

## *Candida albicans* Biofilms Produce Antifungal-Tolerant Persister Cells<sup>∇</sup>

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**Fungal pathogens form biofilms that are highly recalcitrant to antimicrobial therapy. The expression of multidrug resistance pumps in young biofilms has been linked to increased resistance to azoles, but this mechanism does not seem to underlie the resistance of mature biofilms that is a model of in vivo infection. The mechanism of drug resistance of mature biofilms remains largely unknown. We report that biofilms formed by the major human pathogen *Candida albicans* exhibited a strikingly biphasic killing pattern in response to two microbicidal agents, amphotericin B, a polyene antifungal, and chlorhexidine, an antiseptic, indicating that a subpopulation of highly tolerant cells, termed persisters, existed. The extent of killing with a combination of amphotericin B and chlorhexidine was similar to that observed with individually added antimicrobials. Thus, surviving persisters form a multidrug-tolerant subpopulation. Interestingly, surviving *C. albicans* persisters were detected only in biofilms and not in exponentially growing or stationary-phase planktonic populations. Reinoculation of cells that survived killing of the biofilm by amphotericin B produced a new biofilm with a new subpopulation of persisters. This suggests that *C. albicans* persisters are not mutants but phenotypic variants of the wild type. Using a stain for dead cells, rare dark cells were visible in a biofilm after amphotericin B treatment, and a bright and a dim population were physically sorted from this biofilm. Only the dim cells produced colonies, showing that this method allows the isolation of yeast persisters. Given that persisters formed only in biofilms, mutants defective in biofilm formation were examined for tolerance of amphotericin B. All of the known mutants affected in biofilm formation were able to produce normal levels of persisters. This finding indicates that attachment rather than formation of a complex biofilm architecture initiates persister formation. Bacteria produce multidrug-tolerant persister cells in both planktonic and biofilm populations, and it appears that yeasts and bacteria have evolved analogous strategies that assign the function of survival to a small part of the population. In bacteria, persisters are dormant cells. It remains to be seen whether attachment initiates dormancy that leads to the formation of fungal persisters. This study suggests that persisters may be largely responsible for the multidrug tolerance of fungal biofilms.**

*Candida albicans* is an opportunistic pathogen that is a common member of the human oral and gastrointestinal microflora. Biofilms of *C. albicans* often form on indwelling devices, such as blood and urinary catheters and heart valves. These infections are essentially untreatable by antifungals and can develop into life-threatening disease with a mortality rate approaching 40%. The recalcitrance of these infections to antifungals is puzzling, since planktonic populations of the same strain can be susceptible to a range of antifungals, including azole compounds, echinocandins, and amphotericin B. A number of excellent reviews have recently been published on this subject (2, 10, 14, 16, 20, 24, 25, 27, 29, 33, 35, 40, 44).

The biofilm forms when single cells attach to a surface and grow into microcolonies, which then merge and produce a complex three-dimensional (3D) structure that is held together by hyphae and an exopolymer matrix (7). The biofilm contains a mixture of yeast, hyphae, and pseudohyphae. Several factors may account for the high resistance of biofilm cells to antimicrobial drugs. For example, the exopolymer matrix restricts

penetration by immune system components (15, 51). Since in vivo, antimicrobials act in concert with the immune system to eradicate infections, the biofilm exopolymer is evidently an important component of recalcitrance. However, the exopolymer does not appreciably hinder the penetration of antifungal drugs (3, 46). Quiescence can be a factor in drug resistance, but the majority of cells in *C. albicans* biofilms are metabolically active, based on studies with XTT and FUN-1 metabolic indicators (21–23). Genes encoding multidrug resistance (MDR) transporters, *MDR1*, *CDR1* and *CDR2*, are upregulated upon attachment of *C. albicans* cells to a surface, and this accounts for the resistance of young biofilms to azole antibiotics (34). However, the high level of drug resistance of mature biofilms ( $\geq 48$  h) was not affected by deletion of all three of these genes, either singly or in combination, including an *mdr1Δ cdr1Δ cdr2Δ* triple mutant (26, 34, 39). Decreased ergosterol content (26, 34) and a diminished level of ergosterol biosynthetic gene expression (11) have been reported in mature *Candida* biofilms and may contribute to drug resistance. Indeed, azoles act by inhibiting ergosterol biosynthesis, and amphotericin B binds to ergosterol. However, ergosterol is unlikely to be involved in the actions of echinocandins that inhibit the synthesis of cell wall  $\beta$ -glucan (8) or of chlorhexidine, a membrane-active antiseptic that is very effective against bacteria that lack sterols. In essence, the mechanism of *C. albicans* biofilm antifungal resistance remains largely unknown.

