together, these studies demonstrated that *Candida* biofilms are associated with device-related infections. Such reports have fueled a dramatic increase in the volume of scientific research on med-

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1368-7646/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.drup.2004.09.002

ical biofilms, as summarized in recent reviews (Ghannoum, 2004; Donlan and Costerton, 2002; Lane and Matthay, 2002; Habash and Reid, 1999; Douglas, 2003).

Microbial biofilms assume greater significance in the clinical context since these communities are associated with drastically enhanced ability to express resistance against most antimicrobial agents (Donlan and Costerton, 2002; Douglas, 2003; Kuhn and Ghannoum, 2004; Stewart et al., 2004; Hogan and Kolter, 2002; Prince, 2002; Stewart, 2002). These help explain (in part) the persistence of both bacterial and fungal pathogens despite adequate antimicrobial therapy. Numerous antimicrobial agents have been tested for their activity against biofilms. They range from non-specific oxidants, (such as chlorine) to antibiotics targeting specific microbial sites (such as amphotericin B [AmB]). In this review, we will focus on the antifungal resistance exhibited by *Candida* biofilms and discuss the mechanisms underlying this resistance.

2. *Candida* biofilms—different models and drug susceptibility testing methods

Different in vitro models of *Candida* biofilm development have been developed in recent years. Most of these models involve the adhesion of *Candida* cells to an inanimate surface (e.g., denture acrylic, silicone elastomer, polyvinylchloride, etc.) followed by incubation in growth medium. Formation of biofilms can be followed by: (a) incorporation of [^{3}H]-leucine into *Candida* cells in biofilm (Hawser and Douglas, 1995); (b) reduction of tetrazolium salts (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) or 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl amino) carbonyl]-2*H* tetrazolium hydroxide (XTT)) to formazan products that are measured spectrophotometrically (Kuhn et al., 2004; Kuhn and Ghannoum, 2004; Hawser and Douglas, 1994; Chandra et al., 2001a,b; Kuhn et al., 2002a,b); (c) crystal violet staining (Jin et al., 2003), or (d) determination of the dry weight of biofilms at different time points (Kuhn et al., 2004; Kuhn and Ghannoum, 2004; Hawser and Douglas, 1994; Chandra et al., 2001a,b; Kuhn et al., 2002a,b). Among these, metabolic activity assays based on XTT and dry weight determination have been more commonly used to quantitate the formation of *Candida albicans* biofilms. Antifungal susceptibilities of biofilms formed by *Candida* species is commonly determined using a XTT-based assay. The XTT-based assay has also been used previously to determine the susceptibility of planktonic *C. albicans* cells, and it has been shown that data obtained from this colorimetric method correlate well with that obtained from the standardized, M-27A reference method (National Committee for Clinical Laboratory Standards, 1997) for determination of antifungal susceptibility against yeasts (Hawser et al., 1998). Recently, an in vivo model of catheter related biofilm was developed using rabbits, and demonstrated the in vivo activity of antifungal agents against *C. albicans* biofilm (Schinabeck et al., 2004).

3. Development and architecture of *Candida* biofilms

Although research on bacterial biofilms has been fairly advanced (Donlan and Costerton, 2002; Costerton et al., 1987, 1995, 1999) studies on fungal biofilms have only recently been initiated. In vitro and in vivo models of *C. albicans* biofilms formed on bioprosthetic surfaces were recently developed and used to gain insight into fungal biofilm biology (Chandra et al., 2001b; Kuhn et al., 2002b). These studies demonstrate the heterogeneous and highly complex interactions influencing the formation of *Candida* biofilms, a theme also observed in bacterial biofilms. *C. albicans* biofilm formation proceeds in an organized fashion through distinct early, intermediate and maturation developmental phases. Fluorescence and confocal scanning laser microscopy (CSLM) utilizing carbohydrate-specific dyes (e.g., calcofluor and concanavalin A, Con-A) indicated that the *C. albicans* biofilms are encased within a polysaccharide-rich extracellular matrix (ECM) (Chandra et al., 2001b). Similar stages of development and the presence of extracellular polysaccharide matrix have also been reported for bacterial biofilms (Davey and O'Toole, 2000; O'Toole et al., 2000; Sauer et al., 2002). Recent in vivo studies using a rabbit model of biofilms formed by *C. albicans* on intravascular catheter demonstrated the formation of biofilms in vivo (Schinabeck et al., 2004).

