

Cryptococcus neoformans Biofilm Formation Depends on Surface Support and Carbon Source and Reduces Fungal Cell Susceptibility to Heat, Cold, and UV Light[∇]

Luis R. Martinez and Arturo Casadevall*

Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York

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The fungus *Cryptococcus neoformans* possesses a polysaccharide capsule and can form biofilms on medical devices. We describe the characteristics of *C. neoformans* biofilm development using a microtiter plate model, microscopic examinations, and a colorimetric 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium-hydroxide (XTT) reduction assay to observe the metabolic activity of cryptococci within a biofilm. A strong correlation between XTT and CFU assays was demonstrated. Chemical analysis of the exopolymeric material revealed sugar composition consisting predominantly of xylose, mannose, and glucose, indicating the presence of other polysaccharides in addition to glucurunoxylomannan. Biofilm formation was affected by surface support differences, conditioning films on the surface, characteristics of the medium, and properties of the microbial cell. A specific antibody to the capsular polysaccharide of this fungus was used to stain the extracellular polysaccharide matrix of the fungal biofilms using light and confocal microscopy. Additionally, the susceptibility of *C. neoformans* biofilms and planktonic cells to environmental stress was investigated using XTT reduction and CFU assays. Biofilms were less susceptible to heat, cold, and UV light exposition than their planktonic counterparts. Our findings demonstrate that fungal biofilm formation is dependent on support surface characteristics and that growth in the biofilm state makes fungal cells less susceptible to potential environmental stresses.

Cryptococcus neoformans is an encapsulated yeast-like fungus that frequently causes life-threatening meningoencephalitis in immunocompromised individuals. Glucurunoxylomannan (GXM) is the major component of the polysaccharide capsule. GXM is copiously shed in tissues during cryptococcal infection and is believed to contribute to pathogenesis through many deleterious effects on the host's immunity (1, 16). GXM release is essential for cryptococcal biofilm formation (9), a strategy that has been associated with chronic infections as a result of acquired resistance to host immune mechanisms (8) and antimicrobial therapy (11). *C. neoformans* forms biofilms on polystyrene plates (9) and prosthetic medical devices, including ventriculoatrial shunt catheters (17). 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.

Recent technological advances have provided evidence that biofilm formation represents the most common mode of growth of microorganisms in nature, a state that presumably allows microbial cells to both survive hostile environments and disperse to colonize new niches (5). *C. neoformans* has several well-characterized virulence factors that allow the fungus to evade multiple defenses and damage the host, including the polysaccharide capsule, the ability to grow at mammalian temperatures, and melanin production. *C. neoformans* is fre-

quently found in soil contaminated with pigeon droppings (7), where the fungus may be in contact with many soil predators, and this interaction might have influenced the evolution of its virulence factors (15).

Biofilm formation is a major consideration in determining appropriate therapeutic strategies against certain microbes. Biofilm formation is a well-organized process that depends on effects of the surface, conditioning films on the surface, characteristics of the medium, and properties of the microbial cell (3). In this study, we investigated the effect of these factors on cryptococcal biofilm formation and compared the susceptibilities of mature *C. neoformans* biofilms and planktonic cells to environmental stress. Biofilm development and architecture were explored under different conditions of growth using microscopy techniques.

MATERIALS AND METHODS

***C. neoformans*.** *C. neoformans* strain B3501 (serotype D) was acquired from the American Type Culture Collection (Rockville, MD) and used in all experiments. This strain forms strong biofilms on polystyrene surfaces (9). The *cap59* gene deletion mutant (C536) of *C. neoformans* B3501 was acquired from K. J. Kwon-Chung (Bethesda, MD). Yeasts 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).

Biofilm formation. *C. neoformans* cells were collected by centrifugation, washed twice with phosphate-buffered saline (PBS), counted using a hemacytometer, and suspended at 10^7 cells/ml in minimal medium (20 mg/ml thiamine, 30 mM glucose, 26 mM glycine, 20 mM $MgSO_4 \cdot 7H_2O$, and 58.8 mM KH_2PO_4). Then, 100 μ l of the suspension was added to individual wells of polystyrene 96-well plates (Fisher, MA) and incubated at 37°C without shaking. Biofilms were formed for 48 h. 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 using a microtiter plate washer (Skan Washer 400; Molecular Devices, VA). Fungal cells that

* Corresponding author. Mailing address: Department of Medicine, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Phone: (718) 430-2215. Fax: (718) 430-8968. E-mail: casadeva@aecom.yu.edu.

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remained attached to the plastic surface were considered true biofilms. All assays were carried out in six wells.

Measurement of biofilm metabolic activity by XTT reduction assay and CFU count. A semiquantitative measurement of *C. neoformans* biofilm formation was obtained from the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium-hydroxide (XTT) reduction assay. For *C. neoformans* strains, 50 μ l of XTT salt solution (1 mg/ml in PBS) and 4 μ l of menadione solution (1 mM in acetone; Sigma) were added to each well. Microtiter plates were incubated at 37°C for 5 h. Fungal mitochondrial dehydrogenase activity reduces XTT tetrazolium salt to XTT formazan, resulting in colorimetric change that correlates with cell viability (13). The colorimetric change was measured using a microtiter reader (Labsystems Multiskan MS; Labsystems, Finland) at 492 nm. In all the experiments, microtiter wells containing heat-killed *C. neoformans* and minimal medium alone were included as negative controls. For the environmental stress experiments, the percentage of metabolic activity was determined by measuring the optical density of biofilms and planktonic cells exposed to heat, cold, or UV light relative to unexposed biofilms and planktonic cells.

To determine the density of the *C. neoformans* planktonic cells used for comparison to the biofilms, we estimated 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 at 30°C in a rotary shaker at 150 rpm, collected by centrifugation, washed twice with PBS, counted using a hemacytometer, and suspended at various densities (5×10^6 , 1×10^7 , and 5×10^7 cells/ml) in minimal medium. Then, 100 μ l of each suspension was added to 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 XTT reduction assay.

The effects of environmental stress on *C. neoformans* biofilms and planktonic cells were compared by a CFU killing assay. After exposure to environmental stress, *C. neoformans* biofilms were scraped from the bottom of the wells with a sterile 200- μ l micropipette tip to dissociate yeast cells. A volume of 100 μ l of suspension containing dissociated cells was aspirated from the wells, transferred to an Eppendorf tube with 900 μ l of PBS, and vortexed gently for 3 min. Then, serial dilutions were performed, and 100 μ l of diluted suspension was plated on Sabouraud dextrose agar plates. The percentage of CFU survival was determined by comparing stress-exposed *C. neoformans* biofilms and planktonic cells to unexposed fungal cells.

Correlation between XTT and CFU assays in measuring cell viability on biofilms. *C. neoformans* biofilms were formed over a series of time intervals (2, 4, 8, 24, and 48 h) on polystyrene microtiter plates. The metabolic activity and fungal mass were measured by XTT reduction and CFU counts, respectively. The correlation between the XTT reduction and CFU assay results in quantifying cryptococcal biofilm formation was evaluated.

