Dynamics of Mixed-*Candida* Species Biofilms in Response to Antifungals

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Abstract

Oral infections caused by *Candida* species, the most commonly isolated human fungal pathogen, are frequently associated with biofilms. Although *Candida albicans* is the predominant organism found in patients with oral thrush, a biofilm infection, there is an increasing incidence of oral colonization and infections caused by non-*albicans* *Candida* species, including *C. glabrata*, *C. dubliniensis*, and *C. tropicalis*, which are frequently more resistant to antifungal treatment. While single-species *Candida* biofilms have been well studied, considerably less is known about the dynamics of mixed-*Candida* species biofilms and how these dynamics are altered by antifungal treatment. To address these questions, we developed a quantitative polymerase chain reaction-based approach to determine the precise species composition of mixed-*Candida* species biofilms formed by clinical isolates and laboratory strains in the presence and absence of clinically relevant concentrations of 3 commonly used antifungals: fluconazole, caspofungin, and amphotericin B. In monospecies biofilms, fluconazole exposure favored growth of *C. glabrata* and *C. tropicalis*, while caspofungin generally favored significant growth of all species to a varying degree. Fluconazole was not effective against preformed mixed-*Candida* species biofilms while amphotericin B was potent. As a general trend, in mixed-*Candida* species biofilms, *C. albicans* lost dominance in the presence of antifungals. Interestingly, presence in mixed versus monospecies biofilms reduced susceptibility to amphotericin B for *C. tropicalis* and *C. glabrata*. Overall, our data suggest that antifungal treatment favors the growth of specific non-*albicans* *Candida* species in mixed-*Candida* species biofilms.

Keywords: candidiasis, infectious disease medicine, mycology, oral health, microbiology, cell biology

Introduction

Candida species are responsible for a variety of superficial and invasive infections, including oral infections, in immunocompromised individuals, such as patients with HIV/AIDS, recipients of organ transplants, and patients with cancer (Redding et al. 1999; Redding et al. 2000; Pfaller and Diekema 2007; Thein et al. 2009; Dangi et al. 2010). The emergence of *Candida* species as leading oral fungal pathogens is related to certain risk factors, including the administration of immunosuppressive drugs and antibiotics, HIV immunosuppression, prosthetic devices (e.g., dentures), decreased salivary production, atrophic oral mucosa, altered or immature oral flora, and poor oral hygiene (Dangi et al. 2010; Rautemaa and Ramage 2011; Ramage et al. 2012).

Candida infections are frequently associated with formation of biofilms (Ramage et al. 2009; Ramage et al. 2012): highly structured communities of microorganisms that are surface associated and enclosed within a self-produced extracellular matrix (Ramage et al. 2012). This unique environment provides many benefits to microbial pathogens, including protection from host immune defenses, resistance to physical and chemical stresses, nutrient availability, complex community interactions, and metabolic cooperation (Ramage et al. 2012). In many immunocompromised patients, *Candida* biofilms are

associated with oral candidiasis, or thrush (Dangi et al. 2010; Rautemaa and Ramage 2011; Williams et al. 2011). The prevalence of oral thrush in patients with AIDS and cancer is 30% and 20%, respectively (Patel et al. 2012). In the clinic, *Candida* biofilms are of greater significance due to their association with increased resistance to commonly used antifungal agents that can be administered orally, topically, and intravenously, including azoles (e.g., fluconazole [FLC]), polyenes (e.g., amphotericin B [AMPB]), and echinocandins (e.g., caspofungin [CSP]), all of which interfere with fungal membrane or cell wall integrity (Sanglard and Odds 2002; Al-Fattani and

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A supplemental appendix to this article is available online.

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Douglas 2004; Kanafani and Perfect 2008; Thein et al. 2009; Fanning and Mitchell 2012; Ramage et al. 2012). Antifungal resistance represents a major obstacle for clinicians seeking to effectively manage *Candida* infections (Perea and Patterson 2002; Sanguinetti et al. 2015). All 3 classes of antifungals listed above can be used to treat oral and oropharyngeal candidiasis. However, drug toxicity, isolation of non-*albicans* *Candida* species (NACS) that are less susceptible to standard therapies, and recovery of resistant isolates from patients on long-term antibiotic/antifungal prophylaxis are emerging problems for clinicians (Klepser 2001), in part due to the limited arsenal of currently available antifungals.

