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Effects of Iron Chelators on the Formation and Development of *Aspergillus fumigatus* Biofilm

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Iron acquisition is crucial for the growth of *Aspergillus fumigatus*. *A. fumigatus* biofilm formation occurs *in vitro* and *in vivo* and is associated with physiological changes. In this study, we assessed the effects of Fe chelators on biofilm formation and development. Deferiprone (DFP), deferasirox (DFS), and deferoxamine (DFM) were tested for MIC against a reference isolate via a broth macrodilution method. The metabolic effects (assessed by XTT [2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt]) on biofilm formation by conidia were studied upon exposure to DFP, DFM, DFP plus $FeCl_3$, or $FeCl_3$ alone. A preformed biofilm was exposed to DFP with or without $FeCl_3$. The DFP and DFS MIC_{50} against planktonic *A. fumigatus* was 1,250 μM, and XTT gave the same result. DFM showed no planktonic inhibition at concentrations of ≤2,500 μM. By XTT testing, DFM concentrations of <1,250 μM had no effect, whereas 2,500 μM increased biofilms forming in *A. fumigatus* or preformed biofilms ($P < 0.01$). DFP at 156 to 2,500 μM inhibited biofilm formation ($P < 0.01$ to 0.001) in a dose-responsive manner. Biofilm formation with 625 μM DFP plus any concentration of $FeCl_3$ was lower than that in the controls ($P < 0.05$ to 0.001). $FeCl_3$ at ≥625 μM reversed the DFP inhibitory effect ($P < 0.05$ to 0.01), but the reversal was incomplete compared to the controls ($P < 0.05$ to 0.01). For preformed biofilms, DFP in the range of ≥625 to 1,250 μM was inhibitory compared to the controls ($P < 0.01$ to 0.001). $FeCl_3$ at ≥625 μM overcame inhibition by 625 μM DFP ($P < 0.001$). $FeCl_3$ alone at ≥156 μM stimulated biofilm formation ($P < 0.05$ to 0.001). Preformed *A. fumigatus* biofilm increased with 2,500 μM $FeCl_3$ only ($P < 0.05$). In a strain survey, various susceptibilities of biofilms of *A. fumigatus* clinical isolates to DFP were noted. In conclusion, iron stimulates biofilm formation and preformed biofilms. Chelators can inhibit or enhance biofilms. Chelation may be a potential therapy for *A. fumigatus*, but we show here that chelators must be chosen carefully. Individual isolate susceptibility assessments may be needed.

Aspergillus fumigatus, the ubiquitous saprophytic mold, frequently causes respiratory tract infections, including invasive pulmonary aspergillosis and allergic bronchopulmonary aspergillosis (1). *A. fumigatus* disease occurs most frequently in immunocompromised individuals, such as bone marrow transplant and other neutropenic patients, solid organ transplant recipients (2), and in those with cystic fibrosis (1, 3), chronic granulomatous disease, or chronic obstructive pulmonary disease (1, 4). Despite therapeutic advances in the development and administration of antifungals, mortality from *A. fumigatus* infection remains high (5).

A. fumigatus has shown, *in vivo* and *in vitro*, the ability to form biofilms, or complex aggregates of organisms embedded within a polymer-rich extracellular matrix, which demonstrate increased antimicrobial resistance (32). Thus, there is a need for other therapeutic methods with mechanisms that differ from those of the common antifungal targets of ergosterol or cell wall biosynthesis. Given the clinical prevalence of *A. fumigatus* biofilms (6), new therapies should demonstrate efficacy against *A. fumigatus* biofilms.

One potential mechanistic target lies in iron (Fe) acquisition, as it represents a key factor in *A. fumigatus* pathogenicity (7, 8). Previous data indicate that iron chelators can inhibit the planktonic conidial growth of *A. fumigatus in vitro* (9, 10). This has also been shown in murine models to serve as a treatment for aspergillosis or mucormycosis in combination with other antifungals (11). However, some iron chelators have been shown to enhance conidial growth (10), which makes the identification of therapeutic chelators especially important. Because of the lack of information on the effects of Fe chelators on *A. fumigatus* biofilms, we assessed the effects of different Fe chelators on the establishment and development of *A. fumigatus* biofilms *in vitro*.

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MATERIALS AND METHODS

Iron chelators and $FeCl_3$. Three iron chelators were studied. These were deferasirox (DFS) (Novartis Pharmaceuticals, East Hanover, NJ), deferoxamine (DFM) (APP Pharmaceuticals, Schaumburg, IL), and deferiprone (DFP) (Sigma-Aldrich Co., St. Louis, MO). Ferric chloride ($FeCl_3$) was obtained from Sigma-Aldrich Co.

