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A Small Subpopulation of Blastospores in *Candida albicans* Biofilms Exhibit Resistance to Amphotericin B Associated with Differential Regulation of Ergosterol and β-1,6-Glucan Pathway Genes[▽]

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The resistance of *Candida albicans* biofilms to a broad spectrum of antimicrobial agents has been well documented. Biofilms are known to be heterogeneous, consisting of microenvironments that may induce formation of resistant subpopulations. In this study we characterized one such subpopulation. *C. albicans* biofilms were cultured in a tubular flow cell (TF) for 36 h. The relatively large shear forces imposed by draining the TF removed most of the biofilm, which consisted of a tangled mass of filamentous forms with associated clusters of yeast forms. This portion of the biofilm exhibited the classic architecture and morphological heterogeneity of a *C. albicans* biofilm and was only slightly more resistant than either exponential- or stationary-phase planktonic cells. A submonolayer fraction of blastospores that remained on the substratum was resistant to 10 times the amphotericin B dose that eliminated the activity of the planktonic populations. A comparison between planktonic and biofilm populations of transcript abundance for genes coding for enzymes in the ergosterol (*ERG1*, *-3*, *-5*, *-6*, *-9*, *-11*, and *-25*) and β-1,6-glucan (*SKN* and *KRE1*, *-5*, *-6*, and *-9*) pathways was performed by quantitative RT-PCR. The results indicate a possible association between the high level of resistance exhibited by the blastospore subpopulation and differential regulation of *ERG1*, *ERG25*, *SKN1*, and *KRE1*. We hypothesize that the resistance originates from a synergistic effect involving changes in both the cell membrane and the cell wall.

Candida albicans is a commensal opportunistic pathogen that causes both superficial and invasive, disseminated infections (55). Invasive candidiasis is the most prevalent invasive mycosis in humans, with *C. albicans* being the most common etiological agent (58). Superficial forms of candidiasis that are non-life-threatening but are nevertheless considered serious health concerns include relapsing vaginitis and human immunodeficiency virus-associated oropharyngeal candidiasis (64).

C. albicans colonizes various biomaterials and readily forms dense, complex biofilms under a variety of in vitro conditions (60). A particularly high risk of invasive candidiasis is associated with the use of urinary and vascular catheters and ventricular assistive devices (41). The likelihood of bloodstream infections (BSI) resulting from colonization of intravascular catheters by *Candida* species ranks second only to that posed by *Staphylococcus aureus* (19). The prognosis for BSI caused by *Candida* species is poor, with an attributable mortality for nosocomial BSI of nearly 50% (34).

From a rigorous clinical perspective, in vitro biofilm models are most appropriate for testing hypotheses related to biomaterial-centered infections. However, from a broader, fundamental perspective, their utility for advancing understanding of infective *C. albicans* communities extends further. The biofilm paradigm is considered an appropriate model for some tissue infections (18), and superficial tissue infections such as oropharyngeal candidiasis resemble, in many respects, *C. albicans* biofilms that form in vitro (33). Also, some forms of localized invasive candidiasis manifest as a dense pleomorphic community (55), and these infections share conspicuous morphological characteristics with in vitro biofilms.

In general, microbial biofilms exhibit an exceptional ability to survive doses of antimicrobial agents that are many times greater than the dose which is lethal for their planktonic counterparts (18, 22, 45, 68). *C. albicans* biofilms have been shown to be resistant to a variety of azoles, including fluconazole (36), voriconazole (46), miconazole (44), itraconazole (36), ketoconazole (36), the antiseptic chlorhexidine (15, 16), flucytosine (36), and the polyenes nystatin (15, 16) and amphotericin B (AmB) (15, 16, 36, 61, 62). The reasons for biofilm resistance have not been fully elucidated, but the emerging theme is that biofilm resistance cannot be generally traced to traits characteristic of isolated cells but rather originates from special conditions inherent in the biofilm community lifestyle (18, 22, 60, 68). One possibility is that microenvironments in biofilms accommodate subpopulations of cells with physiological traits that render them less susceptible to antimicrobial agents, a phenomenon sometimes referred to as phenotypic adaptation (48). We present evidence here for the existence of such a

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subpopulation in *C. albicans* biofilms cultured in our experimental system.

MATERIALS AND METHODS

***C. albicans* strain and medium.** *C. albicans* CA-1 is a clinical isolate obtained from the culture collection of Diane Brawner (Microbiology Department, Montana State University) (35). The biofilm-forming behavior of CA-1 was characterized in previous studies (69, 71). The strain was stored at −80°C. Planktonic cells were cultured in 2% YEPD medium (2% glucose, 1% Bacto yeast extract, and 2% Bacto peptone), and biofilms were cultured in a sixfold dilution of this medium.

Planktonic cultures. Cultures were grown aerobically in 250-ml Erlenmeyer flasks containing 100 ml growth medium. The flasks were placed in a shaker incubator at 37°C and 160 rpm for the desired period of growth. *C. albicans* grew as a budding yeast under these conditions. Exponential-phase cells were harvested after a 5-h growth period and stationary-phase cells at 120 h.