Isolation of *C. neoformans* biofilm exopolymeric matrix. *C. neoformans* biofilms were formed on a 96-well polystyrene plate for 48 h at 37°C. After the removal of nonadherent cells by washing with Endosafe reagent water (Charles Rivers Laboratories, Charleston, SC), cryptococcal biofilms were scraped from the bottom of the wells with a sterile 200- μ l micropipette tip to dissociate yeast cells and exopolymeric matrix. A volume of 100 μ l of suspension containing dissociated cells and matrix was aspirated from the wells, transferred to an Eppendorf tube, and vortexed vigorously for 5 min. Then, cryptococci and exopolymeric matrix were separated by centrifugation at 2,000 rpm for 5 min. The supernatant containing the carbohydrates was collected and analyzed.

Glycosyl composition analysis of matrix material. Glycosyl composition analysis was performed by combined gas chromatography/mass spectrometry of the per-*O*-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis. The analysis was conducted at the Center for Plant and Microbial Complex Carbohydrates (Athens, GA). Methyl glycosides were first prepared from dry samples by methanolysis in 1 M HCl in methanol at 80°C (18 to 22 h), followed by re-*N*-acetylation with pyridine and acetic anhydride in methanol (for detection of amino sugars). The samples were then per-*O*-trimethylsilylated by treatment with Tri-Sil (Pierce, Rockford, IL) at 80°C (0.5 h). Gas chromatography/mass spectrometry analysis of the TMS methyl glycosides was performed on an HP 5890 gas chromatograph interfaced to an HP 5970 mass selective detector, using an All Tech EC-1 fused silica capillary column (inside diameter, 30 m by 0.25 mm).

Adhesion assay. Adhesion of *C. neoformans* cells was determined by cell counts using a PhotoZoom inverted light microscope (Cambridge Instrument, MA). The number of cryptococcal cells attached to the bottom of each well was averaged per 40-power field. This assay was done in triplicate.

***C. neoformans* capsular polysaccharide isolation.** *C. neoformans* was grown in Sabouraud dextrose broth for 7 days at 30°C in a rotary shaker at 150 rpm. The supernatant was collected and filtered through a 0.45- μ m membrane. Then,

NaCH₃CO₂ was slowly added to produce a 10% weight (per volume) solution and the pH adjusted to 7.0. *C. neoformans* capsular polysaccharide was precipitated by adding 2.5 volumes of 95 to 100% ethanol and incubated at room temperature or 4°C. Afterward, the supernatant was discarded, and the polysaccharide was dissolved in distilled H₂O. Finally, the concentration of polysaccharide was measured by the phenol-sulfuric acid method.

***C. neoformans* capsular polysaccharide binding assay.** *C. neoformans* GXM was serially diluted on the plate and allowed to attach to the bottom of the wells for 2 h. Microtiter polystyrene plates were blocked with 1% bovine serum albumin (BSA) in PBS to prevent non-antibody-specific binding and incubated for 1 h. Next, the immunoglobulin G1 (IgG1) GXM binding monoclonal antibody (MAb) 18B7 (2 μ g/ml) was added, and the plate was incubated for 1 h. The enzyme-linked immunosorbent assay (ELISA) was completed by adding, in successive steps, 1 μ g of alkaline phosphatase-labeled goat anti-mouse IgG1/ml and 50 μ l of *p*-nitrophenyl phosphate (5 mg/ml) in substrate buffer. Finally, the amount of GXM bound to the bottom of the plate was measured using a microtiter reader at 405 nm. Between every step, the wells were washed with 0.05% Tween 20 in TBS. All incubations were done at 37°C for 1 to 2 h or at 4°C overnight.

Effect of physical properties on *C. neoformans* biofilm formation. (i) Surface support effects on *C. neoformans* biofilm formation. *C. neoformans* strain B3501 was grown as described above in 96-well microtiter plates made of different materials, such as polystyrene, polyvinyl, polycarbonate, and glass, to examine the ability of yeast cells to adhere and form biofilms. Biofilms formed over a series of time intervals (2, 4, 8, 24, and 48 h).

(ii) Surface preconditioning. The effect of surface preconditioning on *C. neoformans* biofilm formation was examined. Microtiter plates were preconditioned with PBS, 20% fetal calf serum (FCS), artificial cerebrospinal fluid (ACSF; 25 mM NaCl, 2.5 mM KCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄ · H₂O, 1 mM MgCl₂, 11 mM glucose, and 2 mM CaCl₂), or 1% BSA. Yeast cells grown on unconditioned surfaces were utilized as controls. All of the conditioning solutions (100 μ l) were prepared at a 50% concentration (vol/vol) in sterile PBS (100 μ l) and were individually dispensed (200 μ l) into six wells of a 96-well microtiter plate. The plate was then incubated overnight at 4°C. Wells were then aspirated, and the adsorbed conditioning film was washed once in sterile distilled H₂O. *C. neoformans* strain B3501 cells were washed in PBS and resuspended at a concentration of 10⁷ cells per ml in minimal medium and dispensed (100 μ l per well) into the 96-well microtiter plates and incubated for 0.5, 2, 4, 8, 24, and 48 h at 37°C. For each time point, the wells were aspirated and washed three times in sterile PBS, and adhesion was measured by the XTT reduction assay. Experiments were performed twice with similar results.

(iii) Temperature. To examine the effect of temperature on *C. neoformans* biofilm formation, B3501 cells were grown as described above at various temperatures (4, 23, 30, 37, and 45°C). Biofilms formed over a series of time intervals (2, 4, 8, 24, 48, and 72 h).

(iv) pH. To evaluate the effect of pH on biofilm production by *C. neoformans*, B3501 cells were grown as described above at distinct pHs (3, 4, 5, 6, 7, 8, and 9). Biofilms formed over a series of time intervals (2, 4, 8, 24, and 48 h).

(v) Sugars. To determine the effect of sugars on *C. neoformans* biofilm formation, yeast cells were grown in minimal medium containing 30 mM of glucose, galactose, maltose, lactose, sucrose, or mannose as a sugar source in 96-well plates over a series of time intervals (2, 4, 8, 24, and 48 h).

Imaging of *C. neoformans* biofilms. (i) SEM. *C. neoformans* biofilms were grown on polyvinyl catheters. Catheters were added to a *C. neoformans* culture grown in minimal medium for 48 h in a rotary shaker at 150 rpm. Then, catheters with biofilms were washed three times with PBS, transferred to another 6-well microtiter plate containing 2.5% glutaraldehyde, and incubated for 1 h at room temperature. The samples were serially dehydrated in alcohol, fixed in a critical-point dryer (Samdri-790; Tousimis, Rockville, MD), coated with gold-palladium (Desk-1; Denton Vacuum, Inc., Cherry Hill, NJ), and viewed with a JEOL (Tokyo, Japan) JSM-6400 scanning electron microscope (SEM). Two separate sets of cultures were prepared.

(ii) Light microscopy. Capsular polysaccharide surrounding cryptococcal biofilms was stained using the GXM binding MAb 18B7. After biofilm formation, wells were blocked with PBS (1% BSA). Next, the IgG1 GXM binding MAb 18B7 (2 μ g/ml) was added, and the plate was incubated for 1 h at 37°C. Peroxidase-conjugated goat anti-mouse IgG1 (Fisher Scientific, Orangeburg, NY) was diluted at 1:250 in PBS (1% BSA) and applied for 1 h at room temperature. Color was developed after a 5-min incubation with 3,3'-diaminobenzidine tetrahydrochloride and 0.3% H₂O₂ at room temperature. Microscopic examinations were performed with light microscopy using an Axiovert 200 M inverted microscope (Carl Zeiss MicroImaging, NY).

