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Role of Matrix β-1,3 Glucan in Antifungal Resistance of Non-*albicans Candida* Biofilms

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Candida biofilm infections pose an increasing threat in the health care setting due to the drug resistance associated with this lifestyle. Several mechanisms underlie the resistance phenomenon. In *Candida albicans*, one mechanism involves drug impedance by the biofilm matrix linked to β-1,3 glucan. Here, we show this is important for other *Candida* spp. We identified β-1,3 glucan in the matrix, found that the matrix sequesters antifungal drug, and enhanced antifungal susceptibility with matrix β-1,3 glucan hydrolysis.

Similar to many microbes, *Candida* species exhibit a propensity to grow as biofilms on implanted medical devices such as a central venous catheter (1, 2). Among biofilm-forming pathogens, infection due to *Candida* spp. is associated with the highest nosocomial mortality (3). Treating these infections proves challenging due to high levels of drug resistance (4, 5). Compared to their planktonic counterparts, biofilm cells exhibit up to a 1,000-fold increase in resistance (6, 7). For most patients, removal of the medical device is the only viable treatment option (1).

A number of factors contribute to *Candida albicans* biofilm resistance (8–11). The extracellular matrix that enmeshes the biofilm cells accounts for a large percentage of this phenotype by sequestering antifungal drugs. The matrix polysaccharide β-1,3 glucan has been strongly linked to this mechanism (12–14).

While *C. albicans* remains the most frequently isolated *Candida* species, other members of the genus are increasingly common. The most recent surveillance data in the United States (15) found *C. albicans* comprised far less than 50% of isolates. *Candida glabrata* (29%), *Candida parapsilosis* (17%), and *Candida tropicalis* (10%) as a group represented the majority of infections. Each of these species has been shown to form biofilms with comparable levels of antifungal resistance to *C. albicans* (16–18).

The increasing prevalence of non-*albicans Candida* species and their role in biofilm device infections prompted us to ask if they also exhibit a β-1,3 glucan matrix resistance mechanism. The purpose of this study was to determine if β-1,3 glucan was present in the matrix of these species and, if so, did it play a role in drug resistance similar to that described for *C. albicans*. Specifically, three experiments with three non-*albicans Candida* species were undertaken: (i) determination of matrix β-1,3 glucan content, (ii) assessment of the ability of the extracellular matrix to sequester the antifungal fluconazole, and (iii) examination of the impact of β-1,3 glucan disruption on biofilm antifungal drug susceptibility.

C. glabrata, *C. parapsilosis*, and *C. tropicalis* were chosen for study based upon relative incidence in clinical surveillance and demonstrated propensity for device biofilm formation. With the exception of the *C. albicans* isolate (strain SN250), all strains were clinical isolates from cases of invasive candidiasis (*C. glabrata* strains 570 and 5376, *C. parapsilosis* strains 5986 and CD371, and *C. tropicalis* strains 2058 and 98-234). Biofilms were grown in RPMI-MOPS (morpholinepropanesulfonic acid) medium on a polystyrene substrate for all experiments. Each of the strains formed robust biofilms with an average XTT [2,3-bis-(2-me-thoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt] optical density (OD) of 1.42 for *C. albicans* and 1.40 for the non-*albicans* group after 24 h of incubation. For matrix composition analysis, biofilms were grown for 48 h using 1-liter roller bottles. Matrix was isolated using water bath sonication and vortexing as previously described (19). A β-1,3 glucan enzyme-linked immunosorbent assay (ELISA) was performed on three biologic replicates, and assays were completed in triplicate for each strain as previously detailed. Matrix β-1,3 glucan was normalized by matrix dry weight and expressed as ng/mg matrix. As shown in Fig. 1A, the β-1,3 glucan polymer was identified in the biofilm matrix of each *Candida* strain tested. The concentrations of this polysaccharide among the species were relatively similar.

We utilized a 6-well plate format for assessment of antifungal drug biofilm penetration using [H^3]fluconazole as described previously (13, 19). Briefly, mature biofilms (24 h of incubation) were washed twice with sterile water followed by exposure to a total of 8.48×10^5 cpm of [H^3]fluconazole in RPMI-MOPS medium. Biofilms were incubated for 30 min at 37°C and then chased with 20 μM unlabeled fluconazole in medium. The fluconazole content was measured in intact biofilms, isolated matrix, cell wall, and cell cytoplasm by scintillation counting. Assays were performed in triplicate for each *Candida* isolate. Consistent with previous findings in *C. albicans* (13, 19), the majority of [H^3]fluconazole is present in the extracellular matrix for each of these species, with very little or no drug found intracellularly or in the cell wall (Fig. 1B).