Biofilm cultures. Biofilms were grown in a tubular flow cell (TF). Silicone tubing (Cole-Parmer; catalog no. EW-95802-08) with an inner diameter of 4.78 mm, an outer diameter of 6.35 mm, a wall of 0.79 mm, and a length of 60 cm constituted the biofilm-growing region of the TF. The source of growth medium for the TF was a 2-liter Erlenmeyer flask. A bubble trap was placed between the Erlenmeyer flask and the TF to prevent passage of air bubbles during biofilm growth. Flow rates of 1.17 ml/min (shear rate, 109.5 s^{-1}) were maintained by a peristaltic pump coupled at the effluent end of the TF. The residence time for the volume of liquid contained in the tubular reactor portion of the flow system (20-cm length of tubing) was 3 min. This condition ensured that the contribution to the cell population in the TF from cells in the planktonic mode of growth (doubling time, approximately 80 min) was negligible. After sterilization by autoclaving, the entire setup was placed horizontally on a grilled shelf in an incubator at 37°C. The TF was filled with growth medium before being inoculated with cells. The inoculum was prepared from a 24-h planktonic culture at a concentration of 10^8 cells/ml in 0.1 M phosphate-buffered saline (PBS, pH 7) buffer. It was fed into the TF from the effluent end by reversing the direction of flow. Flow was then discontinued for 1 h. After the 1-h inoculation period, flow was resumed for 36 h. At the end of this culture period, the section of tubing in which the biofilm grew was clamped at both ends and removed by cutting the tubing with a sterile blade. The liquid column was drained into a petri dish by moving the tubing to a vertical position and releasing the clamps. The tubing was then rinsed with PBS buffer equivalent to one tube volume. The fraction of biofilm collected in the petri dish by this procedure is referred to as the shear-removed biofilm. The cells that remained adhering to the walls of the tube are referred to as the basal blastospores or the basal blastospore subpopulation.

Susceptibility testing. AmB was from Biosource International Inc. (Fungizone with 0.00205% sodium deoxycholate solubilizing agent). A standard broth dilution method was used to assess the AmB MIC of CA-1, with ATCC 24433 used as a reference strain (52). Susceptibility of biofilms and planktonic populations used for comparative analysis were assessed by the alamarBlue metabolic assay (BioSource International Inc.; catalog no. DAL1100) in a 96-well plate format (47, 57, 72). AmB treatment of planktonic cells and shear-removed biofilm was performed in 1.5-ml centrifuge tubes. The tubes contained a total working volume of 450 μl. AmB dissolved in 200 μl of 0.1 M PBS (pH 7.0) and cells resuspended in 250 μl of PBS adjusted to an optical density of 0.05 (A_{660}) were added to the 1.5-ml tube. After more than 10 preliminary experiments, the time between recovery of the shear-removed biofilm in the petri dish and exposure to AmB was reduced to approximately 5 min. The positive controls had no AmB, and the negative controls had no cells. For AmB treatment of the basal blastospore population, each sample consisted of a 0.5-cm long section of tubing cut from the TF. Each tubing sample was positioned in the 96-well plate (Corning Inc.; Costar 3370) such that its outer wall snugly fit along the wall of the well in the 96-well plate. This enabled recording of absorbance data for the alamarBlue dye in real time without perturbing the well contents. The 0.5-cm tubing samples were randomly placed in the 96-well plate in order to randomize the distribution between treated samples and untreated controls. Solution (225 μl) containing AmB in 0.1 M PBS was added to wells to completely submerge the 0.5-cm tubing during the AmB treatment phase. Untreated samples were the positive controls. Three independent TF experiments were run, generating a total of 24 samples per AmB concentration and 61 untreated controls for the basal blastospores. Sterile tubing was used as a negative control. In every experiment, a minimum of four negative controls were used. The AmB treatment period was 1 h across all cell populations. During AmB exposure, the 1.5-ml tubes (containing either the planktonic or shear-removed populations) and 96-well plates (containing the basal blastospores) were placed in a shaker incubator at 37°C and 150 rpm. After AmB treatment, cells in 1.5-ml tubes were centrifuged for 5 min at 4,000 × *g*, the supernatant was decanted, and the pellet was resuspended in the same volume of fresh PBS buffer. Cells were then transferred into wells of the 96-well plate system for the metabolic assay. For the 96-well plate containing sections of tubing, AmB was carefully aspirated out of the wells and immediately replaced with reagents of the metabolic assay.

The components of the alamarBlue metabolic assay were at the same concentrations for all populations tested. Each well had a total working volume of 230 μl. It contained 25 μl alamarBlue dye, 30 μl 2% YEPD growth medium, 100 μl of cells (or the 0.5-cm tubing section) in PBS, and 75 μl of a PBS solution containing AmB-quenching reagents and antibacterial reagent (penicillin-streptomycin; Invitrogen catalog no. 15140-122; final concentration, 1% [by volume]). The final concentrations of the reagents included in the assay to quench the action of AmB during the posttreatment metabolic assay period were 40 μM ergosterol (Alfa Aesar; catalog no. 57-87-4), 42.5 μM KCl, and 88 μM $MgCl_2$ (26, 27, 38). The metabolic assay in the 96-well format was run for 24 h at 37°C with continuous shaking in a Synergy-HT plate reader (Biotek Inc.). Absorbance data at 570 nm and 600 nm were collected at the 24-h endpoint using KC4 software (Biotek Inc.). The percent reduction of the alamarBlue dye, which is a function of metabolic activity of viable cells, was calculated as per the manufacturer's formula (product literature; BioSource International Inc.; catalog no. DAL1100). The results were expressed as the mean percent reduced for each triplicate culture (except as otherwise stated) ± the standard error.

Scanning electron microscopy. Biofilm samples from the TF were fixed in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), overnight, at 4°C. They were rinsed with 0.1 M sodium cacodylate buffer twice for 10 min. Samples were postfixed in 2% osmium tetroxide for 45 min on a rotator. Samples were subsequently dehydrated in a series of ethanol washes (twice for 5 min in 50%, twice for 5 min in 70%, and twice for 5 min in 95%, followed by two washes for 10 min in 100%) and then air dried in an open vial. Biofilms were visualized with a scanning electron microscope in high-vacuum mode at 25 kV after sputter coating with gold. The images were processed using Microsoft Photo Editor software.