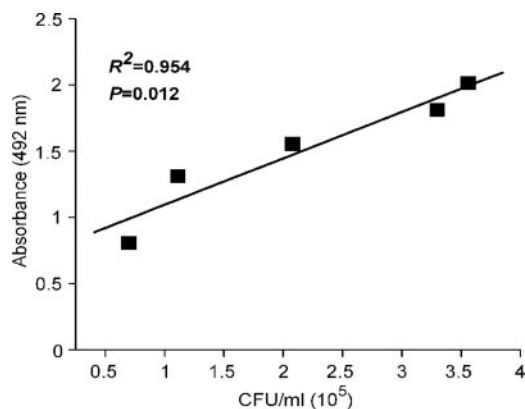


FIG. 1. Correlation between XTT reduction and CFU assays for monitoring *C. neoformans* biofilm formation. The R^2 and P values for each regression are also indicated.

(iii) **CM.** *C. neoformans* biofilms were incubated for 45 min in 75 μ l of PBS containing the fluorescent stain FUN-1 (10 μ M). Then, wells were blocked with PBS (1% BSA) for 1 h. Next, the IgG1 GXM binding MAb 18B7 (2 μ g/ml) was added, and the plate was incubated for 1 h. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG1 (Fisher Scientific) at a 1- μ g/ml concentration in PBS (1% BSA) was applied for 1 h. Between every step, the wells were washed with 0.05% Tween 20 in TBS. All incubations were done at 37 or 4°C overnight. FUN-1 (excitation wavelength, 470 nm; emission, 590 nm) is converted to orange-red cylindrical intravacuolar structures by metabolically active cells, while MAb 18B7-FITC-conjugated goat anti-mouse IgG1 (excitation wavelength, 488 nm; emission, 530 nm) binds to GXM and fluoresces green. Microscopic examinations of biofilms formed in microtiter plates were performed with confocal

microscopy (CM) using an Axiovert 200 M inverted microscope (Carl Zeiss MicroImaging, NY). A 40 \times objective was used (numerical aperture of 0.6). Depth measurements were taken at regular intervals across the width of the device. To determine the structure of the biofilms, a series of horizontal (xy) optical sections with a thickness of 1.175 μ m were taken throughout the full length of the biofilm. Confocal images of green (MAb 18B7-FITC-conjugated goat anti-mouse IgG1) and red (FUN-1) fluorescence were recorded simultaneously by CM using a multichannel mode. Z-stack images and measurements were corrected utilizing Axio Vision 4.4 software in deconvolution mode (Carl Zeiss MicroImaging, NY).

Susceptibility of *C. neoformans* biofilms to environmental stress. (i) **Exposure of *C. neoformans* biofilms to heat.** *C. neoformans* biofilms and planktonic cells in 96-well microtiter plates were placed in an incubator and heated to 47°C. This temperature had previously been shown to be microbicidal to *C. neoformans* (12). After various time intervals (30 and 60 min) of exposure to the heat, the metabolic activity and fungal mass of biofilms and planktonic cells were measured using the XTT reduction and CFU assays.

(ii) **Susceptibility of *C. neoformans* biofilms to cold.** Cryptococcal biofilms and planktonic cells were cooled or frozen by incubation at -80 , -20 , and 4°C for 24 h. After the incubation, the wells containing the biofilms and planktonic cells were thawed at room temperature, and the viability was measured by XTT reduction and CFU assays. Control cells were plated at the same time the experimental cells were cooled or frozen to avoid changes in number.

(iii) **Effect of UV light irradiation on mature *C. neoformans* biofilms.** The susceptibility of *C. neoformans* biofilms and planktonic cells to irradiation by UV light was determined by exposing fungal cells to various doses (100, 200, 300, and 400 μ J \times 100/cm²) of UV light (254 nm) generated by a Stratalinker 1800 (Stratagene, La Jolla, CA). The viability of biofilms and planktonic cells irradiated with UV light was measured by XTT reduction and CFU assays and compared to that of nonirradiated cells.

Statistical analysis. All data were subjected to statistical analysis using Origin 7.0 (Origin Lab Corporation, Northampton, MA). P values were calculated by t test. P values of <0.05 were considered significant.

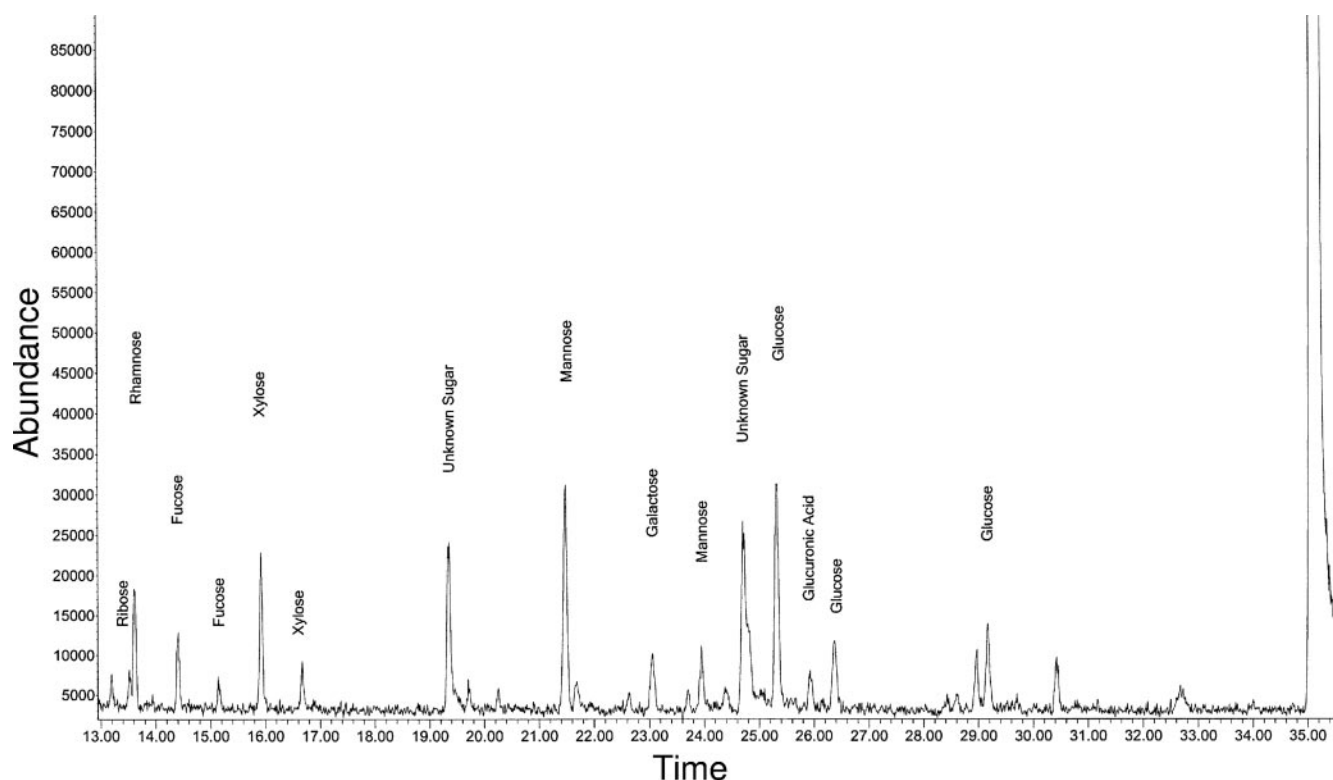


FIG. 2. Gas chromatogram detection profile of the isolated matrix material from the biofilms of *C. neoformans*. Peaks represent different anomers of the TMS derivatives of monosaccharides.