Structural heterogeneity of architecture appears to be a common property of biofilms formed by almost all microbes (Wimpenny et al., 2000), and those formed by *C. albicans* also share this characteristic (Chandra et al., 2001b; Baillie and Douglas, 1999). Different studies have shown that architecture of *C. albicans* biofilms is affected by the substrate surface. For example, biofilms formed on flat hydrophobic surfaces of silicone elastomer or polyvinyl chloride (PVC) discs have a distinct biphasic structure composed of an adherent blastospore layer covered by hyphal elements embedded within layer of ECM (Chandra et al., 2001b; Kuhn et al., 2002a,b). In contrast, *C. albicans* biofilm formed on rough-edged and irregular surfaces of polymethylmethacrylate denture strips appear as dense tracks of cells growing along the raised rough edges of the surfaces at early phase of development, with an overgrowth of cells and ECM in mature biofilms (Chandra et al., 2001b).

Recently, Mukherjee et al. (2004) reported the EDTA-based isolation of ECM from *C. albicans* biofilm grown to different developmental stages, and showed that the isolated ECM consists of protein, carbohydrates, and DNA. Furthermore, the total protein (TP) and total carbohydrate (TC) composition of the ECM varies with different developmental stages of *C. albicans* biofilm formation. TC was significantly lower in ECM of biofilm grown to early phase, when compared with ECM of biofilm grown to mature phase (96.4 mg/g versus 153.0 mg/g, respectively, $P < 0.05$).

This was consistent with earlier studies which revealed that biofilm maturation is accompanied with abundant synthesis of carbohydrate-rich ECM (Chandra et al., 2001b). Size-based analyses revealed that ECM contains two groups (high and low molecular weight) each of TP and TC (Mukherjee et al., 2004). However, the role of the individual protein and carbohydrate components of ECM in *C. albicans* biofilm formation and drug resistance has not been investigated.

Environmental factors (including fluid shear, pH, oxygen availability and redox-gradients) have been proposed to account for heterogeneous architecture in bacterial biofilms (Wimpenny et al., 2000; Rasmussen and Lewandowski, 1998; Stoodley et al., 1997; Stoodley et al., 2001; Xu et al., 1998) Although the role of these factors in *C. albicans* biofilm formation has not been investigated in detail. Garcia-Sanchez et al. (2004) showed that biofilm populations which are formed in different environments display very similar and specific transcript profiles. These investigators also identified two clusters of genes encoding for amino acid biosynthesis, and showed that Gcn4p (a regulator of amino acid metabolism) was required for normal biofilm growth.

The architecture of fungal biofilms can also be species-dependent. Kuhn et al. (2002b) showed that biofilm formed by *C. parapsilosis* do not exhibit biphasic arrangement of discrete layers as seen with *C. albicans*, but rather consist of patches of mushroom-shaped biofilm communities. Additionally, *C. parapsilosis* biofilms consisting of ECM was significantly less than those formed by *C. albicans*.

