Impact of Environmental Conditions on the Form and Function of Candida albicans Biofilms

Karla J. Daniels, Yang-Nim Park, Thyagarajan Srikantha, Claude Pujol, David R. Soll
Developmental Studies Hybridoma Bank, Department of Biology, University of Iowa, Iowa City, Iowa, USA

Candida albicans, like other pathogens, can form complex biofilms on a variety of substrates. However, as the number of studies of gene regulation, architecture, and pathogenic traits of C. albicans biofilms has increased, so have differences in results. This suggests that depending upon the conditions employed, biofilms may vary widely, thus hampering attempts at a uniform description. Gene expression studies suggest that this may be the case. To explore this hypothesis further, we compared the architectures and traits of biofilms formed in RPMI 1640 and Spider media at 37°C in air. Biofilms formed by α/α cells in the two media differed to various degrees in cellular architecture, matrix deposition, penetrability by leukocytes, fluconazole susceptibility, and the facilitation of mating. Similar comparisons of α/α cells in the two media, however, were made difficult given that in air, although α/α cells form traditional biofilms in RPMI medium, they form polylayers composed primarily of yeast cells in Spider medium. These polylayers lack an upper hyphal/matrix region, are readily penetrated by leukocytes, are highly fluconazole susceptible, and do not facilitate mating. If, however, air is replaced with 20% CO2, α/α cells make a biofilm in Spider medium similar architecturally to that of α/α cells, which facilitates mating. A second, more cursory comparison is made between the disparate cellular architectures of α/α biofilms formed in air in RPMI and Lee’s media. The results demonstrate that C. albicans forms very different types of biofilms depending upon the composition of the medium, level of CO2 in the atmosphere, and configuration of the MTL locus.

The opportunistic fungal pathogen Candida albicans, like opportunistic bacterial pathogens (1, 2), forms biofilms on the surfaces of a variety of tissues and synthetic devices introduced into hosts (3–5). Traditionally, biofilms serve the purpose of anchoring populations of pathogens to a surface and providing a conditioned microenvironment that facilitates growth, survival, and intercellular signaling (6, 7). Biofilms also serve to exclude host and therapeutic challenges, such as antibodies, white blood cells, and antibiotics (8, 9). Bacterial biofilms, which were initially the most intensely studied, have been shown to be architecturally complex, adhering at their base to a targeted surface and composed of multiple cell types embedded in a self-generated complex extracellular matrix (10, 11). Many of the characteristics of bacterial biofilms have been shown to be exhibited by biofilms formed by C. albicans, including drug resistance (12–15), impermeability to low- and high-molecular-weight molecules (15), and resistance to penetration by human polymorphonuclear leukocytes (PMNs) (15, 16). However, studies both to characterize the traits of C. albicans biofilms and to elucidate the molecular mechanisms regulating formation do not always provide the same results, most notably transcription profiles, mutational analyses of the signal transduction pathways, and transcription factors regulating their formation. Such inconsistencies interfere with attempts to formulate a unified model for the regulation of biofilm formation, biofilm architecture, and biofilm traits associated with pathogenesis. In trying to make sense of the inconsistencies emerging in the rapidly evolving biofilm literature of C. albicans, it became apparent that many of them might be due to differences in the in vitro conditions used to support biofilm formation, most notably differences in the compositions of the supporting media. Indeed, Mitchell and colleagues (17) recently demonstrated that up to one-third of 302 genes that they analyzed were regulated differently in two media commonly used to generate biofilms in vitro, Spider medium and yeast extract-peptone-dextrose (YPD) medium. To explore just how different biofilms can be formed under different in vitro conditions, we undertook a comparison of biofilm formation in two disparate media used in biofilm studies, RPMI 1640 medium (RPMI medium) and Spider medium. Rather than comparing gene expression and gene regulation, we compared biofilm architecture and function in both α/α and α/α strains at 37°C, in air. In particular, we compared cellular architecture, matrix density, penetrability by human leukocytes, drug resistance, and the facilitation of mating. We also performed a more cursory architectural comparison of α/α biofilms formed in RPMI medium with those formed in Lee’s medium to underscore just how disparate biofilms formed in vitro can be.

Our major results reveal both similarities and differences between α/α biofilms formed in RPMI medium and those formed in Spider medium at 37°C in air. However, a comparison between α/α white cell biofilm formation in RPMI medium and that in Spider medium at 37°C, in air, was problematic, given that cells of several natural α/α strains did not exhibit any of the characteristics of α/α biofilms in Spider medium. While white α/α cells formed a biofilm architecturally similar to that formed by α/α cells in RPMI medium in air, white α/α cells in Spider medium formed polylayers made up predominately of yeast cells, roughly 10 cells thick, that were devoid of an upper region containing hyphae and dense matrix, hallmarks of in vitro biofilms (3). The biofilms formed by
a/a cells in Spider medium were not resistant to leukocyte penetration, were extremely susceptible to fluconazole, and did not facilitate mating of seeded opaque cells. However, when a/a biofilms were formed in Spider medium in 20% CO₂ rather than air, they formed a biofilm architecturally similar to that formed by a/α cells in Spider medium. CO₂-induced a/a biofilms formed in Spider medium facilitated mating at higher frequencies than did a/α biofilms, although not to the same degree as a/a biofilms formed in RPMI medium under otherwise similar conditions.

Our combined results first reveal that both similarities and dissimilarities exist between a/a biofilms formed in RPMI medium and those formed in Spider medium, either in air or in 20% CO₂. They also demonstrate that a/a cells form a biofilm in Spider medium with an upper hyphal/matrix region only in high concentrations of CO₂, not in air. These fundamental differences in biofilm architecture and traits, together with previous observations demonstrating differences in gene expression (17), suggest that more caution must be applied in comparing studies of biofilm regulation under different experimental conditions and, most importantly, in different media. This is a particularly poignant point when comparing the regulation of a/a biofilms formed in RPMI medium with the regulation of those formed in Spider medium in air, given the extreme differences in architecture and function. Most importantly, these results indicate that formulating a single model for the gene circuitry regulating biofilm development in C. albicans may be elusive, given that different in vitro conditions, far more numerous than analyzed here, and different configurations of the MTL locus lead to very different biofilms, in some cases with few of the traits of a pathogenic or sexual biofilm. Recently, Fan- ning et al. (17) and Nobile et al. (18) compared gene regulation between biofilms formed in vitro and in vivo in the former case, and between denture and catheter models in the latter case. They found by mutational analyses that select genes that played a regulatory role under one set of conditions did not do so under an alternative set of conditions. These results again suggest that caution must be applied in comparing results obtained not only under different in vitro conditions but between different in vivo models.

MATERIALS AND METHODS

Strains. The names, genotypes, and origins of the wild-type strains and mutants used in this study are listed in Table S1 in the supplemental material. Strains were maintained at 25°C on agar plates containing modified Lee’s medium (19, 20) prior to use in experiments.