Quantitative RT-PCR. (i) Total RNA isolation and cDNA preparation. Total RNA from planktonic and biofilm populations was isolated using the RiboPure yeast kit (Ambion, Inc.) according to the manufacturer's instructions. Basal blastospores were collected for RNA isolation by treating the tubes with trypsin-EDTA (Invitrogen; catalog no. T25300-120) for 4 min, scraping adherent cells (4 min), concentrating them by centrifugation (2 min), and washing the pellet with PBS (2 min). Total RNA (2.5 μg from biofilm and planktonic samples) was reverse transcribed with oligo(dT) primers using Superscript reverse transcriptase II (Invitrogen) according to the manufacturer's instructions.

(ii) Primer design. Sequences of *C. albicans* genes were downloaded from the Candida Genome Database (5) using Omiga (version 2.0) software. Primer3 software (65) was used to design primers for quantitative RT-PCR. The optimal conditions for choosing efficient primers included: biasing the primers toward the 3′ end of the gene, restricting the amplicon size to between 50 and 200 base pairs, maintaining the GC content of the primers at a range of 40 to 60%, and keeping the difference in melting temperature between the two primers below 1°C. Primers were tested to avoid 3′ self-complementarity and self-annealing using the Omiga software. A BLAST*n* search was performed with the designed primers to ensure that they were unique to the gene of interest within the *C. albicans* genome. The primer efficiencies for each gene were estimated from standard curves using serial dilutions of cDNA (250, 25, 2.5, and 0.25 ng equivalent of RNA). The PCR primers were considered efficient if the coefficient of determination of the standard curve was >0.98 and the target amplification efficiency ($10^{-\text{slope}} - 1$) was equal to 100% (acceptable error of 10%). Additional information about the genes of interest and their primer sequences are available in Table 1. All primer pairs listed in Table 1 had acceptable efficiencies in the cDNA concentration range tested.

(iii) Quantitative RT-PCR protocol. Each PCR mixture contained the following (final concentrations): 1× 20 mM Tris-HCl (pH 8.3) buffer, 50 mM KCl, 3 mM $MgCl_2$, 0.3 μM forward primer, 0.3 μM reverse primer, 1× additive reagent (0.2 mg/ml bovine serum albumin, 150 mM trehalose, and 0.2% Tween-20), 0.25× SYBR green, 1.5 U Platinum *Taq* polymerase, a 200 μM concentration of each deoxynucleoside triphosphate, 2 μl cDNA, and H_2O to bring the final volume to 25 μl. The cDNA was amplified according to the following steps: (i) 95°C for 2 min, (ii) 95°C for 12 s, (iii) 58 to 63°C for 15 s, (iv) 72°C for 20 s, and (v) 80°C for 6 s to detect SYBR green (nonspecific products melt at <80°C and therefore were not detected). Steps 2 to 5 were repeated for 40 cycles, and at the end of the last cycle the temperature was increased 0.2°C/s from 60 to 95°C to produce a melting curve. Smart Cycler software calculated corresponding cycle threshold (C_T) values for a threshold fluorescence of 30. All samples had RNA

TABLE 1. *C. albicans* genes chosen for transcript analysis using quantitative RT-PCR[a]

Gene	Accession no.	Protein product and/or function	Primer sequences[b]
GAP	U72203	Glyceraldehyde-3-phosphate dehydrogenase; enzyme of glycolysis	TGACCACTGTCCACTCCATC AGCAGTTCTACCACCTCTCCA
ACT1	X16377	Actin	GCTGAACGTATGCAAAAGGAA TGTGGTGAACAATGGATGGA
EFB1	X96517	Translation elongation factor EF-1 beta	GAAGGCTGCTAAAGGTCCAAA ACCCCAAGTCAAACCTTCCA
ERG1	U69674	Squalene epoxidase, catalyzes epoxidation of squalene to 2,3(S)-oxidosqualene in the ergosterol biosynthetic pathway	TCGTTGTTCTCCCTTTCCTC CCTTGCTCAACTCCCATTCT
ERG3	AF069752	C-5 sterol desaturase; introduces C-5(6) double bond into episterol in ergosterol biosynthesis	GCTTGTCACACTGTCCATCAC AATCTTCTTTTCTTCTTCTGCCTTT
ERG5	CA4418	Putative C-22 sterol desaturase; catalyzes formation of the C-22(23) double bond in the sterol side chain in ergosterol biosynthesis	TCTTTGAGATACCGTCCACCA GTCGTGCAAAGCAGGATACA
ERG6	AF031941	Delta(24)-sterol C-methyltransferase; converts zymosterol to fecosterol in ergosterol biosynthesis by methylating position C-24	GGTGGTCCTGGTAGAGAAATCACA AGCTTCAATGGCATAAACAGCATC
ERG9	D89610	Putative farnesyl-diphosphate farnesyl transferase (squalene synthase) involved in the sterol biosynthesis pathway	AGTAAAAACCCCTGCCAAAGA GCACAAAATGAGAATGAAGAAGG
ERG11	X13296	Lanosterol 14-alpha-demethylase, member of the cytochrome P450 family that functions in ergosterol biosynthesis	GGATACTGCTGCTGCCAAA GCAAATTGTTCCCCAATACATC
ERG25	AF051914	Putative C-4 methyl sterol oxidase with role in C_4 demethylation of ergosterol biosynthesis intermediates (based on similarity to *S. cerevisiae*)	GCAGCAGAATATGCTCATCCA ATCGGAATACCAACCGTACCC
SKN1	D88491	Protein with predicted role in β-1,6-glucan synthesis; probable N-glycosylated type II membrane protein	TCGATCAGGGATGATTCAAAG ACCATAACCCAGGCCAAAA
KRE1	M81588	Predicted GPI[c] anchor, role in β-1,6-glucan biosynthesis; production in *S. cerevisiae* increases glucan content and complements killer toxin sensitivity of *KRE1* mutant	TTAGCCACCACCCAATCAA ACCCCAATCCAATAGAACCA
KRE5	XM_714894	Predicted UDP-glucose:glycoprotein glucosyltransferase; required for wild-type β-1,6-glucan biosynthesis	TGTCTGCATCGGAAAACAAA AAACTGGCACCTCAATGCTC
KRE6	D88490	Protein of β-1,6-glucan biosynthesis	GTGATGAATTTGATGCTGAAGG GAATGTCAGGAGCGGTGAA
KRE9	AF069763	Protein of β-1,6-glucan biosynthesis	AAAGGCTGGACAACCAAA TGGATATGGAGCAACACTAGCA