TABLE 1. Glycosyl composition analysis of exopolymeric matrix isolated from biofilms of *C. neoformans* B3501

Glycosyl residue	Mass (μg)	mol% of total carbohydrate ^a
Ribose	0.04	4.5
Rhamnose	0.1	9.5
Fucose	0.1	7.4
Xylose	0.2	22.9
Glucuronic acid	0.1	6.0
Galacturonic acid	0.0	0.0
Mannose	0.2	20.0
Galactose	0.1	6.6
Glucose	0.2	23.1
<i>N</i> -Acetylgalactosamine	0.0	0.0
<i>N</i> -Acetylglucosamine	0.0	0.0
Heptose	0.0	0.0
3-Deoxy-2-manno-2-octulosonic acid	0.0	0.0
Sum of carbohydrates	1.04	100.0

^a The total percentage of carbohydrate of this sample is calculated to be 0.2%.

RESULTS

Correlation between XTT reduction and CFU killing assays.

We investigated the correlation between XTT reduction and CFU assays in monitoring *C. neoformans* biofilm formation (Fig. 1). A strong correlation between the results of the two assays was demonstrated ($R^2 = 0.954$, $P = 0.012$). XTT activity was linearly associated with CFU counts. Consequently, XTT colorimetric intensity reflects fungal mass in the biofilm.

Glycosyl composition of exopolymeric matrix from biofilms of *C. neoformans*. Preparations of cryptococcal biofilm matrix material from strain B3501 were analyzed for carbohydrate composition by combined gas chromatography/mass spectrometry (Fig. 2). The glycosyl composition of exopolymeric matrix isolated from biofilms is consistent with the presence of GXM. However, we observed significant quantities of sugars not found in GXM, such as glucose (23.1%), ribose (4.5%), and fucose (7.4%) (Table 1), implying that the exopolymeric matrix of *C. neoformans* biofilms includes polysaccharides other than GXM.

***C. neoformans* forms stronger biofilms on polyvinyl.** The effect of the solid surface support material in the ability of *C.*

neoformans cells to adhere and form biofilms was investigated. Experiments were carried out on microtiter plates composed of polystyrene, polyvinyl, polycarbonate, and glass. *C. neoformans* strain B3501 cells grown on polyvinyl support formed significantly stronger biofilms than cells grown on polystyrene, polycarbonate, or glass support (Fig. 3). *C. neoformans* strain B3501 cells formed biofilms on support material in the following order of strength: polyvinyl > glass > polystyrene > polycarbonate. Fungal cells attached strongly to polyvinyl after 2 h of incubation (Fig. 3A). During the early stage of biofilm formation (0 to 8 h), B3501 cells grown on polystyrene, polycarbonate, and glass showed similar increases in metabolic activity (Fig. 3B). However, yeast cells grown on glass formed better biofilms than did those grown on polystyrene and polycarbonate.

Furthermore, we investigated whether there was a correlation between biofilm formation and the ability of GXM to bind to a specific solid support material. The total capsular polysaccharide of B3501 cells was isolated and added to polystyrene plates, and binding was detected with MAbs. *C. neoformans* GXM adhered best to the polyvinyl and glass materials, followed by polystyrene and then polycarbonate (Fig. 3C). Hence, the relative ability of *C. neoformans* to form biofilms correlated with GXM binding to a specific material.

Influence of temperature on *C. neoformans* biofilm formation.

The effect of temperature on the kinetics of biofilm formation by the *C. neoformans* strain B3501 cells on the surface of polystyrene microtiter plates was quantified (Fig. 4A). The metabolic activities displayed by *C. neoformans* strain B3501 cells during the adhesion period (0 to 8 h) were similar at 23, 30, and 37°C. B3501 cells showed a rapid increase in cellular growth during the first 24 h and then reached a plateau. *C. neoformans* strain B3501 cells formed similar and strong biofilms at 23, 30, and 37°C (Fig. 4A). In contrast, strain B3501 cells did not form biofilms at 4 or 45°C.

Effects of surface conditioning on biofilm formation by *C. neoformans*.

The cryptococcal biofilm development of strain B3501 was tested on untreated surfaces or surfaces preconditioned with different solutions. Stronger biofilm formation was observed in ACSF and PBS (Fig. 4B). After 2 h, *C. neoformans* cells grown on surfaces preconditioned with ACSF and PBS

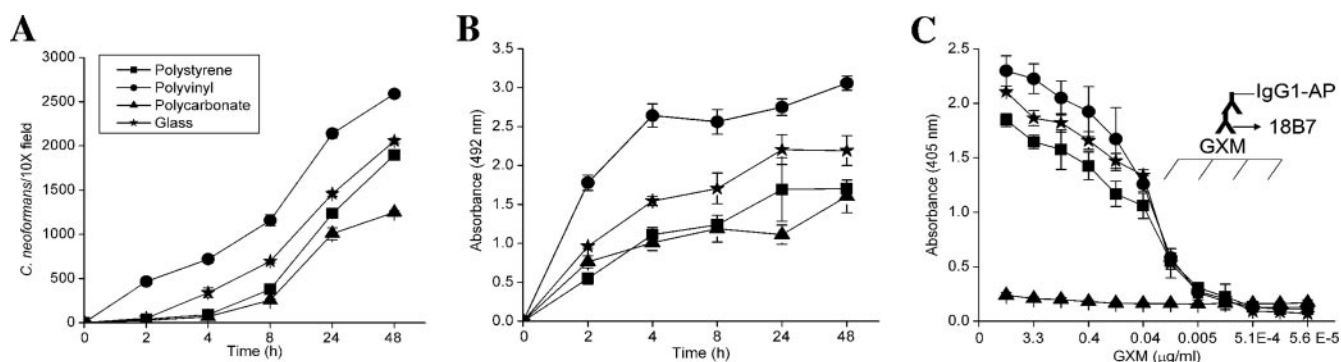


FIG. 3. Kinetics of *C. neoformans* biofilm formation in microtiter plates of different materials, as determined by (A) cell counts and (B) XTT reduction assay. Each point represents the average of six measurements. (C) Binding of *C. neoformans* GXM to microtiter plates of different materials. The inset diagram indicates the ELISA configuration used to detect the polysaccharide bound to the bottom of the plate. AP, alkaline phosphatase.