Media and buffers. Dulbecco’s modified phosphate-buffered saline without CaCl₂ or MgCl₂ (D-PBS) and RPMI 1640 medium were purchased from Gibco-Life Technologies. 3-(N-Morpholino)propanesulfonic acid (MOPS; 0.165 M) was added to RPMI 1640 medium (RPMI medium) to stabilize the pH at 7.0 (21). RPMI medium was used without nic acid (MOPS; 0.165 M) was added to RPMI 1640 medium (RPMI medium) prior to use in experiments.

Biofilm development. Each well containing a disc prepared specifically for alternative media was replaced with 2 ml of a suspension of 2 × 10⁷ stationary-phase cells in either RPMI or Spider medium. The cells were allowed to adhere to the discs for 90 min at 37°C without disturbance. Each disc was rinsed once from its well, gently rinsed with D-PBS to remove nonadherent cells, and placed in a fresh 12-well plate with 2.5 ml of fresh medium. In either medium, approximately 2 × 10⁶ cells adhered to the discs. The discs were incubated with gentle mixing on a rocker (Immunetics) with 6°/s deflection in a 37°C incubator in air or 20% CO₂. RPMI and Spider medium preparations were prepared simultaneously and, in each experiment, were cultured in parallel under identical conditions for 48 h. For biofilm formation in Lee’s medium, the conditions of Lin et al. (24) were rigidly repeated. In brief, cells were grown in Spider medium and then plated on plastic or silicone elastomer in Lee’s medium and incubated for 24 h, without rocking or mixing, at 25°C in air.

Biofilm thickness and cellular architecture. Forty-eight-hour preparations were fixed for 1 h at room temperature by the addition of 16% paraformaldehyde directly into the wells, resulting in a final concentration of 2% (vol/vol) paraformaldehyde. After fixation, the discs were rinsed once in D-PBS and stained with calcofluor white M2R (0.1 mg/ml in TBS [Tris-buffered saline; 20 mM Tris·HCl, 150 mM NaCl], pH 9.0; Fluorescent Brightener 28; Sigma, St. Louis, MO) (25). Biofilm thickness and architecture were analyzed using primarily an inverted Radiance 2100 two-photon laser scanning confocal microscope (LSCM) (Bio-Rad) equipped with a Mai-Tai infrared (IR) laser (Spectra Physics) at an excitation wavelength of 780 nm with emission at 460 nm. In preparation for imaging, biofilms were inverted and placed upon a 10-μl drop of water in the center of a 25-mm glass coverslip held within an open Sykes-Moore chamber (26). The water cushion prevented biofilm compression onto the coverslip surface and also diminished biofilm dehydration while imaging. Fluorescent images were acquired as a z-series at 2-μm intervals through 125 μm or more. For analysis, 100 scans were obtained at 2-μm intervals for a 200-μm z-series and were examined through each biofilm using a 20× objective and LaserSharp software (Bio-Rad). For imaging on a plastic surface, we employed an upright Zeiss LSM710 microscope without a coverslip using a 63× immersion objective. The extent of filamentation was estimated by dividing single optical scans into 16 equal squares (32 by 32 pixels). The numbers of hyphae and yeast in each of 4 squares were counted and averaged. Percent filamentation was determined by dividing the number of hyphae by the total number of cells in the field in three separate biofilms.

Biofilm matrix. The density and architecture of the extrapolymeric substance (EPS), referred to here simply as “extracellular matrix,” was determined by staining paraformaldehyde-fixed samples with FilmTracer Sypro Ruby biofilm matrix stain (Life Technologies). Biofilms were rinsed with D-PBS and stained for 1 h at room temperature with 1 ml of Sypro Ruby solution. The biofilms were then rinsed twice with D-PBS. A z-series of LSCM scans was acquired as described for cellular architecture (457-nm excitation/610-nm emission). The settings that gave optimal matrix intensity for biofilms formed in RPMI medium were then used for both types of biofilms, in comparisons performed within minutes of each other. The same strategy was used to assess matrix density using the cal-
cofluor white-stained preparations. In each case, the intercellular regions were assessed for intensity in single scans.

**Biofilm penetrability.** To assess penetration of alternative biofilms by leukocytes, we used a newly developed method that employs the modified human leukemia cell line HL60-GFP, in which the green fluorescent protein (GFP) is driven by the cytomegalovirus (CMV) promoter in pLNCN vector and expressed only in differentiated cells that have been activated (27, 28), rather than an assay using Dil (1,1'-dioctadecyl 3,3,3',3'-tetramethylindocarbocyanine perchlorate)-stained fresh leukocytes (polymorphonuclear leukocytes [PMNs]), as previously described (15, 16). HL60-GFP cells were a generous gift from O. D. Weiner of the University of California, San Francisco. To differentiate HL60-GFP cells, 2 × 10^6 cells/ml were suspended in RPMI medium supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1.3% dimethyl sulfoxide (DMSO) (Sigma), and 15 ml of this cell suspension was added to cell culture flasks with filtered caps. The flasks were incubated for 5 days at 37°C in 5% CO₂.

**Biofilm drug susceptibility assay.** Fluconazole susceptibility was assayed as previously described (16). Briefly, 1 ml of medium was removed from each mature biofilm preparation (29) and replaced with 1 ml of fresh medium containing fluconazole. Fluconazole was added to a final concentration of 25 µg/ml. Biofilms were incubated for an additional 24 h on the rocker at 37°C. Biofilms were rinsed with D-PBS and replaced in 1.5 ml Eppendorf tubes containing 1 ml of D-PBS supplemented with 20 mM EDTA for 5 min. Biofilms were then vortexed to remove biofilm cells from their silicone elastomer substrate. Cells were rinsed in TBS (pH 7.5) twice. Cell pellets were resuspended in TBS (pH 7.4) containing 5 mM Sytox Green, a dead-cell double-stranded DNA stain (Invitrogen), for 5 min with intermittent mixing. Sytox Green was removed by sequential rinses, followed by 2% paraformaldehyde fixation for 1 h at room temperature. Samples were stored at 4°C. For analysis, all cell nuclei were counterstained with 10 µg/ml Hoechst 33342 (Invitrogen) for 1 h at room temperature. To determine fluconazole susceptibility, microscope fields (20× objective) were sequentially imaged by LSCM for staining by Hoechst 33342 (Mac-Tai IR laser; excitation, 786 nm/emission, 345 nm) to determine total cell number and for staining by Sytox Green (argon laser; excitation, 488 nm; emission, 523 nm) for cells with impaired permeability. TIF images of the 100 optical sections were acquired, and the percent total nuclei and percent Sytox Green-stained nuclei were determined using Image J software (30). Four fields were counted for each of three duplicate biofilms for a total of 12 data points per strain.