Most previous studies on *Candida* biofilms have focused on single-species biofilms formed by *C. albicans*. Although *C. albicans* is the predominant organism found in oral thrush (Rautemaa and Ramage 2011), several reports indicate that NACS are being isolated with increasing frequency and can be found in combination during infection in vivo (Kirkpatrick et al. 1998; Redding et al. 2000; Klepser 2001; Silva et al. 2011; Patel et al. 2012). These reports document the presence of *C. dubliniensis*, *C. tropicalis*, *C. krusei*, and *C. glabrata* in oral thrush samples from patients with HIV, diabetes, periodontitis, and dental prostheses as well as patients receiving long-term immunosuppressive drugs and radiation therapy for head and neck cancer (Sangeorzan et al. 1994; Sullivan et al. 1997; Redding et al. 1999; Redding et al. 2000; Sanglard and Odds 2002; Shaheen and Taha 2006; Meurman et al. 2007; Dangi et al. 2010; Nejad et al. 2011; Patel et al. 2012). These studies also show increased minimum inhibitory concentration values for NACS and, in certain cases, report the isolation of antifungal-resistant strains (Redding et al. 1999; Sanglard and Odds 2002). The emergence of NACS that are inherently resistant or rapidly develop resistance has clinical implications because conventional doses of antifungal therapy are sometimes ineffective at clearing oral infections caused by these species (Klepser 2001; Sanglard and Odds 2002; Patel et al. 2012). Therefore, to more effectively treat oral thrush, it is important that clinicians are aware of shifting antifungal susceptibility patterns as well as the specific composition of *Candida* species in infections (Klepser 2001). Because multiple mechanisms appear to play a role in the antifungal drug resistance of biofilms (Sanglard and Odds 2002; Ramage et al. 2012) and certain *Candida* species are intrinsically resistant or can rapidly develop resistance (Sanglard and Odds 2002; Al-Fattani and Douglas 2004; Meurman et al. 2007), the precise composition of mixed-*Candida* species biofilms may contribute to the overall level of antifungal resistance.

While the frequency of infections with NACS is on the rise and biofilms composed of multiple *Candida* species are more commonly encountered in the clinic (Fidel et al. 1999; Silva et al. 2010; Patel et al. 2012; Al Mubarak et al. 2013; Pannanusorn et al. 2013; Papon et al. 2013; Sardi et al. 2013), very little is known about the dynamics (changes in relative species composition) of mixed-*Candida* species biofilms, particularly in response to antifungals. To address this question, we developed a quantitative polymerase chain reaction (qPCR)-based

approach to determine the precise species composition of these biofilms. Here, using this assay, not only do we demonstrate that growth of NACS is favored following antifungal exposure but, more interestingly, presence in a mixed versus monospecies biofilm reduces susceptibility to AMPB for certain NACS. These studies provide new insights into how the composition of mixed-*Candida* species biofilms is correlated with antifungal drug resistance and why NACS are isolated from immunocompromised patients with increasing frequency.

Materials and Methods

Candida Strains and Growth Conditions

Twelve *Candida* strains were used in this study (Appendix Table 1). Three strains of each of the following species were obtained: *C. albicans*, *C. dubliniensis*, *C. tropicalis*, and *C. glabrata*. These strains consisted of a general laboratory strain and 2 clinical isolates—1 susceptible and 1 resistant to antifungal treatment with FLC and CSP. The clinical isolates were obtained from the Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio, and categorized per planktonic minimum inhibitory concentrations via the broth microdilution method (Clinical and Laboratory Standards Institute 2008; Appendix Table 2).

Biofilm Culture Conditions

Biofilms were formed on 1-cm² silicon elastomer sheets (Cardiovascular Instrument Corp.) as previously described (Uppuluri et al. 2009). For a detailed description of biofilm culture conditions, see Appendix Materials and Methods.

