ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1998, p. 1900–1905 Vol. 42, No. 8
0066-4804/98/$04.00+0

Effect of Growth Rate on Resistance of *Candida albicans* Biofilms to Antifungal Agents

GEORGE S. BAILLIE AND L. JULIA DOUGLAS*

Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, United Kingdom

Received 22 December 1997/Returned for modification 29 January 1998/Accepted 18 May 1998

A perfused biofilm fermentor, which allows growth-rate control of adherent microbial populations, was used to assess whether the susceptibility of *Candida albicans* biofilms to antifungal agents is dependent on growth rate. Biofilms were generated under conditions of glucose limitation and were perfused with drugs at a high concentration (20 times the MIC). Amphotericin B produced a greater reduction in the number of daughter cells in biofilm eluates than ketoconazole, fluconazole, or flucytosine. Similar decreases in daughter cell counts were observed when biofilms growing at three different rates were perfused with amphotericin B. In a separate series of experiments, intact biofilms, resuspended biofilm cells, and newly formed daughter cells were removed from the fermentor and were exposed to a lower concentration of amphotericin B for 1 h. The susceptibility profiles over a range of growth rates were then compared with those obtained for planktonic cells grown at the same rates under glucose limitation in a chemostat. Intact biofilms were resistant to amphotericin B at all growth rates tested, whereas planktonic cells were resistant only at low growth rates ($\leq$0.13 h^{-1}). Cells resuspended from biofilms were less resistant than intact biofilm populations but more resistant than daughter cells; the susceptibilities of both these cell types were largely independent of growth rate. Our findings indicate that the amphotericin B resistance of *C. albicans* biofilms is not simply due to a low growth rate but depends on some other feature of the biofilm mode of growth.

Candida albicans is the major fungal pathogen of humans (7, 25). During the last decade this organism, together with closely related *Candida* species, has become one of the commonest agents of hospital-acquired infection (16). The evolution of these fungi, previously considered to be of low virulence, into important nosocomial pathogens is related to specific risk factors associated with modern medical therapeutics. These include the use of broad-spectrum antibacterial antibiotics, hyperalimentation, cancer chemotherapy, immunosuppression following organ transplantation, and surgical procedures resulting in prolonged, intensive care unit hospitalization.

Implants, particularly indwelling intravascular catheters, represent another very significant risk factor and are almost invariably associated with nosocomial *Candida* infections. These devices can become colonized by microorganisms which form a biofilm of cells embedded within a matrix of extracellular material (5, 6, 9). Detachment of organisms from the biofilm often results in a septicemia which may be responsive to drug therapy. However, the biofilm itself is resistant both to antimicrobial agents and to host defense mechanisms and so constitutes a continuing source of infection. As a result, implant-associated infections are difficult to resolve, and usually the implant must be removed (18, 27).

Recently, a model system was devised for studying *Candida* biofilms growing on the surfaces of small discs of catheter material (19–21). Growth of the biofilms was monitored quantitatively by dry weight measurements and by colorimetric or radioisotope assays. With this system, biofilm formation by 15 different isolates of *C. albicans* and a number of other *Candida* species was investigated (19). Scanning electron microscopy (SEM) showed that the biofilms of *C. albicans* consisted of a dense network of yeasts, hyphae, and pseudohyphae, together with a matrix material whose synthesis increased dramatically when developing biofilms were subjected to a liquid flow (21). The biofilms were resistant to the actions of five clinically important antifungal agents, including amphotericin B and fluconazole (20).

The mechanisms by which *Candida* biofilms resist the actions of antifungal agents are not known. One possible resistance mechanism is related to the slow growth rate of biofilm cells as a result of the limited availability of key nutrients, particularly at the base of the biofilm. Growth rate is one of the major differences between planktonic (dispersed) growth of microorganisms in enriched laboratory media and biofilm growth in natural environments (3). A slow growth rate is frequently associated with the adoption of a different phenotype by microorganisms. With many bacteria, for example, changes in growth rate are accompanied by changes in cell envelope composition (12), and these, in turn, affect the susceptibility of the bacteria to antimicrobial agents. Growth rate may therefore be an important modulator of drug activity in biofilms (3, 4). To investigate this possibility with *C. albicans*, in the present study we used a perfused biofilm fermentor (17) to generate *Candida* biofilms at different growth rates. We then compared the susceptibility of the biofilm organisms to amphotericin B with that of planktonic cells grown at the same rates in a conventional chemostat.

MATERIALS AND METHODS

Organism. *C. albicans* GDH 2346, isolated at Glasgow Dental Hospital from a patient with denture stomatitis, was used in all experiments. It was maintained on slopes of Sabouraud dextrose agar (Difco) and was subcultured monthly. Every 2 months cultures were replaced by new ones freshly grown from freeze-dried stocks.

Growth medium. The growth medium used throughout this study was yeast nitrogen base without amino acids (pH 5.4; Difco) prepared from individual constituents. A limiting glucose concentration of 4 mM was selected; this con-

* Corresponding author. Mailing address: Division of Infection and Immunity, Institute of Biomedical and Life Sciences, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, United Kingdom. Phone: 0141-330-5842. Fax: 0141-330-4600. E-mail: J.Douglas@bio.gla.ac.uk.

centration allowed batch growth of *C. albicans* to a stationary-phase optical density of 1.3 at 540 nm.