**Analysis of mating in biofilms.** Mating in biofilms was assessed by a complementation assay recently described in detail (31). All strains used were auxotrophic (see Table S1 in the supplemental material) and, therefore, were grown to stationary phase at 25°C in suspension in modified Lee’s medium (19) supplemented with 30 µg/ml adenine and 100 µg/ml uridine (nonselection medium). Cells were then collected, washed, and resuspended in either RPMI 1640 or Spider medium supplemented with adenine and uridine. Cells were then mixed in the combinations described in Results. A total of 2 × 10^7 cells were then plated as described previously for biofilms in general, in the alternative media supplemented with adenine and uridine. Incubation was performed under two sets of conditions: 37°C, air; 37°C, 20% CO₂. After 48 h, cells were removed mechanically from the elastomer disc by sonication three times for a 5-s duration at 70% amplitude (Vibra Cell Sonic and Materials, Inc.) in 1 ml of Hanks’ balanced salt solution (HBSS; Gibco, Grand Island, NY) without divalent cations, containing 20 mM EDTA. Aliquots were then plated on agar selection plates (10 cm) containing modified Lee’s medium lacking adenine and uridine (selection medium), which allowed assessment of the frequency of complementation events (i.e., viable colonies). In experiments to compare a/a biofilms formed in RPMI medium versus Spider medium, diluted aliquots were plated on agar plates 10 cm in diameter containing modified Lee’s medium supplemented with adenine and uridine (nonselection plates), as well as phloxine B to assess in parallel the total CFU and the number of opaque CFU, which stained red with phloxine B (32). Each experiment included 3 biofilms under each set of conditions. Each biofilm was analyzed on three selection plates containing Lee’s medium lacking adenine and uridine, resulting in nine measurements for each condition per experiment. The combined number of plates for two independent experiments was six. Since opaque CFU per biofilm were similar under the different conditions, frequency was computed as the number of complementation events per biofilm.

**RESULTS**

**Differences in a/α biofilm architecture.** To generate biofilms, cells of two unrelated a/α strains, SC5314 and P37037 (a/α) (see Table S1 in the supplemental material), were grown in liquid-supplemented Lee’s nutrient medium (19, 20) to stationary phase and distributed on pretreated silicone elastomer discs in wells containing either RPMI 1640 medium (referred to subsequently as RPMI medium) or Spider medium. These preparations were then incubated in air at 37°C for 90 min, conditions used in a majority of biofilm studies using Spider medium. After incubation, an average of 2 × 10⁶ cells adhered to the silicone elastomer in both cases. The original medium and nonadhering cells were then gently withdrawn and immediately replaced with fresh medium. Biofilms were then gently rocked for 48 h, stained with calcofluor white, and scanned by LSCM for cellular architecture. It is important to note that except for preincubation of the silicone elastomers and the alternative media, the origins of the cells and all conditions for biofilm development were identical and performed in parallel. For assessing cellular architecture, the settings of the microscope were set to optimize visualization of the calcofluor white-stained walls of yeast cells and hyphae in biofilms formed in RPMI medium. LSCM was then performed at these same prescribed settings for biofilms formed in the alternative media. One hundred scans were collected beginning at the substrate (Fig. 1B and E, lower scan) and ending past the top of each biofilm. Projection images of the 100 stacked scans of biofilms formed from cells of a/α strains SC5314 and P37037 (a/α) in RPMI medium and in Spider medium were generated and rotated 90°. They were then viewed from this angle (side view), as in Fig. 1A and D, respectively. The intense calcofluor white staining at the top of the biofilms was artificial, due to the rapid surface absorption of dye upon application. This artifact did not affect the analysis of cellular architecture within the biofilm in individual scans. Representative individual scans of the same a/α biofilms formed in RPMI and Spider media are presented in Fig. 1B and E, respectively. Representative individual scans are provided from the lower, middle, and top portions of the biofilm. The results for the two independent a/α strains, SC5314 and P37037 (a/α), proved highly similar, so only those of SC5314 are presented. All results were consistent between multiple biofilms and between repeat experiments.

An analysis of each z-series of 100 scans at 2-µm intervals beginning at the substrate and ending above the biofilm surface revealed that biofilms formed by a/α cells in RPMI medium at 37°C, in air, began at the substrate with a compact basal yeast cell poly-
layer, approximately four cells deep, that adhered tightly to the substrate. This basal polylayer was approximately 10 μm thick (Fig. 1B, lower individual scan). It gave rise to vertically oriented hyphae that appeared in each subsequent scan through most of the upper portion of the biofilm as white dots (Fig. 1B, middle individual scan). The dot pattern indicated vertically orientated hyphae. Vertical orientation was also suggested by the side views of the projection images (Fig. 1A). In the very top individual LSCM scans of these biofilms (Fig. 1B, top), longer, tangential profiles were observed, suggesting that the distal ends of hyphae in an RPMI medium-derived biofilm lost vertical orientation (i.e., the hyphal ends bent). No yeast cell clumps were observed in the upper portion of these biofilms, which represented 80% of their height (Fig. 1B, middle and top scans). A side-view model of the cellular architecture deduced by carefully analyzing LSCM scans of five individual biofilms of both a/α strains formed in RPMI medium at 37°C is presented in Fig. 2A.

The architecture of biofilms formed by a/α cells in Spider medium at 37°C in air differed from that of biofilms formed in RPMI medium under the same conditions. Biofilms formed in Spider medium contained yeast cells dispersed on the substrate at a density below that of a monolayer (Fig. 1E, lower scan). The great majority of these cells had formed hyphae, suggesting that after initial dispersal of yeast cells in Spider medium, those that adhered
to the silicone elastomer substrate immediately underwent the bud-hypha transition with little proliferation in the yeast phase. Therefore, a/α biofilms formed in Spider medium at 37°C in air did not form a uniform yeast cell polylayer at the substrate. Hyphae radiating from cells at the substrate exhibited a higher frequency of tangential profiles in individual scans throughout the biofilm (Fig. 1E, middle and top scans), suggesting that in a/α biofilms formed in Spider medium, hyphae did not have the same degree of vertical orientation as they did in a/α biofilms formed in RPMI medium. This difference was also suggested in comparisons of the middle region of side views of projection images (compare Fig. 1D with Fig. 1A). Small clumps of budding cells were also observed dispersed throughout a/α biofilms formed in Spider medium (Fig. 1E, arrowheads), with more clumps in the middle region, a characteristic not observed in RPMI medium-derived a/α biofilms. A model of the cellular architecture deduced by carefully analyzing each of the LSCM scans of five individual a/α biofilms of both a/α strains formed in air in Spider medium at 37°C is presented in Fig. 2B, and can be compared to the model of an a/α biofilm formed in RPMI medium under similar conditions (Fig. 2A).

The surfaces of representative paraformaldehyde-fixed a/α biofilms formed in RPMI medium and Spider medium, imaged at low magnification through a stereomicroscope, are presented in Fig. 1C and F, respectively. While a/α biofilms formed in RPMI medium were wrinkled and membranous, a/α biofilms formed in Spider medium were unwrinkled and fuzzy. Higher magnification through the same stereomicroscope revealed that the fuzziness of the latter was due to individual hyphae radiating from the surface of Spider medium-derived biofilms. No hyphae radiated out of RPMI medium-derived biofilms.

