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Iron-Limited Biofilms of *Candida albicans* and Their Susceptibility to Amphotericin B

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Biofilms of *Candida albicans* were grown in vitro under iron limitation and at a low growth rate to simulate conditions for implant-associated biofilms in vivo. Their properties were compared with those of glucose-limited biofilms grown under analogous conditions. At steady state, the adherent cell populations of iron-limited biofilms were double those of glucose-limited biofilms, although the growth rates were similar (0.038 to 0.043 h^{-1}). Both biofilm types were resistant to amphotericin B, but daughter cells from iron-limited biofilms were significantly more susceptible to the drug than those from glucose-limited biofilms.

Pathogenic fungi in the genus *Candida* are now widely recognized as important agents of hospital-acquired infection. Implanted devices, particularly indwelling intravascular catheters, are a significant risk factor and are frequently associated with these infections (11, 13). Catheters provide a surface on which microorganisms can form an adherent biofilm of cells embedded in a matrix of extracellular material (7, 8, 24). *Candida* biofilms have been studied in vitro by using a simple model system in which adherent populations are grown on the surfaces of small discs of catheter material (14–16). Biofilms of *Candida albicans* consisted of mixtures of yeasts, hyphae, and pseudohyphae and were resistant to the action of a variety of antifungal agents, including amphotericin B and fluconazole (15). Recently, a more complex model system, the perfused biofilm fermentor (12), was used to investigate whether the resistance of *C. albicans* biofilms to amphotericin B could be attributed to phenotypic changes resulting from the low growth rate characteristic of biofilm cells (3). The findings indicated that the resistance of *C. albicans* biofilms to amphotericin B was not simply due to a low growth rate under the conditions tested, where growth was limited by the availability of the carbon source, glucose.

In vivo, the growth of *C. albicans* is likely to be limited by the availability of a different nutrient, iron (6). To survive in vivo, pathogenic microorganisms, including *C. albicans*, have developed various iron-scavenging mechanisms, notably the secretion of iron-chelating compounds termed siderophores (18, 22, 25). Iron deprivation can affect the surface composition of microorganisms (20, 23), which, in turn, can alter their susceptibility to antimicrobial agents (5). In this investigation, we have grown biofilms of *C. albicans* under conditions of iron limitation and compared their susceptibility to amphotericin B with that of glucose-limited biofilms grown at a similar rate. Biofilms were formed within cylindrical cellulose filters perfused with culture medium by using a modification of a method previously described for bacterial biofilms (17). This system allows the formation of *Candida* biofilms at reproducible, low growth rates. Moreover, the modified apparatus lacks stainless steel components, thus facilitating the production of iron-limited cultures.

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Growth of biofilms under conditions of iron and glucose limitation. *C. albicans* GDH 2346, a denture stomatitis isolate (23), was used in all experiments. Yeast nitrogen base medium for biofilm growth was prepared from individual constituents and deferrated by using Chelex 100 ion-exchange resin, as described previously (23). Glucose (50 mM) was added as the carbon source. Analysis by graphite furnace atomic absorption spectrometry revealed an iron content of <0.036 μM. This concentration limits the growth of *C. albicans* GDH 2346 (23) and resulted in a stationary-phase optical density of 1.3 at 540 nm in batch culture. The medium used for the growth of biofilms under glucose-limiting conditions was yeast nitrogen base, prepared from individual constituents without deferration and containing 4 mM glucose; this allowed batch growth of *C. albicans* to a stationary-phase optical density of 1.3 at 540 nm.