Genomic DNA Isolation

The Maxwell 16 LEV Blood DNA kit (Promega) was used to isolate genomic DNA from *Candida* species biofilms. For a detailed description, see Appendix Materials and Methods.

qPCR Analysis

Species-specific primers and minor groove binder TaqMan probes to *TUB1*, a conserved housekeeping gene (Appendix Table 3), were used to detect qPCR products with high accuracy, and rigorous testing was performed to confirm species specificity and reproducibility of assays (Appendix Materials and Methods, Appendix Table 4). Standard curves for qPCR analysis were performed using serial dilutions (40, 20, 10, 5, 2.5, 1.25, 0.625 ng/μL) of individual pure species genomic DNA. qPCR reactions were placed in 96-well optical microtiter plates (ABI). Each well held a total volume of 20 μL: 10 μL, Applied Biosystems 2X TaqMan Universal PCR Master Mix; 1.6 μL, HPLC-purified forward primer (10 μM); 1.6 μL, HPLC-purified reverse primer (10 μM); 1.6 μL, Taqman Probe (250 nM); and 5 μL, genomic DNA. Amplification was performed with an ABI 7700 sequence detection system with the

Table 1. Fold Change of Monospecies *Candida* Biofilm Quantity in Response to Antifungal Treatment.

Strain/Isolate: Species	FLC, 32 µg/mL	CSP, 1 µg/mL	AMPB, 0.25 µg/mL
General laboratory strains			
<i>C. albicans</i>	0.65 ^a	2.5	0.56 ^a
<i>C. dubliniensis</i>	0.85	4.4 ^a	0.1 ^a
<i>C. tropicalis</i>	30 ^a	4.4 ^a	—
<i>C. glabrata</i>	2 ^a	2	—
Susceptible clinical isolates			
<i>C. albicans</i>	0.58 ^a	3.1 ^a	0.46 ^a
<i>C. dubliniensis</i>	0.88	0.8	0.62
<i>C. tropicalis</i>	86 ^a	4.4 ^a	27 ^a
<i>C. glabrata</i>	1.7 ^a	0.4	—
Resistant clinical isolates			
<i>C. albicans</i>	1.2	2.2	0.78
<i>C. dubliniensis</i>	1.4	2.6 ^a	0.77
<i>C. tropicalis</i>	7 ^a	195	—
<i>C. glabrata</i>	2	0.5 ^a	—

Fold change = biofilm quantity in presence of antifungal / biofilm quantity in the absence of antifungal. Biofilm quantity in the presence and absence of antifungal was determined at the 48-h point (following 24-h biofilm growth plus or minus drug). Dashes (—) indicate that there were no detectable counts following antifungal treatment.

AMPB, amphotericin B; CSP, caspofungin; FLC, fluconazole.

^aP < 0.05 considered statistically significant (analysis of variance); analysis variable is log(fold change).

following cycling parameters: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C for target signaling. The ABI 7700 analysis program determined the absolute DNA quantity (ng) of each *Candida* species within the biofilm per the standard curve method. C_i values from known DNA quantities were used to develop a standard curve for each species for each run. C_i values of samples were compared with this standard curve to determine DNA quantities. All assays were performed in biological and technical triplicate.

Results

Monospecies *Candida* Biofilms Exhibit Varied Growth Patterns in the Presence of Antifungals

We developed a highly specific qPCR-based assay to determine the precise composition of *Candida* species biofilms composed of *C. albicans*, *C. dubliniensis*, *C. tropicalis*, and/or *C. glabrata*. These species were selected because they are most frequently isolated in patients with oral candidiasis (Sangeorzan et al. 1994; Sullivan et al. 1997; Redding et al. 1999; Redding et al. 2000; Shaheen and Taha 2006; Meurman et al. 2007; Dangi et al. 2010; Nejad et al. 2011; Patel et al. 2012; Sardi et al. 2013). We first determined the effect of antifungal treatment on the quantity of monospecies biofilms formed by each species. DNA was used as a quantitative measure of biofilm formation because it would be the most efficient way to distinguish change in biofilm growth among individual species within a mixed biofilm in later experiments. Drug concentrations (32 µg/mL, FLC; 1 µg/mL, CSP; 0.25 µg/mL, AMPB) were based on sessile minimum inhibitory concentrations (data not shown). For general laboratory strains, the total amount of *C. albicans* biofilm formation showed a statistically significant

decrease following FLC treatment (Table 1). The total quantity of *C. dubliniensis* biofilm showed a minor reduction, while the total amount of *C. glabrata* and *C. tropicalis* biofilm increased significantly. This same trend was observed when monospecies biofilms were formed with susceptible clinical isolates. Monospecies biofilms formed with resistant clinical isolates all showed increased quantity in the presence of FLC, as expected, with the *C. tropicalis* biofilm showing the greatest increase. Overall, these results suggest that FLC treatment favors the growth of *C. tropicalis* and *C. glabrata* biofilms in our system.

