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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-

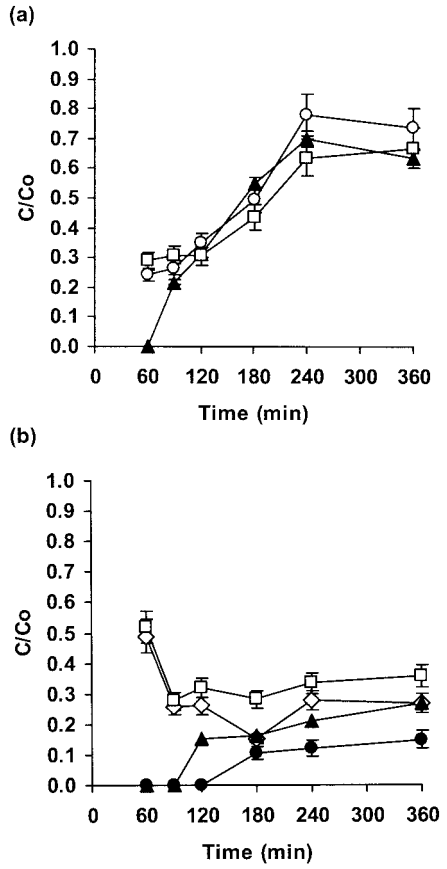

FIG. 1. Penetration of flucytosine through biofilms of *C. albicans* GDH 2346 (closed triangles), *C. albicans* GDH 2023 (open squares), and *C. albicans* GRI 682 (open circles) (a) and through biofilms of *C. krusei* (open squares), *C. glabrata* (open diamonds), *C. parapsilosis* (closed triangles), 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.

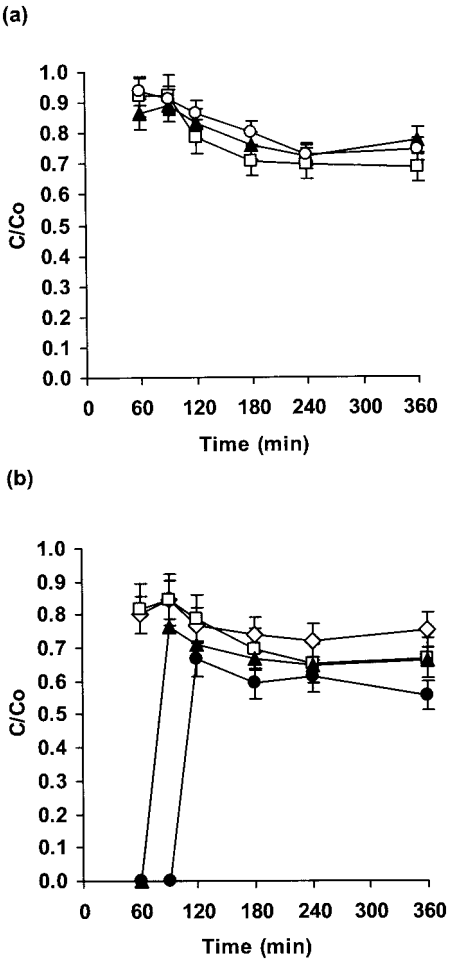

FIG. 2. Penetration of fluconazole through biofilms of *C. albicans* strain GDH 2346 (closed triangles), *C. albicans* GDH 2023 (open squares), and *C. albicans* GRI 682 (open circles) (a) and through biofilms of *C. krusei* (open squares), *C. glabrata* (open diamonds), *C. parapsilosis* (closed triangles), 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

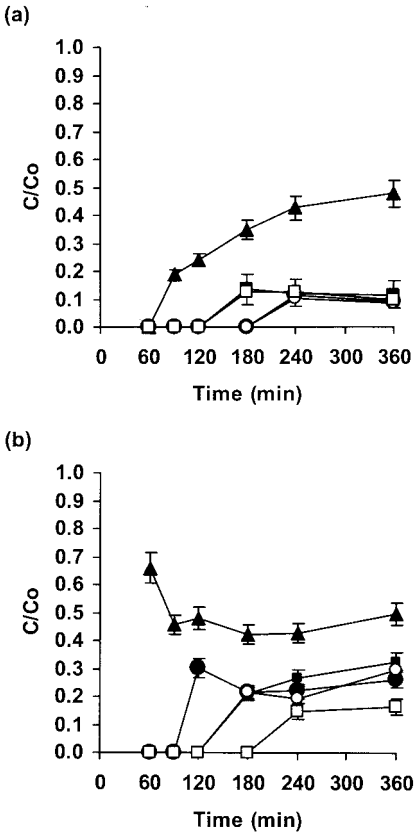

FIG. 3. Penetration of flucytosine (a) and amphotericin B (b) through single- and mixed-species biofilms of *C. albicans* and *S. epidermidis*. Biofilms contained *C. albicans* GDH 2346 (closed triangles), *S. epidermidis* RP62A (closed circles), *S. epidermidis* M7 (closed squares), *C. albicans* GDH 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