[a] Gene descriptions and sequences were downloaded from the Candida Genome Database (5). The primers were designed with Primer3 software (72).
[b] Forward and reverse primers are shown.
[c] GPI, glycosylphosphatidylinositol.

obtained from three independent experiments. To avoid variation between runs, samples of all populations were run simultaneously for each gene. Relative quantification of gene expression was performed using the comparative C_T method (sequence detection systems chemistry guide; Applied Biosystems). The most stable housekeeping genes, *EFB1* and *ACT1* (11), across the sample states were chosen using the statistical technique described by Vandesompele et al. (73). The internal normalization factor was the geometric mean of the stable housekeeping genes (73). The propagated standard error of a set of triplicate samples was estimated using the differential equation of Gauss as described by Muller et al. (51). The change in gene expression was further normalized relative to a calibrator state chosen from the pool of populations being tested. In our study, the exponential-phase planktonic population was selected as the calibrator state.

RESULTS

Biofilm subpopulations. The effluent that was drained from the TF at the end of the 36-h culture period contained masses of material that had the slimy appearance characteristic of many biofilms. Although *C. albicans* biofilms vary in architecture depending on growth conditions (7, 8), many studies have reported an architecture consisting of a pleomorphic mixture of filamentous and yeast forms in which the filaments are intertwined and interspersed with clusters of blastospores (3, 15, 63). In a previous study, we found that the architecture of CA-1 biofilms conformed to this description (69). In these respects, the material displaced from the TF as the tubing was drained resembled a typical *C. albicans* biofilm (Fig. 1).

By holding the tubing vertically and allowing it to drain, we essentially implemented a method previously used to remove biofilms cultured in vitro from biomaterials (32). It was shown that shear forces exerted at the three-phase (solid/liquid/air) interface imparted by passage of an air bubble through a biofilm flow cell are considerable (approximately 2×10^{-7} N) and can peel away biofilms grown under continuous medium flow (32). During growth, our *C. albicans* biofilms were subjected to a constant shear of 109.5 s^{-1}. During this period there was continual detachment of cells (mostly yeast forms) (Fig. 2). However, we did not observe detachment of large aggregates, as has been reported for bacterial biofilms (76).

After the bulk of the biofilm had been removed by draining the tubing, there were still cells left on the surface (Fig. 3). This population consisted entirely of blastospores, arranged in small, fairly dense monolayer domains. Thus, by using this relatively simple method, we separated the biofilm into two subpopulations: a complex mixture of different morphological types that comprised the bulk of the biofilm and a submonolayer of basal blastospores that were more tenaciously attached to the surface of the tubing.

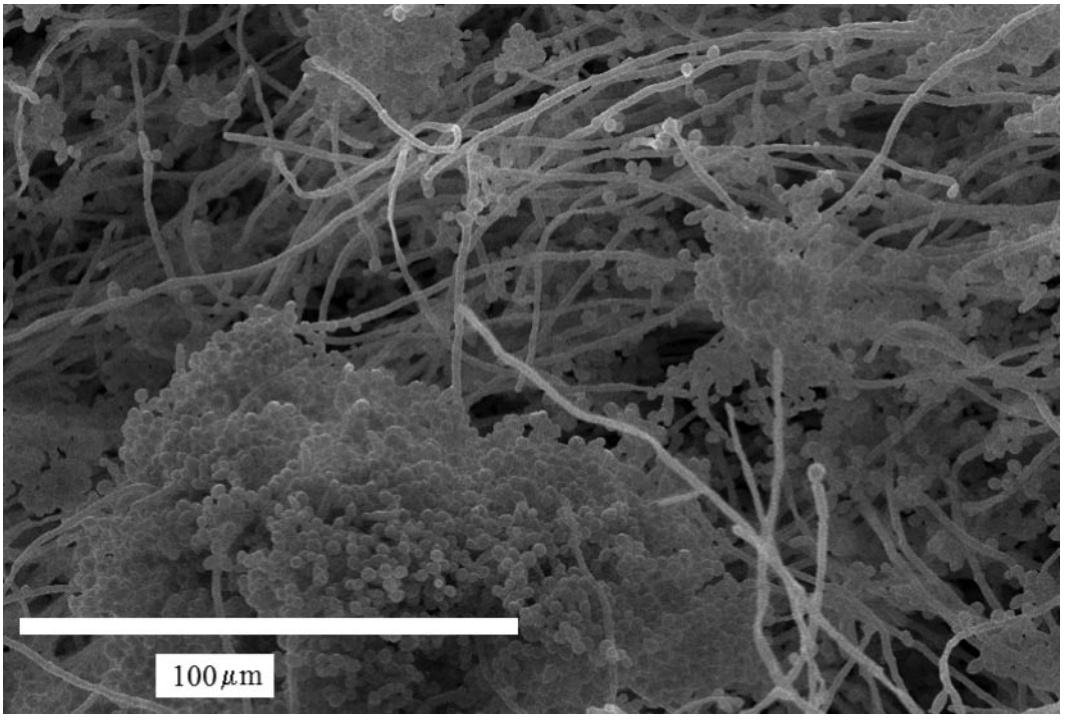

FIG. 1. Scanning electron microscopy image of the shear-removed *C. albicans* biofilm displaced by passage of the three-phase boundary layer.