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TABLE 1. Strains used in this study and tested for persisters

Strain	Genotype	Biofilm architecture	Persisters <sup>a</sup>	Source/reference
3153A	Wild-type laboratory strain	Robust 3D wild type	+++	41
CKY357	CAI-4 <i>mkc1Δ::hisG/mkc1Δ::hisG mkc1::pCK70 (URA3)</i>	Reduced filamentation	++	26
CAI4	SC5314 <i>Δura3::λimm434/Δura3::λimm434</i>	Robust 3D wild type	+	26
CKY136	CAI-4 <i>efg1::hisG/efg1::hisG ade2::pDBI52 (URA3)</i>	Filamentation defect; sparse monolayer of cells	+++	12
CKY138	CAI-4 <i>efg1::hisG/efg1::hisG cph1Δ::hisG/cph1Δ::hisG ade2::pDBI52 (URA3)</i>	Filamentation defect; sparse monolayer of cells	++	12
MC191	<i>ura3Δ::λimm434/ura3Δ::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG flo8::ARG4/flo8::HIS1 ade2::URA3/ADE2</i>	Functionally defective hyphae	+++	Microbia
MC195	<i>ura3Δ::λimm434/ura3Δ::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG flo8::ARG4/flo8::HIS1 ade2::URA3::FLO8-2/ADE2</i>	Robust 3D wild type	+	Microbia
MC245	<i>ura3Δ::λimm434/ura3Δ::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG flo8::ARG4/FLO8 ade2::URA3/ADE2 HIS::his/his</i>	Robust 3D wild type	++	Microbia
DAY185	<i>Δura3::λimm434/Δura3::λimm434 arg4::hisG/arg4::hisG/pARG4-URA3 his1::hisG/his1::hisG/pHIS1</i>	Robust 3D wild type	++	45
DAY286	<i>Δura3::λimm434/Δura3::λimm434 arg4::hisG/arg4::hisG/pARG4-URA3 his1::hisG/his1::hisG</i>	Robust 3D wild type	++	45
GKO443	<i>Δura3::λimm434/Δura3::λimm434arg4::hisG/arg4::hisG his1::hisG/his1::hisG suv3::Tn7-UAU1/suv3::Tn7-URA3</i>	Biofilm defect; decreased biomass	++	45
GKO798	<i>Δura3::λimm434/Δura3::λimm434arg4::hisG/arg4::hisG his1::hisG/his1::hisG kem1::Tn7-UAU1/kem1::Tn7-URA3</i>	Biofilm defect; decreased biomass	++	37
GKO814	<i>Δura3::λimm434/Δura3::λimm434arg4::hisG/arg4::hisG his1::hisG/his1::hisG nup85::Tn7-UAU1/nup85::Tn7-URA3</i>	Biofilm defect; decreased biomass	++	45
GKO9	<i>Δura3::λimm434/Δura3::λimm434arg4::hisG/arg4::hisG his1::hisG/his1::hisG mds3::Tn7-UAU1/mds3::Tn7-URA3</i>	Biofilm defect; decreased biomass	++	45
CJN702	<i>Δura3::λimm434/Δura3::λimm434arg4::hisG/arg4::hisG his1::hisG::pHIS1/his1::hisG bcr1::ARG4/bcr1::URA3</i>	Functionally defective hyphae	++	37
CJN698	<i>Δura3::λimm434/Δura3::λimm434arg4::hisG/arg4::hisG his1::hisG::pHIS1-BCR1/his1::hisG bcr1::ARG4/bcr1::URA3</i>	Robust 3D wild type	++	37

<sup>a</sup> +++, 1 to 2% survival; ++, 0.1 to 1% survival; +, 0.05 to 0.1% survival.

