Fungal biofilm architecture produces hypoxic microenvironments that drive antifungal resistance

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Human fungal infections may fail to respond to contemporary antifungal therapies in vivo despite in vitro fungal isolate drug susceptibility. Such a discrepancy between in vitro antimicrobial susceptibility and in vivo treatment outcomes is partially explained by microbes adopting a drug-resistant biofilm mode of growth during infection. The filamentous fungal pathogen Aspergillus fumigatus forms biofilms in vivo, and during biofilm growth it has reduced susceptibility to all three classes of contemporary antifungal drugs. Specific features of filamentous fungal biofilms that drive antifungal drug resistance remain largely unknown. In this study, we applied a fluorescence microscopy approach coupled with transcriptional bioreporters to define spatial and temporal oxygen gradients and single-cell metabolic activity within A. fumigatus biofilms. Oxygen gradients inevitably arise during A. fumigatus biofilm maturation and are both critical for, and the result of, A. fumigatus late-stage biofilm architecture. We observe that these self-induced hypoxic microenvironments not only contribute to filamentous fungal biofilm maturation but also drive resistance to antifungal treatment. Decreasing oxygen levels toward the base of A. fumigatus biofilms increases antifungal drug resistance. Our results define a previously unknown mechanistic link between filamentous fungal biofilm physiology and contemporary antifungal drug resistance. Moreover, we demonstrate that drug resistance mediated by dynamic oxygen gradients, found in many bacterial biofilms, also extends to the fungal kingdom. The conservation of hypoxic drug-resistant niches in bacterial and fungal biofilms is thus a promising target for improving antimicrobial therapy efficacy.

Significance

Failure of antifungal therapies to clear an infection is in part due to a biofilm mode of bacterial and fungal growth. Understanding how microbial biofilms mediate antimicrobial treatment failure is critical for improving infection outcomes. The mechanisms by which filamentous fungal biofilms mediate drug resistance are particularly ill-defined. We observe that Aspergillus fumigatus biofilms develop hypoxic microenvironments during maturation that facilitate fungal survival in the face of antifungal treatments. Oxygenation of A. fumigatus biofilms, however, reduces fungal survival. These results extend the role of dynamic biofilm oxygen gradients found in bacterial pathogens to the fungal kingdom and provide insights into how to overcome fungal drug resistance through manipulation of biofilm oxygen availability and consumption.

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Failure of antifungal therapies in vivo despite in vitro fungal isolate drug susceptibility is a major clinical problem. Our study reveals how filamentous fungal biofilms form hypoxic microenvironments that drive drug resistance. This understanding provides new targets for developing antifungal therapies.

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Results

Geometry of Oxygen Availability Governs A. fumigatus Biofilm Architecture. To identify filamentous fungal biofilm features that impact antifungal drug resistance, we first explored the impact of oxygen availability on biofilm maturation. Our previous work described oxygen-dependent changes in A. fumigatus biofilm morphology between atmospheric (~21%, normoxia) and low-oxygen (0.2%, hypoxia) conditions (30). This change in architecture, measured as the degree to which hyphae deviate from the axis orthogonal to their base cover slip, increases gradually with a reduction in macroenvironment oxygen tension (Fig. 1A and B). The overall biomass density within the biofilm does not decrease until biofilms are cultured at 2% O2 (Fig. 1C). Biomass distribution is not altered until the biofilms are cultured at 0.2% O2, where biomass localizes completely at the air–liquid interface (Fig. 1C). The inability of the biomass to grow at the coverslip at 0.2% oxygen suggests that oxygen tensions become growth-limiting near the coverslip in this low-oxygen environment. Notably, the binary switch from growth at the coverslip in atmospheric oxygen to growth at the interface at 0.2% O2 is observed in other filamentous fungi including a second strain of A. fumigatus CEA10, a close relative, Aspergillus nidulans, and an evolutionary distant relative, Scedosporium apiospermum (SI Appendix, Fig. S1). In support of this interpretation, growth of the fungus at 0.2% O2 on an oxygen-permeable plate restores growth at the coverslip (Fig. 1A). Together, these data suggest that dynamic oxygen tensions have an important role in shaping filamentous fungal biofilm maturation.

Given the increased growth under oxygen-limiting conditions on an oxygen-permeable plate, we hypothesize that the vertical hyphae formation characteristic of standard laboratory-condition biofilms (in atmospheric oxygen) is driven by a reduction in oxygen tension as the biofilm matures. In support of this hypothesis, growth on oxygen-permeable plates in atmospheric conditions yields a late-stage 24-h biofilm that is largely devoid of vertically aligned hyphae growing toward the air–liquid interface; instead, we observed biofilms with dense hyphal mats directly on and aligned with the permeable surface (Fig. 1D). Together these data indicate that the interaction of hyphal cell growth with local oxygen gradients strongly determines the final architecture of filamentous fungal biofilms, and that this is dependent on the geometry of the surface relative to the source of oxygen. Moreover, these data raise the intriguing hypothesis that hypoxic microenvironments within the filamentous fungal biofilm arise and impact biofilm form and function.

