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Susceptibility of *Cryptococcus neoformans* Biofilms to Antifungal Agents In Vitro

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Microbial biofilms contribute to virulence and resistance to antibiotics by shielding microbial cells from host defenses and antimicrobial drugs, respectively. *Cryptococcus neoformans* was demonstrated to form biofilms in polystyrene microtiter plates. The numbers of CFU of disaggregated biofilms, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide reduction, and light and confocal microscopy were used to measure the fungal mass, the metabolic activity, and the appearance of *C. neoformans* biofilms, respectively. Biofilm development by *C. neoformans* followed a standard sequence of events: fungal surface attachment, microcolony formation, and matrix production. The susceptibilities of *C. neoformans* cells of the biofilm and planktonic phenotypes to four antifungal agents were examined. The exposure of *C. neoformans* cells or preformed cryptococcal biofilms to fluconazole or voriconazole did not result in yeast growth inhibition and did not affect the metabolic activities of the biofilms, respectively. In contrast, both *C. neoformans* cells and preformed biofilms were susceptible to amphotericin B and caspofungin. However, *C. neoformans* biofilms were significantly more resistant to amphotericin B and caspofungin than planktonic cells, and their susceptibilities to these drugs were further reduced if cryptococcal cells contained melanin. A spot enzyme-linked immunosorbent assay and light and confocal microscopy were used to investigate how antifungal drugs affected *C. neoformans* biofilm formation. The mechanism by which amphotericin B and caspofungin interfered with *C. neoformans* biofilm formation involved capsular polysaccharide release and adherence. Our results suggest that biofilm formation may diminish the efficacies of some antifungal drugs during cryptococcal infection.

Cryptococcus neoformans is an encapsulated opportunistic yeast-like fungus that is a relatively frequent cause of meningoencephalitis in immunocompromised patients and also occasionally causes disease in apparently healthy individuals (19). *C. neoformans* capsular polysaccharide is mainly composed of glucuronoxylomannan (GXM), which is a major contributor to its virulence since acapsular strains are not pathogenic (34). Copious amounts of GXM are released during cryptococcal infection, causing deleterious effects on the host immune response (8, 34). Recently, we reported that *C. neoformans* GXM release was necessary for adhesion to a solid support and subsequent biofilm formation (18).

Biofilms are communities of microorganisms attached to a solid surface enclosed in an exopolymeric matrix (12, 15). A cryptococcal biofilm consists of a complex network of yeast cells enmeshed in a substantial amount of polysaccharide matrix (18). Biofilm formation by *C. neoformans* follows a discrete sequence of events, including fungal surface adhesion, microcolony formation, and matrix production (18). *C. neoformans* can form biofilms on polystyrene plates (18, 30) and medical devices after GXM shedding. For instance, Walsh et al. reported that *C. neoformans* could form biofilms in ventriculoatrial shunt catheters (35). In addition, several reports of *C. neoformans* infection of polytetrafluoroethylene peritoneal dialysis fistula and prosthetic cardiac valves highlight the ability of this organism to adhere to medical devices (6, 7, 26). In fact, the increasing use of ventriculoperitoneal shunts to manage intracranial hypertension associated with cryptococcal meningoencephalitis highlights the importance of investigating the biofilm-forming properties of this organism (2, 13).

Biofilm formation is associated with persistent infection since biofilms increase resistance to host immune mechanisms and antimicrobial therapy. Therapy for cryptococcosis remains suboptimal because the infection is difficult to eradicate with antifungal agents. Biofilms constitute a physical barrier that prevents the efficient penetration of antifungal drugs, which confers on microorganisms that form biofilms higher levels of resistance to antifungal activity than that conferred on their planktonic counterparts (1, 10). Various mechanisms of biofilm resistance to antimicrobial agents have been proposed, including the presence of physical barriers that prevent the penetration of the antimicrobial compounds into the biofilm, slow growth of the biofilm due to nutrient limitation, activation of the general stress response, and the existence of a subpopulation of cells within the biofilm known as persisters that are preserved by antimicrobial pressure (17, 27, 28).

Although considerable work on the effect of *Candida albicans* biofilms on susceptibility to antifungal agents has been done (4, 16, 21), no comparable studies have been done with *C. neoformans*. In this study, we exploited the ability of *C. neoformans* to form biofilms in vitro on polystyrene microtiter plates (18, 30) to study the susceptibilities of cryptococcal biofilms to four antifungal drugs. Understanding of the mechanisms of antifungal resistance may lead to the development of novel therapies for biofilm-based diseases and may allow more knowledge about the biology of *C. neoformans* biofilms to be acquired.

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MATERIALS AND METHODS

***C. neoformans*.** *C. neoformans* var. *neoformans* strains 24067 and B3501 (serotypes D) were acquired from the American Type Culture Collection (Manassas, VA). *C. neoformans* var. *grubii* strain H99 (serotype A) was obtained from John Perfect (Durham, NC).

Biofilm formation. *C. neoformans* strains were grown in Sabouraud dextrose broth (Difco Laboratories, Detroit, MI) for 24 h at 30°C in a rotary shaker at 150 rpm (to early stationary phase). The cells were collected by centrifugation, washed twice with phosphate-buffered saline (PBS), counted with a hemacytometer, and suspended at 10^7 cells/ml in minimal medium (20 mg/ml thiamine, 30 mM glucose, 26 mM glycine, 20 mM $MgSO_4 \cdot 7H_2O$, 58.8 mM KH_2PO_4). For each strain, 100 μl of the suspension was added into individual wells of polystyrene 96-well plates (Fisher), and the plates were incubated at 37°C without shaking. The biofilms were allowed to form for 48 h. Three wells without *C. neoformans* cells were used as controls. Following the adhesion stage, the wells containing *C. neoformans* biofilms were washed three times with 0.05% Tween 20 in Tris-buffered saline (TBS) to remove nonadhered cryptococcal cells with a microtiter plate washer (Skan Washer 400; Molecular Devices). Fungal cells that remained attached to the plastic surface were considered true biofilms. All assays were carried out in triplicate.

Measurement of biofilm metabolic activity by XTT reduction assay. A semiquantitative measurement of *C. neoformans* biofilm formation was obtained by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) reduction assay. For *C. neoformans* strains, 50 μl of XTT salt solution (1 mg/ml in PBS) and 4 μl of menadione solution (1 mM in acetone; Sigma Chemical Co.) were added to each well. The microtiter plates were incubated at 37°C for 5 h. The metabolic activity of the yeast cells within the biofilm is measured from mitochondrial dehydrogenase activity, which reduces XTT tetrazolium salt to XTT formazan, resulting in a colorimetric change. The colorimetric change was measured with a microtiter reader (Labsystem Multiskan MS, Finland) at 492 nm. Microtiter wells containing either heat-killed *C. neoformans* or minimal medium without *C. neoformans* cells were included as negative controls.

Killing assay. The toxicities of the antifungal drugs for *C. neoformans* biofilms and planktonic cells were compared by a CFU killing assay. After incubation with amphotericin B or caspofungin, *C. neoformans* biofilms were scraped from the bottoms of the wells with a sterile 200-μl micropipette tip to dissociate the yeast cells. A volume of 100 μl of suspension containing dissociated cells was aspirated from the wells, transferred to an Eppendorf tube with 900 μl of PBS, and vortexed gently for 3 min. Then, serial dilutions were performed and 100 μl of diluted suspension was plated on Sabouraud dextrose agar plates. The percentage of CFU survival was determined by comparing the survival of drug-treated *C. neoformans* biofilms and planktonic cells with the survival of untreated fungal cells.

***C. neoformans* planktonic cells.** To determine the density of *C. neoformans* planktonic cells used for comparison with the biofilms, we estimated the cell numbers from the XTT reduction signal using a dose-response curve. Briefly, cells of *C. neoformans* B3501 were grown in minimal medium for 48 h 30°C in a rotary shaker at 150 rpm, collected by centrifugation, washed twice with PBS, counted with a hemacytometer, and suspended at various densities (5×10^6, 1×10^7, and 5×10^7 cells/ml) in minimal medium. Then, 100 μl of each suspension was added into individual wells of polystyrene 96-well plates to final densities of 5×10^5, 1×10^6, and 5×10^6 cells/ml. The viability was measured by determination of the amount of XTT reduction.

Melanized fungal biofilms. Melanization was induced by growing the biofilms on defined minimal medium broth with the addition of 1 mM L-dopa for 7 days. Nonmelanized controls were obtained by growing the yeast cells on defined minimal medium broth without L-dopa for 7 days.

Antifungal drug susceptibility of *C. neoformans* biofilms. (i) Effects of antifungal drugs in preventing *C. neoformans* biofilm formation. To evaluate the effects of antifungal drugs on biofilm formation, cryptococci were suspended at 10^7 cells per ml in RPMI 1640 medium (Sigma Chemical Co., Cleveland, OH) in the presence or the absence of amphotericin B (Gibco, Carlsbad, CA), caspofungin (Merck, Rahway, NJ), fluconazole (Pfizer, Sandwich, England), or voriconazole (Pfizer, Sandwich, England) at 0.5, 1, 2, 4, 8, 16, 32, or 64 μg/ml. For each strain, 100 μl of the suspension was added into individual wells of polystyrene 96-well plates (Fisher). *C. neoformans* cells and antifungal drugs were mixed for 1 min by use of a microtiter plate reader (Labsystem Multiskan MS) to ensure a uniform distribution and were incubated at 37°C for 24 h. After 24 h of incubation, the wells containing cryptococcal cells were washed and biofilm formation was quantified by the XTT reduction assay. The effects of the antifungal drugs in preventing biofilm formation were determined by comparing the metabolic activities of planktonic yeast cells coincubated with antifungal drugs relative to those of similar planktonic yeast cells grown in PBS.