Biofilm and planktonic resistance to AmB. The dose-response curve for the biofilm subpopulations and planktonic populations used for comparative analysis is shown in Fig. 4. At AmB concentrations greater than or equal to 3.7 μg/ml, the basal blastospores were clearly more active than the other three populations. At these AmB concentrations, the planktonic populations showed no statistically significant difference between AmB-treated and negative (no-cell) controls ($P < 0.0001$, two-tailed

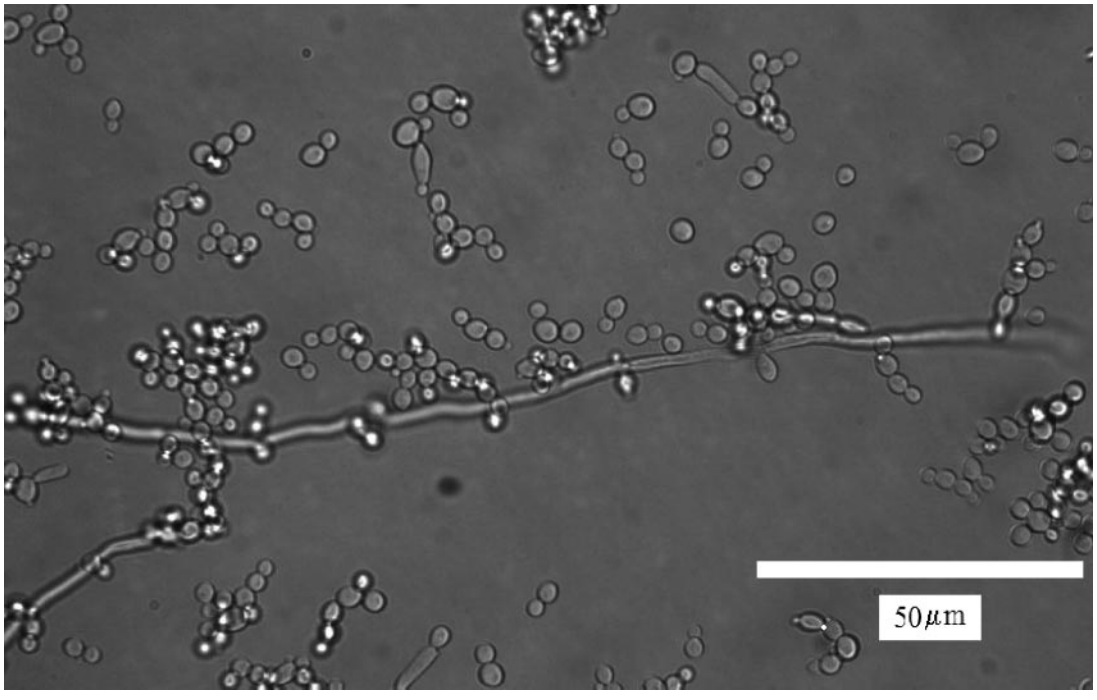

FIG. 2. Transmittance image of *C. albicans* cells that were present in the effluent of the TF during biofilm growth. These cells resemble typical planktonic-phase cells, with an occasional hypha.