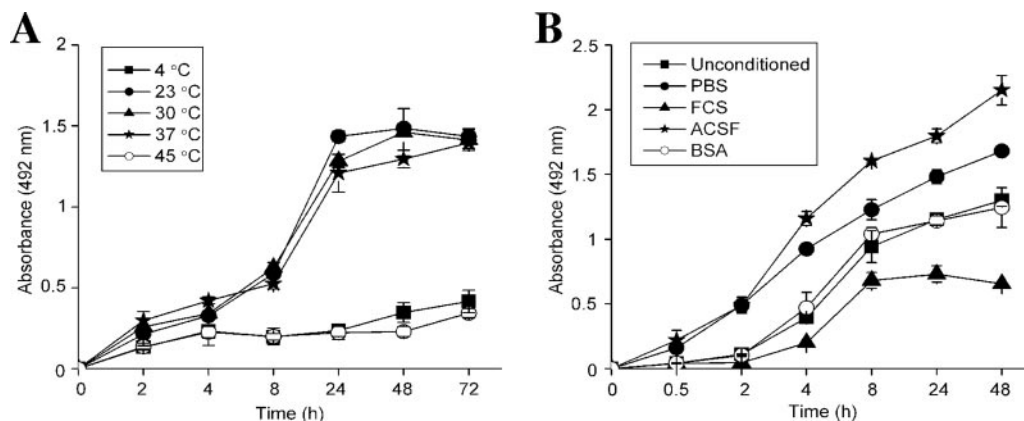


FIG. 4. Kinetics of biofilm formation by *C. neoformans* cells grown at different temperatures (A) and on surface-preconditioned polystyrene microtiter plates (B) as determined by XTT reduction assay. Each point represents the average of six measurements.

adhered rapidly to the support surface, forming strong biofilms. A similar increase in metabolic activity by cryptococci grown on untreated surfaces or on a BSA-conditioned surface was observed after 4 h of incubation. Fungi grown on surfaces precoated with FCS manifested a smaller increase in cellular metabolic activity after 8 h of incubation than did fungi grown on non-FCS-coated surfaces. After 48 h, *C. neoformans* strain B3501 cells formed biofilms on preconditioned surfaces in the following order of strength: ACSF > PBS > untreated surface = BSA > FCS. Therefore, the initial adhesion of *C. neoformans* cells depended on the condition of the surface where the biofilm formed.

C. neoformans forms stronger biofilms at a neutral pH level.

The role of pH level in *C. neoformans* biofilm formation was examined. *C. neoformans* strain B3501 cells formed significantly stronger biofilms under the slightly acidic and neutral pH conditions (pH 5 to 7) tested. Fungal cells grown under neutral conditions attached significantly more strongly to the polystyrene surface of microtiter plates than yeast cells grown in acidic (pH 3 and 4) or alkaline (pH 8 and 9) environments (Fig. 5A). Similarly, B3501 cells within biofilms grown in a neutral milieu showed a significant increase in metabolic activity during the first 24 h (Fig. 5B).

Since GXM release is necessary for cryptococcal biofilm

formation, we purified GXM from *C. neoformans* strain B3501 and investigated whether different pH conditions affect the ability of GXM to bind to plastic. GXM adhered best to the polystyrene material under neutral conditions (Fig. 5C). Hence, the relative ability of *C. neoformans* to form biofilms on polystyrene at various pH levels correlated with GXM binding to that surface.

Effect of sugar type on *C. neoformans* biofilm formation. *C. neoformans* strain B3501 was grown in the presence of different sugars to determine their effects on biofilm formation on polystyrene plates. Strain B3501 was able to attach and form biofilms in the presence of glucose or mannose (Fig. 6A) and to form comparable biofilms with either sugar. During the adhesion stage (0 to 4 h), fungal cells of strain B3501 grown in the presence of sucrose showed metabolic activities that were similar to those of cells grown in the presence of mannose, but after reaching biofilm maturity, the metabolic activity decreased significantly (Fig. 6B). In contrast, no biofilm formation was observed when the cells were grown in the presence of galactose, maltose, or lactose.

SEM visualization of *C. neoformans* biofilms. Biofilm formation was monitored by SEM. Catheters were incubated in a culture of *C. neoformans* in minimal medium on a rotary shaker for 48 h. *C. neoformans* biofilms comprised a dense

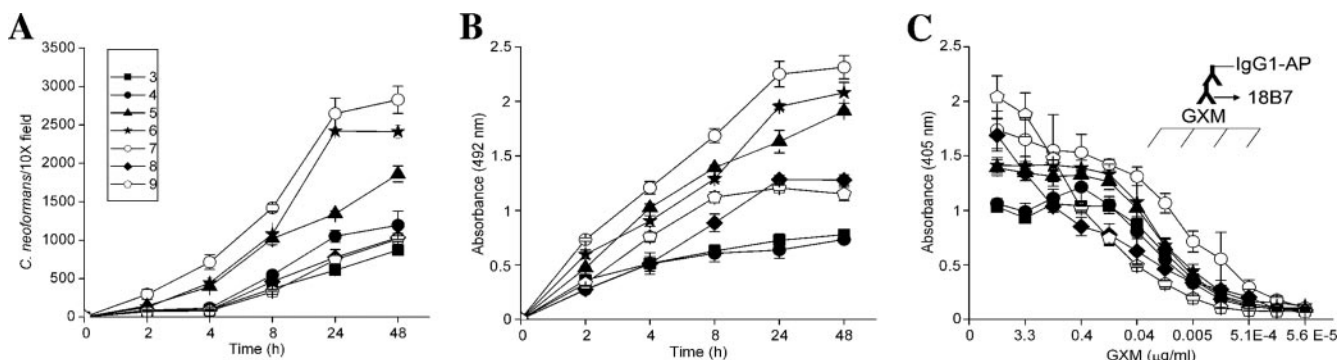


FIG. 5. Kinetics of *C. neoformans* biofilm formation on polystyrene microtiter plates under various pH conditions, as determined by (A) cell counts and (B) XTT reduction assay. Each point represents the average of six measurements. (C) Binding of *C. neoformans* GXM to the plastic surface of a microtiter plate under various pH conditions. The inset diagram indicates the ELISA configuration used to detect the polysaccharide bound to the bottom of the plate. AP, alkaline phosphatase.

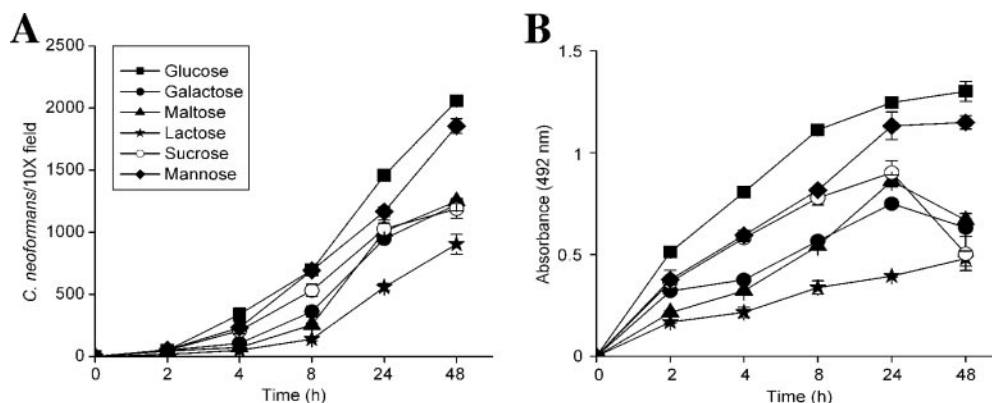


FIG. 6. Kinetics of *C. neoformans* biofilm formation on polystyrene microtiter plates using different sugars as a carbon source in the medium, as determined by (A) cell counts and (B) XTT reduction assay. Each point represents the average of six measurements.

network of yeast cells strongly attached to a piece of catheter that detached after SEM processing (Fig. 7A). Exopolymeric matrix was seen surrounding cryptococcal cells. A closer image of a cryptococcal biofilm showed fine polysaccharide fibers from a yeast cell bound to most of the exopolymeric material (Fig. 7B). Higher-density collections of yeast cells had more extracellular matrix surrounding them, suggesting that close association helped to preserve the polysaccharide matrix from the preparation effects of SEM (Fig. 7C). We evaluated whether the acapsular strain C536 could form biofilm when grown with catheters but found no evidence of a biofilm (data not shown).