**Differences in a/α biofilm architecture.** A similar comparison of cellular architecture was performed between biofilms of white a/α cells formed in RPMI and Spider media in air at 37°C, for two independent a/α strains, P37005 and P37037 (a/α) (see Table S1 in the supplemental material). P37037 (a/α) was a spontaneous derivative of strain P37037 (a/α), resulting from the loss of the chromosome 5 α homolog followed by duplication of the retained homolog (33, 34). Since all of the results obtained for the two a/α strains were highly similar, only those for strain P37005 are described. Just as was the case for a/α biofilms, LSCM scans of calciofluor white-stained biofilms formed by a/α cells in RPMI medium in air at 37°C revealed a basal polylayer of yeast cells (Fig. 3B, lower scan) and an upper region of vertically oriented hyphae (Fig. 3B, middle scan). Again, scans of the very top of the biofilm revealed tangential hyphal profiles, suggesting bending at the hyphal

![Image of biofilm models](https://example.com/biofilm_models.png)
ends (Fig. 3B, top scan). As was the case for \(a/a\) biofilms in RPMI medium, no yeast cell clumps were observed in the upper region. A model of the cellular architecture deduced by carefully analyzing 2-μm-interval z-series scans through five individual biofilms formed in RPMI medium in air at 37°C for both \(a/a\) strains is presented in Fig. 2C. It is similar architecturally to the deduced model for the cellular architecture of \(a/a\) biofilms formed in RPMI medium in air (Fig. 2A).

The cellular architecture of the preparations of both \(a/a\) strains formed in Spider medium in air at 37°C, however, was radically different from that of biofilms formed in RPMI medium and radically different from that of biofilms formed by \(a/\alpha\) cells in Spider medium. After 48 h, cells in Spider medium had formed a yeast cell polylayer approximately 10 cells deep (Fig. 3E, lower, middle, and upper scans). Germ tubes and rare hyphae were observed in the yeast cell polylayer, but an upper region of hyphae and matrix in Spider medium in air, a phenotype quite distinct from \(a/\alpha\) cells in Spider medium. (A and D) A comparison of side views of projection images of stacked LSCM scans. (B and E) A comparison of individual LSCM scans through the lower, middle, and top portions of the alternative fixed, calcofluor white-stained biofilm formed at 37°C in air in RPMI medium and the predominantly yeast cell polylayer formed at 37°C in air in Spider medium, respectively. The vertical orientation of hyphae is suggested by the white dots representing cross sections of hyphae in the middle-portion scans of the biofilm formed in RPMI medium in air, while the compact yeast cell nature of the polylayer formed by \(a/a\) cells in Spider medium in air is apparent in the lower, middle, and top scans. (C and F) A low-magnification comparison of the entire paraformaldehyde-fixed biofilms formed by \(a/a\) cells in RPMI and Spider media, respectively. Notice the wrinkled membranous nature of the \(a/a\) biofilm (C) formed in RPMI medium in air, similar to that formed by \(a/\alpha\) cells in RPMI medium in air (compare with Fig. 1C). Notice the granular pattern of the \(a/a\) biofilm formed in Spider medium in air, reflecting the predominant yeast cell nature. The blow-up panel of cells in a space in the \(a/a\) polylayer formed in Spider medium in air reveals cells only in the budding yeast phase.

FIG 3 Biofilms formed by \(a/a\) cells in strain P37005 in RPMI and Spider media at 37°C in air exhibit dramatic differences in cellular architecture. While \(a/a\) cells form a biofilm in RPMI medium in air similar to that formed by \(a/\alpha\) cells, \(a/a\) cells form a polylayer made up primarily of yeast cells lacking an upper region of hyphae and matrix in Spider medium in air, a phenotype quite distinct from \(a/\alpha\) cells in Spider medium. (A and D) A comparison of side views of projection images of stacked LSCM scans. (B and E) A comparison of individual LSCM scans through the lower, middle, and top portions of the alternative fixed, calcofluor white-stained biofilm formed at 37°C in air in RPMI medium and the predominantly yeast cell polylayer formed at 37°C in air in Spider medium, respectively. The vertical orientation of hyphae is suggested by the white dots representing cross sections of hyphae in the middle-portion scans of the biofilm formed in RPMI medium in air, while the compact yeast cell nature of the polylayer formed by \(a/a\) cells in Spider medium in air is apparent in the lower, middle, and top scans. (C and F) A low-magnification comparison of the entire paraformaldehyde-fixed biofilms formed by \(a/a\) cells in RPMI and Spider media, respectively. Notice the wrinkled membranous nature of the \(a/a\) biofilm (C) formed in RPMI medium in air, similar to that formed by \(a/\alpha\) cells in RPMI medium in air (compare with Fig. 1C). Notice the granular pattern of the \(a/a\) biofilm formed in Spider medium in air, reflecting the predominant yeast cell nature. The blow-up panel of cells in a space in the \(a/a\) polylayer formed in Spider medium in air reveals cells only in the budding yeast phase.
biofilms, formed in RPMI and Spider media in air at 37°C, are imaged at low magnification in Fig. 3C and F, respectively. While the a/α biofilms formed in RPMI medium were wrinkled and membranous (Fig. 3C), as was the case for a/α biofilms formed in RPMI medium in air at 37°C (Fig. 1C), the surfaces of a/α biofilms formed in Spider medium in air at 37°C were punctate rather than membranous and smooth, with no fuzzy surface. An analysis of the frequent spaces observed in the a/α polylayer formed in Spider medium, in which the phenotype of individual cells could be visualized, revealed cells primarily in the yeast phase and a paucity of filamentous cells (Fig. 3F, inset).

Matrix density in a/α biofilms. The uniformly vertical orientation of hyphae in most of the upper region of a/α biofilms formed in RPMI medium versus the less uniformly oriented hyphae in a/α biofilms formed in Spider medium, and the spaces in calcofluor white-stained biofilms formed in Spider medium that were absent from biofilm formed in RPMI medium, suggested to us that a difference may exist in matrix deposition between the two biofilms. To assess matrix density, we stained fixed preparations of a/α biofilms formed in the alternative media with Sypro Ruby, a general protein stain (35), which has previously been used to stain a/α biofilms formed in Spider medium (Fig. 4B) but not in the matrix of a/α biofilms formed in RPMI medium (Fig. 4A). Staining of the matrix in biofilms formed in RPMI medium was more uniform through the biofilm than that of biofilms formed in Spider medium. The biofilms formed in Spider medium exhibited more staining in the lower half (data not shown). The differences in matrix density and uniformity of a/α biofilms formed in RPMI medium versus Spider medium are modeled in Fig. 2A and B, respectively.