Growth of planktonic cells in continuous culture. Conventional (planktonic) continuous cultures were established at 37°C in a 1-liter glass chemostat (500 Series; LH Fermentation, Reading, United Kingdom) with a working volume of 750 ml. The medium in the vessel was prewarmed for 1 h before inoculation with a batch culture of *C. albicans* (20 ml) in the exponential phase of growth. The cells were allowed to grow batchwise until the late exponential phase. Medium flow was then initiated, and the production of a steady state was monitored by determining the optical density of the outflow at 540 nm. Air was pumped through the culture at a rate of 1 liter min^{-1}, and the pH was maintained at a value of between 5.2 and 5.6. The dissolved oxygen in the medium was controlled by the stirring speed (routinely, 250 rpm) and was kept at 80 to 100% saturation. Cultures were tested daily for yeast morphology and bacterial contamination. At steady state, the growth rate (μ) equals the dilution rate (D). Values for D were calculated according to the equation $D = F/V$, where F is the flow rate of the medium and V is the volume of the culture in the vessel.

Growth of biofilms. Biofilms of *C. albicans* were grown on cellulose acetate filters in a perfused biofilm fermentor as described by Gilbert et al. (17) for *Escherichia coli*. A portion (10 ml) of an overnight shake culture of *C. albicans* was added to fresh prewarmed medium (40 ml) and was incubated at 37°C in an orbital shaker at 60 rpm for 3 h until the exponential growth phase had been reached (an optical density of approximately 1 at 540 nm). The cells ($4.5 \times 10^8 \pm 0.6 \times 10^8$ [standard error {SE}]) were collected by pressure filtration on a cellulose acetate membrane (0.2-μm pore size; 47-mm diameter; Whatman), and the membrane was inverted into the base of a perfused biofilm fermentor. Fresh medium was passed into the fermentation chamber at controlled flow rates (18 to 138 ml h^{-1}) via a peristaltic pump. A hydrostatic head develops above the membrane filter and under steady-state conditions perfuses the filter at the rate of medium addition to the vessel (17). The eluate passing through the filter was collected at various time intervals, and viable counts were made by serial dilution in 0.15 M phosphate-buffered saline (pH 7.2) and plating in triplicate on Sabouraud dextrose agar. The plates were incubated at 37°C for 16 h before counting. This gave an estimate of the numbers of newly formed daughter cells. Growth rates of biofilms (divisions $hour^{-1}$) were calculated by dividing the number of daughter cells produced per hour at steady state by the estimated adherent cell population (determined by obtaining the viable counts of the resuspended biofilms). All biofilms were grown for at least 20 h under steady-state conditions before drug treatment.

SEM. The freeze-drying procedure of Hawser et al. (21) was used. Biofilms formed on cellulose acetate filters were removed from the fermentor and were fixed with 2.5% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0). The filters were washed gently three times with distilled water and were then plunged into a liquid propane-isopentane mixture (2:1; vol/vol) at −196°C before freeze-drying under vacuum (10^{-6} Torr). Pieces of each filter were mounted on aluminum stubs and coated with gold before they were viewed under a Philips 500 scanning electron microscope.

Perfusion of biofilms with antifungal agents. The susceptibilities of *C. albicans* biofilms to amphotericin B (Sigma), flucytosine (Sigma), fluconazole (Pfizer), and ketoconazole (Janssen) were tested by perfusing adherent cell populations with yeast nitrogen base medium containing the drugs. Stock solutions of these antifungal agents were prepared as described previously (20). When the biofilms had reached steady state (3 h) at a growth rate of approximately 0.2 h^{-1}, medium containing a high concentration of the test drug (20 times the MIC) was pumped into the vessel for a further 3 h. The MICs used were those previously determined (20) for *C. albicans* GDH 2346 (amphotericin B, 1.3 μg ml^{-1}; flucytosine, 0.2 μg ml^{-1}; fluconazole, 0.4 μg ml^{-1}; ketoconazole, 0.025 μg ml^{-1}). At intervals, the number of viable daughter cells in the perfusate was determined by serial dilution and plating onto Sabouraud dextrose agar.

Susceptibility of planktonic cells to amphotericin B. Steady-state cultures from a conventional chemostat were used. Preliminary experiments were carried out to determine the concentration of amphotericin B which gave an appropriate survival rate following a 1-h contact period. Cells were grown at a rate of 0.23 h^{-1}, which represented a value toward the middle of the growth-rate range tested later. Samples of culture (100 μl; approximately 2×10^7 CFU ml^{-1}) were removed directly from the chemostat and were added to prewarmed distilled water (9.9 ml) containing various concentrations of amphotericin B. These test mixtures were incubated at 37°C for 1 h, and then viable counts were made by serial dilution and plating in triplicate on Sabouraud dextrose agar. The plates were incubated at 37°C for 16 h before counting. Figures for percent survival were calculated by using the counts for untreated control samples processed similarly. An amphotericin B concentration of 0.1 μg ml^{-1} gave a reduction in viability of over 80% with these planktonic cells. This concentration of amphotericin B was used in subsequent experiments in which the susceptibilities of cells grown in the chemostat at rates up to 0.7 h^{-1} were tested by the same procedure.