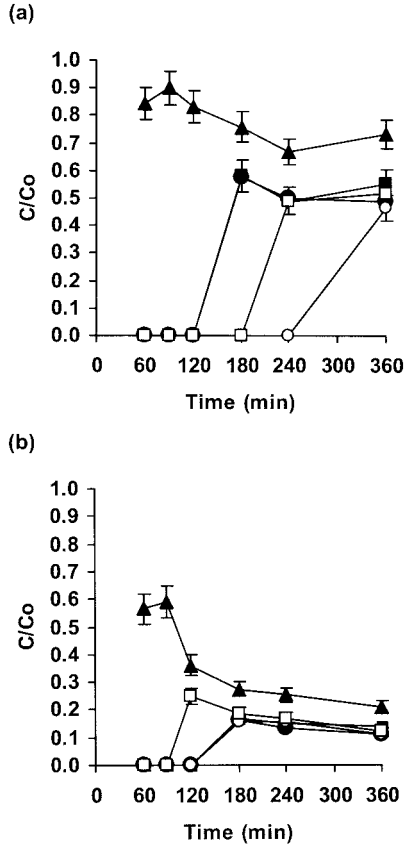

FIG. 4. Penetration of fluconazole (a) and voriconazole (b) through single- and mixed-species biofilms of *C. albicans* and *S. epidermidis*. Biofilms contained *C. albicans* GDH 2346 (closed triangles), *S. epidermidis* RP62A (closed circles), *S. epidermidis* M7 (closed squares), *C. albicans* GDH 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.

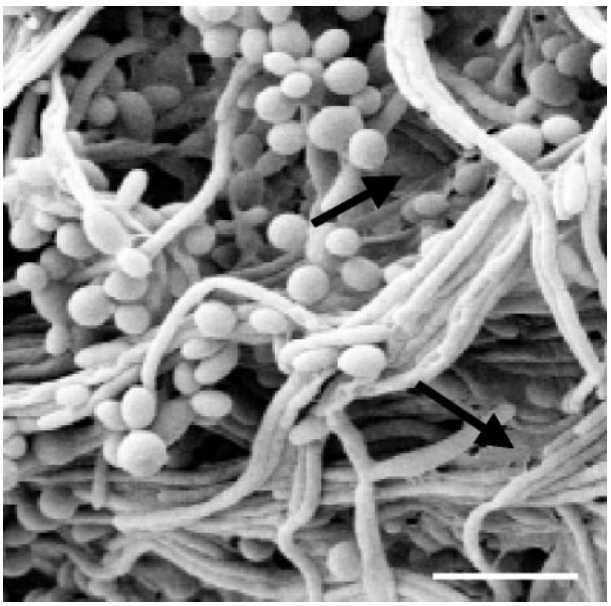

FIG. 5. Scanning electron micrograph of a 48-h-old colony (membrane-supported) biofilm of *C. tropicalis*. Arrows indicate extracellular matrix material. Bar, 10 μm.

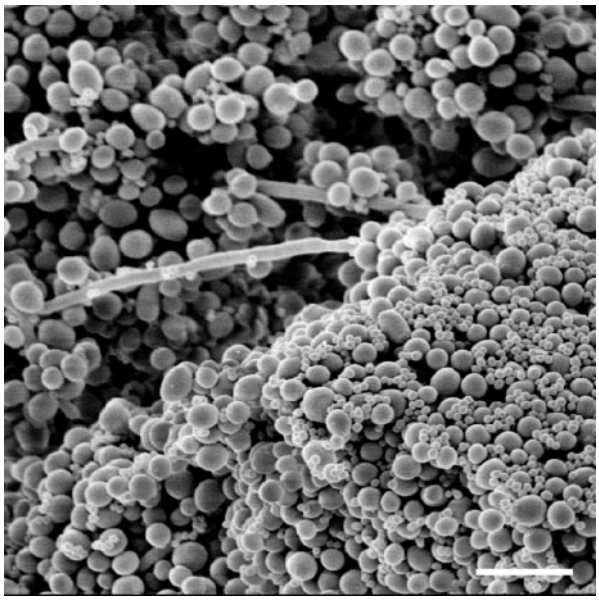

FIG. 6. Scanning electron micrograph of a 48-h-old colony (membrane-supported) biofilm of *C. albicans* GDH 2346 and *S. epidermidis* M7. Bar, 10 μm.

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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