Leukocyte penetrability and fluconazole resistance. We previously demonstrated that MTL-heterozygous (a/α) biofilms formed in RPMI medium are relatively impermeable to low- and high-molecular-weight molecules, highly resistant to penetration by human polymorphonuclear leukocytes, and highly resistant to fluconazole (15, 16, 40). In contrast, MTL-homozygous biofilms (a/a, α/α, or mixed a/a-α/α) are far more permeable, penetrable, and drug susceptible (15, 16, 40). We therefore tested whether a/α biofilms grown in Spider medium in air at 37°C also exhibited differences between these pathogenic traits. Biofilms were first tested for penetrability using a newly developed assay that employs differentiation-dependent DMSO-inducible GFP-fluorescent leukocytes engineered in the cell line HL60 (27, 28). In the assay, fluorescent leukocytes were dispersed on the top of a 48-h biofilm and subsequently incubated for 3 h. Penetration was then assessed in side views of LSCM projection images. As previously reported (15, 16, 40), leukocytes remained at the very top of biofilms of a/α strains SC5314 (Fig. 5A) and P37037 (a/α) (data not shown) grown in RPMI medium in air. Leukocytes, however, penetrated through one-third to one-half of a/α biofilms formed by strains SC5314 (Fig. 5B) and P37037 (a/α) (data not shown) in Spider medium. As previously reported (15, 16, 40), leukocytes penetrated on average through more than 80% of a/a biofilms formed in RPMI medium in air (Fig. 5C). However, leukocytes penetrated through the entire depth of a/a yeast cell polylayers formed in Spider medium in air (Fig. 5D). In these latter yeast cell polylayers, the leukocytes accumulated uniformly on the silicone elastomer substrate (Fig. 5D).

To test for fluconazole resistance, 48-h biofilms were overlaid with 1 ml of fresh medium containing fluconazole, incubated for an additional 24 h, and dispersed and the cells were tested for permeability to Sytox Green, a double-stranded DNA dye that stains only the nuclei of fluconazole-permeabilized cells (i.e., fluconazole-susceptible cells) (15). Total cells, including nucleated hyphal compartments, were assessed by staining nuclei in the fixed cell preparations with a second dye, Hoechst 33342. Only 7% ± 1% of the cells from a/α biofilms of strain SC5314 formed in RPMI medium in air at 37°C were susceptible to fluconazole (Fig. 5E), a value slightly higher than that previously reported (~3%) (15, 16). In contrast, 27% ± 6% of cells in a/α biofilms formed in Spider medium in air at 37°C were susceptible, a 4-fold difference that proved highly significant using Student’s t test (Fig. 5E). In a/a biofilms formed in RPMI medium in air, 38% ± 4% of cells were susceptible to fluconazole, a value very close to that previously reported (15, 16). In contrast, 78% ± 12% of cells in a/a yeast cell polylayers formed in Spider medium were susceptible, which represented a 2-fold increase in susceptibility over that of a/a biofilms.

FIG 4 Staining with Sypro Ruby, a dye that stains both cell wall and matrix, reveals a denser matrix in the areas between the hyphae in an a/α biofilm (SC5314) formed in RPMI medium at 37°C in air (A) than in an a/α biofilm formed in Spider medium at 37°C in air (B). All images were obtained at the same LSCM settings.
formed in RPMI medium (Fig. 5E). This difference proved highly significant using Student's t test.

Facilitation of mating. We recently demonstrated that biofilms formed by a/a, c/α, or 50:50 a/a-α/α cells in RPMI medium facilitate mating between seeded minority opaque a/a and α/α cells at 10 to 100 times the frequency observed in a/α biofilms (31). This difference was found to be true at 28°C or 37°C and in air or 20% CO₂. To test whether MTL-homozygous biofilms (a/a-α/α or a/a) formed in Spider medium similarly facilitated mating, we used the same complementation assay (31). In brief, majority (90%) α/α cells (Δura3/Δura3), majority (90%) white a/a-α/α cell mixtures (Δura3/Δura3 and Δade2/Δade2, respectively), or majority (90%) white a/a cells (Δura3/Δura3) were seeded with majority (10%) opaque cells (5% opaque a/a cells [Δura/Δura] and 5% opaque α/α cells [Δade2/Δade2]), prior to casting biofilms. These cell mixtures were then cast on silicone elastomers in RPMI medium or in Spider medium, both supplemented with uridine and adenine (nonselection medium). These biofilms were referred to as “seeded” biofilms. The seeded a/α population, seeded white a/a-α/α cell population, and seeded white a/a population formed biofilms in RPMI medium in air at 37°C after 48 h similar to those of unseeded a/α cells, white a/a-α/α cells, and a/a cells; the same was true in Spider medium in air at 37°C after 48 h. At 37°C in air, there were no detectable complementation events in seeded a/a biofilms formed either in RPMI medium or in Spider medium in both experimental data sets (Table 1). Under these same conditions, the average frequency (± standard deviation) of complementation events in seeded white a/a-α/α or a/a biofilms formed in RPMI medium was 20.0 ± 3.3 and 22.2 ± 7.5, respectively, per biofilm (Table 1). However, in a/a-α/α or a/a polylayers formed in Spider medium at 37°C in air, there were no detectable complementation events (Table 1), in spite of the presence of opaque cells at similar concentrations. These results demonstrate that at 37°C in air, white a/a-α/α or a/a biofilms formed in RPMI medium facilitate mating between seeded opaque a/a and α/α cells, but the a/a-α/α or a/a yeast cell polylayers formed in Spider medium do not.

a/a strains do form traditional biofilms and facilitate mating in Spider medium in 20% CO₂. Although high concentrations of CO₂ are rarely used in biofilm experiments employing Spider or RPMI medium, we recently observed in an in-depth analysis of mating in RPMI medium that replacing air with 20% CO₂ caused a small, but reproducible, increase in mating frequency in a/a biofilms (31). We therefore tested whether CO₂ affected the facilitation of mating in a/a yeast cell polylayers formed in Spider medium. As in the recent in-depth study (31), we again found that, in RPMI medium in 20% CO₂, the frequency of complementation events per seeded white a/a-α/α biofilm was at least 10 times higher than the frequency per seeded a/α biofilm formed under the same conditions, and the frequency in seeded white a/a biofilms was approximately 15 times higher than that in a/a biofilms in 20% CO₂ (Table 1). However, the results obtained in seeded white a/a-α/α and a/a preparations incubated in Spider medium in 20% CO₂ were unexpected. First, we found that in contrast to the polylayers of predominantly yeast cells (Fig. 6A and B) and the absence of mating facilitation (Table 1) observed in white a/a-α/α cell and white a/a cell populations in Spider medium in air, MTL-homozygous cell preparations incubated in Spider medium in 20% CO₂ formed biofilms with upper regions of hyphae and matrix (Fig. 6C and D) and facilitated mating (Table 1). Although the MTL-homozygous biofilms formed in 20% CO₂ were slightly thinner, they exhibited cellular and matrix architecture (Fig. 6C and D) highly similar to that of a/a biofilms formed in Spider medium in air (Fig. 1D and E) or 20% CO₂ (data not shown). The architecture and matrix of these biofilms are modeled in Fig. 2E. The a/a-α/α and a/a biofilms formed at 37°C in 20% CO₂, like a/a biofilms, contained a diminished basal yeast cell polylayer, less vertically oriented hyphae, pockets of yeast cells, and matrix. But most importantly, seeded white a/a-α/α and a/a biofilms formed in 20% CO₂ facilitated complementation, and at a frequency significantly higher than that in a/a biofilms formed in Spider medium at 37°C in 20% CO₂ (Table 1). In other words, a/a-α/α and a/a biofilms formed in Spider medium in 20% CO₂...
facilitated mating, just as \( a/a-\alpha/\alpha \) and \( a/a \) biofilms formed in RPMI medium facilitated mating (Table 1). However, the complementation frequencies attained in \( a/a-\alpha/\alpha \) and \( a/a \) biofilms formed in Spider medium in 20% CO\(_2\) were not as high as those in \( a/a-\alpha/\alpha \) or \( a/a \) biofilms formed in RPMI medium in 20% CO\(_2\) (Table 1). Therefore, when incubated in Spider medium in 20% CO\(_2\), but not in air, \( MT L \)-homozygous cells will form a biofilm with an upper hypha/matrix region like \( a/\alpha \) biofilms, and these biofilms have an advantage over \( a/\alpha \) biofilms formed in Spider medium in 20% CO\(_2\) in the facilitation of mating.