For general laboratory strains, all species showed increased biofilm formation following CSP treatment (Table 1). This increase was also observed for monospecies biofilms of susceptible strains (with the exception of *C. dubliniensis* and *C. glabrata*) and resistant strains (with the exception of *C. glabrata*). In general, consistent with previous observations (Chamilos et al. 2007), the increase was most apparent for *C. tropicalis*, followed by *C. albicans* and *C. dubliniensis*, and was least apparent for *C. glabrata*. These results are indicative of the paradoxical growth effect, a poorly understood in vitro phenomenon that has been widely documented for echinocandins, especially CSP (Chamilos et al. 2007; Shields et al. 2011). Taken together, these findings also validate our biofilm growth conditions and qPCR assay.

We generally observed that monospecies biofilms for all 4 species in each set of strains were susceptible to AMPB, with *C. glabrata* and *C. tropicalis* being most sensitive (Table 1), although 1 of the susceptible clinical isolates (*C. tropicalis*) demonstrated significant growth following AMPB exposure. Altogether, the observed differences in growth patterns of monospecies *Candida* biofilms in the presence of antifungals suggested that precise species composition may change in a mixed biofilm following drug exposure.

Figure 1. Species ratio of mixed-*Candida* species biofilms containing *C. albicans* (A), *C. dubliniensis* (D), *C. tropicalis* (T), and *C. glabrata* (G) in the presence and absence of antifungal treatment. AMPB, amphotericin B; CSP, caspofungin; FLC, fluconazole. Initially, equal ratios of the indicated *Candida* strains were allowed to grow on silicone elastomer sheets (1 × 1 cm) in fresh RPMI at 37 °C. The indicated antifungal drug was added following 24 h of mixed-biofilm formation. At 48 h, DNA quantity of each species (ng) was determined using our quantitative polymerase chain reaction-based approach. Results are expressed as the percentage of quantity of each species compared to total quantity of mixed biofilm ($n = 3$). Fold change in total amount of biofilm in response to treatment with the indicated drug is shown at the bottom of each histogram. Arrows indicate statistically significant changes in total biofilm quantity following antifungal treatment ($P < 0.05$, paired samples t test). *Statistically significant change in the amount of the indicated species within biofilm following antifungal treatment ($P < 0.05$, analysis of variance [restricted maximum likelihood], model-based fixed effects method). Note that 3 separate no-drug controls ($n = 3$ each) were performed (no statistically significant changes were observed) and that the average values are shown for purposes of display.

For a Mixed Biofilm, Species Composition Changes Following Antifungal Exposure to Favor NACS

To determine how species composition of mixed-*Candida* species biofilms changes following antifungal exposure, we formed biofilms using equal ratios of species to allow for a good basis of comparison. Even in the absence of antifungal treatment, for all combinations, we observed that mixed-biofilm species composition is not equally distributed (Figs. 1, 2; Appendix Fig., Appendix Data Sets 1–3). Interestingly, *C. albicans* showed greater representation in a mixed biofilm composed of general laboratory strains versus clinical isolates.

Following FLC treatment, we observed a general increase in the presence of *C. glabrata* in nearly all combinations of mixed biofilms containing this species for all sets of strains (Figs. 1, 2; Appendix Fig., Appendix Data Set 1). Mixed biofilms exposed to CSP showed an increase in the presence of *C. tropicalis* in nearly all combinations containing this species for all sets of strains (Figs. 1, 2; Appendix Fig., Appendix Data Set 2). While AMPB treatment was quite effective against certain mixed-*Candida* species biofilms (e.g., GD, GT, DT, AGT, and GDT for general laboratory strains and GT for clinical resistant strains), there was no consistent trend seen among all 3 sets of strains (Appendix Data Set 3). For certain susceptible clinical isolate combinations containing *C. tropicalis* (AT, AGT), there was an increase in the percentage of this species (Fig. 2B; Appendix Fig. B), which correlated with our previous monospecies biofilm results (Table 1). In general, we observed a significant decrease in *C. glabrata* following AMPB treatment for all 3 sets of strains (Figs. 1, 2; Appendix Data Set 3).