4. Drug susceptibility of *Candida* in biofilms

4.1. In vitro susceptibility

Candida spp. contained in biofilms exhibit significant reductions in susceptibility against commonly used antifungal agents, and several investigators have studied this resistance phenotype. Hawser and Douglas (Hawser and Douglas, 1995) used an in vitro model of *C. albicans* biofilms formed on discs of PVC catheter to determine their susceptibilities against amphotericin B deoxycholate (AmB), flucytosine (5FC), fluconazole (FLU), itraconazole (ITRA), and ketoconazole (KETO). Susceptibilities were determined by using two methods: (a) [^{3}H]-leucine incorporation in candidal cells in biofilm; and (b) reduction of tetrazolium salt (MTT) to formazan product by metabolically active cells in biofilms, in the presence of different concentrations of drug compared to control. The concentration of drug which gives 50% inhibition in [^{3}H]-leucine incorporation (IL_{50}) or in the formation of MTT formazan product (IF_{50}), by candidal cells in biofilms as compared to the control was considered as minimum inhibitory concentration (MIC) of that drug. In biofilms the MICs for all the antifungals calculated on the basis of IL_{50} and IF_{50} was 30–2000 times higher than when it was calculated for planktonic cells (free-floating cells) using the same approach. This shows that *Candida* cells in biofilms were more highly resistant than planktonic cells. In separate studies, in vitro models of *C. albicans* biofilms grown on two different surfaces (denture and silicone elastomer) were used to determine the antifungal susceptibility of biofilm- and planktonically grown *C. albicans* (Chandra et al., 2001a,b; Kuhn et al., 2002a,b). The MICs of a variety of antifungals against biofilms and planktonic *C. albicans* were determined using: (a) M-27A method (National Committee for Clinical Laboratory Standards, 1997), and (b) 50% reduction in metabolic activity (RMA_{50}) (Chandra et al., 2001a,b; Kuhn et al., 2002a,b). Biofilm metabolic activity was measured as described earlier (Chandra et al., 2001a,b; Kuhn et al., 2002a) using a colorimetric based assay that involves reduction of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl amino) carbonyl]-2*H* tetrazolium hydroxide to a water-soluble brown formazan product that is measured spectrophotometrically at 492 nm. The concentration of drug that shows 50% reduction in metabolic activity was considered MIC (Chandra et al., 2001a,b). As shown in Table 1, *C. albicans* biofilms grown on either surface demonstrated significant increase in MICs to the antifungals tested when compared to the planktonic cells. Investigations of the effect of different drug concentrations on biofilm development showed that antifungal resistance increases during various stages of biofilm formation (Chandra et al., 2001a,b). In a subsequent study, Kuhn et al. (2002a) reported that increasing resistance of biofilms is also exhibited by non-*albicans* species of *Candida*. Biofilms formed by two C. *albicans* and two *C. parapsilosis* isolates were resistant to commonly used antifungal agents including FLU, AmB, nystatin (NYT), chlorhexidine (CHX), terbinafine (TERB), voriconazole (VORI) and ravuconazole (RAVU). The MICs of fungal biofilms were calculated using the same approach as described earlier (Chandra et al., 2001a,b; Kuhn et al., 2002a,b, 2004) and were compared with their planktonic counterparts. Susceptibility testing of FLU, NYT, CHX, TERB, AMB, and the triazoles revealed antifungal resistance in biofilms formed by all *Candida* isolates examined. In contrast, lipid formulations of AMB (liposomal AMB and AMB lipid complex [ABLC]) and echinocandins (caspofungin [CAS] and micafungin [MICAF]) showed activity against *Candida* biofilms. Preincubation of *C. albicans* cells with sub-MIC levels of antifungals decreased the ability of cells to subsequently form biofilm (measured by dry weight determination; $P < 0.0005$). CSLM analysis of planktonic and biofilm-associated blastospores showed treatment with VRC, CAS, and ABLC resulted in morphological alterations, which differed with each agent. These studies demonstrated that *Candida* biofilms are resistant to most antifungal agents, but are susceptible to echinocandins and AMB lipid formulations (Kuhn et al., 2002a). However, lipid complex-NYT (LNYT) failed to inhibit biofilms formed by *C. albicans* (Table 1), indicating that incorporation of lipids is not the deciding factor in imparting anti-biofilm efficacy to polyenes. Other than LAmB and ABLC, only the echinocandins [caspofungin (CAS), micafungin (MICAF), and anidulafungin] have been shown to