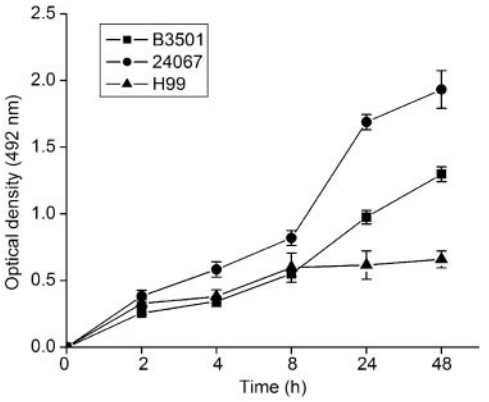


FIG. 1. Kinetics of *C. neoformans* biofilm formation in polystyrene microtiter plates, as determined by the colorimetric XTT reduction assay. The average of three XTT assay measurements was taken. This experiment was done twice, with similar results each time.

(ii) Susceptibilities of *C. neoformans* biofilms to antifungal drugs. To evaluate the susceptibilities of the *C. neoformans* biofilms to antifungal drugs, 200 μl of RPMI 1640 medium containing amphotericin B, caspofungin, fluconazole, or voriconazole (0, 2, 4, 8, 16, 32, or 64 μg/ml) was added to each well. Mature biofilms and antifungal drugs were mixed for 1 min by use of a microtiter plate reader to ensure a uniform distribution and were incubated at 37°C for 24 h. After 24 h of incubation, biofilm metabolic activity was quantified by the XTT reduction assay. The susceptibilities of the mature cryptococcal biofilms to antifungal drugs were determined by comparing the metabolic activities of the biofilms coincubated with antifungal drugs with those of the biofilms grown in PBS.

(iii) Comparison of biofilm and planktonic cryptococcal cell susceptibility to antifungal drugs. *C. neoformans* biofilms were incubated with 200 μl of PBS containing amphotericin B or caspofungin (0.5, 1, 2, 4, 8, 16, 32, or 64 μg/ml). Wells containing cryptococcal biofilms treated with PBS alone were used as a control. *C. neoformans* planktonic cells were suspended at a density of 5×10^6 cells per ml in PBS alone or in the presence of similar concentrations of amphotericin B or caspofungin. *C. neoformans* biofilms or planktonic cells and antifungal drugs were mixed to ensure a uniform distribution and were incubated at 37°C for 24 h. XTT reduction and CFU killing assays were used to determine the metabolic activity and fungal mass, respectively.

(iv) Comparison of melanized and nonmelanized fungal biofilm susceptibilities to antifungal drugs. *C. neoformans* biofilms were incubated with 200 μl of PBS containing amphotericin B or caspofungin (0.5, 1, 2, 4, 8, 16, 32 or 64 μg/ml). Wells containing melanized and nonmelanized biofilms treated with PBS alone were used as a control. Melanized or nonmelanized biofilms and antifungal drugs were mixed to ensure a uniform distribution and were incubated at 37°C for 24 h. The XTT reduction assay was used to determine viability.

Antifungal drug susceptibility. The susceptibilities of the biofilm and the planktonic cryptococcal phenotypes of strain B3501 to amphotericin B and caspofungin were determined by three independent methods. First, the MICs for planktonic cells were determined by the M27-A protocol developed by the CLSI (formerly the National Committee for Clinical Laboratory Standards) (22). Second, the XTT reduction assay was used to measure the diminution in metabolic activity (50% reduction in metabolic activity [RMA]) for biofilms and planktonic cells. Third, cell survival in the biofilm and the planktonic suspension was evaluated by the CFU killing assay.

Spot enzyme-linked immunosorbent assay (ELISA). *C. neoformans* strains were suspended at 10^4 cells per ml in minimal medium. For each strain, 100 μl of the suspension was added into individual wells of polystyrene 96-well plates and the plates were incubated at 37°C. *C. neoformans* cells were exposed to 2, 4, 8, 16, 32, and 64 μg/ml of amphotericin B, caspofungin, voriconazole, or fluconazole for 2 h at 37°C. Following the adhesion stage, the wells containing *C. neoformans* biofilms were washed three times with 0.05% Tween 20 in TBS to remove nonadherent cryptococcal cells by using a microtiter plate washer. All assays were carried out in five wells for each strain. The wells were then blocked for nonspecific binding by adding 200 μl of 1% bovine serum albumin (BSA) in

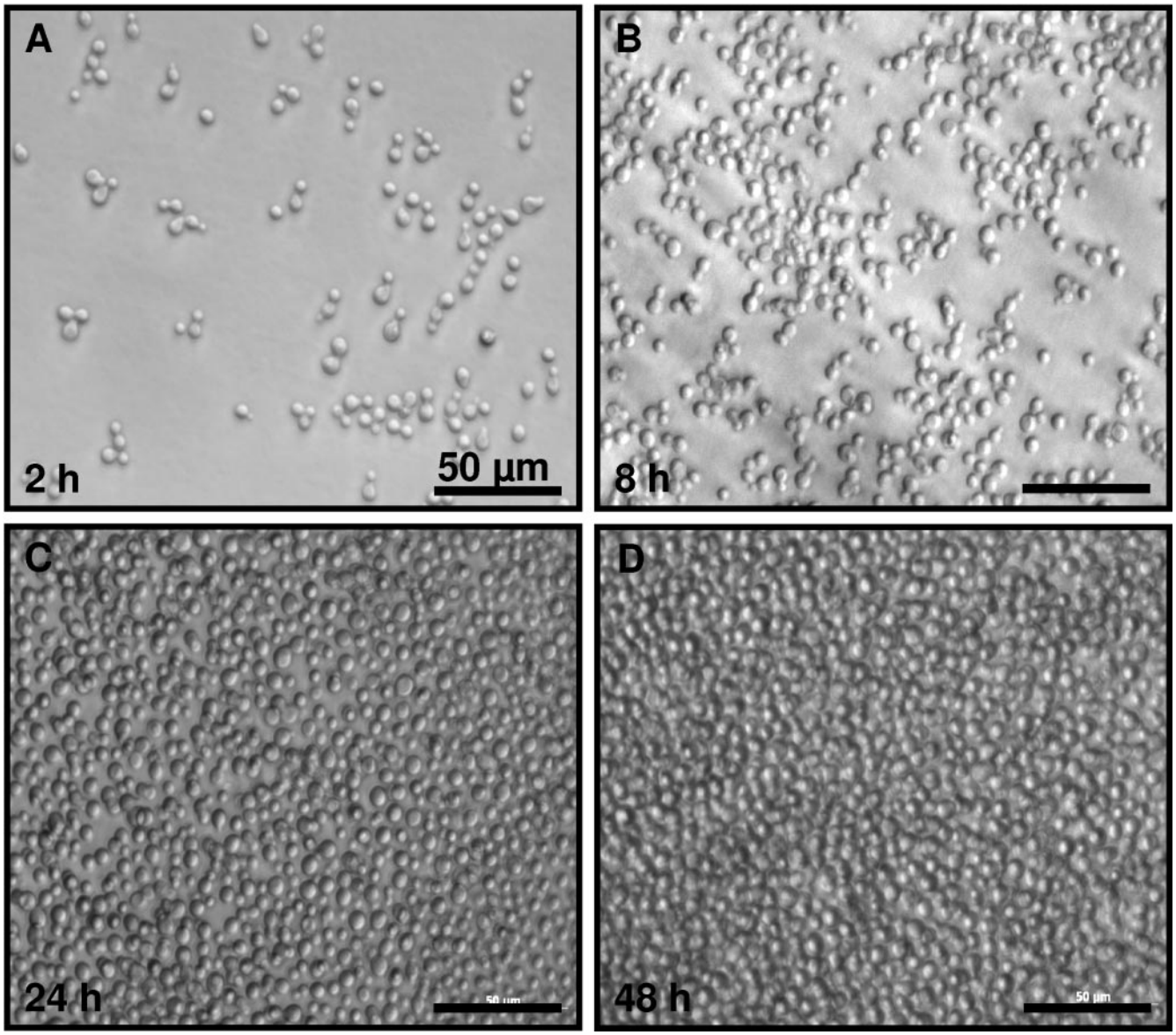


FIG. 2. Light microscopy images of *C. neoformans* strain B3501 biofilms after forceful washing with a microtiter plate washer. (A) Adhesion phase (2 h). The cryptococcal cells adhered to the bottom of the wells. At this stage the early biofilm is composed of cells undergoing budding or fungal growth in a monolayer fashion. (B) Intermediate phase (8 h). After attachment of the cryptococcal cells to the polystyrene plate, fungal growth involves the formation of microcolonies consisting of clustered cells. (C and D) Mature phase (24 to 48 h). A dense network of yeast cells bound to each other is formed by a combination of capsular polysaccharide fibers and extracellular material, creating a tenacious layer consisting of cells enmeshed in a polysaccharide matrix. At this point the thickness of the biofilm consists of several layers of cells. The pictures were taken by using a ×40 power field. Scale bars, 50 μm.