Adaptation to Hypoxic Microenvironments in the Biofilm Contributes to A. fumigatus Biofilm Maturation. To test for the presence of hypoxic zones within mature filamentous fungal biofilms, we measured dissolved oxygen using microelectrodes within submerged fungal biofilms from inoculation (time = 0 h) to maturity (time = 50 h) (Fig. 2A). At a depth from the air–liquid interface of 2.5 mm, dissolved oxygen gradually and significantly decreases as the biofilm matures (Fig. 2B). A limitation of the microelectrode is that oxygen cannot be measured directly at the coverslip. To circumvent this, we applied two independent approaches. First, we measured biomass accumulation and distribution over time (SI Appendix, Fig. S2) to parameterize a reaction–diffusion model of biofilm development and oxygen tensions (SI Appendix, Fig. S3). These data, combined with the oxygen measurements above the biomass (Fig. 2A) and published rates of A. fumigatus oxygen consumption at ambient and low oxygen (41), were used to generate models to calculate oxygen tensions further into the biofilm. These models robustly predict the emergence of stratified zones of normoxia (<5% O2), hypoxia (5% > O2 > 0), and anoxia (0% O2) within the developing biofilm (Fig. 2C and SI Appendix, Fig. S4), with hypoxic zones beginning to develop between 14 h and 16 h when cultured under ambient oxygen conditions (~21% O2).

If hypoxic microenvironments indeed arise early in filamentous fungal biofilm development and contribute to verticalized growth of hyphal filaments, we reasoned that null mutants of genes critical for the A. fumigatus hypoxia response would fail to form this characteristic architecture. The mutant strain ΔsrbA is able to grow in low oxygen, but with a ~50% growth reduction in liquid batch cultures (42). A 24-h biofilm with ΔsrbA grown at ambient oxygen (21% O2) is stunted with a clear reduction in overall biomass and vertical growth (Fig. 2D). Additionally, a mutant completely unable to grow in low-oxygen conditions, ΔsrbA Δ (43), fails to develop a mature biofilm with vertically aligned hyphal growth by 24 h in two independent strain backgrounds when grown at ambient oxygen (Fig. 2D). Conversely,

![Fig. 1. Oxygen gradients within submerged fungal biofilms are necessary for maturation. (A) Representative 3D renderings from n = 3 independent biological samples depicting the side view (XZ) of mature biofilms cultured at various oxygen tensions on normal and oxygen-permeable plates. (Scale bar, 200 μm.) (B) Heat map illustrating the alteration in hyphal architecture within biofilms cultured on normal plates as a result of reduced oxygen tensions, where architecture is defined as the angle hyphae deviate from a vertical axis. Each column is representative of n = 3 independent biological samples. (C) Heat map illustrating the altered distribution of fungal biomass as a function of height in biofilms at various oxygen tensions, as shown in A. Each column is representative of n = 3 independent biological samples. (D) Representative 3D renderings of 24-h biofilms from n = 3 independent biological samples have a collapsed architecture when cultured on an oxygen permeable surface and sealing of the surface with an oxygen-impermeable seal restores the normal plate architecture. (Scale bar, 200 μm.)](image-url)
expression of an allele that confers increased low-oxygen fitness (hrmA<sup>Δ<sub>ER</sub></sup>) (30) generates a more robust 24-h biofilm compared to the control strain AF293 (Fig. 2D). These mutant strains support the observation that hypoxic microenvironments develop during filamentous fungal biofilm maturation and that hyphal growth in the context of these oxygen gradients drives the emergence of mature fungal biofilm architecture including vertically aligned branching networks.

**A Hypoxic Response Is Spatially Induced in Filamentous Fungal Biofilms and Associated with Reduced Translational Activity in Individual Hyphae.** Our results indicate that *A. fumigatus* biofilm architecture is highly dependent on prevailing oxygen concentrations, and that as these biofilms mature they deplete the local oxygen supply in the basal layers of their own structure. Consequently, these data suggest that basal areas of the biofilm may induce a physiological hypoxic response to the changing micro-environment they occupy. To examine the spatial distribution of a hypoxia response in a filamentous fungal biofilm, we generated a hypoxia response transcriptional reporter with green fluorescent protein (GFP) and counterstained all biomass with the cell-wall-binding compound calcofluor white. The hypoxia reporter was generated using the erg25A promoter, an ergosterol biosynthetic gene encoding a C4-sterol methylxidase that has low transcript counts at ~21% oxygen and high transcript counts at 0.2% oxygen (*SI Appendix*, Fig. S5A) (30). Introduction of the reporter did not impact growth of *A. fumigatus* (*SI Appendix*, Fig. S5B), and GFP signal could be differentiated from background signal by 40 min of exposure to 0.2% oxygen in swollen conidia (*SI Appendix*, Fig. S5C). Twelve-hour biofilms of the hypoxia reporter strain (erg25A-GFP) have no detectable signal in normal oxygen, but when shifted to 0.2% oxygen for 1 h all hyphal fluorescentsce (Fig. 3 A and B and *SI Appendix*, Fig. S6A). Modeling predicts that hypoxic zones occur between 14 h and 16 h (Fig. 2C), and we observe GFP fluorescence concentrated at the base of 16-h, 18-h, and 24-h biofilms (Fig. 3 A and B and *SI Appendix*, Fig. S5A). Mature 16-h biofilms cultured on oxygen-permeable plates are devoid of signal, indicating the absence of a detectable hypoxic zone under these conditions (Fig. 3 C and D and *SI Appendix*, Fig. S6B).