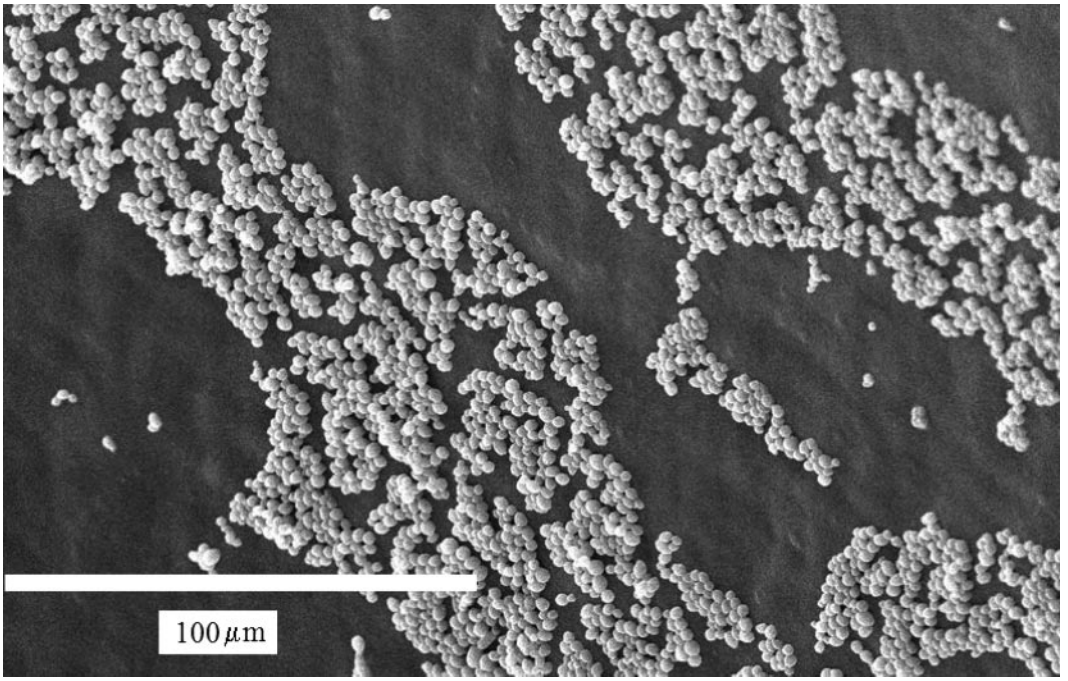

FIG. 3. Scanning electron microscopy image of the *C. albicans* basal blastospore subpopulation that remained on the silicone tubing surface of the TF after passage of the three-phase boundary layer.

Student's *t* test), and the shear-removed biofilm exhibited significant inhibition of metabolic activity (>90%).

As assessed by the broth dilution method, CA-1 exhibited an AmB MIC (0.5 μg/ml) within a standard range for susceptible *C. albicans* strains. Based on previous studies (26, 28), it was anticipated that stationary-phase planktonic cells would exhibit greater AmB resistance than exponential-phase planktonic cells when evaluated by a metabolic assay sensitive to membrane integrity. This difference was evident only at 1.77 μg/ml AmB. Data not shown here indicated that when replication efficiency on solid medium was used to assess AmB action, the greater tolerance of stationary-phase batch cultures than ex-

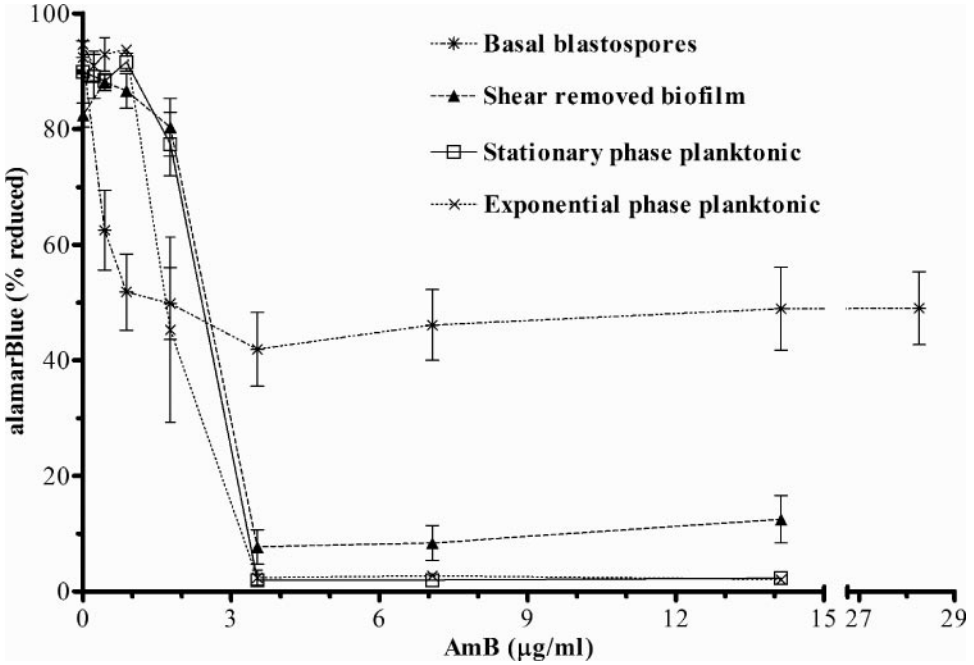

FIG. 4. AmB dose-response curves for *C. albicans* biofilm and planktonic populations assessed by an alamarBlue metabolic assay. The data are means ± standard errors for all populations considered in the study and are from three independent experiments.

TABLE 2. Relative transcript abundance for selected genes of the ergosterol pathway in *C. albicans*

Gene[a]	Change (fold) in gene expression[b]			
	Planktonic forms		Shear-removed biofilm	Basal blastospore subpopulation
	Exponential phase	Stationary phase		
ACT1	1.0 ± 0.05	1.61 ± 0.15	0.85 ± 0.31	0.9 ± 0.04
EFB1	1.0 ± 0.10	0.62 ± 0.05	1.18 ± 0.41	1.11 ± 0.11
ERG1	1.0 ± 0.72	35.55 ± 24.03	−2.06 ± 1.67	−11.93 ± 4.69
ERG3	1.0 ± 0.06	3.67 ± 0.6	5.31 ± 1.74	0.91 ± 0.22
ERG5	1.0 ± 0.06	2.1 ± 0.55	2.11 ± 0.96	1.48 ± 0.27
ERG6	1.0 ± 0.12	0.72 ± 0.23	3.95 ± 1.95	1.32 ± 0.55
ERG9	1.0 ± 0.15	1.05 ± 0.21	1.89 ± 0.45	−2.2 ± 0.78
ERG11	1.0 ± 0.06	0.98 ± 0.29	2.0 ± 0.96	0.88 ± 0.27
ERG25	1.0 ± 0.18	7.1 ± 1.13	13.28 ± 3.83	3.7 ± 0.73

[a] See Table 1 for descriptions of genes. *ACT1* and *EFB1* were internal normalization controls.
[b] Values were estimated relative to those for the exponential-phase planktonic population (calibrator state) and are given as means ± propagated standard errors.