Susceptibilities of biofilm cells to amphotericin B. The susceptibilities of biofilm cells, resuspended biofilm cells, and newly formed daughter cells to amphotericin B were tested by the method of Evans et al. (15), as follows. After growth of the organism in the perfused biofilm fermentor at rates up to 0.7 h^{-1}, cellulose acetate filters with adherent biofilms were removed from the apparatus and cut in half. One half of each filter was immersed in amphotericin B solution (0.1 μg ml^{-1}; 10 ml) for 1 h at 37°C, and the adherent cells were then resuspended by vigorous shaking for 10 min. The cells on the other half of the filter were first resuspended by shaking in sterile water (10 ml) in an identical fashion, samples of the suspension (100 μl; approximately 2×10^6 CFU ml^{-1}) were added to an amphotericin B solution (9.9 ml; final concentration, 0.1 μg ml^{-1}), and the mixtures were incubated at 37°C for 1 h. Samples of the perfusate (1 ml) containing newly formed daughter cells (approximately 2×10^5 CFU ml^{-1}) were also added to an amphotericin B solution (9 ml; final concentration, 0.1 μg ml^{-1}) and were incubated similarly for 1 h. Viable counts were made for all samples by serial dilution in 0.15 M phosphate-buffered saline (pH 7.2) and plating in triplicate on Sabouraud dextrose agar. The plates were incubated at 37°C for 16 h before the colonies were counted. Values of percent survival were calculated by using counts for untreated control samples processed similarly. Colony counts for control samples before and after the 1-h incubation period showed only very small increases in cell numbers.

RESULTS

Growth of *C. albicans* biofilms using a perfused biofilm fermentor. Controlled biofilm growth was produced with a perfused biofilm fermentor in which a biofilm is formed on the underside of a cellulose acetate membrane inserted into the base of the vessel. The membrane is perfused with medium from the sterile side. A steady state is developed when the size of the biofilm population remains constant and dispersed cells are collected in the spent medium. At steady state, the rate of perfusion with fresh medium controls the overall growth rate of the culture (17).

Since studies of fungal biofilms with a perfused biofilm fermentor have not been reported previously, a preliminary investigation of the suitability of this technique for work with *C. albicans* was performed. Exponential-phase organisms growing in yeast nitrogen base medium containing a limiting glucose concentration (4 mM) were collected on a prewashed cellulose acetate membrane filter (0.2-μm pore size). The cell-impregnated membrane was then inverted and was carefully placed in the base of the fermentor, which was maintained at 37°C with a water jacket. Initially, all filters were perfused at a rate of 1.12 ml min^{-1}. Eluate passing through the filter was periodically collected, and viable counts were determined by serial dilution and plating. Loosely attached cells were dislodged from the membrane by the perfusing medium for up to 80 min after the initiation of flow (Fig. 1); this also occurs with bacterial biofilms (17). More than 90% of the cells added to the filter were removed during this period. After the initial cell loss, organisms were eluted from the filter at a constant rate. These represent newly formed daughter cells budding from the biofilm. Steady state could be maintained for approximately 30 h, after which the organism appeared to grow through the cellulose acetate support. The mean population size of a *C. albicans* biofilm was $3.5 \times 10^7 \pm 0.3 \times 10^7$ (SE) cells, which represented 7.8% of the cell number originally added to the membrane (4.5×10^8 cells). As expected, *C. albicans* biofilms contained fewer organisms than those of *E. coli* or *Staphylococcus epidermidis* (10^8 and 10^9, respectively) (10, 17) due to their greater cell size. Consequently, the number of daughter cells shed from the biofilm during steady state was also reduced compared with the number of cells shed from bacterial systems.

In subsequent experiments, the rate of flow of fresh medium through the biofilm was altered after steady state had been reached, and the number of viable cells in the eluate was estimated 24 h later. As in a conventional chemostat, increasing the medium flow rate and hence the availability of the limiting nutrient resulted in a greater yield of newly formed daughter cells. This was demonstrated up to a flow rate of 1.7 ml min^{-1} (the critical medium flow rate), above which the production of daughter cells decreased and growth rate control under steady-state conditions was lost (Fig. 2). Calculation of specific growth rates for different medium flow rates showed that there was a significant correlation ($r = 0.85$) between the