A. fumigatus. One patient isolate that is known to be virulent, *A. fumigatus* 10*AF*, was used as a reference isolate (13, 14). Nine other clinical isolates were studied in comparison (see Results); all 10 were confirmed by molecular methods to be *A. fumigatus sensu stricto* (15). Long-term storage was performed as described previously (16). Four-day-old conidia were harvested and stored in 0.05% Tween 80 (J. T. Baker Chemical Co., Phillipsburg, NJ) in saline (0.9% NaCl) (Baxter Healthcare Corp., Deerfield, IL), as described previously (14).

Assessment of iron chelator effects on planktonic *A. fumigatus* growth. MIC testing of iron chelators against planktonic *A. fumigatus* growth was performed using the CLSI M38-A2 protocol for broth macrodilution (17). In brief, an inoculum of 3×10^5 conidia was added to fresh RPMI 1640 medium in 5-ml polystyrene round-bottom tubes (BD Biosciences, Durham, NC). A 2-fold dilution series of DFP, DFS, DFM, or sterile H_2O only (controls) in the medium was tested. The tubes were incubated at 37°C with shaking (100 rpm) for 24 h, and growth was determined visually. Measurement of metabolic activity by XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt) assay was also performed.

***In vitro* model of examining iron chelator and/or $FeCl_3$ effects on *A. fumigatus* biofilm formation.** We chose to first test the effects of DFP and DFM on the development of *A. fumigatus* biofilms. Our laboratory first utilized and reported on a 12-well assay for studies of *A. fumigatus* biofilm development (18). In later studies, an assay using 96-well plates was explored. We found that our 96-well assay gives the same results if the conditions in both systems are closely controlled, as described in the studies. The 12-well assay is useful for the recovery of sufficient materials from the wells in various analyses at the conclusion of the experiments. The 96-well assay is both sparing of reagents and allows more replicates to be studied concurrently, which increases the chances of statistical significance occurring when differences between groups are small, e.g., at the inflection point of a dose-response curve. An analogous 96-well assay used to assay antifungal drugs, and differing in specifics from ours, was previously described (19). The interpretations made here are based on 7 experiments on biofilm formation in the 12-well format and 9 in the 96-well format, which included 10*AF* as a reference control.

For the 12-well assay, to form biofilms, sterile polystyrene disks (BioSurface Technologies, Bozeman, MT) were placed in 12-well tissue culture plates (Corning, Inc., Corning, NY). Each well contained 2.7 ml of fresh RPMI 1640 medium plus 0.3 ml of DFP or DFM in phosphate-buffered saline (PBS) (pH 7.3 to 7.5) (Lonza) or PBS only (controls), and 10^5 conidia/ml. The final chelator concentrations were 2-fold dilutions ranging from 2,500 μM to 39 μM. The disks were incubated for 16 h at 37°C to allow *A. fumigatus* cells to form biofilm in the presence of chelators. After this initial 16 h, nonadherent cells were removed through washing with PBS, and the disks were transferred to 3 ml of fresh RPMI 1640 medium with or without 10% fetal bovine serum (FBS) without iron chelators for an additional 24 h of growth at 37°C before quantification of fungal growth by the XTT assay. Experiments with $FeCl_3$ supplementation involved growth of *A. fumigatus* for 16 h in the presence of 625 μM DFP alone, 625 μM DFP plus $FeCl_3$ (2,500 μM to 156 μM), or $FeCl_3$ alone (2,500 μM to 156 μM) and removal of the disks to fresh medium alone, followed by 24 h of additional growth at 37°C with shaking (70 rpm).

For the 96-well assay, after removing the Tween from the conidia by two washes in PBS, biofilms were formed on the flat-bottom wells (Costar 3596; Corning), and the same sequences, time intervals (with washing of the wells with PBS before the introduction of fresh medium at 16 h), chelator concentrations, media (FBS studies not done), and shaking protocols were used. The well volume was 0.2 ml, and the inoculum contained 2×10^3 conidia/well.

Preformed (i.e., established) biofilm experimental design. In the 12-well assay, to allow the *A. fumigatus* to form biofilms, 10^5 conidia/ml in 3 ml of fresh RPMI 1640 medium were grown on flat polystyrene disks in 12-well tissue culture plates for 16 h. Preformed *A. fumigatus* biofilms were washed and then transferred to new wells containing 2.7 ml of RPMI 1640 medium and 0.3 ml of various concentrations of DFP (2,500 μM to 39 μM), DFM (2,500 μM to 156 μM), or PBS (controls) for an additional 24 h at 37°C with shaking (70 rpm). After incubation, an XTT assay was performed to measure biofilm metabolic activity. The experiments with $FeCl_3$ were performed by exposing the preformed biofilms to 625 μM DFP, 625 μM DFP plus $FeCl_3$ (2,500 μM to 39 μM), or $FeCl_3$ alone (2,500 μM to 39 μM).