When all 4 species are present in a mixed biofilm, the precise composition changes following antifungal exposure to generally favor NACS, particularly for both sets of clinical isolates (Fig. 1). For these strains, we observed an increase in *C. glabrata* following FLC treatment, an increase in *C. tropicalis* following CSP exposure, and an increase in the presence of *C. dubliniensis* following AMPB treatment. Although the general laboratory strains exhibited the same trend for *C. glabrata* and *C. tropicalis* following FLC and CSP treatment, respectively, we did not observe an increase in *C. dubliniensis* following AMPB exposure. Interestingly, we also observed that *Candida* species may show a different composition in a mixed biofilm, depending on the other species present and the type of drug exposure (Figs. 1, 2; Appendix Fig., Appendix Data Sets 1–3).

In general, there were no statistically significant differences in the total amount of biofilm in the presence versus absence of FLC (Figs. 1, 2; Appendix Fig., Appendix Data Set 1), suggesting that FLC is not effective against preformed biofilms or that FLC does not influence the growth of these biofilms. Generally, the quantity of a mixed biofilm significantly increases following CSP treatment, confirming the paradoxical growth effect phenomenon and validating our assay and in vitro system (Figs. 1, 2; Appendix Fig., Appendix Data Set 2). Finally, we observed in general that the overall quantity of a mixed biofilm significantly decreased following AMPB treatment, suggesting that this drug is effective against preformed mixed biofilms (Figs. 1, 2; Appendix Fig., Appendix Data Set 3).

In Mixed-Candida Species Biofilms, *C. albicans* Loses Dominance in Response to Antifungal Treatment

For all mixed-biofilm combinations containing *C. albicans*, species were ordered from those present in highest to lowest quantity (Fig. 3). For biofilms composed of several combinations of clinical isolates (including GA for resistant strains and DA, DGA, TDA, and GTA for susceptible strains), *C. albicans* was already least dominant prior to antifungal treatment. For other clinical isolate mixed-biofilm combinations, *C. albicans* generally lost dominance following treatment with all 3 antifungals. In contrast, for general laboratory strains, *C. albicans* lost dominance in only certain combinations upon CSP exposure. Overall, however, *C. albicans* generally appeared to lose dominance or remain least dominant in mixed-*Candida* species biofilms in response to antifungals.

Presence in Mixed versus Monospecies Biofilms Reduces Susceptibility to AMPB for Certain NACS

For all 3 sets of strains, and particularly for general laboratory strains and resistant clinical isolates, we observed higher amounts of *C. tropicalis* and *C. glabrata* biofilm following AMPB exposure in a mixed biofilm containing *C. tropicalis*, *C. glabrata*, *C. albicans*, and *C. dubliniensis*, compared to those in monospecies counterparts (Table 2). A similar trend was observed for biofilms composed of 2 or 3 *Candida* species (Appendix Table 5). These findings suggest that presence in a mixed versus monospecies biofilm reduces susceptibility of certain NACS to AMPB.

Discussion

Based on a recently developed and highly accurate qPCR-based assay, our studies provide new and important information about the effect of antifungals on the dynamics of *Candida* species growth in an in vitro monospecies and mixed-species biofilm environment. Following FLC exposure, we observed an increase in *C. glabrata* and *C. tropicalis* monospecies biofilms

Figure 2. Species ratio of mixed-*Candida* species biofilms containing (A) *C. albicans* (A), *C. glabrata* (G), and *C. dubliniensis* (D) or (B) *C. albicans*, *C. glabrata*, and *C. tropicalis* (T) in the presence and absence of antifungal treatment. AMPB, amphotericin B; CSP, caspofungin; FLC, fluconazole. Initially, equal ratios of the indicated *Candida* strains were allowed to grow on silicone elastomer sheets (1 × 1 cm) in fresh RPMI at 37 °C. The indicated antifungal drug was added following 24 h of mixed biofilm formation. At 48 h, DNA quantity of each species (ng) was determined using our quantitative polymerase chain reaction-based approach. Results are expressed as the percentage of quantity of each species compared to total quantity of mixed biofilm ($n = 3$). Fold change in the total amount of biofilm in response to treatment with the indicated drug is shown at the bottom of each histogram. Arrows indicate statistically significant changes in total amount of biofilm quantity following antifungal treatment ($P < 0.05$, paired samples t test). *Statistically significant change in the amount of the indicated species within biofilm following antifungal treatment ($P < 0.05$, analysis of variance [restricted maximum likelihood], model-based fixed effects method). Note that 3 separate no-drug controls ($n = 3$ each) were performed (no statistically significant changes were observed) and that the average values are shown for purposes of display.