**Biofilm formation in Lee’s medium.** To emphasize the effects of medium and incubation conditions on biofilm formation, we performed a cursory comparison of the architecture of biofilms formed in RPMI medium with the architecture of those formed in Lee’s medium. In 2006, we found that supplemented Lee’s medium (19, 20) did not support the formation of robust biofilms (K. Daniels and D. R. Soll, unpublished observations) and instead selected RPMI medium, which we found, as did Douglas (3), was a very good supporting medium for biofilm formation. Recently, Lin et al. (24) used Spider medium—grown inoculum to generate Lee’s medium—derived cluster-well–based adhesive cells incubated for 24 h as biofilms to challenge the conclusion by Soll and colleagues (15, 41) that Tec1 was the target of the mitogen-activated protein (MAP) kinase pathway in \( a/a \) biofilm formation. However, whereas the \( a/a \) biofilms studied by Soll and colleagues were always developed in RPMI medium for 48 h with slow rocking (15, 16, 29, 40–44), those studied for comparison by Lin et al. (24) were developed in Lee’s medium for 24 h in stationary, nonrocking cultures, conditions very close to the adhesion assay, not the biofilm assay, used by Soll and colleagues (29, 44). A comparison of biofilm formation in RPMI medium with that in Lee’s medium thus provided an excellent test for the hypothesis, explored here, that differences under conditions used in *in vitro* can lead to biofilms that are quite different in architecture and regulation.

In the comparison, the strain used by Lin et al. (24), P37005 \( (a/a) \), which was obtained from Soll and colleagues (45), was employed. The five conditions that differed between the two studies on pheromone-stimulated biofilms included (i) the growth conditions of the original cell inoculum, (ii) the use of Lee’s medium for biofilm development by Lin et al. (24) versus the use of RPMI 1640 medium by Soll and colleagues (15, 16, 29, 40–44, 46–48), (iii) gentle rocking to mix medium during biofilm development versus stationary cultures applied in the methods of Lin et al. (24), (iv) a 48-h incubation period versus a 24-h incubation period used by Lin et al. (24), and (v) biofilms cast on plastic by Lin et al. (24) versus the exclusive use of silicone elastomer for RPMI medium–derived biofilms.

The differences in the architectures of the two biofilms that formed were extreme. Whereas RPMI medium–derived biofilms were on average 72 ± 3 \( \mu \)m thick (Fig. 7B and C), Lee medium–derived preparations were on average 8 ± 2 \( \mu \)m thick, both those on elastomer, which were visualized with an inverted LSCM (Fig. 7E and F), and those on plastic, which were visualized with an

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**TABLE 1** Facilitation of mating by \( MT L \)-homozygous (\( a/a-\alpha/\alpha \) or \( a/a \)) biofilms formed in Spider medium at 37°C in air or in 20% CO\(_2\).

| Expt set | Population genotype | Conditions | RPMI medium | Spider medium |
|----------|---------------------|------------|-------------|---------------|
| 1        | \( a/a (90\%) + op \) | 37°C, air | 0.0         | 0.0           |
|          | \( a/a-\alpha/\alpha (90\%) + op \) | 37°C, air | 20.0 ± 3.3  | 0.0           |
|          | \( a/a (90\%) + op \) | 37°C, 20% CO\(_2\) | 3.3 ± 0.0  | 1.1 ± 1.9     |
|          | \( a/a-\alpha/\alpha (90\%) + op \) | 37°C, 20% CO\(_2\) | 30.0 ± 3.3  | 5.6 ± 1.9     |
| 2        | \( a/a (90\%) + op \) | 37°C, air | 0.0         | 0.0           |
|          | \( a/a (90\%) + op \) | 37°C, 20% CO\(_2\) | 22.2 ± 7.5 | 0.0           |
|          | \( a/a-\alpha/\alpha (90\%) + op \) | 37°C, 20% CO\(_2\) | 2.2 ± 1.7  | 1.7 ± 1.8     |
|          | \( a/a (90\%) + op \) | 37°C, 20% CO\(_2\) | 29.4 ± 6.5 | 11.1 ± 7.5    |

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"op" (10%) represents 5% \( a/a \) opaque plus 5% \( \alpha/\alpha \) opaque.

See Table S1 in the supplemental material for genotypes of strains used.

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**FIG 6** While \( a/\alpha \) cells form a thick biofilm with an upper hyphal/matrix region in Spider medium in 20% CO\(_2\), they form a much thinner, predominantly yeast cell polylayer, approximately 10 cells thick, in Spider medium in air. (A and C) Comparisons of top view (highest LSCM scan) of the alternative calcfluor white-stained \( a/a \) biofilms formed in air and 20% CO\(_2\), respectively. The former is comprised of yeast cells and the latter of hyphae. (B and D) Orthogonal 90° view of a single z-slice through LSCM projected scans in air and 20% CO\(_2\), respectively. The means and standard derivations of thickness in micrometers are presented below panel B and D.
upright LSCM (Fig. 7H and I). It is important to note that in Lee’s medium on plastic, attached cells were easily dislodged from the substrate by gentle mechanical disruption. The cellular architecture of RPMI medium-derived biofilms has been described in detail above (Fig. 2 and 3). A top view is shown in Fig. 7A, and a side view is shown in Fig. 7B. The top view reveals an exclusively hyphal upper region (Fig. 5A). Higher-magnification images are presented in Fig. S1A in the supplemental material. The architecture of Lee’s medium-derived preparations formed on the elastomer substrate is shown in Fig. 7D and E, and the architecture of those formed on the plastic substrate is shown in Fig. 7G and H, respectively. Higher-magnification pictures on the two substrates are presented in Fig. S1C and D and S1E and F, respectively. On silicone elastomer, Lee’s medium-derived biofilms consisted of...
92% yeast cells and about 8% filamentous cells (Fig. 7F). The latter included germ tubes, pseudohyphae, and short hyphae. On plastic, the percent filamentous cells was below 0.1% (Fig. 7I). Neither the Lee’s medium-derived preparations on silicone elastomer nor those on plastic exhibited the upper hyphal and matrix architecture of RPMI medium-derived biofilms (Fig. 7E and H, respectively). They also did not form the thick polylayer of predominantly budding yeast cells that was formed by a/a cells in RPMI medium.