Table 1
Antifungal susceptibilities of *Candida* isolates grown as planktonic cells or biofilms on different substrates

Antifungal	Organism	Strain	Substrate	Biofilm MIC (μg/ml)	Planktonic MIC (μg/ml)	Reference
AmB	*C. albicans*	GDH	Denture	8	0.25	Chandra et al. (2001)
	C. albicans	M61	Catheter	4	0.5	Kuhn et al. (2002)
	C. parapsilosis	P/A71	Catheter	8	0.25	Kuhn et al. (2002)
FLU	*C. albicans*	GDH	Denture	>64	0.25	Kuhn et al. (2002)
	C. albicans	M61	Catheter	>256	1	Kuhn et al. (2002)
	C. parapsilosis	P/A71	Catheter	>256	8	Kuhn et al. (2002)
VORI	*C. albicans*	M61	Catheter	>256	0.5	Kuhn et al. (2002)
	C. parapsilosis	P/A71	Catheter	128	0.125	Chandra et al. (2001)
RAVU	*C. albicans*	M61	Catheter	128	0.1	Kuhn et al. (2002)
	C. parapsilosis	P/A71	Catheter	n.d.	0.125	Kuhn et al. (2002)
NYT	*C. albicans*	GDH	Denture	16	1	Chandra et al. (2001)
	C. albicans	M61	Catheter	16	2	Kuhn et al. (2002)
	C. parapsilosis	P/A71	Catheter	16	0.5	Kuhn et al. (2002)
CHX	*C. albicans*	GDH	Denture	128	8	Kuhn et al. (2002)
	C. albicans	M61	Catheter	32	8	Kuhn et al. (2002)
	C. parapsilosis	P/A71	Catheter	16	8	Kuhn et al. (2002)
CAS	*C. albicans*	M61	Catheter	0.25	0.125	Kuhn et al. (2002)
	C. parapsilosis	P/A71	Catheter	0.125	1	Kuhn et al. (2002)
MICAF	*C. albicans*	M61	Catheter	0.25	0.001	Kuhn et al. (2002)
	C. parapsilosis	P/A71	Catheter	0.125	0.25	Kuhn et al. (2002)
LAmB	*C. albicans*	M61	Catheter	0.25	0.5	Kuhn et al. (2002)
	C. parapsilosis	P/A71	Catheter	1	0.06	Kuhn et al. (2002)
ABLC	*C. albicans*	M61	Catheter	0.25	0.25	Kuhn et al. (2002)
	C. parapsilosis	P/A71	Catheter	0.25	0.06	Kuhn et al. (2002)
LNYT	*C. albicans*	M61	Catheter	8	0.5	Kuhn et al. (2002)
	C. parapsilosis	P/A71	Catheter	32	0.5	Chandra et al. (2001)
TERB	*C. albicans*	M61	Catheter	128	32	
	C. parapsilosis	P/A71	Catheter	n.d.	4	

n.d., not determined.

have activity against *Candida* organisms in biofilms (Table 1) (Kuhn et al., 2002a).