PBS. Next, 2 μg/ml of GXM binding monoclonal antibody 18B7 in PBS (1% BSA) was added, followed by the addition of 1 μg of biotin-labeled goat anti-mouse immunoglobulin G1/ml. Between every step, the wells were washed with 0.05% Tween 20 in TBS. All incubations were done at either 37 or 4°C overnight. After the biotinylated monoclonal antibody step, a 50-μl volume of 1 mg of bromo-4-chloro-3-indolyl phosphate (Amersco, Solon, OH) per ml diluted in AMP buffer (95.8 ml of 2-amino-2-methyl-1-propanol, 0.1 ml of Triton X-405, and 0.2 g of $MgCl_2 \cdot 6H_2O$ in 800 ml of double-distilled water [pH 8.6]; Sigma Chemical Co.). After 1 h the wells were washed five times with distilled water and air dried. Light microscopy was used to determine the area involved in the binding of the GXM released by the *C. neoformans* cells on the spot of attachment. The surface area of the spots was measured by tracing the circumference of the whole spot left by the organism at the equatorial plane (area = πr^2, where r is the radius of the spot).

Light microscopy. Microscopic examinations of the biofilms formed in microtiter plates were performed by light microscopy with an Axiovert 200 M inverted microscope (Carl Zeiss MicroImaging).

Confocal microscopy (CM). Mature *C. neoformans* biofilms were incubated for 45 min at 37°C in 75 μl of PBS containing the fluorescent stains FUN-1 (10 μM) and concanavalin A–Alexa Fluor 488 conjugate (ConA; 25 μM) (Molecular Probes, Eugene, OR). FUN-1 (excitation wavelength, 470 nm; emission wavelength, 590 nm) is converted to orange-red cylindrical intravacuolar structures by metabolically active cells, while ConA (excitation wavelength, 488 nm; emission wavelength, 505 nm) binds to the glucose and the mannose residues of cell wall and capsule polysaccharides and fluoresces green. Microscopic examinations of the biofilms formed in microtiter plates were performed by confocal microscopy with an Axiovert 200 M inverted microscope. A ×40 objective (numerical aperture, 0.6) was used. Depth measurements across the width of the device were

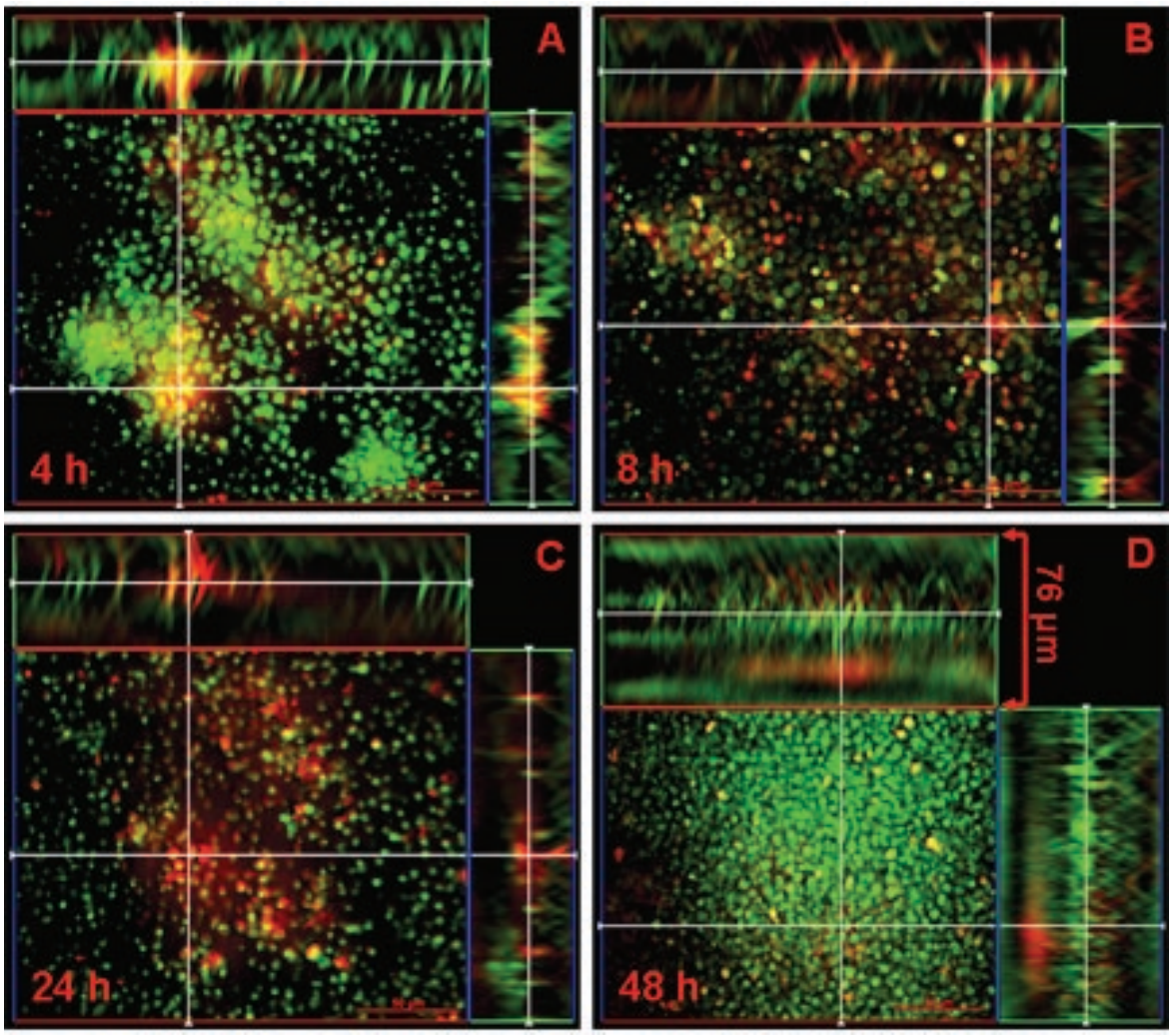


FIG. 3. Confocal microscopic images of *C. neoformans* strain B3501 biofilm grown on polystyrene plates reveal the organization of biofilm development. Orthogonal images of *C. neoformans* biofilm formation showed metabolically active (red, FUN-1-stained) cells embedded in the polysaccharide extracellular material (green, ConA-stained). For each panel (A to D), a top view of the biofilm sections is shown below the red line, and to the left of the blue line the images denote a *Z*-stack reconstruction. White lines indicate the location of the *Z*-stack sections in relation to the top view. The thickness of the biofilm can be observed in the upper and right side views of the *Z*-stack reconstruction. (A) Adhesion phase (4 h). The cryptococcal cells adhere to the bottom of the wells in a monolayer arrangement. Metabolically active fungal cells are concentrated in a small region of the field. (B) Intermediate phase (8 h). After attachment of the cryptococcal cells to the polystyrene plate, fungal growth and the increase in metabolic activity involve microcolony formation. (C) Early maturation phase (24 h). Exopolymeric matrix production of the cryptococcal biofilm begins. The metabolic activity of the biofilm remains high and steady. (D) Mature phase (48 h). The mature *C. neoformans* biofilm reveals a complex structure with internal regions of metabolically active cells interwoven with extracellular polysaccharide material. The thickness of a mature biofilm is approximately 76 μm. The pictures were taken by using a ×40 power field. Scale bars, 50 μm.

taken at regular intervals. To determine the structure of the biofilms, a series of horizontal (*x-y*) optical sections with a thickness of 1.175 μm were taken throughout the full length of the biofilm. Confocal images of green (ConA) and red (FUN-1) fluorescence were recorded simultaneously by using a multichannel mode. *Z*-stack images and measurements were corrected by using Axio Vision 4.4 software (Carl Zeiss MicroImaging) in the deconvolution mode.

Statistical analysis. All data were subjected to statistical analysis by using the Primer of Statistics—The Program (McGraw Hill Co., New York, NY). *P* values were calculated by analysis of variance and were adjusted by use of the Bonferroni correction. *P* values of <0.05 were considered significant.

RESULTS

***C. neoformans* biofilms.** The kinetics of biofilm formation by *C. neoformans* strains B3501, 24067, and H99 on the surfaces of polystyrene microtiter plates over 48 h was quantified by the colorimetric XTT reduction assay (Fig. 1). *C. neoformans* strain 24067 produced biofilms more efficiently than strains B3501 and H99. However, both serotype D strains were better biofilm producers than strain H99. The metabolic activity of the biofilm increased over time as the cellular mass increased.