TABLE 3. Relative transcript abundance for selected genes of the β-1,6-glucan pathway in *C. albicans*

Gene[a]	Change (fold) in gene expression[b]			
	Planktonic forms		Shear-removed biofilm	Basal blastospore subpopulation
	Exponential phase	Stationary phase		
SKN1	1.0 ± 0.15	9.09 ± 4.23	2.49 ± 1.63	30.70 ± 4.48
KRE1	1.0 ± 0.34	12.92 ± 2.06	17.01 ± 6.03	29.86 ± 7.35
KRE5	1.0 ± 0.2	7.35 ± 2.58	1.11 ± 0.42	−2.22 ± 0.84
KRE6	1.0 ± 0.1	7.83 ± 2.12	4.88 ± 2.24	1.9 ± 1.28
KRE9	1.0 ± 0.07	2.92 ± 0.86	2.08 ± 0.7	2.62 ± 0.30

[a] See Table 1 for descriptions of genes. *ACT1* and *EFB1* (Table 2) were internal normalization controls.
[b] Values were estimated relative to those for the exponential-phase planktonic population (calibrator state) and are given as means ± propagated standard errors.

ponential-phase batch cultures was more apparent for a range of AmB concentrations (0.88 to 3.53 μg/ml).

The basal blastospore subpopulation exhibited metabolic activity up to a tested concentration of 28.26 μg/ml AmB. Even at this highest AmB concentration, microscopic observation revealed the appearance of budding planktonic yeast forms during the metabolic assay, suggesting that the basal blastospores produced viable cells. At lower concentrations (<3.53 μg/ml), the basal blastospore subpopulation showed a greater loss of metabolic activity than the other samples. This result would be expected if a portion of the cells in the basal blastospore subpopulation were more susceptible to AmB than the other populations and suggests that this subpopulation is itself not completely homogeneous with respect to its susceptibility to AmB.

Biofilm and planktonic gene expression. Transcript analysis using quantitative RT-PCR indicated differential gene expression of several genes in the ergosterol (Table 2) and β-1,6-glucan (Table 3) pathways. Data for biofilm and planktonic populations were obtained from three independent culture experiments. *GAP*, *ACT1*, and *EFB1* were chosen as potential housekeeping candidates to estimate an internal normalization factor. Based on the statistical algorithm proposed by Vandesompele et al. (73), only *ACT1* and *EFB1* proved to be stable across the four cell populations tested in this study. Their geometric mean was used to estimate the internal normalization control. Gene expression values were calculated relative to the calibrator state, which in our study was the exponential-phase planktonic state.

The basal blastospore subpopulation had a unique transcript profile for both the pathways compared to the other cell states. *ERG1* (−11.93 ± 4.68) and *ERG25* (+3.70 ± 0.73) were highly differentially regulated in this resistant fraction. For the β-1,6-glucan pathway, the resistant basal blastospores showed a high degree of differential up regulation of the *SKN1* (+30.7 ± 4.48) and *KRE1* (+29.86 ± 7.39) genes. The *KRE1* gene exhibited an interesting trend of increasing transcript abundance for populations exhibiting increased resistance at higher concentrations of AmB.

DISCUSSION

We have demonstrated that *C. albicans* biofilms cultured in our in vitro system harbored a subpopulation of blastospores that were substantially more resistant to AmB than both planktonic cells and the bulk of the biofilm that had been displaced from the substratum. It is not surprising to find a subpopulation of microbes that exhibit superior tolerance to an antimicrobial challenge in a biofilm. In contrast to suspended cultures, biofilms are not well-mixed systems; thus, any substance that is consumed by the organisms at a substantial rate (such as the carbon source or electron acceptor) will be relatively depleted in certain regions of any given biofilm to some degree, with a consequent effect on metabolic activity. This phenomenon has been well documented for bacterial biofilms (24, 37, 77). The level of metabolic activity is known to influence susceptibility of microbes to antimicrobials (30) as well as other stresses (31), and in situ characterization of bacterial biofilms has confirmed that regions with reduced access to nutrients correspond to regions exhibiting tolerance to an antimicrobial (2).

Our results have broad implications if similar subpopulations of highly resistant blastospores are present in other types of *C. albicans* biofilms, or in infective *C. albicans* communities that have salient features in common with *C. albicans* biofilms. There is sufficient suggestive evidence that our biofilm has some universally relevant characteristics to justify entertaining this hypothesis. The biofilms that formed on the TF had an architecture that resembled that reported by others, including, in particular, the presence of a basal layer of blastospores (9, 15, 42). We showed previously that, in early-stage biofilms, attached blastospores were less susceptible to membrane disruption by the antiseptic chlorhexidine than filamentous forms (70). The basal layer of blastospores was located furthest away from the medium source and thus may have experienced nutrient limitations similar to that of stationary-phase cultures. Similar nutrient-deprived microenvironments are likely to exist in many *C. albicans* biofilms, as well as in infective *C. albicans* communities, and it has been demonstrated that resistance to membrane disruption by AmB increases dramatically when yeast forms transition into stationary phase (26, 28).

The transcript analysis provides a starting point for investigating the molecular basis of the resistance exhibited by the

basal layer of blastospores. The data indicated possible association of genes involved with both the ergosterol and β-1,6-glucan synthetic pathways (Table 1). The results indicated that the basal blastospore subpopulation was a distinct population at this level of molecular characterization.