It is noteworthy that many features of fungal biofilms are shared by the better-studied bacterial biofilms. In both cases, biofilms are often formed by merging of initial founder microcolonies, are protected from the immune system by exopolymers, are composed mainly of slowly growing cells, and exhibit multidrug tolerance (27, 28). Bacterial biofilms produce dormant persister cells that are largely responsible for multidrug tolerance (6, 19, 48). Persisters are able to survive despite the presence of antibiotics at concentrations well above the MIC and are phenotypic variants of the wild type, rather than mutants (19). Persisters are formed by all bacterial species studied and are present at 0.1 to 1% in the biofilms of *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus*, for example (19).

Given their similarity to bacterial biofilms, we reasoned that fungal biofilms may similarly produce persister cells. In this study, we report that *C. albicans* biofilms contain highly drug-tolerant persisters that may largely account for biofilm multidrug tolerance.

## MATERIALS AND METHODS

**Yeast strains and growth conditions.** The yeast strains used in this study are described in Table 1. *C. albicans* was streaked onto yeast extract, peptone, dextrose (YPD) agar from frozen stocks (−80°C) and incubated at 37°C for 24 to 48 h. When necessary (CAI4 derivatives), the growth medium was supplemented with 80 μg/ml uridine. Biofilms were grown essentially as described by Ramage et al. (41). Briefly, cells were grown overnight at 30°C in YPD liquid medium. Samples were harvested by centrifugation, washed twice in sterile phosphate-buffered saline (PBS), pH 7.4, and resuspended in RPMI 1640 with L-glutamine and 0.165 M MOPS (morpholinepropanesulfonic acid) (BioWhittaker). The optical density of the culture (at 600 nm) was adjusted to 0.1, which is equivalent to approximately  $1 \times 10^6$  cells/ml, by dilution with RPMI 1640. One hundred microliters of this suspension was aliquoted into 300-μl wells of a flat-bottom microtiter plate (Corning Costar 3370). The plates were incubated for 48 h at 37°C on a microtiter plate shaker (Lab-Line Instruments; model 4625) at approximately 100 rpm. Exponential- and stationary-phase cultures inoculated from single colonies were grown in RPMI 1640 with L-glutamine and 0.165 M MOPS at 37°C for 12 and 72 h, respectively.

**Antibiotic susceptibility testing.** Amphotericin B and chlorhexidine were obtained from Fisher Scientific, and stock solutions were prepared in dimethyl sulfoxide. Caspofungin was obtained from Merck. Antifungals were dissolved in growth medium. Biofilms were washed twice with sterile PBS in order to remove nonadherent cells prior to antifungal challenge. Antifungals were then added at

100  $\mu$ l per well. Biofilms were disrupted by scraping them and were vortexed vigorously for 30 seconds before serial dilution and plating. Microscopic observation showed that the resulting suspension consisted of clumps and individual cells in approximately equal numbers, with the size of clumps being  $\leq 10$  cells. The plates were incubated for 24 to 48 h prior to counting of the colonies.

One hundred-microliter samples of exponentially growing (12-h) or stationary-phase (72-h) planktonic cultures were harvested by centrifugation, washed twice with PBS, and challenged in microtiter plates with antifungals in 100  $\mu$ l of RPMI 1640 with L-glutamine and 0.165 M MOPS growth medium. The cultures containing antifungals were incubated at 37°C for 24 h, washed twice with PBS by centrifugation and aspiration of the supernatant, and resuspended in 100  $\mu$ l PBS. The cultures were then 10-fold serially diluted in PBS and plated for CFU determination.

For sequential challenge, biofilms were treated with 100  $\mu$ g/ml amphotericin B or chlorhexidine as described above. After 24 h, the medium was aspirated, 100  $\mu$ l RPMI 1640 with L-glutamine and 0.165 M MOPS containing 100  $\mu$ g/ml of amphotericin B and chlorhexidine was added to each well, and the plates were incubated for an additional 24 h.