Anoxic regions are also predicted to occur as the biofilm develops, which may contribute to the reduction in hypoxia reporter signal at 18 h and 24 h relative to 16 h (44). To further examine this observation, a translational activity reporter strain was constructed. GFP translation and resulting fluorescence following exogenous induction of the reporter serves as a marker for translationally active cells. Cells in a quiescent or translationally inactive state will not express GFP, or will more slowly express it, following transcriptional induction (37). We constructed a xylose-inducible GFP reporter system (45), as the small size of this monosaccharide allows for simple diffusion through hyphae within the biofilm (where space between hyphae ranges from 20 to 30 μm). The induced GFP signal is specific for addition of xylose and observed above background after 45 min of induction (*SI Appendix*, Fig. S7A). Xylose induction in a mature biofilm with the translational reporter system p<sup>3o f1 1</sup>-GFP

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**Fig. 2.** Hypoxia occurs during maturation of *A. fumigatus* biofilms. (A) Measurements of dissolved oxygen within developing fungal biofilms from *n* = 3 independent biological samples. Error bars depict SEM. (B) Extrapolation from data in A at 2,500 μm depth shows oxygen tensions gradually and significantly reduce throughout biofilm maturation. Error bars indicate SEM of *n* = 3 independent biological samples (one-way ANOVA with Tukey’s multiple comparisons test). (C) Computational modeling of oxygen zones within a fungal biofilm as a function of oxygen consumption and biomass. White zone: 5% < O<sub>2</sub> ≤ 21%, red/hypoxic zone: 0% < O<sub>2</sub> < 5%, gray/anoxic zone: O<sub>2</sub> = 0%. (D) Representative images (*n* = 3 independent biological samples) of mutant strains of *A. fumigatus* with partial (CEA10 ΔsrbB) or absolute (CEA10 ΔsrbA; AF293 ΔsrbA) low-oxygen growth defects form stunted 24-h biofilms in normoxia. The hypoxia-fit strain hrmA<sup>Δ<sub>REV</sub></sup> forms a more robust 24-h biofilm compared to the control AF293 (representative of *n* = 3 independent biological samples). (Scale bar, 500 μm.)
(18 h plus 3-h xylose induction) results in GFP signal localized to the vertically aligned hyphae within the predicted normoxic zone. In the hypoxic zone at the base, signal was largely, but not completely, absent (Fig. 3 E and F and SI Appendix, Fig. S7B). The signal is specific for induction with xylose (SI Appendix, Fig. S7B) and absent in an isogenic parent strain lacking the reporter (Fig. 3 E and F). Importantly, for a strain containing a constitutive GFP-expressing construct (AF293GFP), GFP signal is visible at all depths of the biofilm, indicating the presence of enough oxygen throughout the biofilm for GFP folding and fluorescence (Fig. 3 E and F and SI Appendix, Fig. S7B). These data indicate that reduced translational activity occurs within the basal hypoxic microenvironments in mature filamentous fungal biofilms.

Fungal-Induced Hypoxic Microenvironments Facilitate Biofilm Antifungal Drug Resistance. Because current antifungal agents target metabolically active fungal cells, we hypothesized that self-induced hypoxic microenvironments facilitate antifungal drug resistance. As an important note, we use the term “resistance” herein to describe the biofilm phenotype of persistent fungal metabolic activity and reduced drug susceptibility in the face of antifungal drug treatment. We alternatively considered the use of “tolerance” that has been more rigorously defined in the context of planktonic yeast cell populations, but it remains unclear if the reduced drug susceptibility of hypoxic hyphae within the biofilm meet the accepted tolerance definition for planktonic cells (9, 46).