In the 96-well assay, the formation of preformed biofilm was performed as described for the biofilm formation assay. The formed biofilm was then washed in fresh medium and exposed to the same chelator concentrations, times, temperatures, and shaking conditions as described above. The interpretations made here are based on 15 experiments on preformed biofilm in the 12-well format and 20 in the 96-well format, including 10*AF* as a reference control.

XTT assay. An assessment of the effects of iron chelators alone or in the presence of $FeCl_3$ on the growth of *A. fumigatus* biofilm was done using an XTT assay. The tetrazolium salt XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt) (Sigma, St. Louis, MO) was used to measure the metabolic activity of *A. fumigatus*. Whereas XTT is a direct measure of the metabolic activity of cells, previous studies of *A. fumigatus* have indicated that XTT results are linear with mass and equated the XTT result with dry weight (19–21). Our confocal microscopy studies also address this point.

In the 12-well assay, in brief, disks, as described above, were rinsed 3 times in sterile saline and transferred to wells containing 3 ml of sterile PBS. Menadione (Sigma) (0.85 g) was added to 5 ml of acetone, mixed in a 1:11 ratio of menadione to XTT (1 mg/ml). The XTT-menadione solution (180 μl) was added to each well, and the plates were incubated in the dark for 2 h at 37°C. Following incubation, the contents of the wells were collected and centrifuged at 13,300 × *g* for 10 min, and the absorbance at 490 nm of the supernatant was determined with a spectrophotometer (Genesys 20; Thermo Scientific, Waltham, MA). For the XTT assay of planktonic *A. fumigatus* growth, 180 μl of the XTT-menadione solution was added directly to the 3 ml of RPMI 1640 in which the *A. fumigatus* was growing, and the tubes were incubated for 2 h at 37°C in the dark. For the 96-well biofilm assays, after washing, the concentrations used for the assay were 200 μg/ml XTT with 40 μM menadione (0.2 ml/well). After the 2 h incubation, the plates were centrifuged, and 0.15 ml of supernatant/well was transferred to a microtiter plate and the A_{490} determined with a microplate reader (Dynex, Chantilly, VA).

Confocal laser scanning microscopy. To determine the effects on the morphology and architecture of *A. fumigatus* biofilms, biofilms were formed on disks as described above. The biofilms were challenged with chelators. After incubation at 37°C, the disks were washed three times in sterile PBS and stained using a fluorescent stain (FUN 1; Invitrogen Molecular Probes, Eugene, OR), prepared according to the manufacturer's instructions. One microliter of FUN 1 from a 10 mM stock was mixed in 1 ml of PBS. Staining was performed as previously described (22, 23). Three drops of the mixture was added on the top of the biofilm, which was then mounted on a glass slide and covered with a glass coverslip (22 by 22 mm). The disks were incubated for 45 min at 37°C in the dark. The FUN 1 stain was used to visualize the morphology of *A. fumigatus* biofilm, a bright green cytoplasmic stain produced after passive diffusion; viability by FUN 1 staining was not assessed.

Sections on the *xy* plane were taken at 1-μm intervals along the *z* axis to determine the depth of the biofilms. Microscopic visualization and acquisition of biofilm images were conducted at the Stanford Biofilm Research Center using an upright Leica TCDSP2 confocal laser scanning