Since Yi et al. (15) showed a significant increase in thickness of a/a cell preparations in RPMI medium when seeded with opaque cells, a source of pheromone, and Lin et al. (24) demonstrated an increase in thickness of preparations in Lee’s medium when treated with pheromone, we analyzed α-pheromone-treated preparations formed in Lee’s medium to see if the complex hyphal/matrix upper region was established. When treated with α-pheromone with a finite half-life or, in the case of RPMI medium-derived biofilms, when seeded with a minority (10%) of a/a or α/α opaque cells, both RPMI medium-derived and Lee’s medium-derived biofilms increased in thickness, by 17% in the former case and by over 100% in the latter case (Fig. 7K, L, N, O, P, R, and Q). However, the stimulated thickness of Lee’s medium-derived biofilms was still one-fourth of that of stimulated RPMI medium-derived biofilms (Fig. 7K and L versus Fig. 7N and O and Fig. 7Q and R). Pheromone-stimulated Lee’s medium-derived biofilms on either substrate still contained no upper hyphal region, but rather a majority of budding yeast cells and a minority of short filamentous cells (Fig. 7M and P; see also Fig. S1D and F in the supplemental material).

To assess matrix deposition, paraformaldehyde-fixed biofilms were stained with Sypro Ruby. The LSCM settings were set to visualize the extracellular matrix of biofilms formed in RPMI medium between cells, and these settings were then used to compare biofilms formed in the matrix of RPMI medium-derived and Lee’s medium-derived biofilms. The cells and the regions between cells in all of the Lee’s medium-derived biofilms, both untreated and pheromone treated, stained far less intensely than the cells and the regions between cells at the substrate and hyphae in the upper region in RPMI medium-derived biofilms (compare Fig. S2A and B in the supplemental material with S2C and D and S2E and F). These results indicate that in the absence of pheromone, there is very little to no matrix deposition in Lee’s medium-derived biofilms, and in pheromone-induced Lee’s medium-derived biofilms, there is more matrix deposition but far less than that in RPMI medium-derived biofilms. Together with the cellular architecture differences, these results underscore just how disparate the architecture of MTL-homozygous biofilm preparations can be, depending on the supporting medium, mixing conditions, and time of incubation.

**DISCUSSION**

The assumption, or perception, that *C. albicans* forms one type of biofilm *in vitro* places a burden upon attempts to develop a single model for biofilm regulation, architecture, and function that includes the results of experiments performed in a large number of laboratories using different conditions. This problem has also been discussed by Joo and Otto (49) as it relates to biofilms formed by bacterial pathogens. Recently, Fanning et al. (17) showed quite convincingly that gene expression patterns can differ dramatically between a/α biofilms formed in two quite common media, thus arguing against the idea that “one shoe fits all.” In addition, both Fanning et al. (17) and Nobile et al. (18) have shown that regulation can be different between *in vitro* and *in vivo* models and between different *in vivo* models. Here, we present additional evidence that the assumption that *C. albicans* forms a single biofilm is invalid and that, under some conditions, particularly pertaining to MTL-homozygous cells, different media can result in biofilms with dramatically different architectures and functional characteristics. Here, our challenge to the assumption is based not on gene expression but rather upon a comparison of the cellular architecture, matrix, pathogenic traits, and facilitation of mating between biofilms formed in two commonly used but compositionally different media, and a more cursory analysis of a medium recently employed that results in an even more disparate biofilm.

**The compositions of RPMI 1640 and Spider media.** We have based most of our arguments on differences between biofilms formed in RPMI and Spider media. RPMI medium was first developed by Moore et al. (50) for growing human leukocytes. It is defined and includes essential amino acids, vitamins, salts, and glucose as the main carbon source (50). The pH of RPMI medium is normally 7.4 and stabilized by the addition of MOPS when used in the absence of CO2. Spider medium was first used with *C. albicans* for studies of hypha induction (22). It is an undefined medium that includes nutrient broth and mannitol as the main carbon source. Nutrient broth contains peptone and beef extract (51). Beef extract contains peptone, including those found in blood. The pH of Spider medium has ranged from 6.7 to 7.2 in biofilm experiments. Here, we have compared in detail biofilm formation by both a/α and a/a cells after 48 h in RPMI medium versus Spider medium, under conditions commonly used in biofilm experiments using the latter medium. The conditions for biofilm development included cells similarly grown to stationary phase in the same medium, the same silicone elastomer surface, a temperature of 37°C, an atmosphere of air, similar pH (7.0 versus 7.2, respectively), the same incubation time (48 h), and the same gentle rocking procedure for mixing the medium in the biofilm cultures.

**Differences in a/α architecture.** Given that Spider medium was originally used to induce a/α yeast cells to extend germ tubes, which elongate to form compartmentalized hyphae (52–59), it was no surprise to find that the addition of a/α yeast cells from the stationary phase of planktonic cultures to this medium resulted in germ tube formation by a majority of the cells that had adhered to the substrate after 90 min. These germ tubes subsequently grew into narrow, long hyphae. We therefore found that, unlike biofilms formed in RPMI medium, those formed in Spider medium had a far less pronounced basal yeast cell polylayer than did biofilms formed in RPMI medium as well as in a number of other rich media, such as YPD medium (yeast extract-peptone-dextrose) (60). Ernst and coworkers showed that the induction of hyphae by Spider medium was not due to mannitol as the carbon source, since mannitol could be replaced with glucose without changing hypha induction (61, 62). Ernst and coworkers (62) also showed that addition of ammonia salts to Spider medium did not inhibit hyphal induction, thus demonstrating that induction was not due to nitrogen starvation (62). The only other ingredient in Spider medium is nutrient broth, which is composed of beef extract and peptone. Since peptone is an ingredient of other media, like YPD, which do not immediately induce hypha formation and which facilitate the formation of a basal yeast cell polylayer during bio-
film formation (3; Daniels and Soll, unpublished), it seems unlikely that the peptide in Spider medium causes the rapid induction of hyphae that results in the absence of a basal yeast cell polylayer. Finally, it has been demonstrated that coating silicone elastomer with bovine serum, a step used to prepare the elastomer for biofilm adhesion in Spider medium, does not inhibit the formation of a yeast cell polylayer (3, 63). Therefore, we have tentatively concluded that it is the beef extract peptides in Spider medium that rapidly induce hypha formation and that result in the absence of a basal yeast cell polylayer.

In contrast to Spider medium, the addition of stationary-phase a/α yeast cells to RPMI medium results in yeast cell proliferation over the first 12 h after adhesion to the substrate, resulting in the formation of a cohesive yeast cell polylayer, roughly four cells thick. After 12 h, cells at the distal edge of this polylayer (i.e., the top layer) form germ tubes that elongate into hyphae. In Spider medium, the hyphae formed at the elastomer surface grow upward, but not in a uniformly vertical orientation. The hyphae formed in RPMI medium, however, are oriented vertically throughout the major portion of the upper hyphal region of the biofilm, losing vertical orientation only at the very top, where they bend. One last difference between biofilms formed in RPMI and Spider media is the presence of yeast cell clumps in the latter. While clumps are formed throughout biofilms formed in Spider medium, they are absent from the upper 80% of biofilms formed in RPMI medium. Clumped yeast cells, at even higher proportions and frequently at the substrate, were first demonstrated in a/α biofilms formed in Spider medium by Mitchell and coworkers (64). Several of their conditions differed from those used here, demonstrating that parameters other than medium affect the final architecture of a biofilm.