Progeny biofilms were formed by inoculating samples from antibiotic-challenged, resuspended biofilms into fresh YPD medium and repeating the biofilm growth procedure described above.

**Live-dead-cell determination using fluorescein diacetate staining and epifluorescence microscopy.** Biofilms of strain 3153A were grown and resuspended in RPMI 1640 with L-glutamine and 0.165 M MOPS with 100  $\mu$ g/ml fluorescein diacetate alone (control) or with amphotericin B (100  $\mu$ g/ml) and fluorescein diacetate (100  $\mu$ g/ml) for 24 h. One-microliter samples of biofilms, scraped from the microtiter well at various time points, were placed onto a glass slide under a glass coverslip and viewed with a 100 $\times$  oil immersion lens using a Zeiss Axioskop 2 plus microscope with a standard fluorescein isothiocyanate filter. Photographs were taken using an AxioCam (Carl Zeiss) black-and-white charge-coupled device camera with an accompanying 0.63 $\times$  lens using Axiovision version 4.5. Live and dead control populations of budding yeast cells were obtained from an overnight 3153A culture grown in YPD medium at 30°C. Samples were pelleted and resuspended in YPD medium with 100  $\mu$ g/ml fluorescein diacetate alone or 100  $\mu$ g/ml amphotericin B and fluorescein diacetate for 24 h. Planktonic samples were viewed and photographed in a manner similar to the biofilm cells described above.

**Isolation of persisters.** Biofilms from strain MC 191 were grown and resuspended in RPMI 1640 with L-glutamine and 0.165 M MOPS with fluorescein diacetate (Fisher Scientific; 100  $\mu$ g/ml) with or without amphotericin B (100  $\mu$ g/ml). The biofilms were disrupted by scraping and vortexing them and were washed five times with PBS. Samples were analyzed using a MoFlo (Dako) cell sorter. A homogeneous population of cells was isolated using a gate based on forward and side scatter. Cells that passed the forward and side scatter gate were counted as single events and subsequently sorted into two distinct populations based on high and low green fluorescence. Single events from these populations were then physically sorted directly onto separate YPD agar plates in the form of a 96-well grid and were incubated for 48 h. Photographs of the plates were taken using an Olympus Camedia C-4040 digital camera.

## RESULTS

**Detection of persisters in *C. albicans* biofilms.** Dose-dependent killing provides a straightforward approach to detect persisters. A biphasic killing curve revealing a subpopulation of cells that survive low doses of antimicrobials indicates the presence of persisters. Several compounds, including amphotericin B, chlorhexidine, and caspofungin, have been reported to kill *Candida* biofilms (23, 42, 49).

Mature, 48-h biofilms formed in wells of microtiter plates were rinsed with fresh growth medium and challenged with antifungals for 24 h. In parallel, antifungals were added to exponentially growing and stationary planktonic cultures, as well. Caspofungin, a  $\beta$ -glucan synthesis inhibitor of the cell wall, had a rather limited effect on biofilms, producing  $\leq 10$ -fold killing (data not shown), and was therefore unsuitable for this study.

Amphotericin B, a compound that binds to ergosterol and forms an oligomeric pore in the membrane (1), effectively killed exponentially growing and stationary cells, with little