Reports that *C. albicans* prostaglandins (synthesized by the action of cyclooxygenase isoenzymes) play an important role in fungal colonization (Noverr et al., 2001, 2002, 2003) prompted investigations into the effect of cyclooxygenase inhibitors (e.g. aspirin) on *C. albicans* biofilms (Stepanovic et al., 2004; Alem and Douglas, 2004). Aspirin has a short half-life of about 20 min in circulating blood, and is rapidly converted to its deacetylated derivative salicylate, with plasma levels between 10^{-3} and 10^{-7} M (Vane and Botting, 1987; Wu, 2000). Stepanovic et al. (Alem and Douglas, 2004) determined the effects of aspirin on biofilms produced by two strains of *Candida guilliermondii*, and one strain each of *Candida kefyr*, *Candida glabrata*, *C. albicans*, and *C. parapsilosis*. The concentrations of aspirin which induced significant decrease in biofilm formation ranged from 0.43 to 1.73 mM (which are much higher than therapeutic levels of this drug) (Stepanovic et al., 2004). In a separate study, Alem and Douglas (2004) showed that aspirin concentrations of 0.1–1 mM, caused more than 70% inhibition of metabolic activity in *Candida* biofilms. The clinical relevance of the role of aspirin in inhibiting biofilm formation by *C. albicans* needs to be examined in further detail.

4.2. *In vivo susceptibility*

Schinabeck et al. (2004) showed that *C. albicans* biofilms formed in vivo in an animal model exhibited a resistance phenotype similar to that exhibited by biofilms formed in vitro. The in vivo biofilms were resistant to FLU but susceptible to LAmB, as revealed by counting the colony forming units (CFUs) obtained from catheter cultures, and scanning electron microscopic analyses. Quantitative cultures revealed that catheters exposed to LAmB were completely sterilized, which was statistically different than untreated controls ($P < 0.001$) and the FLU-treated group ($P = 0.0079$). Although FLU treatment tended to have lower CFU compared to untreated controls, there was no difference in mean colony counts between these two groups (1.128 ± 0.764 and 1.841 ± 1.141 $\log_{10}$ CFU/catheter segment, respectively; $P = 0.297$). Scanning electron microscopy revealed abundant biofilm in the control and FLU groups, while the LAmB group was virtually cleared.

5. Mechanisms of drug resistance in fungal biofilms

A number of resistance mechanisms, including overexpression of membrane-localized drug efflux pumps, alteration in drug targets, metabolic salvage pathways, and changes in membrane sterol composition have been proposed to account for the antifungal resistance of planktonic *C. albicans* (Ghannoum and Rice, 1999; Sanglard et al., 1995; White et al., 1998; Prasad et al., 1995). Recent studies have suggested that *C. albicans* can also exhibit resistance against CAS, mediated by mutation of genes encoding 1,3-β-D-glucan synthase enzyme (Douglas et al., 1997), overexpression of Sbe2p, a Golgi protein involved in transport of cell wall components (Osherov et al., 2002), or drug efflux mediated by Cdr2p, a membrane-localized ATP-binding cassette (ABC) transporter (Sanglard et al., 1995; White et al., 1998; Schuetzer-Muehlbauer et al., 2003; Balan et al., 1997; Perea et al., 2002). In contrast to planktonic *C. albicans*, the mechanisms of antifungal resistance of biofilm-grown *C. albicans* have not been investigated in much detail, although several factors contributing to resistance have been proposed. These factors include the physiological state of fungal cells, steric hindrance or barrier function of ECM, overexpression of drug efflux pumps, variations in fungal membrane sterol composition, and different developmental phases. The role of these factors is discussed in the next few sections.

5.1. Role of physiological state

In bacterial biofilms, the existence of metabolically active and inactive (or dormant) layers of cells has been demonstrated (Mah and O'Toole, 2001; Xu et al., 2000). However, such metabolic heterogeneity does not appear to exist in *C. albicans* biofilms, since basal layer blastospores observed in *C. albicans* biofilms also exhibit high metabolic activity (Chandra et al., 2001a,b). Moreover, metabolic activity of fungal biofilms increases with the development phases (Chandra et al., 2001b), suggesting that the resistance observed in these biofilms is not due to the presence of dormant or dead cells (Fig. 1). Growth in anaerobic and aerobic conditions within bacterial biofilms has also been reported to be involved in growth and resistance. Since azoles appear to work in anaerobic environments (Zimmermann et al., 2002), and because *C. albicans* does not grow optimally under anaerobic conditions, such environmental conditions are not expected to contribute significantly to antimicrobial resistance in *C. albicans* biofilms. A sub-population of bacterial biofilm cells, the persister cells, do not grow or adapt as rapidly as the regular biofilm cells, but can survive and reseed the community if required (Keren et al., 2004; Spoering and Lewis, 2001; Stewart, 2003). These persister cells can contribute to metabolic quiescence in bacterial biofilms and contribute to antimicrobial resistance. However, the existence of such persister cells has not been documented in *C. albicans* biofilms so far. These studies indicate that the physiological state of *C. albicans* biofilms plays a minimal role in imparting resistance against a majority of antifungal agents.