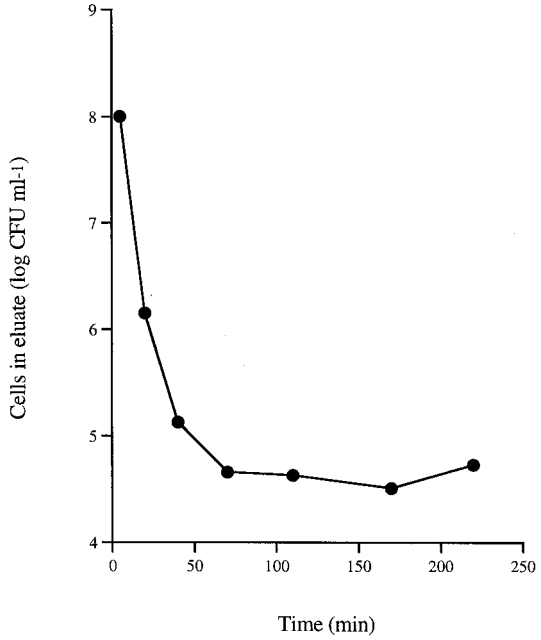

FIG. 1. Elution of *C. albicans* from a cellulose acetate filter perfused with medium in the biofilm fermentor at a rate of 1.12 ml min^{-1}. Organisms eluted during the initial 80 min correspond to loosely attached cells. Thereafter, newly formed daughter cells are eluted under steady-state conditions. The results are from a representative experiment repeated at least three times.

two (Fig. 3). As expected, the maximum specific growth rate (μ_{max}) of 0.70 h^{-1} was reached at the critical medium flow rate (1.7 ml min^{-1}), after which there was a decrease. This value is comparable to that obtained for μ_{max} with the same strain under steady-state conditions in a conventional chemostat

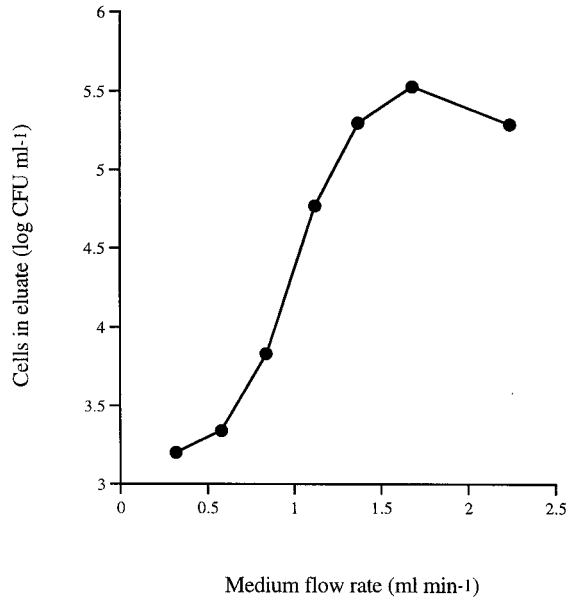

FIG. 2. Determination of the critical medium flow rate for a *C. albicans* biofilm in the perfused biofilm fermentor. The number of daughter cells released from the biofilm increased with flow up to 1.7 ml min^{-1}. This represents the critical medium flow rate at which the growth rate is maximum (μ_{max}). The results are from a typical experiment repeated at least three times.

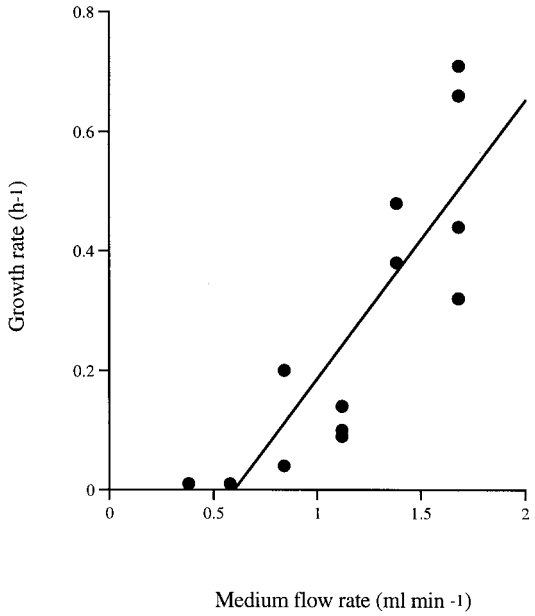

FIG. 3. Relationship between flow rate and growth rate for a *C. albicans* biofilm in the perfused biofilm fermentor up to the critical medium flow rate (1.7 ml min^{-1}).

(0.73 h^{-1}), suggesting that the entire adherent population is under growth rate control.

SEM. Scanning electron micrographs of steady-state biofilms on filters revealed a complex mixture of yeasts and hyphae enmeshed in a dense matrix material (Fig. 4). Each biofilm consisted mainly of a cell monolayer, but in some areas biofilms were three cells deep. There were also small regions where the underlying filter could be seen due to the complete absence of cells (Fig. 4).