Distribution of GXM surrounding *C. neoformans* biofilms.

The distribution of capsular polysaccharide surrounding a *C. neoformans* biofilm was investigated by staining with a specific MAb to GXM and visualizing by light microscopy. *C. neoformans* mature biofilms showed that GXM is copiously released and profusely distributed throughout the exopolymeric matrix (Fig. 8A). Individual fungal cells were not distinguishable due to encasement in extracellular capsular polysaccharide material. A closer view of a biofilm displayed darker regions of MAb 18B7-stained GXM where yeast cells were densely packed within a thick layer of GXM (Fig. 8B).

CM of mature *C. neoformans* biofilms using a GXM-specific MAb. A mature *C. neoformans* biofilm was investigated using CM, because this technique preserved the structural integrity of the biofilms. *C. neoformans* biofilm reconstruction was done by the compilation of a series of individual *xy* sections taken

across the *z* axis. Red color due to FUN-1 staining localized in dense aggregates in the cytoplasm of metabolically active cells (Fig. 9A), while the intense green fluorescence resulted from specific MAb 18B7-FITC-conjugated goat anti-mouse IgG1 bound to GXM (Fig. 9B). Orthogonal images of a mature *C. neoformans* biofilm in polystyrene 96-well plates were analyzed to determine biofilm thickness and architecture. Vertical (*xz*) sectioning (side view) of three-dimensional reconstructed images showed that mature *C. neoformans* biofilms consisted of a highly organized architecture (~56- μ m-thick biofilm) with red spots representing metabolically active cells interwoven with extracellular polysaccharide material (Fig. 9C).

***C. neoformans* cells in biofilms are less susceptible than planktonic cells to environmental stress.** The susceptibility of fungal biofilms to harsh environmental factors, such as high and low temperatures and UV light, was investigated and compared with that of planktonic yeast cells. XTT reduction and CFU killing assays were utilized to quantify fungal metabolic activity and cellular mass, respectively.

C. neoformans cells in biofilms were significantly more resistant to thermal stress than their planktonic counterparts (Fig. 10A). The metabolic activity of biofilms was not affected after exposures to a relatively high temperature. However, planktonic cells showed a significant reduction in metabolic activity after being exposed to 47°C for 30 ($P = 0.004$) and 60 ($P = 0.0001$) min. To confirm the results obtained by XTT reduction assay, the percent survival of cells in biofilms or in planktonic form was determined by counting the numbers of CFU in wells

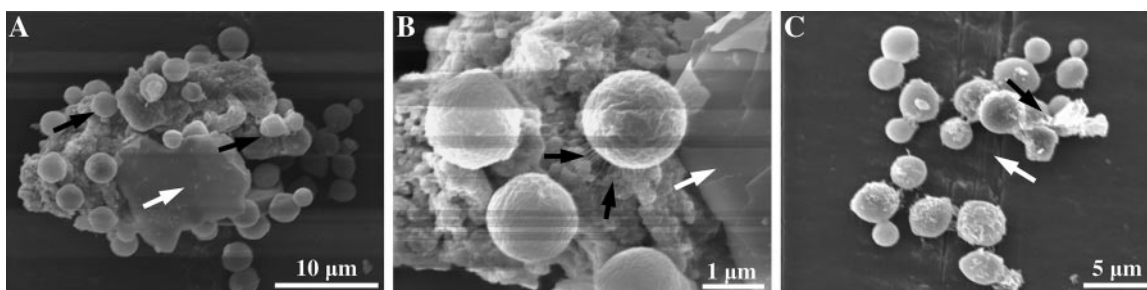


FIG. 7. SEM images of a mature *C. neoformans* B3501 biofilm formed on polyvinyl catheters in vitro revealed the strong attachment of cryptococcal cells to the substrate. Black and white arrows denote polysaccharide and polyvinyl substrate, respectively.

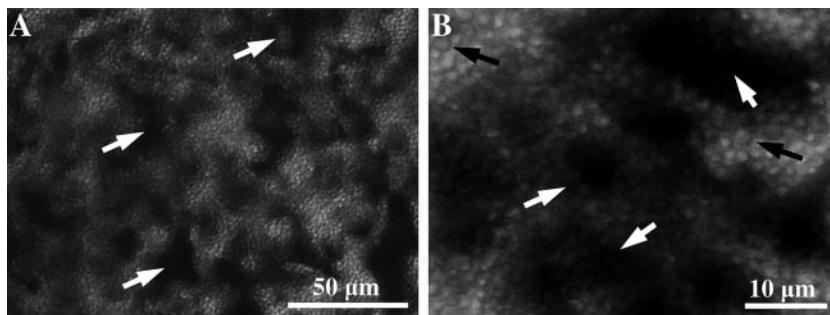


FIG. 8. Light microscopy images of the exopolymeric matrix of a mature *C. neoformans* biofilm stained with GXM-specific MAb 18B7. Images of a mature biofilm show that capsular binding MAb 18B7 binds and darkly stains shed capsular polysaccharide. (A) Picture was taken using a 10 \times power field. (B) Picture was taken using a 40 \times power field. Black and white arrows denote yeast cells and exopolymeric matrix, respectively.

treated with heat and comparing these to the numbers of colonies obtained from unexposed cells (Fig. 10B). After a 60-min exposure to heat, *C. neoformans* cells within biofilms displayed approximately 50% survival, whereas most of the planktonic cells were killed.

C. neoformans biofilms were more resistant to cold than planktonic cells (Fig. 10C and D). In both assays, biofilm and planktonic cells did not show statistically significant differences when yeast cells were incubated at 4 $^{\circ}$ C. Cryptococcal biofilms were less susceptible to damage by freezing to -20° C than planktonic cells, regardless of whether the effect was measured by XTT reduction ($P = 0.005$) or CFU enumeration ($P = 0.0001$). Moreover, biofilms and planktonic cells exhibited an approximately 80% reduction in viability after freezing at -80° C, but the survival of cells within biofilms was significantly higher ($P = 0.004$) than for planktonic cells.