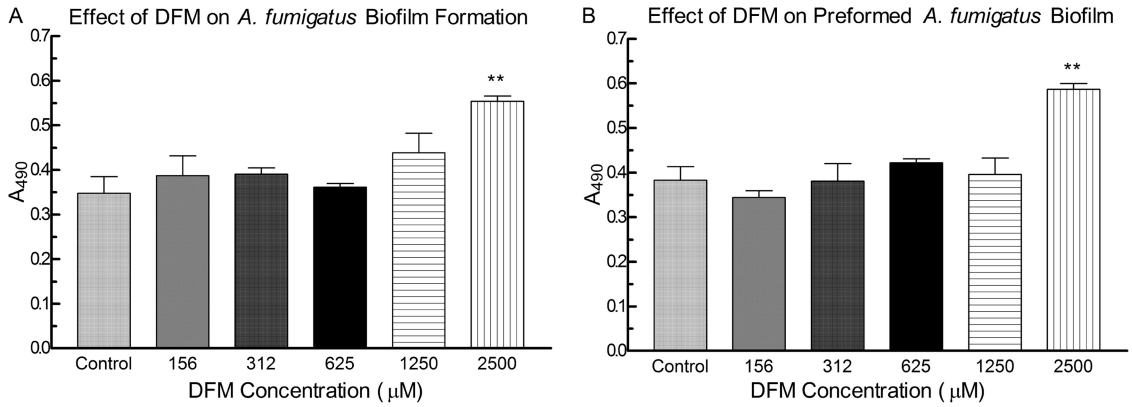

FIG 1 Effect of deferoxamine (DFM) on *A. fumigatus* biofilm formation and preformed *A. fumigatus* biofilm. (A) *A. fumigatus* conidia were exposed to DFM for 16 h, the disk was moved to new medium and incubated for an additional 24 h, and growth was assayed using XTT reduction, which was assessed by measuring the absorbance at 490 nm. (B) Preformed *A. fumigatus* biofilms were exposed to DFM for 24 h, and the resulting growth was assessed by XTT reduction by absorbance at 490 nm. Twelve-well assays were used. Each condition was performed in triplicate, and the results are pooled from two separate experiments on different days. Two asterisks indicate a significant *P* value (<0.01) compared to *A. fumigatus* control. (A) A DFM concentration of 2,500 μM enhanced *A. fumigatus* biofilm formation significantly more than 156 μM ($P < 0.05$), 312 μM ($P < 0.05$), and 625 μM ($P < 0.01$). (B) For preformed biofilms, 2,500 μM DFM was significantly more stimulatory than all other concentrations tested ($P < 0.001$, 156 μM; $P < 0.01$, 312 μM to 2,500 μM). The values shown are the means ± the standard deviations.

microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) equipped with an argon/krypton laser and detectors and filter sets for monitoring green fluorescence (excitation, 480 nm; emission, 517 nm). Images were obtained using 63× 1.4 Plan-Apochromat differential interference contrast (DIC) (Leica, Heidelberg, Germany) objectives. Multichannel simulated fluorescence projection (SFP) (a shadow projection) images and vertical cross sections of the biofilm were generated using the IMARIS software package (Bitplane AG, Zürich, Switzerland). The images were processed for display using the Photoshop software (Adobe, Mt. View, CA). Representative images were taken.

Serum effect. Our studies presented here were, as were previously published studies of chelators on planktonic growth (10), done in serum-free medium to avoid the varied amounts of iron that would be present in various sera and the presence of competing endogenous iron-binding moieties that might be present in serum. However, preliminary experiments using medium supplemented with 10% FBS (Gibco, Grand Island, NY) (data not shown) indicated that although the presence of serum sharply reduced the chelation effect, there was some residual activity on the formation or further development of *A. fumigatus* biofilm only at the upper end of the range tested, 2,500 μM DFP ($P < 0.01$, conidia forming biofilm; $P < 0.05$, preformed biofilm). This preliminary result can be compared with the studies of biofilm formation or the growth of preformed biofilm in RPMI 1640 medium without added serum, detailed in Results.

Statistical analysis. The 12-well assays included triplicate wells for each study group for analysis, and the 96-well assays included 7 replicate wells per group. Statistical differences were evaluated with one-way analysis of variance (ANOVA), followed by a Tukey *post hoc* test. GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) was used for calculations. Statistical significance was considered at a *P* value of <0.05.

RESULTS

Chelator effects on planktonic *A. fumigatus* growth. MIC tests of DFP and DFS against planktonic *A. fumigatus* showed 50% and 100% inhibition of growth by visual assessment at 1,250 μM and >2,500 μM, respectively. An evaluation of the inhibition of growth by the XTT assay showed identical results. MIC testing and the XTT assay with DFM showed no inhibition or stimulation of planktonic growth at any concentration tested (2-fold dilutions from 2,500 μM to 4.8 μM) (data not shown).

DFM effects on *A. fumigatus* biofilm. Fig. 1A and B show the effects of DFM on the metabolic activity of *A. fumigatus* forming biofilm and preformed *A. fumigatus* biofilm, respectively. Compared to untreated controls, DFM at concentrations of <1,250 μM did not have a positive or negative impact, whereas 2,500 μM significantly increased the metabolic activity of *A. fumigatus* forming biofilm or preformed biofilm ($P < 0.01$, both groups). At a concentration of 2,500 μM, DFM augmented *A. fumigatus* biofilm formation significantly compared to that with concentrations ranging from 156 μM to 625 μM ($P < 0.05$, 156 μM or 312 μM; $P < 0.01$, 625 μM) (Fig. 1A) and showed greater stimulation of preformed *A. fumigatus* biofilm than that with all other concentrations tested ($P < 0.001$, 156 μM; $P < 0.01$, 312 μM to 1,250 μM) (Fig. 1B).