We next determined the effect of matrix β-1,3 glucan hydrolysis on biofilm susceptibility to fluconazole. Using a 96-well plate format, biofilm cell metabolic activity was assayed following exposure to fluconazole and β-1,3 glucanase alone and in combination using a tetrazolium salt XTT reduction assay (20–23). Briefly, after 24 h of biofilm growth, medium was replaced by fresh RPMI-MOPS with dilutions of fluconazole at 1 mg/ml, β-1,3 glucanase (Zymolyase 20T; MP Biomedicals) at 0.7 U/ml, or a combination of the two. The β-1,3
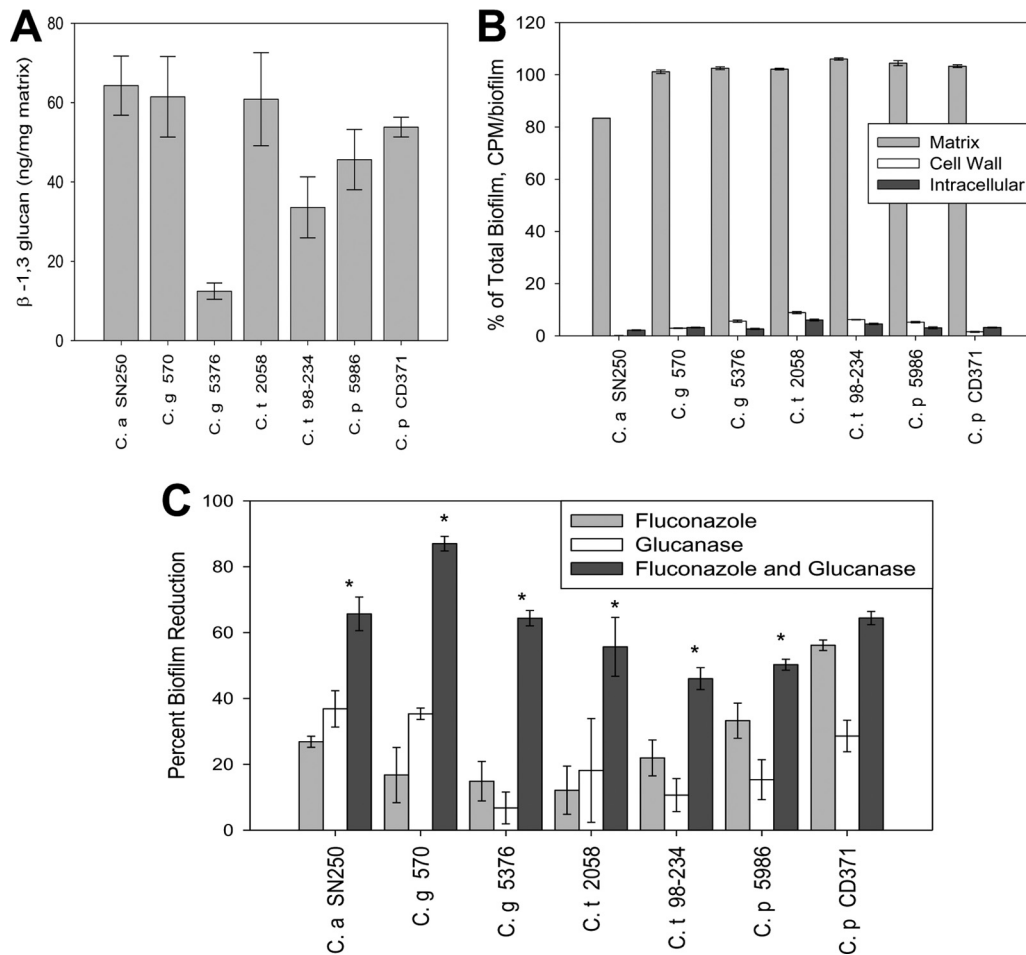
FIG 1 (A) The amount of β-1,3 glucan in a 1-mg/ml sample of purified *in vitro* biofilm matrix, measured by ELISA. Samples are measured in triplicate. Standard errors are shown. (B) [H^3]fluconazole in the matrix, cell wall, and cytoplasm of *in vitro* biofilms. Shown as a percentage of [H^3]fluconazole in the total biofilm, measured as CPM/biofilm. Standard errors are shown. (C) Biofilm susceptibility to fluconazole and/or β-1,3 glucanase as measured by the XTT reduction assay. Fluconazole dosed at 1 mg/ml and β-1,3 glucanase dosed at 0.7 U/ml. Data shown are representative examples, each read in triplicate. *, $P < 0.05$, comparing the combined drug values to either of the single-drug values using a one-way ANOVA.

glucanase concentration was chosen based upon our previous studies with *C. albicans* demonstrating synergy with fluconazole and no effect on cell viability for the enzyme alone (12, 19). Experiments were performed in triplicate. Drug effect is expressed at the percent biofilm reduction relative to growth of untreated controls. The statistical significance of differences among therapies was determined using analysis of variance (ANOVA). Similar to previous reports, fluconazole alone exhibited minimal activity against biofilms for each strain and species (12, 19). The low concentration of β-1,3 glucanase also produced little change in cell metabolic activity. However, fluconazole caused marked biofilm reduction in the presence of the β-1,3 glucan-hydrolyzing enzyme. This effect was observed for all strains tested (Fig. 1C).

The results of the present study with non-*albicans Candida* species are similar to those from *C. albicans*, which demonstrate the contribution of biofilm matrix β-1,3 glucan for the antifungal drug resistance phenomenon linked to this common infection lifestyle (12–14). The relative impact of the mechanism for these other common *Candida* species appears congruent with that shown for *C. albicans* based upon comparable concentrations of matrix β-1,3 glucan, antifungal drug sequestration, and influence

of β-1,3 glucanase treatment on fluconazole efficacy. The prevalence of these non-*albicans Candida* species continues to rise. Insight to the mechanisms responsible for resistance to therapy is critical for design of new treatment strategies. The present study suggests that drug development targeting matrix β-1,3 glucan may potentiate the activity of the currently available antifungal option.

ACKNOWLEDGMENT

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