Biofilms were grown on cylindrical filters consisting of compacted cellulose fibers (Gilson safety filters, 22 by 8 mm; Anachem, Luton, United Kingdom) by using a modification of the method described by Hodgson et al. (17) for bacteria. Each filter was inserted into silicone tubing attached to the bottom of a disposable syringe body (2 ml) from which the plunger had been removed. Medium was pumped directly into the vertically clamped syringe body via silicone tubing. In this modified method there was no requirement for a stainless steel syringe needle, thus removing a possible source of iron contamination. Filters were prewetted with sterile saline (5 ml) and then inoculated with an exponential-phase batch culture (10 ml) grown under glucose-limiting or iron-limiting conditions. Inocula were prepared by addition of samples (10 ml) of an overnight culture in the appropriate medium to fresh batches (40 ml) of prewarmed medium and incubation at 37°C with shaking for 3 h. After perfusion of the inoculum, the filters were perfused with medium at a flow rate of 0.87 ml min^{-1}. 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 viable counts of resuspended biofilms). All biofilms were grown for at least 24 h under steady-state conditions before drug treatment.

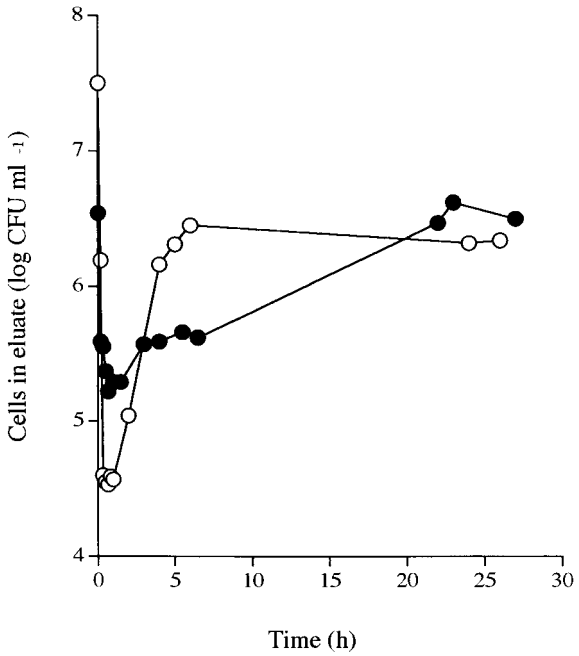

FIG. 1. Elution of *C. albicans* from cylindrical cellulose filters perfused with iron-limited (●) or glucose-limited (○) growth medium at a flow rate of 0.87 ml min^{-1}. Results are means from two independent experiments carried out with duplicate sampling. Standard errors were less than 10% of means.

Properties of iron-limited and glucose-limited biofilms. Elution profiles of cells released from the biofilms (Fig. 1) were highly reproducible but differed from those previously reported for glucose-limited populations maintained in a perfused biofilm fermentor (3). The number of cells eluted from each cylindrical filter decreased over the first hour, but this was followed by a period of biofilm growth during which the adherent culture attained an optimal density (Fig. 1). Iron-limited biofilms took much longer to reach this stage (24 h) than glucose-limited biofilms maintained at the same flow rate (6 h), although both populations shed daughter cells at a constant rate thereafter. By contrast, glucose-limited biofilms in a perfused biofilm fermentor achieved a steady state after only 80 min (3).

The additional time required for iron-limited biofilms to attain an optimal density may correspond to a period of prolonged adaptation to iron limitation of growth. *C. albicans* GDH 2346 synthesizes a siderophore of the hydroxamate type when grown planktonically in yeast nitrogen base medium containing low concentrations of iron (22). The same growth conditions also produce alterations in yeast cell wall composition, as revealed by sensitivity to the muralytic enzyme Zymolyase and by ^{125}I labelling of surface proteins (23). These physiological changes were all apparent after incubation of batch cultures for 48 h at 37°C (22, 23). A detailed study of the kinetics of siderophore synthesis by *C. albicans* GDH 2346 has not been reported. However, a recent investigation of this type with *Staphylococcus aureus* (9) showed that although siderophore activity could be detected after 24 h, maximal levels of activity were not reached until cultures had been incubated for 5 days. A similarly low rate of siderophore production by *C. albicans*, with its consequences for yeast physiological processes, might explain the delay in attainment of a steady state by iron-limited biofilms.