Effects of DFP on *A. fumigatus* biofilm. In these studies, we examined the effects of DFP on the formation of biofilm by *A. fumigatus* and its effect on preformed *A. fumigatus* biofilm. In representative experiments, DFP at concentrations from 156 μM to 2,500 μM significantly inhibited the metabolic activity of *A. fumigatus* forming biofilm in a dose-responsive manner ($P < 0.01$, 156 μM and 312 μM; $P < 0.001$, 625 to 2,500 μM) (Fig. 2A). Concentrations of <156 μM were rarely inhibitory in repeated experiments. Like the effect on formation of *A. fumigatus* biofilm, DFP at 1,250 μM and 2,500 μM significantly inhibited preformed *A. fumigatus* biofilms ($P < 0.01$ and 0.001, respectively) (Fig. 2B). In repeats of this experiment with this isolate, significant inhibition by 625 μM was commonly seen.

Supplemental $FeCl_3$ and DFP inhibition of *A. fumigatus* biofilm. To confirm that the inhibition of biofilm by DFP was a result of the chelation of iron, we sought to overcome inhibition by adding supplemental iron. Figure 3A illustrates the effects of $FeCl_3$ supplementation on the inhibition of *A. fumigatus* biofilm formation by a DFP concentration we had found to be inhibitory. When exposed to $FeCl_3$ alone at any concentration tested, the metabolic activity of *A. fumigatus* forming biofilm significantly increased compared to that with *A. fumigatus* controls growing without supplemental iron ($P < 0.05$, 156 μM and 312 μM; $P < 0.01$, 625 μM;

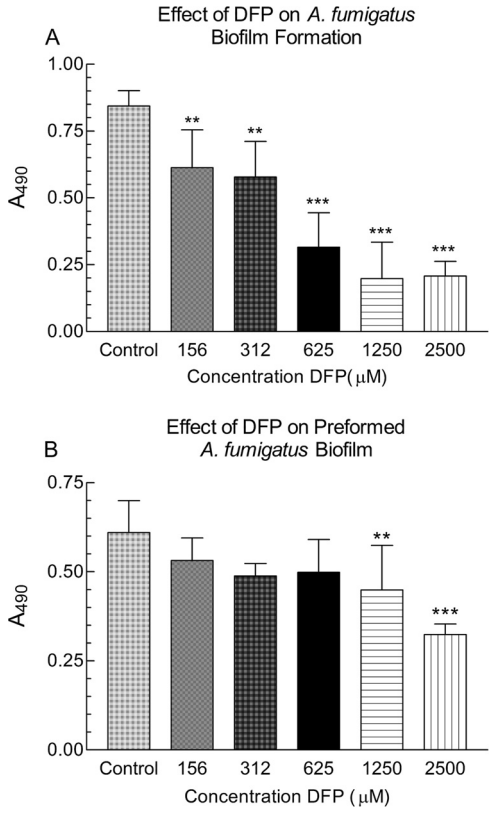

FIG 2 Effect of deferiprone (DFP) on the formation or further development of *A. fumigatus* biofilm. (A) *A. fumigatus* conidia were exposed to DFP (or not exposed [control]) for 16 h, before 24 h of further growth in fresh DFP-free medium. (B) Preformed *A. fumigatus* biofilms were exposed to DFP (concentrations shown) for 24 h or not exposed (control). Ninety-six-well assays were used. Two asterisks denote a significant *P* value of <0.01, and three asterisks denote a significant *P* value of <0.001, compared to the experimental condition containing *A. fumigatus* alone. The values shown are the means ± the standard deviations.

$P < 0.001$, 1,250 μM and 2,500 μM $FeCl_3$) (Fig. 3A). In the presence of 625 μM DFP plus any concentration of $FeCl_3$, *A. fumigatus* biofilm formation was significantly lower than that in the *A. fumigatus* controls (i.e., absence of DFP or $FeCl_3$) ($P < 0.001$, 156 μM and 312 μM $FeCl_3$; $P < 0.01$, 625 μM; $P < 0.05$, 1,250 μM or 2,500 μM). However, $FeCl_3$ concentrations of ≥625 μM partially overcame inhibition of biofilm formation by DFP compared to *A. fumigatus* plus 625 μM DFP alone ($P < 0.05$, 625 μM $FeCl_3$; $P < 0.01$, 1,250 μM and 2,500 μM $FeCl_3$).

Figure 3B shows the effects of various concentrations of $FeCl_3$ (2,500 μM to 39 μM) on preformed *A. fumigatus* biofilms growing with or without 625 μM DFP. In the presence of DFP plus ≤312 μM $FeCl_3$, preformed biofilm had lower metabolic activity than that of the untreated controls ($P < 0.001$, all comparisons). However, $FeCl_3$ at ≥312 μM partially or fully overcame DFP inhibition of preformed biofilm ($P < 0.05$, 312 μM $FeCl_3$; $P < 0.001$, ≥625 μM $FeCl_3$, compared to *A. fumigatus* plus DFP). Although 312 μM $FeCl_3$ only partially overcame DFP inhibition, $FeCl_3$ at concentrations of ≥625 μM completely reversed inhibition, restoring preformed biofilms to levels similar to those of the *A. fumigatus* controls. In the presence of $FeCl_3$ alone, only the 2,500 μM concentration significantly augmented further development of preformed *A. fumigatus* biofilm ($P < 0.05$).