To test the hypothesis that fungal-induced hypoxic microenvironments facilitate antifungal drug resistance, we first treated biofilms with the contemporary drugs voriconazole and amphotericin B at different stages of growth that correspond with different levels of oxygen depletion. Twelve-hour biofilms are significantly more sensitive to antifungal treatment and oxidative stress (menadione) compared to 16-h and 20-h biofilms, as measured using the tetrazolium dye XTT (where percent damage corresponds to the reduction in metabolic activity of the biofilm) (Fig. 4A). Drug sequestration by ECM is the current known mechanism of fungal biofilm drug resistance in Candida
albicans (19). Therefore, we examined the drug resistance of mature 18-h A. fumigatus biofilms deficient in the major ECM component exopolysaccharide galactosaminogalactan. A null mutant of the UDP-glucose 4-epimerase (Δuge3) critical for ECM formation surprisingly remains resistant to antifungals (Fig. 4B). Therefore, other filamentous fungal biofilm-dependent phenotypes contribute to the antifungal recalitrance of these surface-adhered populations. We next turned our attention to the hypoxic microenvironments generated during biofilm maturation.

To determine if a reduction in environmental oxygen availability is sufficient to generate antifungal drug resistance as it affects biofilm architecture, we grew 12-h and 18-h biofilms at ambient oxygen (21% O2) or at 0.2% O2 and then treated with voriconazole, amphotericin B, or menadione. The 12-h biofilms show significantly reduced damage when treated at 0.2% O2 for all three agents (Fig. 4C). This trend was observed using two independent methods for cell viability (SI Appendix, Fig. S8) and across three additional strains of A. fumigatus (SI Appendix, Fig. S9). Eighteen-hour biofilms, which have zones of naturally deoxygenated oxygen and intrinsically exhibit drug resistance, show no significant changes in antifungal-induced damage when treated at 0.2% O2 versus at 21% O2 (Fig. 4C). However, 18-h biofilms cultured and treated on oxygen-permeable surfaces, where oxygen is significantly increased (Fig. 4D), are significantly more sensitive to all three agents (Fig. 4E). Additionally, when grown on oxygen permeable plates mature biofilms of the polymorphic yeast C. albicans also have significantly increased oxygen compared to growth on normal plates (SI Appendix, Fig. S10). Accordingly, C. albicans biofilms grown on these oxygen-permeable plates are also significantly more sensitive to amphotericin B (SI Appendix, Fig. S10).

Hypoxic zones at the biofilm base are predicted through modeling to result from oxygen consumption by biomass closer to the air–liquid interface, as opposed to physical obstacles to oxygen diffusion. To test whether fungal oxygen consumption self-induces hypoxia that protects specific hyphae from antifungal drug treatment, we cultured biofilms in conditions predicted to increase oxygen consumption: incubation with the mitochondrial uncoupler FCCP [carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone] and growth in media supplemented with tri-carboxylic acid cycle (TCA) intermediates. FCCP was previously shown to increase A. fumigatus oxygen consumption rates (41), and FCCP treatment of 24-h biofilms significantly reduces dissolved oxygen at 2.5 mm below the air–liquid interface compared to controls after only 30 min of exposure (Fig. 5A). Cotreatment of 12-h biofilms with FCCP significantly reduces the metabolic activity inhibition (percent damage) caused by amphotericin B treatment (Fig. 5B). Shifting 24-h biofilms to media with TCA intermediates (respiratory minimal media) results in significantly reduced dissolved oxygen at 2.5 mm below the air–liquid interface compared to media with 1% glucose (Fig. 5C). Treatment of 12-h biofilms with amphotericin B in respiratory minimal media remarkably results in significantly less damage, as measured by an increase in metabolic activity, than treatment in minimal media with glucose (Fig. 5D). Together these data support a model where hypoxic microenvironments occur due to fungal oxygen consumption within mature biofilms, and once established they facilitate resistance to antifungal treatment.