A summary of some important features of glucose- and iron-limited biofilms grown on cylindrical filters is presented in Table 1. Iron-limited biofilms at steady state had an adherent cell population which was double that of glucose-limited biofilms. Similarly, eluates from iron-limited biofilms contained almost twice as many cells as those from glucose-limited biofilms. These quantitative determinations were confirmed by scanning electron microscopy, which showed that iron-limited biofilms were denser and more confluent than those formed under glucose limitation (Fig. 2). The medium flow rate was identical for both biofilm types, and unsurprisingly, the calculated growth rates were similar (Table 1). These growth rates were only slightly lower than that reported for *S. aureus* (0.06 h^{-1}) with the same biofilm system (17). However, they were considerably lower than that previously determined for glucose-limited *Candida* biofilms (0.2 h^{-1}) growing in a perfused biofilm fermentor at a comparable flow rate (3). Biofilms produced in the fermentor also contained 100-fold fewer cells ($3.5 \times 10^7 \pm 0.30 \times 10^7$ CFU [mean ± standard error]), presumably because of the two-dimensional nature of the filter support.

The increased adherent cell population of iron-limited biofilms may be related to cell surface hydrophobicity. Hydrophobic interactions are considered to be important in the formation and maintenance of stable biofilm communities, principally by promoting cell adhesion. Indeed, there is some evidence that dispersal of daughter cells may be regulated by changes in surface hydrophobicity (1, 2). The cell surface hydrophobicity of *C. albicans* is significantly enhanced when the yeast is grown planktonically in iron-restricted medium (21). An analogous change in iron-limited adherent populations might produce a denser biofilm that is better able to resist sloughing due to medium flow.

Unexpectedly, both iron-limited and glucose-limited biofilms consisted exclusively of yeast cells. Hyphae and pseudohyphae appeared to be completely absent (Fig. 2). By contrast, biofilms grown without nutrient limitation on polyvinyl chloride catheter discs (14) and glucose-limited biofilms grown on cellulose acetate filters in the perfused biofilm fermentor (3) contain mixtures of morphological forms. Iron deprivation has been shown to inhibit the formation of germ tubes by *C. albicans* (23), and this may partly account for the yeast morphology of iron-limited biofilms. The reason for the absence of hyphae from glucose-limited biofilms is less obvious; it could be related to a possible oxygen deficiency inside the cylindrical filter, although such conditions usually favor hyphal development (19). Alternatively, if morphogenesis in biofilms is dependent on contact-induced gene expression, as suggested previously (14), the precise nature of the surface may be a crucial determinant in triggering the response.

Susceptibility of biofilm cells to amphotericin B. The susceptibility to amphotericin B of steady-state biofilm cells, resuspended biofilm cells, and newly formed daughter cells was determined by a method based on that of Evans et al. (10). Biofilm cells grown under iron-limiting conditions were compared with those grown under glucose-limiting conditions. An

TABLE 1. Analysis of steady-state *C. albicans* biofilms grown on cylindrical cellulose filters under conditions of glucose or iron limitation[a]

Limiting nutrient	Adherent population (10^9 CFU)	Cells in eluate (10^6 CFU ml^{-1})	Growth rate (h^{-1})
Glucose	3.41 ± 1.18	2.94 ± 0.77	0.043 ± 0.012
Iron	7.48 ± 0.17	4.76 ± 1.05	0.038 ± 0.005

[a] Data are means ± standard errors of the means from four (iron-limited biofilms) or six (glucose-limited biofilms) determinations.