Confocal microscopy analysis. The effect of the chelator DFP on *A. fumigatus* biofilm thickness and morphology was assessed using confocal microscopy. The thickness results for the *A. fumigatus* biofilm after exposure of the conidia to DFP for 16 h is shown (Fig. 4). DFP resulted in a significant reduction in the fungal biofilm thickness compared to the untreated control (Fig. 4A, C, and D). Untreated biofilms showed an architecture formed by a dense filamentous multicellular structure with acute-angle dichotomous branching (Fig. 4A). Figure 4C shows the effect of DFP on *A. fumigatus* biofilm formation. Treatment with DFP resulted in a decreased number of hyphae, the presence of some "bulging" structures distributed throughout the disperse filaments, resulting in a reduction in filamentation, and the presence of some "glued" hyphae without a clear separation of the filamentous elements (Fig. 4C).

As was indicated by the biofilm thickness data (Fig. 4D), treatment of preformed *A. fumigatus* biofilm with DFP resulted in thinning of the hyphal mat. Many round structures, many of which occurred at the tips, were noted (Fig. 4B).

Survey of *A. fumigatus* isolates. The effects of chelators on several *A. fumigatus* isolates, with respect to biofilm inhibition, were studied (Table 1). This revealed a polymorphism in susceptibility to DFP over a broad range of DFP concentrations. Of note, biofilm formation was almost always more susceptible than preformed biofilm.

DISCUSSION

The availability of iron and the ability to take up and utilize available iron are essential for the viability of *A. fumigatus*. Iron homeostasis is related to ≥24 genes in *A. fumigatus*, and iron uptake is done through a low-affinity ferrous iron system or one of two high-affinity systems, reductive assimilation or siderophores (24). Although essential for growth, iron also plays a critical role in the virulence of the organism, particularly in its resistance to reactive oxygen species produced by phagocytic cells, and in its capacity to adapt to hypoxic conditions during infection (24). The host response to infection includes mechanisms for the limitation of available iron. *A. fumigatus* can remove iron from serum transferrin (25), and lactoferrin produced in polymorphonuclear leukocytes (PMNs) has been shown to be statically inhibitory to *A. fumigatus* (9).

The critical role of iron to the viability and virulence of *A. fumigatus* makes it an attractive target for the control of infection, particularly through the use of iron chelators (26). *In vivo* studies have shown that the choice of chelator is crucial. DFM therapy has proven to be detrimental in models of mucormycosis and, to a lesser degree, of aspergillosis (8, 11, 27, 28). However, the use of DFS or DFP has been shown to limit fungal growth *in vivo* (8, 29).

Because of the potential importance of biofilms of *A. fumigatus* during infection (6, 30), particularly in airways, we have examined how iron depletion via a chelator affects biofilm formation and development *in vitro*. We show that iron chelators have differential effects on the establishment and further development of *A. fumigatus* biofilms, a finding that is consistent with previous reports of *A. fumigatus* growing planktonically (10, 11). We found that DFM at high concentrations stimulated biofilm formation and development in a manner similar to that with the supplementation of the medium with $FeCl_3$. In contrast, we found that DFP inhibited both biofilm formation and the continued development of preformed biofilm. Furthermore, our results demonstrate that