The biofilms were metabolically active during the early and intermediate stages, which included the adhesion period (2 to 4 h) and the period of microcolony formation (8 to 12 h). During the adhesion period (2 h), the cryptococcal cells became firmly attached to the plastic surface in a monolayer arrangement. The cryptococcal cells adherent to the plastic support consisted of growing cells, as indicated by the frequent

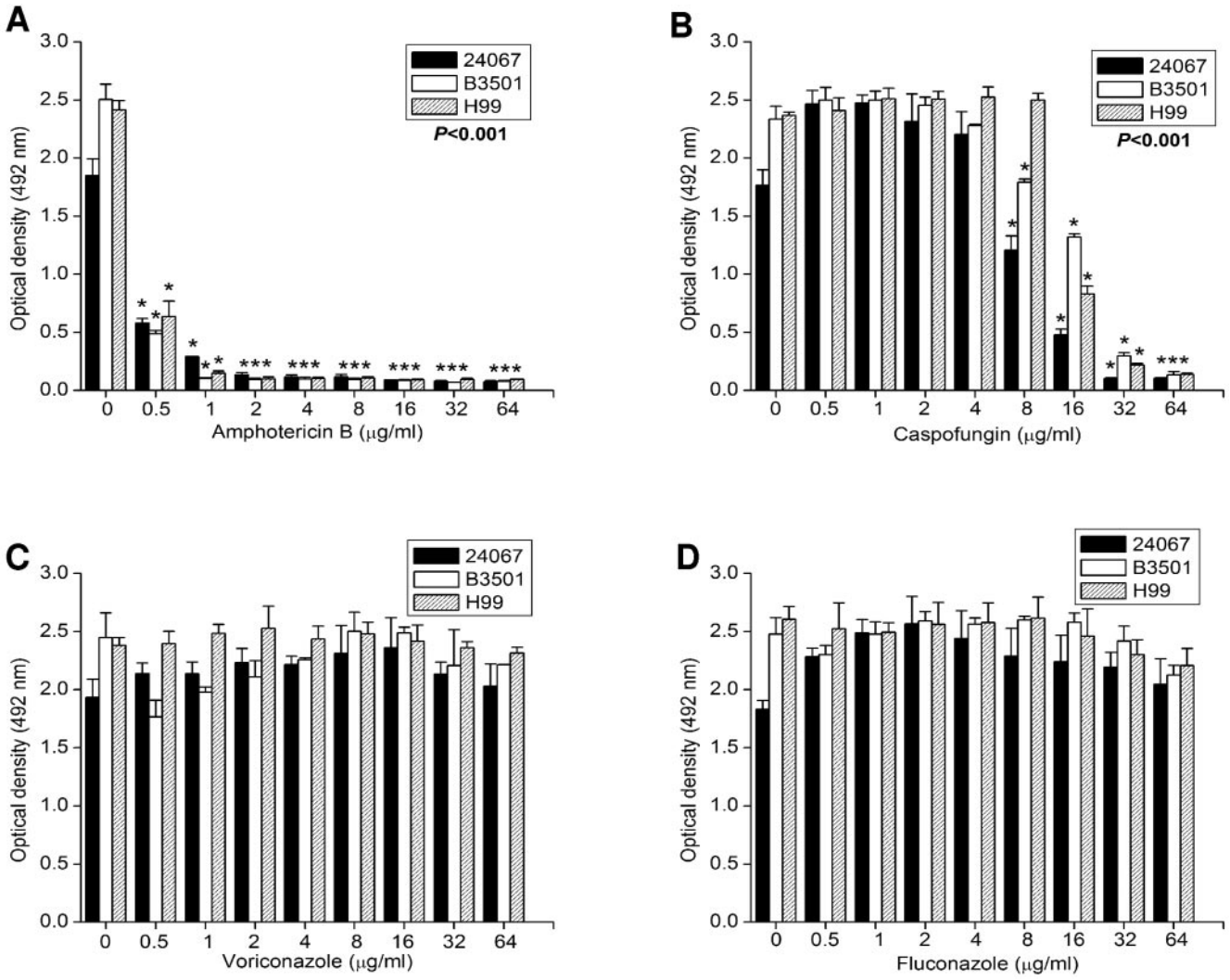


FIG. 4. Effects of four antifungal drugs on *C. neoformans* biofilm formation. The metabolic activities of *C. neoformans* strains 24067, B3501, and H99 were measured by the XTT reduction assay. Yeast cells were exposed to various concentrations (0.5, 1, 2, 4, 8, 16, 32, and 64 μg/ml) of amphotericin B (A), caspofungin (B), voriconazole (C), or fluconazole (D) for 24 h; and their biofilm production was compared to that of fungal cells incubated in PBS. Bars are the averages of three XTT measurements, and brackets denote standard deviations. Asterisks denote *P* value significance, calculated by analysis of variance and adjusted by use of the Bonferroni correction. This experiment was done twice, with similar results each time.

occurrence of budding (Fig. 2A). At the intermediate stage (8 h), the fungal population had increased and consisted of yeast cells spread uniformly throughout the plastic support, forming microcolonies (Fig. 2B). During the maturation stage (24 to 48 h), the microarchitecture of the *C. neoformans* biofilms became more complex due to an increase in the amount of extracellular material surrounding the cells and resulted in compact structures that tenaciously adhered to the plastic support (Fig. 2C and D).

Structural development of *C. neoformans* biofilm. Orthogonal images of *C. neoformans* biofilm growth in polystyrene 96-well plates were analyzed to determine biofilm structural development (Fig. 3). An intense green fluorescence resulting from ConA binding to polysaccharides outlined the cell walls of the yeast, while the red color, due to FUN-1 staining, localized to dense aggregates in the cytoplasm of metabolically active cells. During the adhesion stage of biofilm progression (4 h), individual yeasts could be distinguished at the top image due to a lack of exopolymeric matrix. Fungal cells attached to the plastic surface of the 96-well plate in a monolayer fashion with a relatively small area of metabolically active yeast cells (Fig. 3A). At the intermediate stage (8 h), a top view showed uniformly spread yeasts stained red, indicating an increase in the metabolic activity of the fungal population and microcolony formation (Fig. 3B). At the early maturation stage (24 h), individual fungal cells were indistinguishable due to the aggregation of exopolymeric material (Fig. 3C). At this time, the metabolic activity of the cryptococcal cells on the biofilms remained high and steady. Vertical (*x-z*) sectioning (side view) of three-dimensional reconstructed images showed that mature (48-h) *C. neoformans* biofilms had a highly organized structure (an ~76-μm-thick biofilm), with thin areas of metabolically active cells interwoven with extracellular polysaccharide material (Fig. 3D).

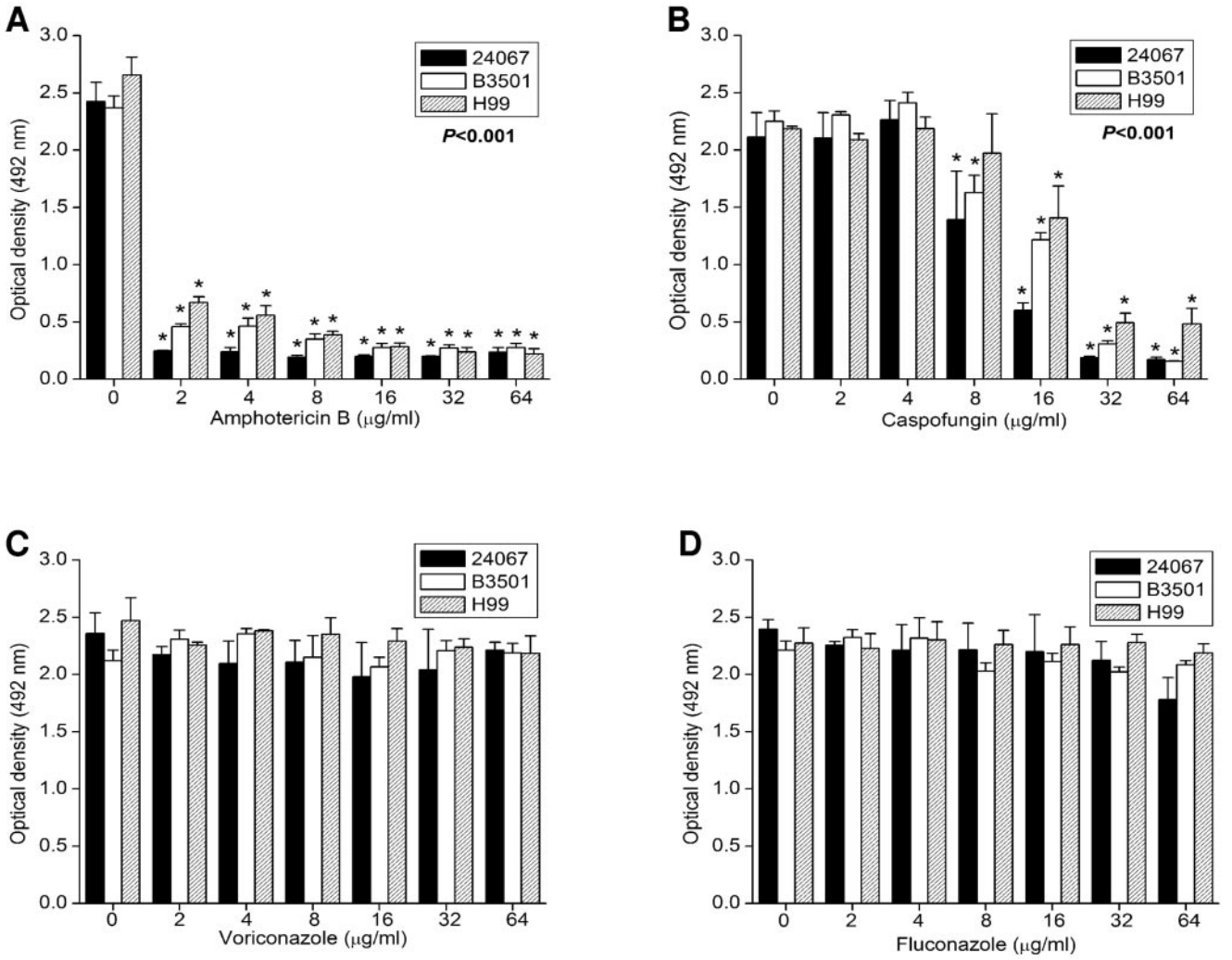


FIG. 5. Susceptibilities of *C. neoformans* biofilms to antifungal drugs. The metabolic activities of the biofilms produced by *C. neoformans* strains 24067, B3501, and H99 were measured by the XTT reduction assay. Biofilms were exposed to various concentrations (2, 4, 8, 16, 32, and 64 μg/ml) of amphotericin B (A), caspofungin (B), voriconazole (C), or fluconazole (D) for 24 h; and their susceptibilities were compared to those of biofilms incubated in PBS. Bars are the averages of three XTT measurements, and brackets denote standard deviations. Asterisks denote *P* value significance, calculated by analysis of variance and adjusted by use of the Bonferroni correction. This experiment was done twice, with similar results each time.