**Cells within the Hypoxic Microenvironment Endure Treatment and Reinitiate Growth.** Our data suggest that hyphal oxygen consumption in the biofilm contributes to the phenotype of biofilm antifungal drug resistance. Therefore, we hypothesized that antifungal treatment would reduce oxygen consumption and increase dissolved oxygen within the biofilm. After 1.5 h of treatment with either voriconazole (fungistatic) or amphotericin B (fungicidal), we observed an increase in dissolved oxygen for
Fig. 5. Respiration-induced hypoxic zones reoxygenate after treatment to facilitate biofilm growth and drug resistance. (A) FCCP (2.5 μM) significantly reduces dissolved oxygen in 24-h biofilms after 30 min. Error bars indicate SEM of n = 3 independent biological samples. Student’s unpaired two-tailed t test performed. (B) FCCP significantly reduces amphotericin B damage in 12-h biofilms. Error bars indicate SEM of n = 4 independent biological samples. One-way ANOVA with Sidak’s multiple comparison test performed. (C) Incubation in RMM that contains TCA intermediates significantly reduces dissolved oxygen in 24-h biofilms compared to incubation in GMM. Error bars indicate SEM of n = 3 independent biological samples. Student’s unpaired two-tailed t test performed. (D) Treatment of 12-h biofilms in RMM significantly reduces amphotericin B damage compared to treatment in GMM. Error bars indicate SEM of n = 4 independent biological samples. Student’s unpaired two-tailed t test performed. (E) Antifungal treatment with voriconazole (1 μg/mL) or amphotericin B (1 μg/mL) significantly increases dissolved oxygen within the 24-h biofilms after 90 min of treatment. Error bars indicate SEM of n = 3 independent biological samples. Comparisons were made using a multiple t test approach with the Holm–Sidak method to determine statistical significance: (a) P = 0.003190, (b) P = 0.003288, (c) P = 0.000443, (d) P = 0.001435, (e) P = 0.003119, (f) P = 0.000312, (g) P = 0.003331. Comparisons without a P value listed are P > 0.05 and not statistically significant. (F) Voriconazole (1 μg/mL) treatment increases the xylose-inducible signal for translation activity at the base of the biofilm. Images representative of n = 3 independent biological samples. (Scale bar, 100 μm.) (G) Heat map quantifying GFP fluorescence relative to overall biomass from images in F shows visible signal at the base of the 18-h biofilm following voriconazole treatment. Each column is representative of n = 3 independent biological samples. (Scale bar, 100 μm.) Each column is representative of n = 3 independent biological samples. (H) Heat map quantifying GFP fluorescence relative to overall biomass from images in H shows visible signal at the base of the 18-h biofilm following amphotericin B treatment. Each column is representative of n = 3 independent biological samples. (I) Heat map quantifying GFP fluorescence relative to overall biomass from images in H shows visible signal at the base of the 18-h biofilm following amphotericin B treatment. Each column is representative of n = 3 independent biological samples. (J) Consecutive treatments with voriconazole or amphotericin B in normoxia significantly increases the damage to 18-h AF293 biofilms, while consecutive treatments in hypoxia cause significantly less damage than in normoxia. Error bars indicate SEM of n = 6 independent biological samples. One-way ANOVA with Tukey’s multiple comparisons test performed. (ns: P > 0.05, not significant).

both treatment groups (Fig. 5E). Two hours after drug removal, only those biofilms that received amphotericin B treatment had significantly increased dissolved oxygen (SI Appendix, Fig. S11 A and B). To determine if increased dissolved oxygen during treatment allows for the hyphae at the base of the biofilm to become more translationally active, we utilized the xylP-GFP
metabolic reporter in the presence and absence of voriconazole and amphotericin B. Following 3-h voriconazole treatment of 18-h biofilms we induced GFP with xylose and observed an increase in the translational activity at the base of the biofilm (Fig. 5 F and G and SI Appendix, Fig. S11 C). Similarly, treatment with amphotericin B dramatically reduces fluorescence within vertically aligned hyphae, and the only visible signal for active GFP translation is concentrated at the base of the biofilm (Fig. 5 H and I and SI Appendix, Fig. S11 D). Importantly, untreated or vehicle-treated biofilms grown on oxygen-permeable surfaces have homogenous signal for active GFP translation indicating homogenous increased rates of translation (SI Appendix, Fig. S12). Treatment of these biofilms with the fungistatic agent voriconazole mildly increases translation reporter signal after 3 h of recovery, while treatment with the fungicidal agent amphotericin B largely reduces overall signal from the translational reporter (SI Appendix, Fig. S12).

To investigate if increased translational activity within the biofilm sensitizes the biofilm to antifungal treatment, we performed consecutive dosing of antifungal drugs with 18-h biofilms. Consecutive doses of voriconazole or amphotericin B at ambient oxygen (normoxia, 21% O2) significantly increases the damage to the biofilms (reduces metabolic activity) compared to the single-dose control (Fig. 5 F). In contrast, a consecutive dose at 0.2% O2 (hypoxia) results in a significantly smaller reduction in metabolic activity than in normoxia (Fig. 5 F). This was true for two independent A. fumigatus strains (SI Appendix, Fig. S13). Taken together, our results highlight that self-induced hypoxic microenvironments arise as a result of fungal oxygen consumption in the maturation of A. fumigatus biofilms. The hypoxia response of fungal cells that manifests as a result of these self-generated hypoxic zones leads to reduced metabolic activity in the basal biofilm layers; the cells in these basal layers then serve as a drug-resistant reservoir of viable biomass that reseeds the fungal biofilm once drug treatment diminishes or is ceased (SI Appendix, Fig. S14).

**Discussion**

Filamentous fungal biofilms present with unique morphology and architecture that is distinct from bacterial and yeast biofilms (29). How these unique filamentous fungal morphologies impact fungal disease relevant outcomes remains unclear. Here we observed that A. fumigatus biofilms consistently develop spatial gradients of hypoxic microenvironments during their maturation. The formation of these hypoxic zones contributes not only to the emergent architecture of the growing biofilm but also functionally in the form of antifungal drug resistance. The spatially defined hypoxic microenvironments in A. fumigatus biofilms occur as a natural consequence of the metabolic activity of the growing hyphal network away from the surface and toward the oxygen supply. These hypoxic regions coincide with spatially distinct hyphae at the biofilm base that are in what is likely a reduced or quiescent metabolic state. The spatial constriction of these quiescent hyphae to the hypoxic zone suggests that they arise as the result of the growing filamentous network geometry in relation to their basal anchoring surface and location of the oxygen source. Thus, the fundamental mechanism of hypoxic region generation begins with what appears to be aerotrophic hyphal growth away from an origin point and toward an oxygen source. This concerted growth toward the oxygen source leads to oxygen depletion within the inner layers of the growing network. In our experiments, the oxygen source was the air-liquid interface of culture dishes, but this principle applies to any arbitrary environment in which hyphal networks begin to grow away from a seeding spore and toward a source of oxygen elsewhere (47).