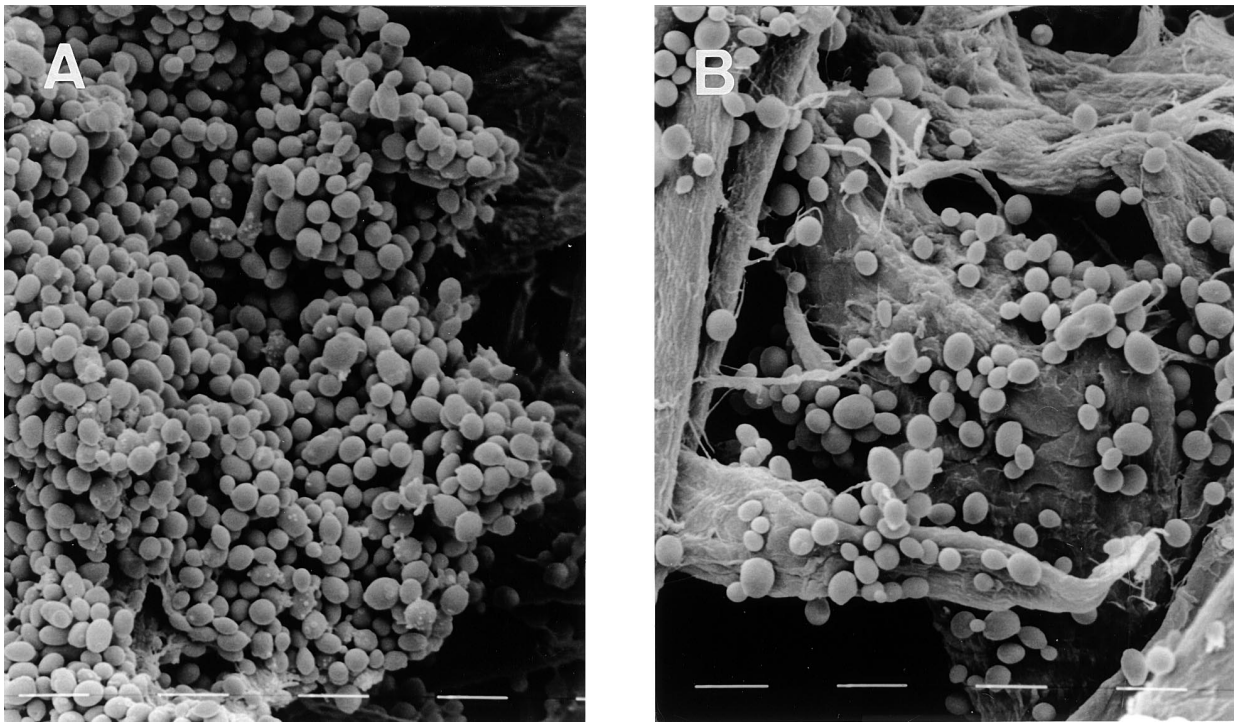

FIG. 2. Scanning electron micrographs of *C. albicans* biofilms grown for 24 h on cylindrical cellulose filters perfused with iron-limited (A) or glucose-limited (B) growth medium. Biofilms were prepared for microscopy as described previously (14) except that they were air dried overnight. Bar, 10 μm.

amphotericin B concentration of 0.1 μg ml^{-1} was used, since this concentration gave a reduction in viability of more than 80% when planktonic cells of *C. albicans* GDH 2346 were tested by the same procedure (3). Cylindrical cellulose filters with adherent biofilms were removed from the apparatus and cut in half longitudinally. One-half of each filter was immersed in amphotericin B solution (0.1 μg ml^{-1}; 10 ml) for 1 h at 37°C; the adherent cells were then resuspended by breaking up the filter with a sterile aluminum rod, followed by vigorous vortexing for 1 min. Cells on the other half of the filter were first resuspended in sterile water (10 ml) in an identical fashion, and then samples of the suspension (25 μl; approximately 3.5×10^8 CFU ml^{-1} for glucose-limited biofilms and 7.5×10^8 CFU ml^{-1} for iron-limited biofilms) were added to amphotericin-B solution (9.975 ml; final concentration, 0.1 μg ml^{-1}) and incubated at 37°C for 1 h. Samples of the eluate (1 ml) containing daughter cells (approximately 3×10^6 CFU ml^{-1} for glucose-limited cells and 4.8×10^6 CFU ml^{-1} for iron-limited cells) were also added to amphotericin-B solution (9 ml; final concentration, 0.1 μg ml^{-1}) and incubated similarly for 1 h. Viable counts were made on 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 counting. Figures for percent survival were calculated by using counts for untreated control samples processed similarly. Colony counts of control samples before and after the 1-h incubation period showed only very small increases in cell numbers.