5.2. Role of extracellular matrix

Since candidal biofilm formation is characterized by the presence of visible ECM, and because the exopolysaccha-

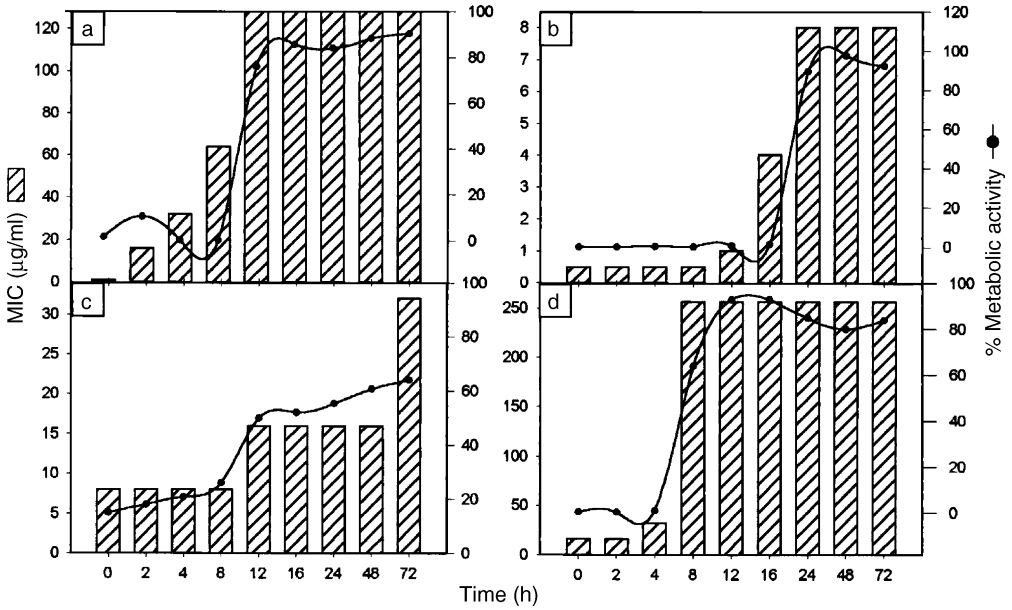

Fig. 1. Correlation of biofilm development and metabolic activity with antifungal resistance. The susceptibilities of *C. albicans* at different stages of biofilm development to fluconazole (a), amphotericin B (b), nystatin (c), and chlorhexidine (d) are represented as histograms. The line curves show percent metabolic activity of growing *C. albicans* biofilms exposed to fluconazole (64 μg/ml), amphotericin B (4 μg/ml), nystatin (8 μg/ml), or chlorhexidine (64 μg/ml). Metabolic activity was normalized to the control without drugs, which was taken as 100%. Reprinted with permission from American Society for Microbiology.