Despite the well-established occurrence of oxygen depletion in densely occupied bacterial and yeast biofilms, this observation in a filamentous fungal biofilm is surprising to us given the unique open architecture of hyphal cell arrangements. How A. fumigatus and other filamentous fungi are able to sense changes in oxygen and potentially polarize growth along oxygen gradients remains an intriguing question and is the focus of ongoing research. Notably, in A. nidulans, the hypoxia response transcription factor SrbA has been implicated in microtubule depolarization within mature biofilms in response to altered gaseous environments (48). These data may suggest that the directionality of hyphal growth toward the air-surface liquid interface is driven in part by SrbA-mediated regulation of microtubule depolarization, and this remains to be tested in A. fumigatus.

An outstanding question from the results presented here concerns the mechanism(s) of hypoxic microenvironment-mediated drug resistance within biofilms. Despite the fact that low-oxygen regions appear common in bacterial and fungal biofilms, mechanistic studies linking the hypoxic response within biofilms and antimicrobial resistance are largely absent among eukaryotic human pathogens (35, 37, 39, 49). To date, biofilm antifungal drug resistance has been best-studied with the polymorphic yeast C. albicans, and while low-oxygen environments have been detected within these fungal biofilms, a mechanistic link between low oxygen and antifungal resistance has not been explicitly shown. Instead a number of other aspects of C. albicans biofilm growth have been linked to antifungal resistance, including β-glucan in the ECM (19), expression of efflux pumps (23, 25, 50), metabolic changes (23, 51), cell-wall stress responses (52), and the occurrence of putative yeast persister cells (however, the existence of yeast persister cells remains controversial) (13, 53). Our observation here that amphotericin B sensitivity of C. albicans biofilms is significantly increased on oxygen-permeable plates poses the intriguing question of how the hypoxic microenvironments within a biofilm may impact these other characterized aspects of fungal physiology linked to antifungal resistance (i.e., efflux pump expression, matrix modulation, cell wall, etc.). Further work, coupled with existing detailed studies of C. albicans biofilm development (51, 54, 55), is required to determine how hypoxic microenvironments in yeast biofilms contribute to drug resistance.

Concerning A. fumigatus, previous studies have not detected increases in resistance to amphotericin B, various azoles, or the echinocandin caspofungin in low-oxygen compared to normal-oxygen conditions (56, 57). These studies, however, were performed with planktonic hyphal cells and were designed such that oxygen limitation was introduced to the culture and at the start of growth by the planktonic hyphal cells of A. fumigatus. Thus, growth of filamentous fungal cells in a low-oxygen environment is not by itself sufficient to drive antifungal drug resistance. This of course raises intriguing questions about the physiological state and phenotypes of the drug-resistant hyphae experiencing hypoxic stress in the biofilm environment. For example, the presence of environmental DNA (20) within biofilms and elevated efflux pump activity (24) has been suggested to contribute to A. fumigatus biofilm drug resistance. It remains unclear if the hypoxic regions within A. fumigatus biofilms increases the spatially restricted expression or activity of efflux pumps that contribute to antifungal resistance, as has been observed with Pseudomonas aeruginosa (37). A recent study has implicated efflux pump activity in A. fumigatus antifungal resistance as a result of mitochondrial dysfunction and aberrant calcium signaling, but this has not yet been investigated in the context of fungal biofilms as a potential mechanism of drug resistance (58). It remains an interesting and important question if altered mitochondria function, as a direct result of low-oxygen stress (41), impacts calcium signaling to contribute to A. fumigatus hypoxia-induced drug resistance in biofilms.

Importantly, we demonstrate in vitro that replenishing oxygen to the fungal biofilms through growth on oxygen-permeable plates or through consecutive antifungal treatment strongly reduces or eliminates the biofilms’ drug resistance. One approach to mitigate this problem in a clinical setting could be to increase...