Glucose-limited biofilms, like those studied previously in the perfused biofilm fermentor (3), were resistant to this treatment with amphotericin B (Fig. 3). Iron-limited biofilms were equally resistant. However, cells resuspended from either biofilm type were some 20% more susceptible than intact biofilm populations (Fig. 3). This partial loss of resistance with resuspended cells was also observed with glucose-limited biofilms from the perfused fermentor (3). It may be due to dispersal of the matrix if the latter acts as a physical barrier to drug penetration, as has been suggested (4).

The major difference observed between the two types of bio-

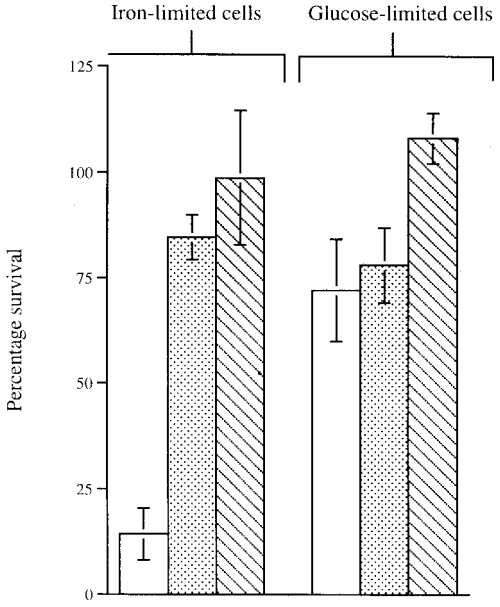

FIG. 3. Survival of biofilm cells of *C. albicans* grown in iron-limited or glucose-limited medium after treatment with amphotericin B. Intact biofilms (▨), resuspended biofilm cells (⊠), and biofilm daughter cells (□) were exposed to amphotericin B for 1 h, and the percent survival was estimated by viable counts. Results are means ± standard errors of the means from three independent experiments with viable counts done in triplicate.

film population lay in the susceptibility to amphotericin B of their daughter cells. Daughter cells eluted from glucose-limited biofilms were more susceptible than either biofilm organisms or organisms resuspended from biofilms (Fig. 3). These results, again, are similar to those obtained with glucose-limited biofilms in the perfused biofilm fermentor (3). However, daughter cells from iron-limited biofilms were significantly more susceptible ($P < 0.05$) than those from glucose-limited biofilms (Fig. 3), with a survival of less than 20% after exposure to the drug.

Microbial biofilms on catheters and other implanted devices are thought to constitute a reservoir of infection, resistant both to host defense mechanisms and to antimicrobial agents (4, 7). Detachment of organisms from the biofilm can give rise to a septicemia which may respond to conventional drug therapy. However, biofilm cells are not killed by such treatment and remain as a potential source of further infection. The results presented here seem to support this view for *Candida* implant infections. Our model system produced slow-growing biofilms of *C. albicans* whose limiting nutrient was iron. These conditions mimic those found in vivo, where growth is slow and the availability of iron is extremely limited (6). Iron-limited *Candida* biofilms were resistant to amphotericin B, whereas daughter cells formed from them were highly susceptible to the drug. In vivo, an acute disseminated infection resulting from the release of such cells would be expected to respond rapidly to therapeutic doses of amphotericin B, possibly leading to the conclusion that the problem had been resolved. The biofilm, however, would persist as a sessile population of viable fungi whose eradication would require removal of the implant.

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