for general laboratory strains as well as drug-susceptible and drug-resistant clinical isolates. For all 3 sets of strains, we also observed an increase in the presence of *C. glabrata* in nearly all mixed-biofilm combinations containing this species. This trend is expected for *C. glabrata* due to its reduced susceptibility to azoles (Sardi et al. 2013). The *C. tropicalis* strains may have rapidly adapted to acquire an increased resistance to FLC, possibly as a result of increased drug efflux pump expression. FLC is one of the first-line treatments for management of local and systemic diseases caused by *C. albicans*, and, as expected,

General Lab Strains				Susceptible Clinical Isolates				Resistant Clinical Isolates			
No drug	FLC	CSP	AMPB	No drug	FLC	CSP	AMPB	No drug	FLC	CSP	AMPB
<u>A</u> G	AG	AG	AG	<u>A</u> G	G <u>A</u>	G <u>A</u>	G <u>A</u>	<u>G</u> A	G <u>A</u>	G <u>A</u>	G <u>A</u>
<u>A</u> D	AD	AD	AD	<u>D</u> A	D <u>A</u>	D <u>A</u>	D <u>A</u>	<u>A</u> D	AD	AD	D <u>A</u>
<u>A</u> T	AT	T <u>A</u>	AT	<u>A</u> T	AT	T <u>A</u>	AT	<u>A</u> T	AT	T <u>A</u>	AT
<u>A</u> GD	AGD	AGD	AGD	<u>D</u> G <u>A</u>	DG <u>A</u>	GD <u>A</u>	DG <u>A</u>	<u>G</u> D <u>A</u>	GD <u>A</u>	G <u>A</u> D	DAG
<u>A</u> TD	ADT	T <u>A</u> D	ADT	<u>T</u> D <u>A</u>	DT <u>A</u>	TD <u>A</u>	DT <u>A</u>	<u>T</u> D <u>A</u>	DAT	TD <u>A</u>	DAT
<u>A</u> GT	AGT	T <u>A</u> G	-	<u>G</u> T <u>A</u>	GTA	TG <u>A</u>	TG <u>A</u>	<u>G</u> A <u>T</u>	GAT	TG <u>A</u>	TG <u>A</u>
<u>A</u> GTD	AGDT	T <u>A</u> GD	AGDT	<u>G</u> D <u>A</u> T	GDT <u>A</u>	TG <u>D</u> A	DTG <u>A</u>	<u>G</u> A <u>D</u> T	GD <u>A</u> T	G <u>A</u> TD	GDT <u>A</u>

C. albicans (A) remains least dominant following antifungal treatment
 C. albicans (A) gains dominance following antifungal treatment
 C. albicans (A) loses dominance following antifungal treatment

Figure 3. Pattern of dominance for mixed-*Candida* species biofilms in the presence and absence of antifungal treatment. The order of species present in largest to smallest DNA quantity (ng), as determined by quantitative polymerase chain reaction, is listed for mixed-*Candida* species biofilms in the presence and absence of the indicated antifungal. AMPB, amphotericin B; CSP, caspofungin; FLC, fluconazole. A, *C. albicans*; D, *C. dubliniensis*; G, *C. glabrata*; T, *C. tropicalis*. Underlined A indicates shift in dominance position of *C. albicans*. $n = 3$.

we observed a significant decrease in the quantity of *C. albicans* biofilm following FLC exposure for both general laboratory and susceptible clinical strains.