Transcript abundance of the *ERG1* gene was significantly reduced in the resistant blastospore subpopulation (Table 2). The ergosterol synthetic pathway has been extensively characterized in *Saccharomyces cerevisiae* and, with the exception of one gene, the genes corresponding to an analogous pathway in *C. albicans* are probably all orthologs (1). Reduction of the ergosterol content of the plasma membrane is the most common mechanism for *C. albicans* to acquire resistance to AmB (29). The *ERG1* gene product catalyzes epoxidation of squalene to 2,3-oxidosqualene, a step that occurs early in the ergosterol synthetic pathway, just after commitment to sterol synthesis, and thus could serve as a point of down-regulation of ergosterol synthesis (21). In a *C. albicans* strain in which both copies of *ERG1* were disrupted, ergosterol was absent from the membrane and was replaced primarily by squalene (56). It is possible that the basal blastospore subpopulation up-regulated the *ERG25* gene in order to shuttle nonergosterol intermediates into the membrane (10).

Transcripts encoded by two genes involved in β-1,6-glucan synthesis (*SKN1* and *KRE1*) were clearly more abundant in the basal blastospore subpopulation than in either the shear-removed biofilm or planktonic populations (Table 3). Along with mannoproteins and chitin, β-1,3-glucans and β-1,6-glucans form the polymeric network that comprises the *C. albicans* cell wall (40, 66). In *C. albicans*, β-1,6-glucans constitute 20% (dry weight) of the cell wall (40). In general, the cell wall plays a protective role. Restructuring of the glucan portion of the cell wall has been implicated in conferring transient AmB resistance to *C. albicans* (26). Although the *SKN1* gene has been implicated in β-1,6-glucan synthesis in *S. cerevisiae*, it has not been shown to be essential for β-1,6-glucan synthesis in *C. albicans* (49). The role of the *KRE1* gene in β-1,6-glucan synthesis was unambiguously demonstrated in *S. cerevisiae* (13), and closely corresponding structural similarities, including the presence of an amino-terminal signal sequence (12), indicate that the corresponding gene in *C. albicans* is an ortholog. A more recent study suggests that the *KRE1* gene product plays a role in both cell wall assembly and architecture in *S. cerevisiae* (14). Thus, the up-regulation of *KRE1* in the basal blastospores suggests either an increase in or restructuring of the β-1,6-glucan portion of the cell wall. In addition, the trend of increasing *KRE1* transcript abundance for populations exhibiting increasing levels of AmB resistance (Table 3; Fig. 4) supports the hypothesis that differential regulation of the *KRE1* gene may confer resistance.

The transcript analysis of both β-1,6-glucan and ergosterol pathways is consistent with a regulatory mechanism that reduced the susceptibility of the subpopulation of basal blastospores to AmB by a synergistic effect of increasing and/or restructuring the glucan portion of the cell wall and reducing the ergosterol content of the plasma membrane. If this is the case, then in the context of *C. albicans* biology, the basal blastospore subpopulation was exploiting fairly generic molecular mechanisms to minimize their susceptibility to AmB which were transiently activated by the biofilm environment. There is a precedent for involvement of the glucan portion of the cell wall in conferring the biofilm resistance phenotype, since echinocandins, which interfere with β-1,3-glucan synthesis, are singularly effective against *C. albicans* biofilms (6, 43). Although strains with mutation-based alterations resulting in a resistant phenotype associated with reduced ergosterol content can be induced to form in the laboratory (10), they are rarely isolated from clinical samples (1, 29, 67, 75). However, this does not preclude the in vivo existence of a similar transient, reversible phenotype in some infective *C. albicans* populations.

A number of reviews outline the various theories proposed to explain resistance of biofilms (18, 22, 25, 68). Hindered transport of an antimicrobial by interaction with biofilm components (cells and the exopolysaccharide matrix) was one of the first theories posed (4, 53, 54). Since the basal layer of blastospores was exposed to AmB after the removal of the bulk of the biofilm, lack of delivery of AmB to the cells is an unlikely explanation for their enhanced resistance. Although our flow cell design precluded any direct measurements of the microenvironment occupied by the basal layer of blastospores that might have lead to increased resistance, the high oxygen permeability of the silicone tubing should have maintained aerobic conditions at base of the biofilm. It seems likely that the basal blastospores experienced a reduced level of medium nutrients. However, the superior resistance of the basal blastospore subpopulation compared to the stationary-phase cultures, as well as the difference in transcript abundance for genes involved in the ergosterol pathway (*ERG1*, *ERG3*, and *ERG25*) and β-1,6-glucan pathway (*SKN1*, *KRE5*, and *KRE6*) between these two populations (Table 2 and Table 3), suggests that there were additional factors, besides nutrient deprivation, that led to the enhanced resistance of this biofilm subpopulation.

One possibility is that these highly resistant blastospores regulated their genes in direct response to their relatively intimate association with the surface. A similar mechanism was proposed to explain AmB resistance of *C. albicans* biofilms grown on PVC catheters (8). Bacterial biofilms have been shown to alter genetic programs in response to surface association (23, 39, 50, 59), and even early-stage (monolayer) biofilms can become more resistant than their planktonic counterparts (17, 20). It is not unreasonable to expect that the resistant blastospores used some of the same mechanisms previously identified in *C. albicans* for sensing surface contact (74).

In summary, we have discovered that *C. albicans* biofilms cultured under our experimental conditions harbored a subpopulation of basal blastospores that were exceptionally resistant to AmB. We have presented arguments that subpopulations with a similar phenotype may exist in *C. albicans* biofilms cultured under different conditions, and perhaps even in infective *C. albicans* communities that share salient features with *C. albicans* biofilms. The molecular mechanisms underlying the resistance of the basal blastospore subpopulation were probed by performing a comparative analysis of transcript abundance for key genes in the ergosterol and β-1,6-glucan pathways. These results show that at this level of genetic regulation the basal blastospore subpopulation was distinct both from the bulk of the biofilm and from exponential- and stationary-phase planktonic populations and suggest possible involvement of these pathways in conferring the resistant phenotype.

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