A comparison of the densities of the extracellular matrixes of a/α biofilms, using Sypro Ruby to stain fixed preparations, suggested that the matrix formed in RPMI medium was denser than that formed in Spider medium. Sypro Ruby is a general protein dye (35) that has been extensively used for staining the matrix of fixed bacterial biofilms (36–39). A comparison of matrix staining with calcofluor white, a chitin stain (25), was less effective because of the trapping effect at the top of both types of biofilms during staining. Even so, the results of calcofluor white staining also suggested the same difference in general matrix density (K. Daniels and D. R. Soll, personal observations). A more interesting comparison was between low-magnification images of a/α biofilms fixed with 2% paraformaldehyde, a fixative which maintains biofilm integrity. Biofilms formed in RPMI medium were membranous and wrinkled, with no hyphae protruding from the surface of what we assume may be a dense mixture of hyphae encapsulated in matrix. Biofilms formed in Spider medium, however, were looser with a hairy surface texture, suggesting that hyphae at the biofilm surface may be less encapsulated in matrix. LSCM comparison of Sypro Ruby-stained preparations revealed a uniform matrix in RPMI medium-derived biofilms and a less uniform matrix disproportionately deposited proximally in Spider medium-derived biofilms.

Differences in a/α biofilm architecture. Perhaps our most surprising observation was that cell preparations from three different, unrelated natural a/a strains incubated in Spider medium in air at 37°C for 48 h did not form an upper region of hyphae and matrix. Rather, a/a cell preparations were comprised predominantly of yeast cells, approximately 10 cells thick, with protruding germ tubes and rare hyphae. In contrast, RPMI medium-derived a/a biofilms exhibited the same complex architecture as did RPMI medium-derived a/α biofilms formed in RPMI medium in air. Mixtures of white a/a and a/α cells incubated in Spider medium on silicone elastomers for 48 h in air also formed primarily a yeast cell polylayer. Therefore, the biofilms formed by a/a or mixtures of a/a and a/α cells in air in Spider medium lacked a structured hyphal network encapsulated in matrix, the architecture observed in a/α biofilms formed in Spider medium in air. Interestingly, we observed that a/a cells do form such a structured biofilm in Spider medium if air is replaced with 20% CO₂. The a/a and a/a-α biofilms formed in Spider medium in 20% CO₂ contained hyphae encapsulated in a dense matrix, like Spider medium-derived a/α biofilm formed in air or 20% CO₂.

Differences in pathogenic traits. We previously demonstrated that although a/α and a/a biofilms formed in RPMI medium exhibit similar architectures, only a/α biofilms exhibit the pathogenic traits of impermeability, leukocyte impenetrability, and drug resistance (15, 16, 40). Here, we show that Spider medium-derived a/α biofilms are also more resistant to leukocyte penetration and slightly more resistant to fluconazole than RPMI medium-derived a/a biofilms but are less resistant to leukocytes and more susceptible to fluconazole than a/α biofilms formed in RPMI medium in air. The Spider medium-derived a/a yeast cell polylayers formed in air, however, were completely penetrated by leukocytes and highly susceptible to fluconazole. The yeast cells in the a/a polylayers formed in air were over 10 times as susceptible to fluconazole as cells in a/α biofilms formed in RPMI medium and over three times as susceptible as a/α biofilms formed in Spider medium. This extraordinary level of fluconazole susceptibility of Spider medium-derived a/a cell biofilms formed in air raises the bar for susceptibility and suggests that even though white a/a biofilms formed in RPMI medium are more susceptible to fluconazole than a/α biofilms (15), they are still more resistant than a/a yeast cell polylayers formed in Spider medium in air. These results, therefore, demonstrate that a/α biofilms formed in Spider medium do exhibit pathogenic traits, but not to the extent exhibited by a/α biofilms formed in RPMI medium. These results also demonstrate that the a/a yeast cell polylayers formed in Spider medium in air exhibit virtually no pathogenic traits.

Facilitation of mating. We previously showed that a/a biofilms formed in RPMI medium at 28°C or 37°C, and in air or 20% CO₂ facilitate mating at frequencies 10 to over 100 times that of a/α biofilms formed under the same conditions (31). Here, we show that a/a biofilms formed in Spider medium at 37°C in 20% CO₂, but not in air, also facilitate mating at frequencies six to 10 times higher than those of a/α biofilms formed in 20% CO₂. We have no explanation for why mating occurs in a/α biofilms, albeit at low frequency, and at higher levels in a/a biofilms formed in Spider medium in CO₂, but not in a/α biofilms or a/a yeast cell polylayers formed in Spider medium in air.

Biofilms formed in Lee’s medium. Recently, Lin et al. (24) challenged the identity of the target transcription factor of the MAP kinase pathway in a/a biofilm formation identified by Soll and colleagues (15, 41, 43). However, they used very different conditions for biofilm development. They generated white a/a cell biofilms in Lee’s medium for 24 h, as opposed to generating them in RPMI medium for 48 h. The biofilms that formed were noticeably thinner, even when pheromone was applied to stimulate thickness (24), a stimulatory effect first demonstrated by Yi et al.
The use of Lee's medium and the different conditions employed to underscore, in an even more dramatic manner, derived biofilms to a source of pheromone. We therefore performed a comparison of RPMI medium-derived biofilms seeded with minority yeast cells, with few hyphae and little matrix in the absence of pheromone, and a biofilm consisting of approximately 10% filamentous cells when treated with pheromone. However, even the latter biofilms had no organized upper hyphal/matrix region. This cursory comparison underscores just how different biofilm preparations may be when developed in different media and under different conditions.

**Conclusion.** The results presented here, based on cellular architecture, matrix deposition, pathogenic traits, and the facilitation of mating, reinforce previous mutational and gene expression studies (17, 18), indicating that biofilms formed under different conditions can exhibit different phenotypes and can be regulated by different regulatory pathways and transcription factors. Although we have limited the study presented here to differences in media and, in the case of a/a biofilms, to air versus CO2, just about every condition involved in a biofilm preparation may have an effect on gene regulation and biofilm phenotype. These include temperature, atmosphere (air versus 20% CO2), growth of the initial inoculum, the medium used for biofilm development, mixing conditions, and substrate. And in addition to phenotypic differences, biofilms can exhibit the same architecture, as do RPMI medium-derived a/a and a/a biofilms, yet can be regulated by alternative signal transduction pathways and can differ functionally (15, 65). Therefore, in comparing data obtained under different conditions, one can consider the differences in a comparative framework, but one must be extremely cautious in trying to interpret the combined data within a single contextual framework, in this case within the framework of a single C. albicans biofilm model. This argument has also been made for bacterial biofilm formation (49).

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