Furthermore, cryptococcal biofilms were less susceptible to killing by UV irradiation than planktonic cells as measured by CFU killing assay (Fig. 10E). There were no statistically significant differences between biofilms and planktonic cells when fungal cells were irradiated with doses of 100 and 200 μ J \times 100/cm 2 . Conversely, after UV light irradiation of 300 μ J \times 100/cm 2 , the percent survival of planktonic cells was significantly reduced, by approximately 80%, versus 40% for cryptococcal biofilms ($P = 0.008$). In the XTT reduction assay for UV light susceptibility, there was no statistically significant differ-

ence in the metabolic activities of biofilms and planktonic cells (data not shown).

DISCUSSION

Biofilm development by *C. neoformans* follows a standard sequence of events, including fungal surface attachment, microcolony formation, and matrix production (9). *C. neoformans* biofilm development is dependent on the release of capsular polysaccharide to the solid surface to create an exopolysaccharide matrix (9). Sugar composition analysis was remarkable for the predominance of xylose, mannose, and glucose and the presence of several minor sugars not found in either of the *C. neoformans* capsular polysaccharides, GXM or galactoxylo-mannan. Since neither GXM nor galactoxylo-mannan contains glucose, we infer that the exopolymeric matrix is composed of types of polysaccharides that are different from those used to assemble the capsule. Detailed SEM imaging revealed that *C. neoformans* strongly attached to polyvinyl catheters, suggesting that the polysaccharide capsule conferred an advantage during the colonization process, given that acapsular cryptococci did not adhere to the plastic material. Since GXM is the major component of the extracellular polysaccharide surrounding fungal cells within a mature *C. neoformans* biofilm, we utilized an IgG1 GXM-specific MAb, 18B7, as a reagent to visualize the exopolymeric matrix by light microscopy. A secondary

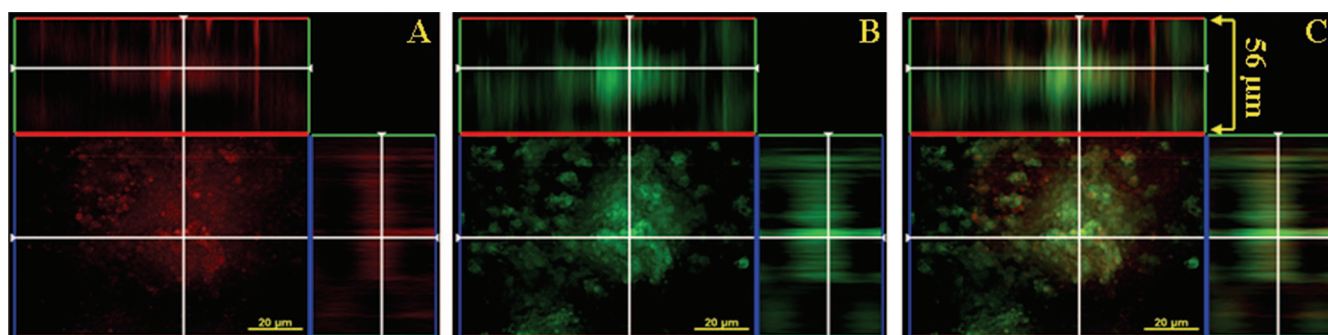


FIG. 9. CM image of a mature *C. neoformans* biofilm stained with GXM-specific MAb 18B7. The orthogonal image of a mature *C. neoformans* biofilm shows (A) metabolically active (red, FUN-1-stained) *C. neoformans* cells, (B) extracellular polysaccharide material stained with capsular binding MAb 18B7 (green, goat anti-mouse IgG1-FITC stained), and (C) a merged image of panels A and B. The mature *C. neoformans* biofilm showed a complex structure with metabolically active cells interwoven with extracellular polysaccharide material. The thickness of a mature biofilm is approximately 56 μ m. The pictures were taken using a 40 \times power field. Scale bar, 20 μ m.