ride of bacterial biofilms has been implicated in resistance against some antimicrobial agents (Boyd and Chakrabarty, 1995; Davies et al., 1993; Davies and Geesey, 1995; Gacesa, 1998; Hentzer et al., 2001; May et al., 1991), different investigators have studied the relation between antifungal resistance and ECM of fungal biofilms. In one of the early studies, the contribution of ECM to antifungal resistance of *C. albicans* biofilms was investigated by determining the susceptibility profiles of biofilms grown statically (exhibiting minimal ECM) or with shaking (maximal ECM synthesis) (Baillie and Douglas, 2000). Biofilms grown with or without shaking did not show significant differences in susceptibility to any of the drugs. More recently, it was shown that the resistance phenotype of *C. albicans* biofilms was maintained by sessile cells when resuspended as free-floating cells, suggesting that biofilm integrity and the presence of ECM are not the sole determinants of biofilm resistance (Ramage et al., 2002a). Further evidence against the role of ECM in biofilm resistance were presented by Ramage et al. (2002b), who showed that ECM-deficient biofilms formed by filamentation defective mutants of *C. albicans* retain high level of antifungal resistance. A previous study reported that *C. parapsilosis* biofilms are as resistant as those formed by *C. albicans*, despite being considerably less complex and forming much less ECM (Kuhn et al., 2002a,b).

5.3. Role of slowed or limited drug penetration

Preliminary studies performed by our group using equilibrium dialysis and diffusion bioassays have suggested that the penetration through biofilm may play only a minimal role in biofilm resistance to FLU (Chandra et al., 2003). At a concentration of 64 μg/ml, FLU was equally distributed in the two chambers of equilibrium dialyzer, indicating that the drug freely penetrated the biofilm formed on cellulose membrane. In contrast, when *C. albicans* biofilm was incubated with higher concentration of FLU (256 μg/ml or 1024 μg/ml), very low amounts (2–5%, respectively) of FLU was bound to the biofilm, indicating saturation of biofilm at high concentrations. This study suggested that drug binding or penetration through the membrane does not play a major role in FLU resistance of *C. albicans* biofilm. In a recent study, Al-Fattani and Douglas (2004) used a filter disk assay to investigate the rates of diffusion and penetration of FLU or 5FC through *Candida* biofilms, and that of FLU, 5FC, VORI, or AmB through mixed-species (*C. albicans–C. glabrata*, *C. albicans–C. krusei* and *Candida–Staphylococcus*) biofilms. These investigators showed that the rates of diffusion of FLU and 5FC through biofilms of *C. glabrata* or *C. krusei* were faster than those through biofilms of *Candida parapsilosis* or *Candida tropicalis*. For mixed-species biofilms, all four antifungal agents showed very slow diffusion. Thus, although the presence of bacteria and bacterial matrix material appeared to affect the diffusion of antifungal agents, poor penetration does not account for the drug resistance of *Candida* biofilm cells. In view of the limited number of studies and because the ECM has not been purified from *Candida* biofilms, the role of ECM in fungal biofilm resistance is yet to be determined unequivocally.

5.4. Role of drug efflux pumps

Drug resistance in planktonic forms of *C. albicans* has long been associated with the overexpression of membrane-localized drug efflux pumps (Sanglard et al., 1995, 1997; White et al., 1998; Prasad et al., 1995; Vanden Bossche et al., 1998). Recently, the role of these pumps has been explored in biofilm-associated resistance (Ramage et al., 2002a; Mukherjee et al., 2003). Ramage et al. (2002a) evaluated resistance in biofilms formed by *C. albicans* mutants deficient for Cdr1p, Cdr2p or Mdr1p, at intermediate and mature phases (24 and 48 h) of growth. These investigators demonstrated that the *CDR1* and *CDR2* genes were up-regulated in intermediate and mature *C. albicans* biofilms. Moreover, isogenic *C. albicans* mutants carrying single and double deletion mutations (Δ*cdr1*, Δ*cdr2*, Δ*mdr1*, Δ*cdr1*/Δ*cdr2* and Δ*mdr1*/Δ*cdr1*) were hyper-susceptible to FLU when planktonic, but still retained the resistant phenotype during biofilm growth.