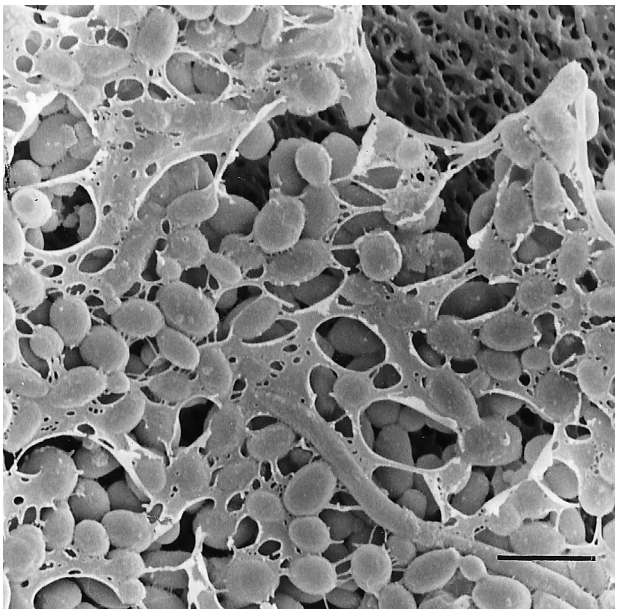

FIG. 4. Scanning electron micrograph of a *C. albicans* biofilm grown on a cellulose acetate filter in a perfused biofilm fermentor. The growth rate was 0.2 h^{-1}. Bar, 10 μm.

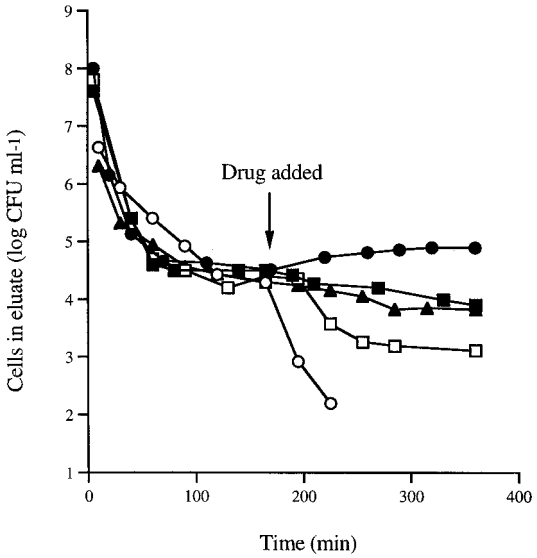

FIG. 5. Perfusion of *C. albicans* biofilms with antifungal agents. Biofilms were maintained at a growth rate of 0.2 h^{-1} and were perfused with medium containing amphotericin B (○), flucytosine (■), ketoconazole (□), fluconazole (▲), or no antifungal agent (●). The antifungal agents were used at the following concentrations (20 times the MIC): amphotericin B, 26 μg ml^{-1}; flucytosine, 4 μg ml^{-1}; fluconazole, 8 μg ml^{-1}; and ketoconazole, 0.5 μg ml^{-1}. Results represent mean values from two independent experiments carried out with duplicate samples. SEs were less than 10% of the mean values. The viable counts of eluates from biofilms perfused with amphotericin B were zero after 225 min.

Perfusion of biofilms with antifungal agents. Initially, the susceptibilities of the *C. albicans* biofilms to antifungal agents were assessed by perfusing the biofilms with the drugs. Four agents in common clinical use were added separately to the growth medium at a high concentration (20 times the MIC) and were allowed to perfuse steady-state biofilms maintained at a growth rate of approximately 0.2 h^{-1}. Ketoconazole appeared to be more effective than either fluconazole or flucytosine at reducing the number of viable daughter cells in the eluate (Fig. 5), but the differences were not statistically significant. Exposure to amphotericin B for 1 h resulted in a decrease in the viable count from 3.2×10^4 to 1.6×10^2 CFU ml^{-1}; after 225 min, no viable cells were detected in the eluate (Fig. 5).

Subsequent experiments were confined to investigating the effect of amphotericin B on biofilms. By adjusting the flow rate, the biofilm growth rate was varied from approximately 0.02 to 0.2 h^{-1} and 0.4 h^{-1}. Following exposure to amphotericin B at a high concentration (20 times the MIC), there were similar decreases in the numbers of viable daughter cells eluted at all three growth rates (Fig. 6). At a growth rate of 0.02 h^{-1}, however, the biofilm continued to shed steady-state numbers of daughter cells for up to 30 min after drug addition, suggesting a delayed effect with very slow growth.

Susceptibility of biofilms and planktonic cells to amphotericin B at equivalent growth rates. Our perfusion experiments yielded only preliminary information on the production of viable daughter cells by biofilms treated with amphotericin B at different growth rates. No results on the viability of the biofilm cells on the filter were obtained. To investigate possible growth-rate effects in detail, biofilms formed at different growth rates were removed from the fermentor and were exposed to amphotericin B at a concentration of 0.1 μg ml^{-1} for 1 h at 37°C. Cells resuspended from biofilms and daughter cells eluted from biofilms were treated similarly. For comparison, *C. albicans* cells grown planktonically at equivalent growth rates in a chemostat were also exposed to the drug. In all cases, viable counts were determined, and the percent survival of treated cells was calculated by reference to the counts obtained with unexposed, control cells.