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oxygen tension to damaged tissue; hyperbaric oxygen treatment has typically been used in the clinic to achieve this goal. Hyperbaric oxygen treatment has fungal biofilm growth-arresting capabilities in vitro, but this treatment surprisingly failed to synergize with the antifungals voriconazole or amphotericin B in vitro and in vivo (59). The reduced growth rate in response to oxygen supplementation perhaps counterintuitively drives a biofilm hypoxia-related phenotype, preventing increased efficacy of the antifungals. Future work investigating how A. fumigatus senses oxygen will be important in understanding the mechanisms of oxygen-mediated antifungal drug resistance and critical to identifying potential targets and/or approaches to sensitize the entire fungal biofilm to antifungal drugs regardless of the oxygen environment and fungal metabolic state. One seminal example of this concept is the observation that P. aeruginosa aggregate biofilms display oxygen-dependent differential sensitivity to tobramycin and chlorhexidine, with chlorhexidine selectively targeting anaerobic cells (60). Thus, the extension of oxygen gradient-mediated microbially biofilm drug resistance to the fungal kingdom paves the way for future therapeutic approaches that are expected to impact both bacterial and fungal biofilms in vivo.

Materials and Methods

Strains and Growth Culture Conditions. All strains utilized in this study are listed in Table 1. Suspensions were cultured on 1% w/v Sabouraud solid media (GM1, 1% glucose; 6 g NaNO3, 0.52 g KCl, 0.52 g MgSO4·7H2O, 1.52 g KH2PO4 monobasic, 2.2 ml ZnSO4·7H2O, 1.1 mg/L H3BO3, 0.5 mg/mL MnCl2·4H2O, 0.5 mg/mL FeSO4·7H2O, 0.16 mg/mL CoCl2·5H2O, 0.16 mg/mL CuSO4·5H2O, 0.11 mg/mL (NH4)6Mo7O24·4H2O, and 5 mg/mL Na2EDTA; pH 6.5). For solid agar media was added to 1.5%. Conidia were collected for experiments after growth at 37 °C and 5% CO2 for 72 to 96 h by flooding the plate with 0.01% Tween-80 at the center of the plate (Afu8g02440) start codon in AF293 identified using FungiDB.org (61). The erg25A gene was selected as the 672 base pairs (bp) immediately upstream of the xylanase (α10 mM glutamate, 10 mM malate, 10 mM pyruvate, and 10 mM glutamate) (Penicillium chrysogenum) promoter was ordered as a gBlock (IDT, San Diego) and incorporated into the chromosomal genome of A. nidulans for nonadherent strain ΔhrmAR-EV, using protoplasting and transformation protocols as previously described (63). All constructs were integrated ectopically into the chromosomal genome of A. nidulans ΔhrmAR-EV. Simultaneous transduction of the dominant marker for pyrithiamine resistance, hmgB (Fla 1.3) promoter was selected as the 15% conidia was collected for experiments after growth at 37 °C and 5% CO2 for 72 to 96 h by flooding the plate with 0.01% Tween-80 at the center of the plate for the times listed. For the nonadherent strain Δerg25A and the AF293 control, the 24-well Falcon polystyrene microplate was coated for 12 h with collagen coating solution (125-50; Sigma) at room temperature to facilitate adherence.

For C. albicans biofilm strain SC5314 was utilized. SC5314 was cultured in yeast extract–peptone–dextrose overnight at 30 °C on a rotating culture wheel and then subcultured for 6 h in the same conditions. Cells were washed three times with 1× PBS and diluted to 10⁶ cells per mL in RPMI 1640 (1× glutamine, –phenol red). Biofilms were seeded with 0.5 mL per well of a normal polystyrene 24-well culture plate or an oxygen-permeable 24-well gas-permeable plates (8602000; Coy Lab Products) (permeable plates) with 10⁵ conidia per mL of GMM broth (0.5 mL per well at 37 °C, 5% O2 and 21% O2 for the times noted. For the nonadherent strain Δerg25A and the AF293 control, the 24-well Falcon polystyrene microplate was coated for 12 h with collagen coating solution (125-50; Sigma) at room temperature to facilitate adherence.

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Fluorescence Microscopy of Fungal Biofilms. Microscopy equipment. Fluorescence confocal microscopy was performed on either an inverted Nikon Eclipse Ti inverted microscope equipped with Perfect Focus with a CFI Plan Apochromat 10X GCy objective (Nikon) or CFI Plan Fluor 20X CM (Nikon) or a Zeiss LSM800 with a C-Apochromat 10×0.45 W M27 objective (Zeiss).

Biofilm sample preparation. A. fumigatus biofilms were cultured for imaging in MatTek dishes (“normal plates,” P35G-1.0-14-C, MatTek) or in gas-permeable slide plates (“permeable plates,” 960203; Coy Lab Products), with or without a gas-permeable seal, in GMM broth inoculated with 10⁵ conidia per mL with 2 mL per dish. A. nidulans and S. apiospermum biofilms were imaged similarly but in Czapec-duck media (Difco). For A. fumigatus biofilms at 21% O2, the incubation time was 24 h unless otherwise noted.
Resazurin assay. After 3 h of treatment, the drug or vehicle was removed and the biofilms were washed as described for the XTT assay above. A 10% resazurin (TOX8; Sigma) solution was prepared in 1× PBS and added to the biofilms at 0.3 mL per well. The biofilms were incubated at 37 °C, 5% CO₂ and 21% O₂ until the control untreated wells turned blue (unreduced) to red (reduced) (2 to 4 h). The resazurin supernatant was analyzed spectrophotometrically at 690 nm (reference optical density) and 600 nm (unreduced resazurin dye). Percent damage, a metric of change in metabolic activity, was calculated by comparing the drug-treated wells to the vehicle-treated controls and resazurin-only wells.