A separate study investigated the antifungal resistance of *C. albicans* biofilms in a triple deletion background (with no known functional efflux pump), and whether efflux pumps play any role in resistance at early phase of biofilm formation (Mukherjee et al., 2003). The effect of deletion of all three efflux pump-encoding genes and the role of developmental phase on biofilm resistance was investigated in biofilms formed by a *C. albicans* strain lacking all three major efflux pumps (Δ*cdr1*/Δ*cdr2*/Δ*mdr1*). This study revealed that the azole susceptibilities of biofilms formed by efflux pump mutants varied with both the number of pumps deleted, and the developmental phase of biofilms. Mature phase biofilms formed by the triple knock-out mutant strain had drastically reduced susceptibilities to FLU, compared to planktonic cells, while early phase biofilm formed by this strain was susceptible to azoles. Functional studies of efflux pumps using Rhodamine123 (Rh123, a fluorescent substrate for these proteins) demonstrated that compared to the early phase biofilm, the level of Rh123 was significantly reduced in intermediate and mature phases, while no significant differences in Rh123 levels were found at 12 and 48 h. These studies suggested that: (a) efflux pumps are differentially expressed during biofilm formation, and (b) these pumps contribute to biofilm-associated resistance at the early phase of development but not during later stages. Studies performed with bacterial biofilms, especially those formed by *Escherichia coli* (Maira-Litran et al., 2000a,b) and *Pseudomonas aeruginosa* (De Kievit et al., 2001; Brooun et al., 2000) also do not support a role of existing drug efflux pumps in the biofilm defense. However, temporal regulation of efflux pumps has not been investigated so far in bacterial biofilms, and it is possible that phase-specific contribution of efflux

pumps may be a common mechanism of drug resistance in fungal and bacterial biofilms.

5.5. Role of sterol composition

Fluconazole and others azoles target the fungal biosynthesis of ergosterol by inhibiting the cytochrome P-450 hemoprotein involved in the biosynthetic pathway (Ghannoum and Rice, 1999). The resulting decrease in ergosterol levels and variation in membrane sterol composition has been previously postulated to result in antifungal resistance of planktonic *C. albicans* cells. In a recent study, we determined whether *C. albicans* biofilms have variations in sterol composition at different developmental phases, and whether drug resistance of *C. albicans* biofilms can be related to such variations (Mukherjee et al., 2003). Total membrane sterols were isolated from biofilms and planktonic cells, and analyzed by gas liquid chromatography. The ergosterol levels of biofilms grown to intermediate and mature phases were reduced by 41 and 50%, respectively, compared to early phase *C. albicans* biofilm. These results showed that the level of sterols is modulated during *C. albicans* biofilm formation and suggested that such modulation may contribute to drug resistance in a phase-specific manner.

6. Conclusions

Candida biofilms exhibit enhanced resistance against most antifungal agents, except echinocandins and lipid formulations of AmB. The expression of drug efflux pumps during the early phase of biofilm formation and alterations in membrane sterol composition contribute to resistance of these biofilms against azoles. Metabolic dormancy and ECM do not appear to contribute to resistance, although in a mixed-species biofilm, ECM does retard the diffusion of drugs across biofilm. These multifactorial mechanisms of resistance in fungal biofilms constitute a broad-spectrum defense that is effective against many types of antifungal agents, and bears a close resemblance to the mechanisms existing in bacterial biofilms. Characterization of the components of biofilm ECM holds the potential of identifying unique mechanisms of resistance in *Candida* biofilms. New strategies including genomic sequencing, microarrays, and proteomics are likely to result in the identification of genes and proteins critical for biofilm-associated drug resistance, potentially revealing additional, new mechanisms of resistance in fungal biofilms.

Acknowledgements

Funding from the National Institutes of Heath (1R01 DE13932-01A1), American Heart Association (Scientist Development Grant # 0335313N), NIH/NIAMS/Skin Diseases Research Center (P30-AR-39750), and the Dermatology Foundation is gratefully acknowledged.

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