Azoles do not prevent *C. neoformans* biofilm formation. We investigated whether coincubation of antifungal agents, such as amphotericin B, caspofungin, fluconazole, or voriconazole, with yeast cells prevented *C. neoformans* biofilm formation in vitro using the XTT reduction assay (Fig. 4). Amphotericin B and caspofungin at concentrations greater than 0.5 and 8 μg/ml, respectively, prevented *C. neoformans* strains 24067 and B3501 from forming biofilms. *C. neoformans* strain H99 was able to form biofilms in RPMI 1640 medium and was similarly susceptible to amphotericin B with regard to biofilm production. However, a concentration of 16 μg/ml of caspofungin was necessary to affect *C. neoformans* strain H99 biofilm formation. Neither fluconazole nor voriconazole prevented biofilm formation at the concentrations tested.

***C. neoformans* biofilms are resistant to azoles.** To evaluate the susceptibilities of fungal cells in mature biofilms, cryptococcal biofilms were incubated with antifungal drugs and metabolic activity was measured by the XTT reduction assay (Fig. 5). *C. neoformans* strain 24067, B3501, and H99 biofilms were susceptible to amphotericin B and caspofungin at concentrations greater than 2 and 16 μg/ml, respectively. However, *C. neoformans* biofilms incubated with fluconazole and voriconazole at different concentrations displayed metabolic activities similar to those observed in the control biofilms. Hence, the *C. neoformans* biofilms were resistant to both fluconazole and voriconazole.

Spot ELISA. A spot ELISA was used to investigate the effects of amphotericin B, caspofungin, voriconazole, and fluconazole on *C. neoformans* biofilm formation, particularly during the adhesion stage. In Fig. 6, light microscopy was used to capture images of the spots formed by *C. neoformans* serotype D strain B3501 after treatment with antifungal drugs.

Amphotericin B and caspofungin prevented the binding of the *C. neoformans* GXM released on the spot of adhesion. The sizes

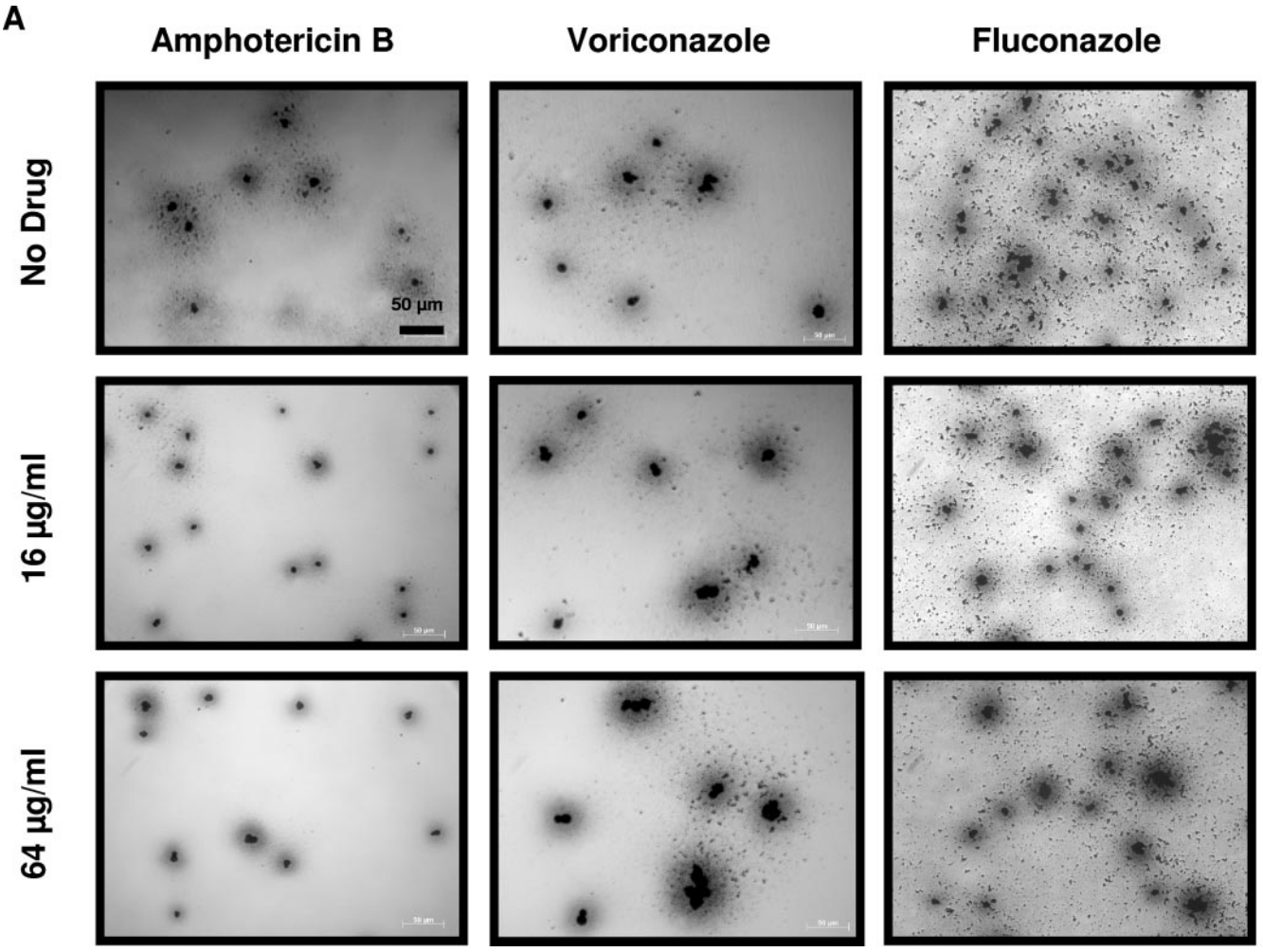


FIG. 6. Effects of antifungal drugs during the adhesion stage of *C. neoformans* biofilm formation. (A) Light microscopic images of spots formed by *C. neoformans* strain B3501 during the spot ELISA. Images were obtained after 2 h of exposure of the fungal cells to various concentrations (16 and 64 μg/ml) of amphotericin B, voriconazole, or fluconazole; and the images were compared with those of yeast cells incubated in presence of PBS. The pictures were taken by using a ×20 power field. Scale bars, 50 μm. The results are representative of those of two experiments. (B) The release of *C. neoformans* strain B3501 GXM was visualized by the spot ELISA after exposure of the yeast cells for 2 h to various concentrations (2, 4, 8, 16, 32, and 64 μg/ml) of amphotericin B, voriconazole, or fluconazole. Fungal cells incubated in the presence of PBS were used as a control. Bars are the averages of the areas of 20 spots per power field, with the area being calculated by the equation πr^2. Five power fields were observed for each time interval. Brackets denote standard deviations. Asterisks denote *P* value significance, calculated by analysis of variance and adjusted by use of the Bonferroni correction.

of the spots of *C. neoformans* serotype D strain B3501 decreased significantly after incubation of the fungal cells in the presence of amphotericin B (Fig. 6A). The surface areas of the strain B3501 spots were dramatically reduced after exposure of the yeast cells to 2 μg/ml of amphotericin B ($P < 0.001$) (Fig. 6B). All *C. neoformans* strains incubated in the presence of caspofungin were unable to form spots, whereas fungal cells grown in the absence of the drug were able to form spots. *C. neoformans* strain B3501 incubated in the presence of fluconazole or voriconazole formed similar spots at all concentrations tested (Fig. 6A and B). Results similar to those obtained for strain B3501 were observed for serotype D strain 24067 (data not shown). No spots were observed for strain H99 cells incubated in the presence of any of the drugs tested (data not shown).