Dissolved Oxygen Quantification. **Unisens equipment and software.** Dissolved oxygen was quantified using a Unisense OXYSensor Type 1-CH (Unisense OXY METER) equipped with a microsensor (Unisense MM33), motorized micromanipulator stage (Unisense MMS), motor controller (Unisense MC-232), and a 25-μm Clark-type/amperometric oxygen sensor (Unisense OX-25). The readers were automated and analyzed through SensorTrace Suite Software v3.1.15I (Unisense STSUITE).

**Biofilm sample preparation.** Biofilms were prepared at 10⁵ conidia per mL in 4 mL GM broth unless otherwise noted and incubated at 37 °C, 5% CO₂ and 21% O₂ for the described time. As noted, biofilms were prepared in Falcon 35-mm polystyrene Petri dishes (normal plates, 351008; Fisher) or gas-permeable silicone plates (permeable plates, 8620393; Coy Lab Products). *C. albicans* biofilms were prepared as described above for the drug treatment assays.

**Oxygen quantification.** The microelectrode was manually positioned at the air–liquid interface of the biofilm with the micromanipulator (Unisense MM33). This starting position is ~3 mm above the fungal growth at 24 h and is considered a depth of 0 μm. The motorized stage was set to record oxygen measurements in technical duplicates at 200-μm or 500-μm intervals from this starting position. At each position the microelectrode waited 10 s to equilibrate before measuring for an additional 10 s and recording the average. Three separate XY positions in the biofilm were measured in technical duplicates at each depth. This was repeated for a minimum of three biological replicates per condition. Samples where the biofilm had become detached or visually disrupted were omitted. Due to the fragility of the microelectrodes, depths greater than 2.5 mm to 3 mm were not attempted.

**Modeling of Fungal Biofilm Maturation.**

**Estimation of biomass density.** Here we estimate the distribution of biomass density along the biofilm’s height over time, based on microscopy data from the bottom 570 μm of the biofilm at different time points during development and from the full mature biofilm at 24 h. For each imaged horizontal layer of the biofilm (biofilm height), we use a sigmoidal model \( d(t) = d_1 \left[ \frac{1}{1 + \exp(-m(t-t_0))} \right] \) to fit the biomass density growing in time until it reaches the mature biomass density, with parameters \( t_0 \), \( m \), \( d_1 \) indicating the time at which that height is reached, \( m \) indicating the speed at which the biofilm matures at that height, and \( d_1 \) indicating the final biomass density (SI Appendix, Fig. S3A). We use these layers’ biomass densities samples of three to five biological replicates. Heat maps display data from the representative sample of three replicates.

**Flow Cytometry Reporter Characterization.**

**erg25A-GFP reporter.** Conidia of AF293 (no fluorophore) or *erg25A-GFP* strain were swollen in GM broth supplemented with 0.5% yeast extract (210929; Gibco) at 10⁵ conidia per mL for 5 h at 37 °C and 100 rpm agitation. The AF293 swollen conidia were analyzed from 21% O₂ after 5 h. The *erg25A-GFP* swollen conidia were analyzed from 21% O₂ after 5 h (0 min hypoxia) or after additional incubation at 0.2% O₂ (hypoxia) for the noted time. The population was gated for cells that were singlets and swollen and analyzed using proprietary GATITC sigui (488 nm) and GATI easyCyte BHT benchtop flow cytometer. Data were analyzed using Flowjo v.9.9.6.

**KlyP-GFP reporter.** Conidia of AF293 (no reporter system) or *KlyP-GFP* strain were swollen as described above. The swollen conidia were washed once with 1× PBS and resuspended in 4% xylose in minimal media (GMW without 1% glucose) for the times as noted. The populations were gated for cells that were singlets and swollen and were analyzed for GFP/FITC (488 nm) using a MaciQuant VYB flow cytometer. Data were analyzed using Flowjo v.9.9.6.

**Statistics.** Unless otherwise noted, statistics were performed in GraphPad Prism 8. Unless otherwise noted, comparisons for \( n = 2 \) utilized a Student’s unpaired two-tailed \( t \) test and comparisons for \( n > 3 \) utilized a one-way ANOVA with a Tukey’s multiple comparisons test. Fluorescent micrographs and three-dimensional (3D) renderings were prepared from representative samples of three to five biological replicates. Heat maps display data from the representative sample of three replicates.

Data Availability. All study data are included in the paper and **SI Appendix**.

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