Biofilm organisms were resistant to amphotericin B at all growth rates tested. By contrast, the susceptibility of planktonic cultures to the drug was highly dependent on growth rate (Fig. 7). At very low growth rates, planktonic cells were just as resistant as biofilm cells, but their sensitivity increased sharply at growth rates above 0.13 h^{-1}. Cells resuspended from biofilms were more resistant than planktonic organisms at growth rates in excess of 0.2 h^{-1} but were less resistant than intact biofilm populations. The daughter cells eluted from the biofilms, on the other hand, were more susceptible than either biofilm organisms or organisms resuspended from biofilms (Fig. 7). These findings indicate that the amphotericin B resistance of *Candida* biofilms is attributable not simply to a low growth rate but also to the biofilm mode of growth at a surface.

DISCUSSION

A number of experimental systems for studying bacterial biofilms have been devised. Continuous-growth models which have been widely used with bacteria include the Robbins device (23) and submerged test-piece systems (22). However, these systems lack effective growth-rate control and therefore do not differentiate between microbial properties attributable to growth rate and those associated with adhesion (3). With the perfused biofilm fermentor, on the other hand, a biofilm is established on the underside of a cellulose membrane, and a steady state develops in which the rate of perfusion with fresh medium controls the rate of biofilm growth (17). Although this model has been used to investigate a variety of bacterial biofilms (10, 11, 13–15, 17), the present report describes its first successful application to a study of fungal biofilms. A complex network of yeasts and hyphae was generated on each cellulose membrane; these biofilms lacked the overall depth of those

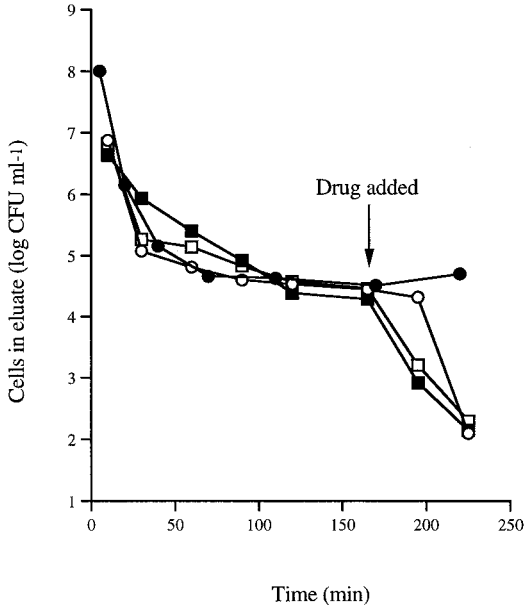

FIG. 6. Perfusion of *C. albicans* biofilms grown at different rates with amphotericin B. Biofilms were maintained at a growth rate of 0.02 h^{-1} (○), 0.2 h^{-1} (■), or 0.4 h^{-1} (□). Control biofilms (●) were grown at a rate of 0.2 h^{-1} in medium without amphotericin B. Results represent mean values from two independent experiments carried out with duplicate samples. SEs were less than 10% of the mean values.

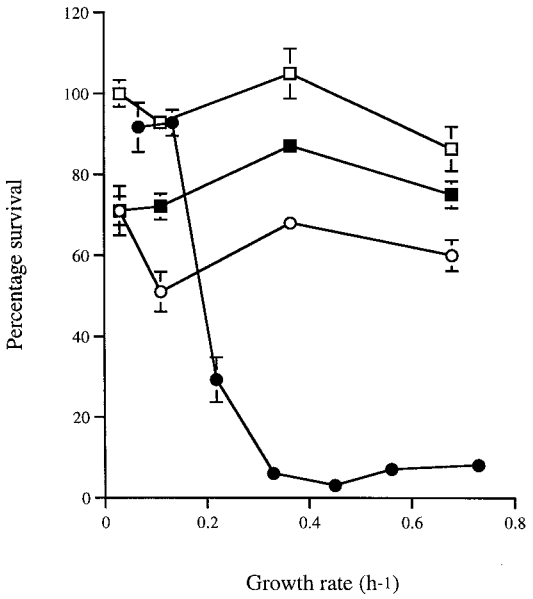

FIG. 7. Survival of planktonic and biofilm cells of *C. albicans* grown at different rates after treatment with amphotericin B. Intact biofilms (□), resuspended biofilm cells (■), biofilm daughter cells (○), and planktonic cells (●) were exposed to amphotericin B for 1 h, and the percent survival was estimated by determining the viable counts. Results represent mean values (± standard error of the mean) from two independent experiments with viable counts determined in triplicate.

formed on discs of catheter material in previous investigations (19–21) but were otherwise similar in appearance. A substantial amount of matrix material was present, which is consistent with earlier findings that synthesis of the matrix increases when developing *Candida* biofilms are subjected to a liquid flow (21).

Our initial approach to investigating drug resistance involved the perfusion of biofilms with medium containing different antifungal agents at concentrations representing 20 times the MIC. Of the drugs tested, amphotericin B proved to be the most effective at reducing the number of daughter cells shed from steady-state biofilms. The apparent failure of two azole compounds (ketoconazole and fluconazole) and flucytosine to reduce greatly the numbers of eluted cells may be a consequence of the relatively short contact time used, in conjunction with the fungistatic nature of these drugs.