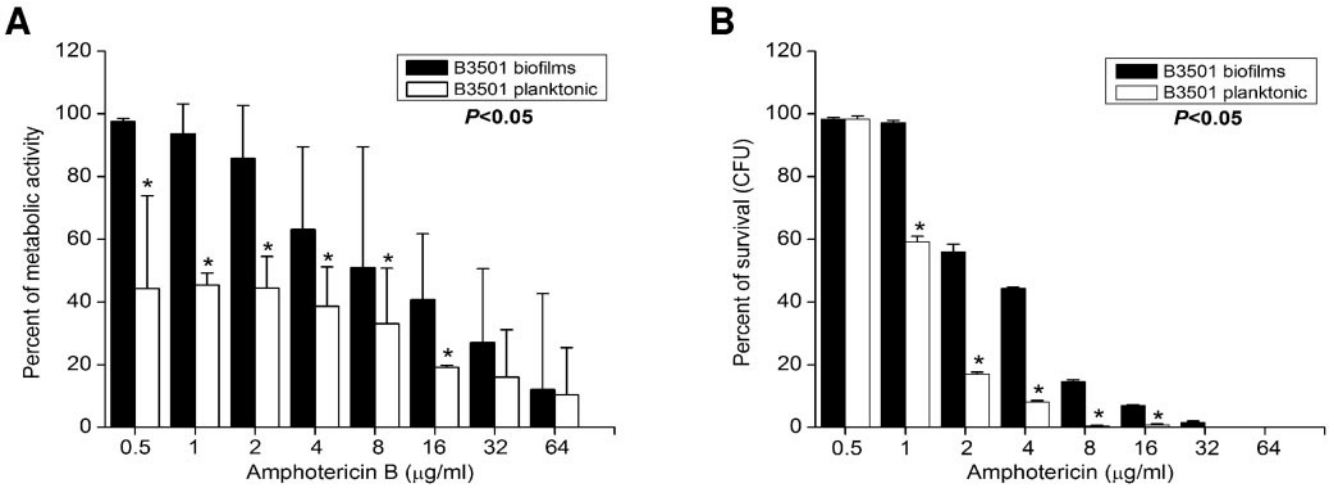


FIG. 7. *C. neoformans* biofilms are more resistant to amphotericin B than planktonic cells. (A) The percentage of metabolic activity of *C. neoformans* strain B3501 biofilms and planktonic cells was measured by the XTT reduction assay. Both phenotypes were exposed to various concentrations (0.5, 1, 2, 4, 8, 16, 32, and 64 μg/ml) of amphotericin B for 24 h; and their metabolic activities were compared to those of fungal cells incubated in PBS. (B) The percent survival of *C. neoformans* strain B3501 biofilms and planktonic cells was measured by determination of the numbers of CFU. Both phenotypes were exposed to various concentrations (0.5, 1, 2, 4, 8, 16, 32, and 64 μg/ml) of amphotericin B for 24 h; and their rates of survival were compared to those of fungal cells incubated in PBS. For panels A and B, the bars are the averages of three measurements, and brackets denote standard deviations. Asterisks denote *P* value significance, calculated by analysis of variance and adjusted by use of the Bonferroni correction. This experiment was done twice, with similar results each time. (C) CM of *C. neoformans* B3501 biofilms and planktonic cells treated with amphotericin B. Orthogonal images of mature *C. neoformans* biofilms and planktonic cells showed metabolically active (red, FUN-1-stained) cells embedded in the polysaccharide extracellular material (green, ConA stained), while the yellow-brownish areas represent metabolically inactive or nonviable cells. Images were obtained after 24 h of exposure of the fungal cells to various concentrations (4 and 16 μg/ml) of amphotericin B, and the images were compared with those of yeast cells incubated in presence of PBS. The pictures were taken by using a ×40 power field. Scale bars, 50 μm. The results are representative of those of two experiments.

***C. neoformans* biofilms are more resistant to amphotericin B than planktonic cells.** *C. neoformans* biofilms were significantly more resistant to amphotericin B than planktonic cells when viability was measured by the XTT reduction assay (Fig. 7A). For instance, the metabolic activities of cryptococcal biofilms were reduced approximately 35 and 50% only when biofilms were treated with 4 and 8 μg/ml of amphotericin B, respectively. In contrast, the metabolic activities of planktonic cells were significantly reduced after treatment with 0.5 μg/ml of amphotericin B.

To confirm the results obtained by the XTT reduction assay, the percent survival of the cells in the biofilm or the planktonic form was determined by counting the numbers of CFU in wells treated with amphotericin B and comparing these to the numbers of colonies obtained from untreated wells (Fig. 7B). *C. neoformans* planktonic cells were more susceptible to amphotericin B than biofilms after treatment with 1 μg/ml. The survival of biofilms was reduced approximately 40 and 50% after treatment with 2 and 4 μg/ml of amphotericin B, respectively. *C. neoformans* cells in the biofilm and planktonic forms were killed significantly (80% of cells) after treatment with 8 and 2 μg/ml of amphotericin B, respectively.

Confocal microscopic examination was used to correlate the XTT reduction and CFU killing assay results with the visual effects on biofilm metabolism and structure (Fig. 7C). Regions of red fluorescence (FUN-1) represent metabolically active cells, the green fluorescence (ConA) indicates cell wall or capsule polysaccharides, and yellow-brownish areas represent metabolically inactive or nonviable cells. *C. neoformans* biofilms and planktonic cells grown in the presence of PBS alone showed regions of high metabolic activity. Biofilms treated with 4 μg/ml of amphotericin B manifested a decrease in the thickness of the exopolymeric matrix and metabolic activity. Biofilms treated with 16 μg/ml of amphotericin B manifested architectural disruption. Planktonic cells treated with 4 and 16 μg/ml of amphotericin B had a significant reduction in metabolic activity.

***C. neoformans* cells in biofilms are more resistant to caspofungin than planktonic cells.** *C. neoformans* biofilms were less susceptible to caspofungin than planktonic cells, as measured by the XTT reduction and CFU killing assays. In both assays, *C. neoformans* biofilms and planktonic cells showed 50% reductions in viability after treatment with 32 and 8 μg/ml of caspofungin, respectively (Fig. 8A and B). Confocal microscopic examination of the effects of caspofungin on planktonic cells and biofilms revealed regions of high metabolic activity (Fig. 8C). Biofilms treated with 16 μg/ml of caspofungin showed distinguishable viable and nonviable cells due to partial disruption and a decreased thickness of the exopolymeric matrix. Biofilms treated with 64 μg/ml of caspofungin showed a monolayer arrangement of clustered metabolically active cells and disruption of the exopolymeric architecture. Planktonic cells treated with 16 and 64 μg/ml of caspofungin showed a decrease in metabolic activity and a lack of capsular polysaccharide.

Relationship of MIC, 50% RMA, and 50% RS methods for drug susceptibility. Table 1 summarizes the in vitro susceptibilities of biofilm versus planktonic cells of *C. neoformans* strain B3501 to amphotericin B and caspofungin. Three independent methods demonstrated that the cryptococcal biofilms were more resistant than the planktonic cells to amphotericin

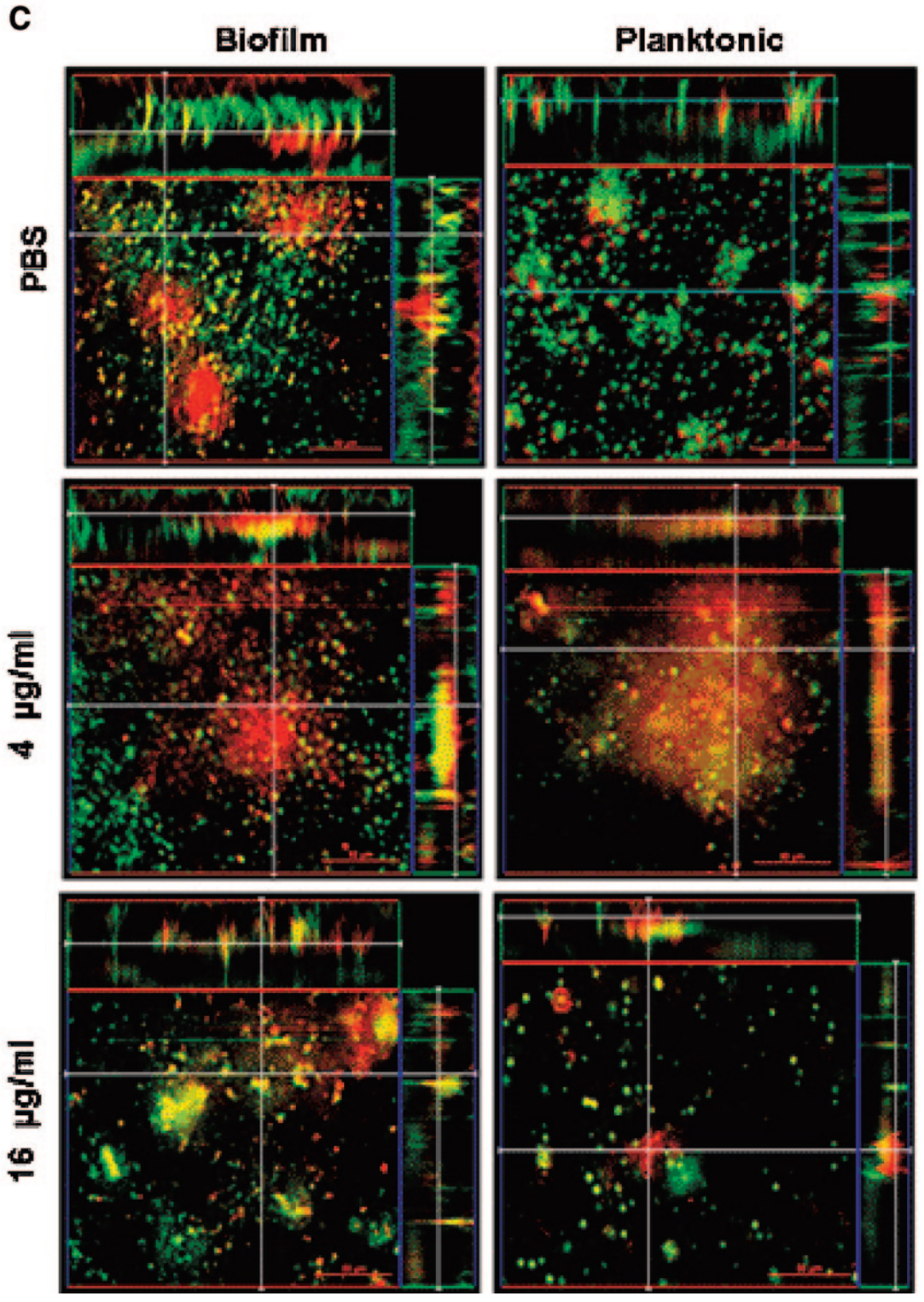


FIG. 7—*Continued.*

B and caspofungin. However, the results obtained for MIC, RMA, and reduction in survival (RS) for planktonic cells and the results obtained for RMA and RS for biofilms treated with amphotericin B varied, depending on the method. In contrast, similar results for MIC, RMA, and RS for both phenotypes were obtained after treatment with caspofungin.