Our observation of the paradoxical growth effect upon CSP treatment is consistent with previous findings (Chamilos et al. 2007; Shields et al. 2011) and validates our qPCR-based system for determining *Candida*-species biofilm composition; while the molecular mechanisms responsible for this effect are unknown, they are thought to involve the PKC cell wall integrity pathway and increases in chitin content (Cota et al. 2008; Wiederhold 2009). For *C. dubliniensis* and *C. glabrata* monospecies

biofilms, the overall fold change in biofilm quantity in response to CSP is higher for general laboratory strains as compared with clinical isolates (Table 1; Appendix Data Set 2). *C. albicans* also appeared to be a more dominant species in mixed biofilms composed of general laboratory strains versus clinical isolates (Fig. 3). These findings suggest that the dynamics of mixed-*Candida* species biofilms formed by general laboratory strains may not always accurately predict those observed in biofilms composed of clinical isolates.

As compared with the other antifungals tested, AMPB was generally highly effective against monospecies and mixed-*Candida* species biofilms, although no consistent trends were observed among general laboratory strains and drug-susceptible and drug-resistant clinical isolates. Altogether, certain antifungals appear to favor

growth of NACS following exposure in our system. The observation that *C. albicans* generally loses dominance or remains least dominant following antifungal treatment of mixed-*Candida* species biofilms further supports this conclusion. While our observations are very consistent with the recent increase in frequency of NACS infections observed in the clinic (Klepser 2001; Silva et al. 2011; Patel et al. 2012), a limitation of our system is that *Candida* biofilms formed in vitro may not be identical to those formed on epithelial cells in vivo. In addition, drug concentrations used in our in vitro assay may not be identical to those found in oral biofilms grown in saliva in vivo. Also,

Table 2. Presence in Mixed versus Monospecies Biofilms Reduces Susceptibility to Amphotericin B for Certain Non-*albicans Candida* Species.

Strain/Isolate: Species	+ Amphotericin B		– Amphotericin B	
	Monospecies	Mixed (AGDT)	Monospecies	Mixed (AGDT)
General laboratory strains				
<i>C. albicans</i>	3.9	1.5	6.9	4.4
<i>C. dubliniensis</i>	0.38	0.15	2.2	0.5
<i>C. tropicalis</i>	0	0.05	0.6	1.6
<i>C. glabrata</i>	0	0.3	4.4	1.6
Susceptible clinical isolates				
<i>C. albicans</i>	1.6	0.3	3.4	0.4
<i>C. dubliniensis</i>	2.1	0.8	3.4	0.6
<i>C. tropicalis</i>	0.3	0.4	0.016	1.2
<i>C. glabrata</i>	0.03	0.3	3.4	0.9
Resistant clinical isolates				
<i>C. albicans</i>	5.7	1.7	7.3	3.6
<i>C. dubliniensis</i>	10.3	2.8	13.3	3.5
<i>C. tropicalis</i>	0	1.7	1.53	3.3
<i>C. glabrata</i>	0	4.1	8.7	6.3

Quantity values are listed in nanograms of DNA. All quantities were determined at the 48-h point (following 24-h biofilm growth in the presence or absence of drug).

A, *C. albicans*; D, *C. dubliniensis*; G, *C. glabrata*; T, *C. tropicalis*.

although our qPCR-based assay can accurately determine the quantity of each *Candida* species within a mixed biofilm, it cannot distinguish between live and dead cells and thus does not provide a direct measure of viability (although dead cells are less likely to adhere to the biofilm, resulting in reduced quantity). These limitations are important to bear in mind when interpreting data obtained with this assay.

Interestingly, our results demonstrate that presence in a mixed versus monospecies *Candida* biofilm significantly reduces susceptibility to AMPB for certain NACS, particularly *C. tropicalis* and *C. glabrata*. Several factors may contribute to the observed reduction in susceptibility, including shielding by a dense mat of hyphae and upregulation of drug efflux pumps, as well as proteins involved in other drug-resistance mechanisms, when *Candida* species are present in mixed versus monospecies biofilms. Because multispecies *Candida* biofilms are likely to be encountered more often in clinical situations, monitoring of changes in biofilm species composition will likely provide greater insight into the generation of antifungal resistance.

Author Contributions

G. Vipulanandan, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; M. Herrera, contributed to design, data acquisition, and analysis, critically revised the manuscript; N.P. Wiederhold, contributed to data acquisition and interpretation, critically revised the manuscript; X. Li, J. Mintz, contributed to data analysis and interpretation, critically revised the manuscript; B.L. Wickes, contributed to conception, design, data analysis, and interpretation, critically revised the manuscript; D. Kadosh, contributed to conception, design, data analysis, and interpretation, drafted and critically revised the manuscript.

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