Similar perfusion experiments were carried out by Ashby et al. (1) to study the effects of various antibiotics on biofilms of *E. coli*. They found that two compounds, imipenem and ciprofloxacin, which were active against nongrowing planktonic cells also showed some activity against steady-state biofilms when the compounds were tested at 20 times the MIC. However, neither antibiotic completely eradicated the biofilms. Only one flow rate was used in that study (1). Here, the flow rate was adjusted to allow the effect of perfused amphotericin B to be investigated at three biofilm growth rates. These experiments suggested that growth rate did not have a major influence on the susceptibilities of the biofilms to the drug.

More detailed studies on the effect of biofilm growth rate on resistance to amphotericin B were performed by using the approach taken by Evans et al. (15), in which biofilms, resuspended biofilm cells, and daughter cells are separately tested for drug susceptibility after growth at different rates in the perfused biofilm fermentor. The results are then compared with those obtained for planktonic cells grown at identical rates in a chemostat. Our findings demonstrated that intact *C. albicans* biofilms are resistant to the drug over a range of growth rates, whereas planktonic cells are resistant only at very low growth rates. Very similar results have been reported for biofilms and planktonic cells of a mucoid strain of *Pseudomonas aeruginosa* tested by the same protocol for susceptibility to the quinolone ciprofloxacin (14). By contrast, analogous studies with *E. coli* (13–15), *S. epidermidis* (10, 11), and nonmucoid *P. aeruginosa* (14) do suggest some relationship between growth rate and biofilm susceptibility to antimicrobial agents.

With *P. aeruginosa* biofilms, extensive production of matrix material or glycocalyx by mucoid strains may mask growth-rate effects (14). Drug exclusion by the matrix is regarded as another possible resistance mechanism for bacterial biofilms. The potential of the matrix to act as a physical barrier to penetration will depend on a number of factors, including the nature of the drug and the binding capacity of the matrix toward it (3, 24). Substantial amounts of matrix material were observed in electron micrographs of perfused *C. albicans* biofilms in this study (Fig. 4). Moreover, resuspended biofilm cells (which presumably have lost most of their matrix) were some 20% less resistant to amphotericin B than intact biofilms. The matrix may therefore play a relatively minor role in the drug resistance of *Candida* biofilms. However, it is unlikely to mask any growth-rate effect since the resistance of resuspended biofilm cells to amphotericin B was largely independent of growth rate.

Newly formed daughter cells eluted from *Candida* biofilms also displayed a susceptibility to amphotericin B that was independent of growth rate. These cells were significantly more susceptible to the drug than either intact biofilms or resuspended biofilm cells. Daughter cells dislodged from bacterial biofilms grown in the perfused biofilm fermentor are known to be even more drug sensitive. Such cells eluted from biofilms of *P. aeruginosa* or *E. coli*, for example, showed a susceptibility to ciprofloxacin equal to that of planktonic cells grown at the maximum rate in a chemostat (14). Again, drug sensitivity was unaffected by the growth rate of the daughter cells.

The drug sensitivity of daughter cells in this system, coupled with the drug resistance of the biofilms, suggests that the perfused biofilm fermentor represents a useful model for *Candida* implant infections in vivo. Implant-associated microorganisms often cause systemic infections by releasing daughter cells into the bloodstream. Although the septicemia can often be successfully treated with antibiotics, the source of the infection—the biofilm—is difficult to eliminate. One drawback of the perfused biofilm fermentor as applied to *C. albicans* is the relatively short period of continuous biofilm growth possible due to hyphal penetration of the cellulose acetate filter. Growth of *Candida* hyphae over a membrane surface and through the pores has been reported previously (26). This behavior was attributed to contact guidance (thigmotropism), an important property which allows the organism to sense changes in surface topography and is likely to facilitate hyphal penetration of epithelia in vivo.

Overall, the results of this study have confirmed earlier findings (20) that biofilms of *C. albicans* are resistant to the actions of antifungal agents and have established that resistance is not simply attributable to the low growth rate typical of biofilms. The resistance mechanism(s) remains unknown. There is some indication from our experiments on the drug sensitivity of resuspended biofilms that the matrix may play a minor role in excluding antifungal agents from the biofilm. Further studies are required to explore this possibility. Work with bacterial systems points increasingly to specific contact-induced gene expression as the mechanism by which biofilms acquire their characteristic properties (6, 8). The same may be true for

C. albicans since it is already known that synthesis of new proteins occurs following attachment of the yeast to certain surfaces (2).

ACKNOWLEDGMENTS

This work was supported by grant 94/22A from the Sir Jules Thorn Charitable Trust.

We are very grateful to I. A. Critchley for a gift of two perfused biofilm fermentors. We are also indebted to the Janssen Research Foundation for a supply of ketoconazole and to M. D. Richardson for fluconazole. Assistance with SEM was kindly provided by L. Tetley and M. Mullin.