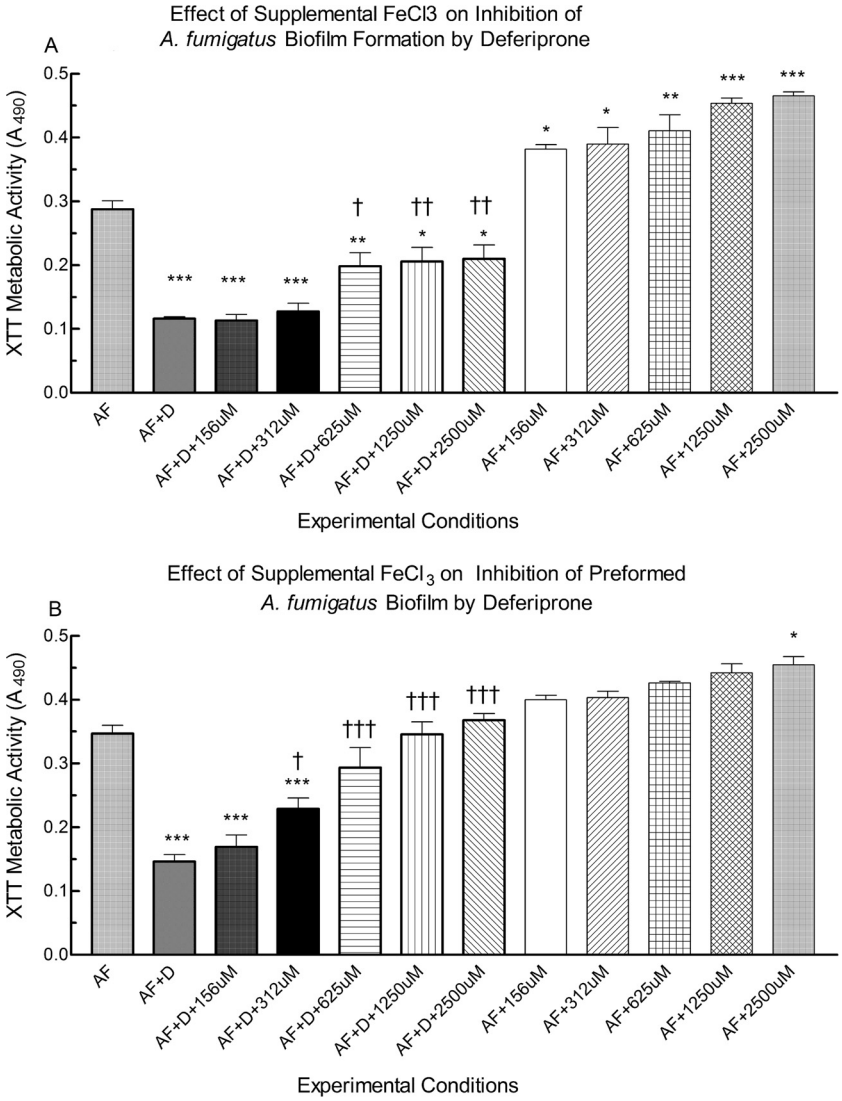

FIG 3 Effect of iron on the DFP inhibition of formation or further development of *A. fumigatus* biofilm. (A) *A. fumigatus* conidia were exposed to 625 μM DFP (D) alone, 625 μM DFP plus various concentrations of $FeCl_3$ (156 to 2,500 μM, shown as μM), or $FeCl_3$ alone for 16 h or not exposed (AF), before 24 h of further growth in fresh medium without added DFP or $FeCl_3$. (B) Preformed (16 h) *A. fumigatus* biofilms were exposed to 625 μM DFP alone, 625 μM DFP plus various concentrations of $FeCl_3$ (assays with 39 to 78 μM $FeCl_3$ not different than those with 156 μM; not shown), or $FeCl_3$ alone for 24 h or not exposed. Biofilms were quantified via XTT assay with absorbance at 490 nm. Twelve-well assays were used. Each condition was performed in triplicate, and the results were pooled from two to five separate experiments on different days. A single asterisk or dagger denotes a significant *P* value of <0.05, two asterisks or daggers denote a significant *P* value of <0.01, and three asterisks or daggers denote a significant *P* value of <0.001, compared to the experimental condition containing *A. fumigatus* alone (AF) (asterisks) or *A. fumigatus* plus DFP (daggers). The values shown are the means ± the standard deviations.

this inhibition is the result of iron chelation by the DFP, in that supplementation of the medium with $FeCl_3$ reversed the inhibition. Serum, likely due to the presence of iron-containing molecules, such as transferrin, blunts the inhibitory effects of iron chelation by DFP at DFP concentrations of <2,500 μM. Last, excess Fe^{3+} enhanced biofilm, and concentrations sufficient to augment biofilm establishment were lower than those necessary to further stimulate preformed biofilm.

Overall, we found that iron chelators can either inhibit or enhance the formation and growth of biofilm, and the magnitude of these effects differs between preformed biofilm of and biofilm formation by *A. fumigatus*. However, the inhibition of biofilm formation and growth by an iron chelator can be overcome by supplementing the medium with Fe^{3+}. We believe that the enhancement of biofilm by DFM is due to an ability of *A. fumigatus* to acquire the Fe-chelator complex via siderophore-like mechanisms, allowing the organism to access greater amounts of iron.

A range of susceptibilities of biofilm to DFP was discovered. This is similar to the polymorphism seen when surveying random clinical *A. fumigatus* isolates for susceptibility to antifungal drugs (2, 17). The greater susceptibility of biofilm formation than that of preformed biofilm to a chelator is consistent with the differential effects of *Pseudomonas aeruginosa* cells (or culture filtrates) on *A. fumigatus* biofilm demonstrated in our previous studies (18).