Melanization enhances biofilm resistance to antifungal drugs. Melanized *C. neoformans* strain B3501 biofilms were signifi-

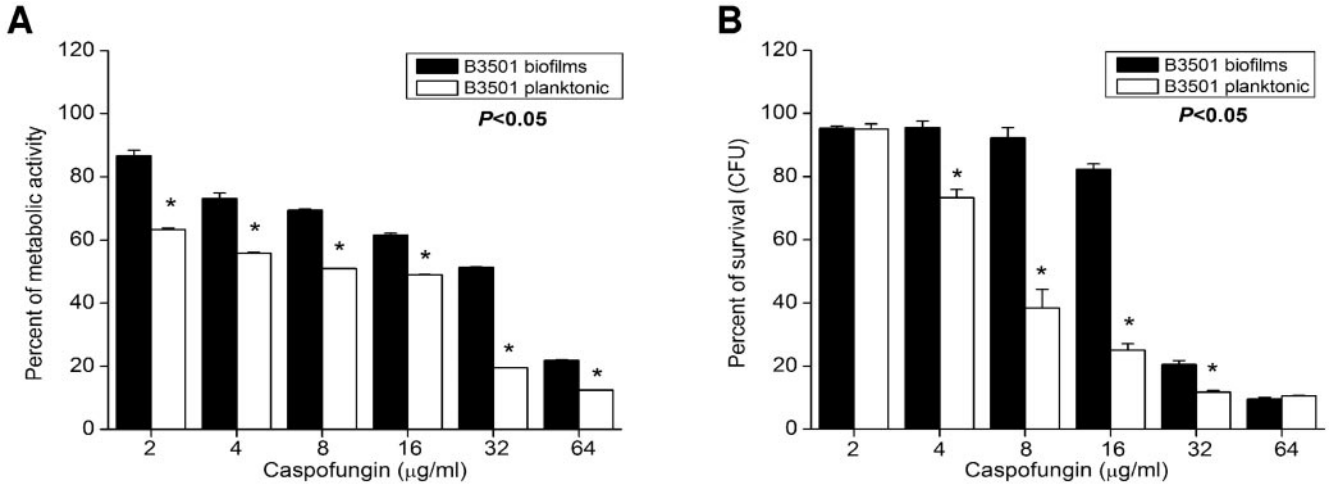


FIG. 8. *C. neoformans* biofilms are more resistant to caspofungin than planktonic cells. (A) The percent metabolic activity of *C. neoformans* strain B3501 biofilms and planktonic cells was measured by the XTT reduction assay. Cells in biofilms and planktonic forms were exposed to various concentrations (0.5, 1, 2, 4, 8, 16, 32, and 64 μg/ml) of caspofungin for 24 h, and their metabolic activities were compared to those of fungal cells incubated in PBS. (B) The percent survival of *C. neoformans* strain B3501 biofilms and planktonic cells was measured by determination of the numbers of CFU. Both phenotypes were exposed to various concentrations (0.5, 1, 2, 4, 8, 16, 32, and 64 μg/ml) of caspofungin for 24 h, and their rates of survival were compared to those of fungal cells incubated in PBS. For panels A and B, bars are the averages of three measurements, and brackets denote standard deviations. Asterisks denote *P* value significance, calculated by analysis of variance and adjusted by use of the Bonferroni correction. This experiment was done twice, with similar results each time. (C) CM of *C. neoformans* B3501 biofilms and planktonic cells treated with caspofungin. Orthogonal images of mature *C. neoformans* biofilms and planktonic cells showed metabolically active (red, FUN-1-stained) cells embedded in the polysaccharide extracellular material (green, ConA stained), while the yellow-brownish areas represent metabolically inactive or nonviable cells. Images were obtained after 24 h of exposure of fungal cells to various concentrations (4 and 16 μg/ml) of caspofungin, and the images were compared with those of yeast cells incubated in presence of PBS. The pictures were taken by using a ×40 power field. Scale bars, 50 μm. The results are representative of those of two experiments.

cantly less susceptible to amphotericin B or caspofungin than nonmelanized biofilms. Light microscopy revealed thick dark cell walls indicative of melanin production in cryptococcal biofilms after incubation with L-dopa (Fig. 9A). Melanized biofilms were less susceptible to amphotericin B and caspofungin than nonmelanized biofilms at concentrations >2 and >16 μg/ml, respectively (Fig. 9).

DISCUSSION

We evaluated the antifungal activities of amphotericin B, caspofungin, voriconazole, and fluconazole against *C. neoformans* biofilms. Amphotericin B was the most effective agent in preventing *C. neoformans* biofilm establishment and against mature biofilms, and the activity of this drug was closely followed by that of caspofungin. *C. neoformans* biofilms were significantly less susceptible than planktonic cells to both amphotericin B and caspofungin. These results correlate with those presented in other reports that have suggested that the biofilm phenotype confers resistance to antifungal drug therapy (11, 16). Both drugs mediated a significant reduction in the metabolic activity of *C. neoformans* cells, consistent with their fungicidal properties; but the concentrations used were high and were above the levels achievable in vivo after systemic administration. Hence, none of the agents tested had activity at physiological levels. However, the drug concentrations used here should be achievable inside intravascular catheter lumens or after local infusion.

Amphotericin B is a strong fungicidal compound with a broad spectrum of activity, since this agent binds to ergosterol and destabilizes the cellular membranes of diverse fungi (4, 11, 27). Based on light microscopy after the spot ELISA, it is most likely that amphotericin B interferes with *C. neoformans* GXM shedding, since there was a reduction in the area of the spots when the concentration of this compound was increased (14). In fact, there is evidence that amphotericin B affects capsule formation even at a concentrations below the MIC (23, 33). The release of the cryptococcal polysaccharide capsule is important for adhesion of the yeast and biofilm development in *C. neoformans* (18).

Caspofungin inhibits the synthesis of β-1,3-glucan, a major component of the fungal cell wall. Our results showed that addition of caspofungin to *C. neoformans* biofilms significantly reduced metabolic activity and prevented the adhesion of the yeast cells to the polystyrene surface. The cell wall is the structure that mediates the cell's interactions with the environment, and it is involved with the adhesive properties of the fungus *Candida albicans* (9). Bachmann et al. proposed the use of the cell wall as an attractive target for the development of strategies that combat biofilm-associated infections (3). Caspofungin has antiadherent activity and prevents *C. albicans* biofilm development (29). Other studies have proposed the treatment of medical devices with antifungal agents before they are implanted in patients (3, 31). Caspofungin may be a good candidate for this endeavor, due to its antiadherent properties against fungal biofilms. However, caspofungin has not been demonstrated to have clinical utility against *C. neoformans* infections, possibly because the melanin deposited in the cell wall protects the fungus (32). Consistent with prior observations showing that melanized planktonic cells are less suscep-