REFERENCES

1. **Ashby, M. J., J. E. Neale, and I. A. Critchley.** 1994. Effect of antibiotics on non-growing planktonic cells and biofilms of *Escherichia coli*. J. Antimicrob. Chemother. **33:**443–452.
2. **Bailey, A., E. Wadsworth, and R. A. Calderone.** 1995. Adherence of *Candida albicans* to human buccal epithelial cells: host-induced protein synthesis and signaling events. Infect. Immun. **63:**569–572.
3. **Brown, M. R. W., and P. Gilbert.** 1993. Sensitivity of biofilms to antimicrobial agents. J. Appl. Bacteriol. Symp. Suppl. **74:**87S–97S.
4. **Brown, M. R. W., D. G. Allison, and P. Gilbert.** 1988. Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? J. Antimicrob. Chemother. **22:**777–789.
5. **Costerton, J. W., K. J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie.** 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. **41:**435–464.
6. **Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott.** 1995. Microbial biofilms. Annu. Rev. Microbiol. **49:**711–745.
7. **Cox, G. M., and J. R. Perfect.** 1993. Fungal infections. Curr. Opin. Infect. Dis. **6:**422–426.
8. **Davies, D. G., A. M. Chakrabarty, and G. G. Geesey.** 1993. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **59:**1181–1186.
9. **Dougherty, S. H.** 1988. Pathobiology of infection in prosthetic devices. Rev. Infect. Dis. **10:**1102–1117.
10. **Duguid, I. G., E. Evans, M. R. W. Brown, and P. Gilbert.** 1992. Growth-rate-independent killing by ciprofloxacin of biofilm-derived *Staphylococcus epidermidis*: evidence for cell-cycle dependency. J. Antimicrob. Chemother. **30:**791–802.
11. **Duguid, I. G., E. Evans, M. R. W. Brown, and P. Gilbert.** 1992. Effect of biofilm culture upon the susceptibility of *Staphylococcus epidermidis* to tobramycin. J. Antimicrob. Chemother. **30:**803–810.
12. **Ellwood, D. C., and D. W. Tempest.** 1972. Effects of environment on bacterial cell wall content and composition. Adv. Microb. Physiol. **7:**83–117.
13. **Evans, D. J., D. G. Allison, M. R. W. Brown, and P. Gilbert.** 1990. Effect of growth rate on resistance of gram-negative biofilms to cetrimide. J. Antimicrob. Chemother. **26:**473–478.
14. **Evans, D. J., D. G. Allison, M. R. W. Brown, and P. Gilbert.** 1991. Susceptibility of *Pseudomonas aeruginosa* and *Escherichia coli* biofilms towards ciprofloxacin: effect of specific growth rate. J. Antimicrob. Chemother. **27:** 177–184.
15. **Evans, D. J., M. R. W. Brown, D. G. Allison, and P. G. Gilbert.** 1990. Susceptibility of bacterial biofilms to tobramycin: role of specific growth rate and phase in the division cycle. J. Antimicrob. Chemother. **25:**585–591.
16. **Fridkin, S. K., and W. R. Jarvis.** 1996. Epidemiology of nosocomial fungal infections. Clin. Microbiol. Rev. **9:**499–511.
17. **Gilbert, P., D. G. Allison, D. J. Evans, P. S. Handley, and M. R. W. Brown.** 1989. Growth rate control of adherent bacterial populations. Appl. Environ. Microbiol. **55:**1308–1311.
18. **Gristina, A. G.** 1987. Biomaterial-centered infection: microbial adhesion versus tissue integration. Science **237:**1588–1595.
19. **Hawser, S. P., and L. J. Douglas.** 1994. Biofilm formation by *Candida* species on the surface of catheter materials in vitro. Infect. Immun. **62:**915–921.
20. **Hawser, S. P., and L. J. Douglas.** 1995. Resistance of *Candida albicans* biofilms to antifungal agents in vitro. Antimicrob. Agents Chemother. **39:** 2128–2131.
21. **Hawser, S. P., G. S. Baillie, and L. J. Douglas.** 1998. Production of extracellular matrix by Candida albicans biofilms. J. Med. Microbiol. **47:**253–256.
22. **Keevil, C. W., D. J. Bradshaw, A. B. Dowsett, and T. W. Feary.** 1987. Microbial film formation: dental plaque deposition on acrylic tiles using continuous culture techniques. J. Appl. Bacteriol. **62:**129–138.
23. **McCoy, W. F., J. D. Bryers, J. Robbins, and J. W. Costerton.** 1981. Observations in fouling biofilm formation. Can. J. Microbiol. **27:**910–917.
24. **Nichols, W. W., S. M. Dorrington, M. P. E. Slack, and H. L. Walmsley.** 1988. Inhibition of tobramycin diffusion by binding to alginate. Antimicrob. Agents Chemother. **32:**518–523.
25. **Odds, F. C.** 1988. *Candida* and candidosis, 2nd ed. Bailliere Tindall, London, United Kingdom.
26. **Sherwood, J., N. A. R. Gow, G. W. Gooday, D. W. Gregory, and D. Marshall.** 1992. Contact sensing in *Candida albicans*: a possible aid to epithelial penetration. J. Med. Vet. Mycol. **30:**461–469.
27. **Tunney, M. M., S. P. Gorman, and S. Patrick.** 1996. Infection associated with medical devices. Rev. Med. Microbiol. **7:**195–205.