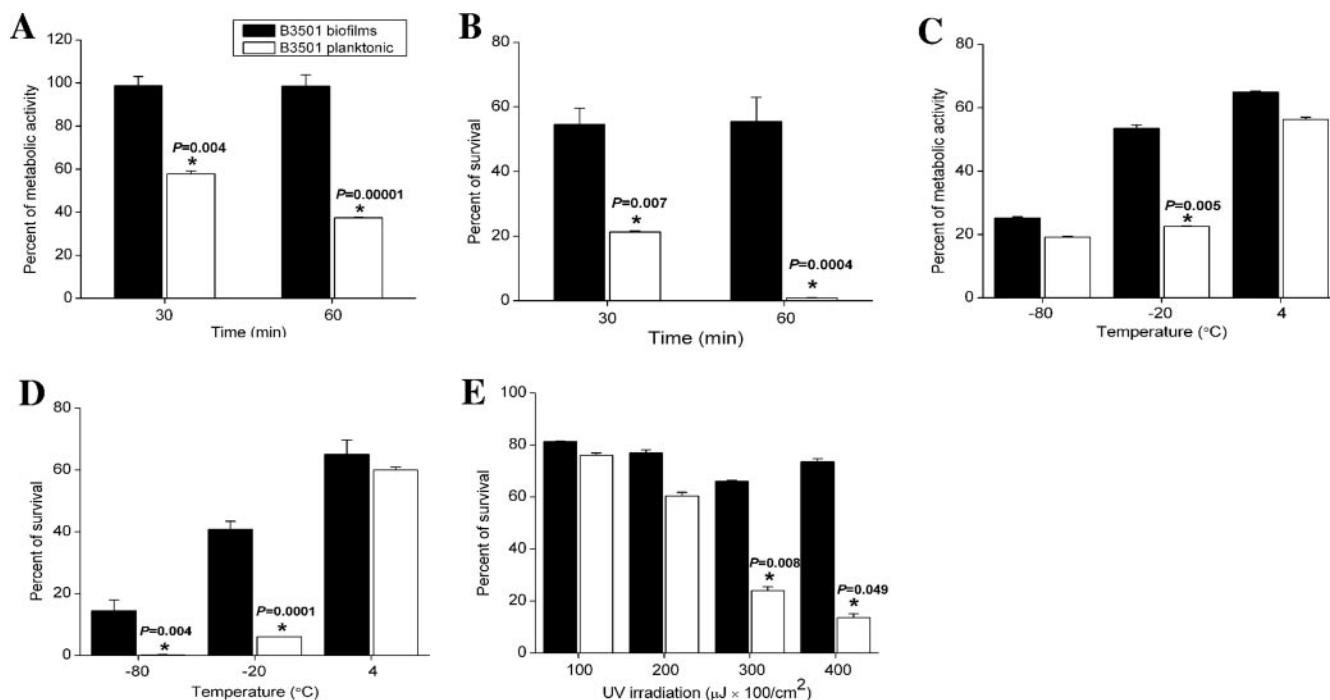


FIG. 10. *C. neoformans* biofilms are less susceptible to environmental stress than planktonic cells. (A and B) *C. neoformans* biofilms are less susceptible to heat (47°C) than are planktonic cells. (A) The metabolic activity of *C. neoformans* biofilms and planktonic cells was measured using an XTT reduction assay and for the treated group is shown here as a percentage of the activity measured in unexposed cells. (B) The percent survival of *C. neoformans* strain B3501 biofilms and planktonic cells was determined by CFU counts and for the treated group is shown here as a percentage of CFU counted in unexposed cells. For graphs A and B, both cell types were exposed to 47°C for 30 and 60 min, and the metabolic activity and CFU counts were compared to those of fungal cells unexposed to heat as a function of time. (C and D) *C. neoformans* biofilms are less susceptible than planktonic cells to cold. (C) The metabolic activity of *C. neoformans* biofilms and planktonic cells was measured using the XTT reduction assay and for the treated group is shown here as a percentage of the activity measured in unexposed cells. (D) The percent survival of *C. neoformans* strain B3501 biofilms and planktonic cells was determined by CFU counts and for the treated group is shown here as a percentage of CFU counted in unexposed cells. For graphs C and D, both cell types were exposed to cold stress for 24 h, and the metabolic activity and CFU count were compared to those of fungal cells unexposed to cold. (E) *C. neoformans* biofilms are less susceptible to UV light exposure than planktonic cells. The percent survival of *C. neoformans* strain B3501 biofilms and planktonic cells was determined by CFU counts and for the treated group is shown here as a percentage of the CFU counts for cells unexposed to UV light. For each graph, bars show the averages of six measurements, and brackets denote standard deviations. Asterisks denote P -value significances calculated by t test.

IgG1 MAb conjugated to horseradish peroxidase was used to identify the MAb 18B7 bound to the biofilm matrix. *C. neoformans* GXM was copiously released to the medium and accumulated, and it encased fungal cells within an exopolymeric matrix that could not be removed by the shear forces generated by an extensive and tenacious washing. The use of specific MAbs is a simple and effective method to study microbial biofilm exopolymeric matrix development by light microscopy.

Similarly, the combination of dye FUN-1 and MAb 18B7 binding and detection with FITC-conjugated goat anti-mouse IgG1 was utilized to visualize mature cryptococcal biofilms using confocal microscopy. MAb 18B7-FITC-conjugated goat anti-mouse IgG1 was used to stain the GXM in the exopolymeric matrix and fluoresced green, while FUN-1 entered yeast cells and fluoresced red, identifying metabolically active cryptococci. Mature *C. neoformans* biofilm displayed a complex structure, with internal regions of metabolically active cells interwoven with extracellular polysaccharide material and interspersed with water channels. Our result suggests that most of the extracellular polysaccharide comprising the matrix enclosing cryptococci within a mature biofilm includes shed

GXM and can be stained by a specific MAb. Concanavalin A binds to mannoproteins in the matrix of cryptococcal biofilms (8, 9, 11). Costaining with concanavalin A and MAb 18B7 revealed different colocalization, strongly suggesting that these compounds bind to different moieties (10).

We compared the XTT assay and CFU quantification for evaluating the biofilm development of *C. neoformans*. Traditionally, CFU determination was used to measure cell viability despite the laborious work involved and the difficulties in disrupting cell aggregates without affecting viability. Recently, the XTT reduction assay, which is based on metabolic activity rather than viability, has been developed for biofilm quantification. The XTT reduction assay is a colorimetric method that quantifies the number of living cells in a biofilm. Our data showed that the CFU assay positively correlates with the XTT readings, suggesting that both methods can be reliably used for quantification of cryptococcal biofilm mass. Nevertheless, we noted that for the experiments where biofilms were exposed to heat and cold stress, the reduction in CFU number was disproportionately greater than the reduction in XTT activity. This may reflect lethal damage to cells by heat and cold stress

without a concomitant temporally related reduction in enzymatic activity. Hence, the slope of the correlation between the CFU and XTT assay results may be a function of the type of experiment performed.

Our studies revealed that the type of solid surface plays an important role in biofilm formation. Surface support roughness robustly promotes microbial adhesion by diminishing shear forces, whereas physicochemical properties promote the tenacious attachment of microorganisms to a solid support (2). *C. neoformans* strain B3501 fairly adhered to all materials tested but formed the strongest biofilms on polyvinyl support. The dynamic nature of the capsule could allow *C. neoformans* cells to interact favorably with a variety of substrata and promote fungal attachment. In contrast to many other microorganisms that preferably form biofilms on hydrophobic surfaces, *C. neoformans* cells strongly attached to glass and formed a mature biofilm. The polysaccharide capsule apparently plays an important role in the adhesion of fungal cells to a glass surface. For instance, the O-antigen component of lipopolysaccharide has also been shown to confer hydrophilic properties to gram-negative bacteria (18).

Artificial cerebrospinal fluid contains high concentrations of cations that could increase the interactions of the microbe with the support surface. When polystyrene material was conditioned with ACSF, the *C. neoformans* strain B3501 cells attached tenaciously to the solid support. Surface conditioning and the characteristics of the medium are important for microbial biofilm development, because these variables affect the rate and the extent of cryptococcal attachment. For example, Mittelman reported that a number of host fluids, such as blood, tears, urine, saliva, intervascular fluid, and respiratory secretions, influenced the attachment of bacteria to biomaterials (14). Ventriculoperitoneal shunts in patients with cryptococcosis are constantly irrigated by cerebrospinal fluid and may be highly susceptible to coating by cryptococcal biofilms. Furthermore, this possibility increases when taking into account that the cerebrospinal fluid is in constant motion, and this situation influences the adhesion of microorganisms to biomaterials (3).

C. neoformans strain B3501 formed a strong biofilm in minimal medium supplemented with either glucose or mannose as a carbon source. During initial attachment, fungal cells grown in the presence of glucose, galactose, sucrose, and mannose adhered similarly to polystyrene. Conversely, galactose and sucrose did not stimulate *C. neoformans* biofilm maturation. These results suggest that the carbon source available for nutrition can have an important effect on biofilm maturation. For instance, sugar differences may affect fungal growth rate and the expression of proteins involved in adhesion or matrix scaffolding (6). Furthermore, temperature and pH level are other characteristics of the medium that may influence microbial biofilm formation. High or low temperature affected *C. neoformans* biofilm formation. Previous studies have shown that seasonal changes may affect the rates of attachment and biofilm formation (4). *C. neoformans* was able to form biofilms under neutral pH conditions comparable to those that would be expected in body fluids. This environment stimulates the ability of GXM to bind a solid support. However, lower or higher pH conditions did not allow *C. neoformans* to develop mature biofilms, possibly by interfering with growth and/or

influencing the charge of the GXM that is conferred by the state of protonation of glucuronic acid groups.

The ability to form biofilms provides a fungus with survival advantages in the environment, e.g., anchorage at a location where growth is favorable, protection from desiccation or predation, and resistance to biocides and detergents. *C. neoformans* is found ubiquitously in the environment in association with pigeon excreta (7) and is exposed to many environmental changes. *C. neoformans* biofilms were significantly less susceptible to each of these stress conditions than their planktonic counterparts. Biofilm establishment may provide protection from environmental shifts due to the facilitation of cell-to-cell interactions, and the exopolymeric matrix may act as a shield against stress conditions.

In conclusion, our results indicate that biofilm development by *C. neoformans* depends on various characteristics, such as capsular production, the physical properties of the substrate material, composition of the medium, etc. Characterization of microbial biofilms may be critically important for designing therapies against biofilm-related diseases. Furthermore, the utilization of a GXM-specific MAb to study the production of exopolymeric material by *C. neoformans* during biofilm formation adds a potential and useful tool in biofilm-forming-microbe research.

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