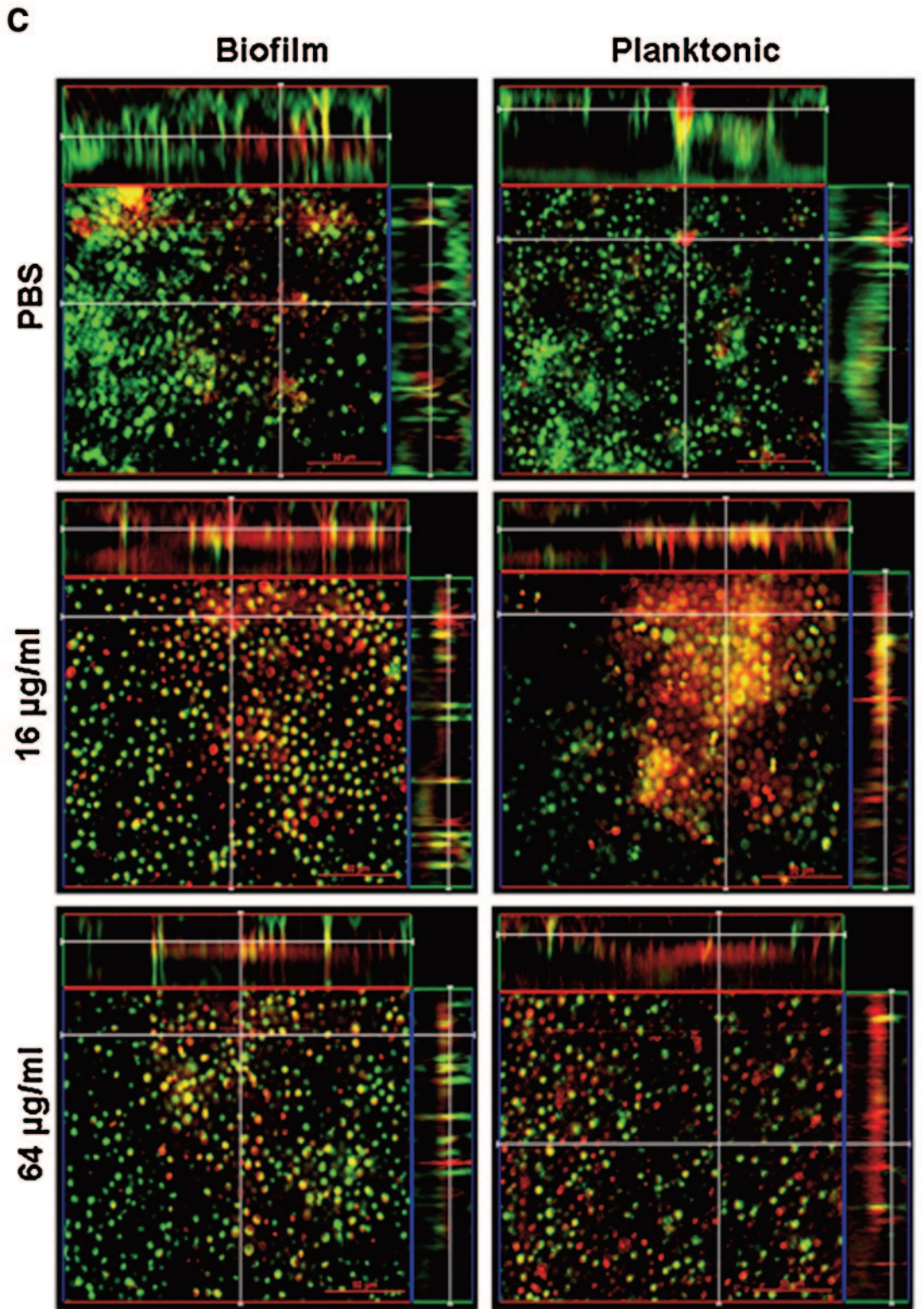
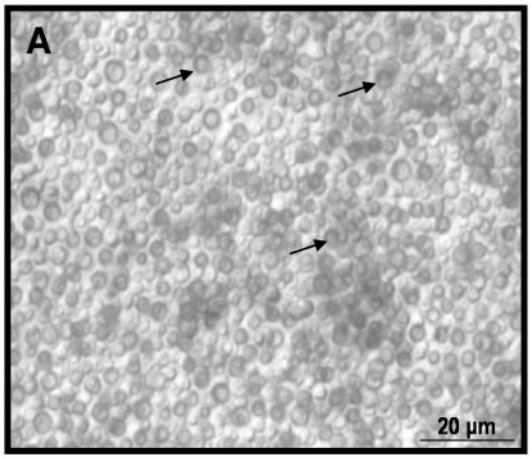
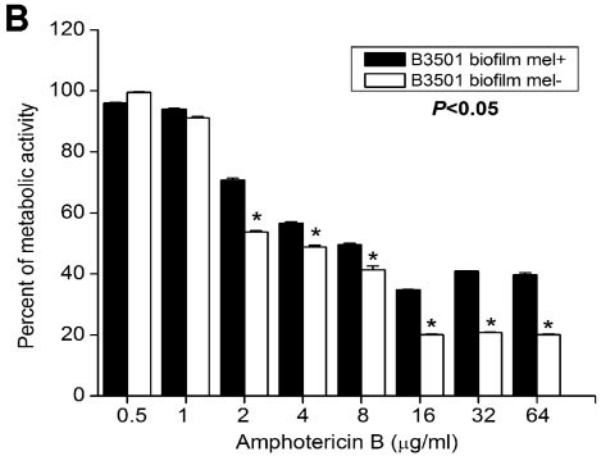


FIG. 8—*Continued.*

tible to amphotericin B and caspofungin (32), melanized biofilms also manifested reduced susceptibilities to these drugs.

The inability of the two azole compounds, voriconazole and fluconazole, to significantly inhibit the metabolic activity of *C. neoformans* cells in biofilms may be a consequence of the fungistatic nature of these drugs. Recently, van Duin et al. suggested that voriconazole is a promising candidate for use against cryptococcosis due to its penetration into the cerebro-





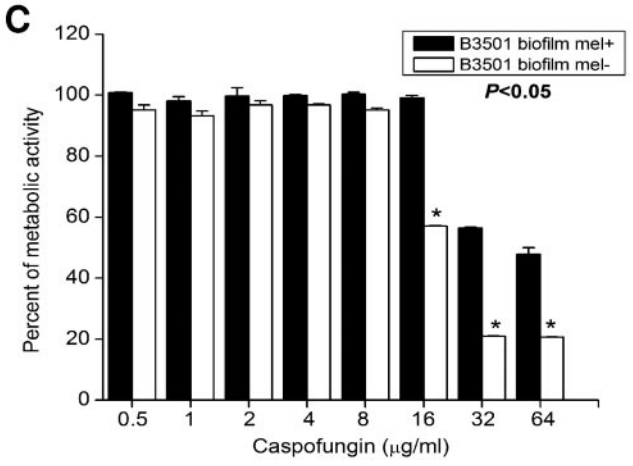


FIG. 9. Melanized *C. neoformans* biofilms were less susceptible to antifungal drugs. (A) Light microscopic image of a melanized *C. neoformans* strain B3501 biofilm. Arrows denote melanin deposition in the cell wall of cryptococcal cells. Magnification ×400. Scale bar, 20 μm. (B and C) Percent metabolic activity of melanized and nonmelanized *C. neoformans* strain B3501 biofilms measured by the XTT reduction assay. The cells in biofilms were exposed to various concentrations (0.5, 1, 2, 4, 8, 16, 32, and 64 μg/ml) of amphotericin B (B) or caspofungin (C) for 24 h, and their metabolic activities were compared to those of fungal cells incubated in PBS.

spinal fluid and because its antifungal activity can be fungicidal for planktonic cells (33). In contrast, *C. neoformans* cells in biofilms were not killed by voriconazole. Light microscopy of spot ELISA plates suggested that neither azole compound prevented *C. neoformans* GXM release, which is the first step in the process of yeast adhesion and subsequent biofilm formation. It has been proposed that the exopolymeric matrix confers antimicrobial resistance to microbial biofilms (5). Individual fungal cells encased in a biofilm may sacrifice proliferation by lowering their growth rate, activating quorum sensing, and coordinating the collective production of an exopolymeric matrix that may act as a physical barrier that prevents the penetration of antifungal agents. Hence, the lack of activity of the azole drugs in this system may reflect the fact that the cryptococcal cells can attach to polystyrene in their presence and rapidly become enmeshed in a polysaccharide matrix.

TABLE 1. MIC, RMA, and RS for *C. neoformans* B3501 biofilm and planktonic cells

Drug	Planktonic cells			Biofilms	
	MIC_{50}[a] (μg/ml)	50% RMA[b] (μg/ml)	50% RS[c] (μg/ml)	50% RMA (μg/ml)	50% RS (μg/ml)
Amphotericin B	0.125	0.5	2	8	4
Caspofungin	8	8	8	32	32

[a] MIC_{50}, the MIC at which 50% of isolates are inhibited, as determined by the CLSI M27-A method (22).
[b] 50% RMA, a 50% reduction in metabolic activity, as determined by the XTT reduction assay.
[c] 50% RS, a 50% reduction in survival, as determined by the CFU killing assay.

C. neoformans strain H99 did not form a strong biofilm in minimal medium with glucose as a carbon source. However, this serotype A strain formed biofilms as strong as those of serotype D strains 24067 and B3501 when it was grown in RPMI 1640 medium. RPMI 1640 medium is rich in nutrients and vitamins and allows the growth of many types of cells. The solid-liquid interface between a surface and an aqueous medium provides an ideal environment for the attachment and growth of microorganisms. When polystyrene material is exposed to RPMI 1640 medium, it becomes conditioned or coated by compounds of the medium and may affect the rate and the extent of *C. neoformans* strain H99 attachment. For instance, Mittelman reported that a number of host-produced conditioning films, such as blood, tears, urine, saliva, intervascular fluid, and respiratory secretions, influence the attachment of bacteria to biomaterials (20). Furthermore, we have

previously observed that when polystyrene plates are treated with bovine serum albumin (1%) and *C. neoformans* strain H99 is grown in minimal medium, fungal cells are able to form mature biofilms (data not shown). These results suggest that factors such as substrate conditioning and the characteristics of the medium are important for microbial biofilm development.

In conclusion, this is the first report in which the susceptibility of *C. neoformans* biofilms to antifungal agents has been investigated. *C. neoformans* biofilms were more resistant than planktonic cells to amphotericin B and caspofungin and were completely resistant to the two azole compounds, fluconazole and voriconazole. The observations with the *C. neoformans* system are consistent with those of studies with other fungi, indicating that fluconazole is not a potent agent against fungal biofilms (3, 7, 8, 16, 17). Additionally, exposure of *C. neoformans* cells or preformed cryptococcal biofilms to voriconazole did not inhibit subsequent biofilm formation or affect the metabolic activity of biofilms, respectively. These findings now need to be validated with animal models of cryptococcosis, with the caveat that no in vivo models of cryptococcal biofilm formation currently exist. Although one must be extremely cautious in extrapolating in vitro observations to clinical situations, our results suggest that amphotericin B may be a superior agent in those situations in which biofilm formation is expected to occur, such as in cryptococcal shunt infections.

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