Iron chelation is a potential beneficial therapy for aspergillosis infections, including those involving biofilms, as in chronic pul-

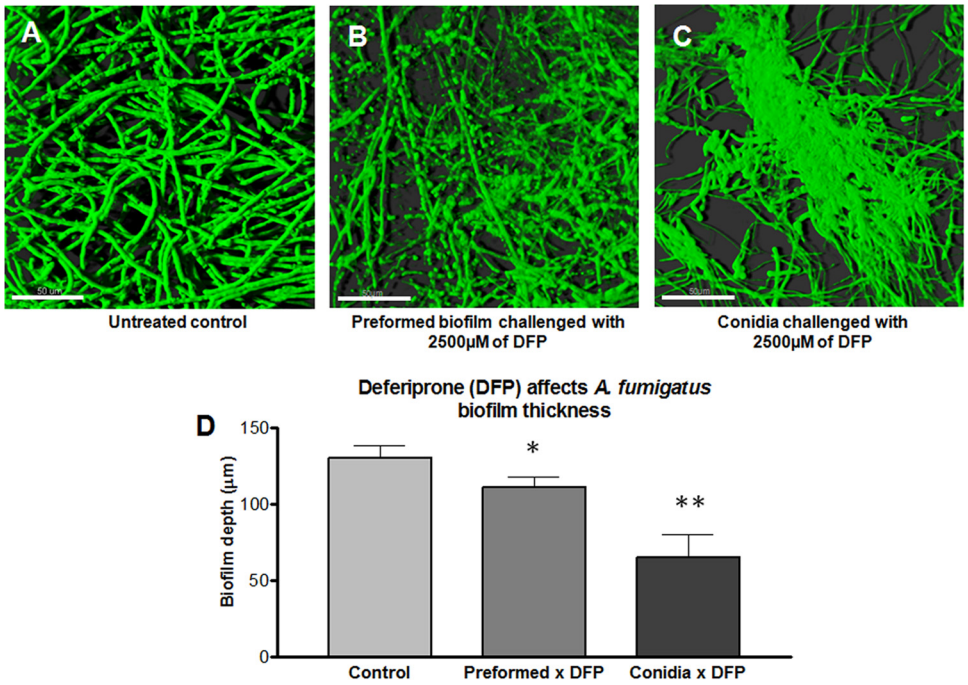

FIG 4 Confocal laser scanning microscope (CLSM) images of *A. fumigatus* biofilm grown on polycarbonate disk surface. (A) Horizontal (*xy*) view of a reconstructed three-dimensional (3D) image of *A. fumigatus* biofilm (untreated control). (B) Horizontal (*xy*) view of a reconstructed 3D image of *A. fumigatus* preformed biofilm challenged with 2,500 μg/ml DFP. (C) Horizontal (*xy*) view of a reconstructed 3D image of *A. fumigatus* biofilm formation challenged with 2,500 μg/ml DFP. (D) Effect of DFP on *A. fumigatus* biofilm thickness. Assays were performed in triplicate, and images were taken from three different fields from each sample stained with FUN 1. The results are representative of two different experiments for each condition tested. The values shown are the means ± the standard deviations. The control, preformed × DFP, and conidia × DFP correspond to the conditions shown in panels A to C, respectively. One asterisk indicates a *P* value of <0.01, and two asterisks indicate a *P* value of <0.001 for the biofilm thickness compared to the untreated control. Magnification, ×63. Scale bar, 50 μm.

monary infections, a situation in which local, (i.e., nonsystemic) use may be a possibility. An attractive advantage is the clinical experience already shown with chelators in the therapy of iron overload disorders, including their pharmacology and toxicology (31). That favorable systemic chelator experience has been accumulated even in the presence of the complicated *in vivo* iron-containing and iron-denying environments. Use in lung infections may have the prospect of fewer host iron-denying competitors locally and an ancillary therapeutic effect on other concurrent pathogens. However, the specific chelators must be carefully chosen, as shown in this study, so as to mitigate fungal pathogenicity. Moreover, susceptibility testing *in vitro* may be required to predict which isolates are the best candidates for chelation therapy. A study of the interaction of chelators with antifungals against *A. fumigatus* biofilm is of considerable interest.

TABLE 1 Survey of chelator effect on *A. fumigatus* isolates

Isolate	Lowest concn of DFP (μM) causing significant inhibition of:	
	A. fumigatus biofilm formation	Preformed *A. fumigatus* biofilm
10*AF*	156	625–1,250
2	625	1,250
3	≤156	625
4	1,250	>2,500
5	≤156	625
6	1,250	1,250
7	156	1,250
8	156	312
9	312	1,250
10	312	2,500

The results are based on 1 or 2 experiments for each isolate (except for 10*AF*, with ≥7 experiments).

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