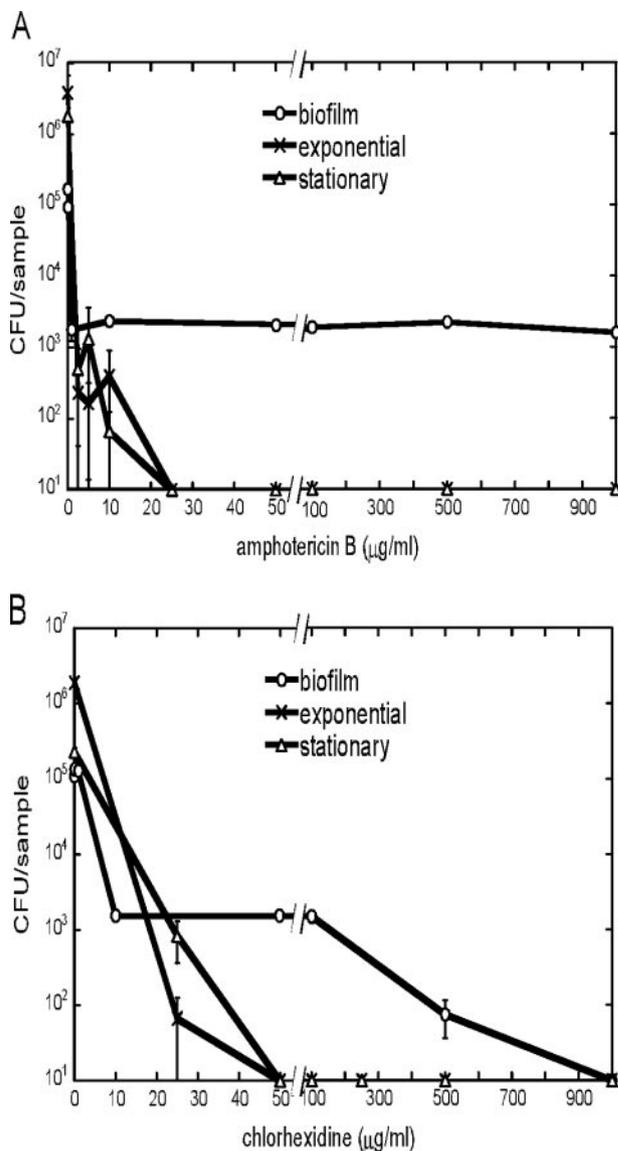


FIG. 1. Survival of *C. albicans* 3153A biofilm and exponential- and stationary-phase cells. Biofilms were cultured in RPMI medium for 48 h, scraped, vortexed, resuspended in 100  $\mu$ l PBS, and plated for CFU determination. Exponential- and stationary-phase cultures were obtained by growth in the same medium. The experiment was performed in triplicate, and the error bars represent standard deviations. (A) Amphotericin B. (B) Chlorhexidine.

indication of surviving cells (Fig. 1A). By contrast, strikingly biphasic killing was observed in *Candida* biofilms, with the majority of the population killed at low concentrations (but above the MIC) while the remaining cells were unaffected by higher concentrations of the drug (Fig. 1A). About 1% of the cells appeared completely invulnerable to amphotericin B, indicating the presence of persisters in the yeast biofilm. This is very different from our previous observations with bacteria, where stationary planktonic populations produce more persisters than the biofilm (48). It was also rather surprising to see complete resistance to killing by amphotericin B, which makes “holes” in the membrane.

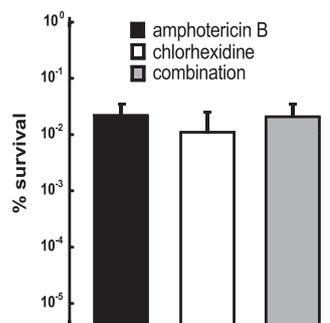


FIG. 2. Survival of *C. albicans* biofilms challenged with amphotericin B and chlorhexidine. Biofilms were treated with 100  $\mu\text{g/ml}$  amphotericin B, 100  $\mu\text{g/ml}$  chlorhexidine, or a combination of the two antifungals for 24 h. The biofilms were washed and sampled for CFU determination before and after antibiotic treatment. The experiment was performed in triplicate, and the error bars indicate standard deviations.

Similarly to amphotericin B, chlorhexidine, a membrane-acting antiseptic used in oral antifungal applications (5), produced distinctly biphasic killing of the biofilm, while cells in both exponential- and stationary-phase cultures were completely eliminated (Fig. 1B). At higher concentrations (above 100  $\mu\text{g/ml}$ ), killing of persisters became evident, and the biofilm was completely sterilized at 1,000  $\mu\text{g/ml}$ , a concentration twofold lower than what is commonly used in mouthwash and as a therapy for treatment of oral thrush caused by *C. albicans* (0.2%).

**Persister cells are multidrug tolerant.** In bacteria, persisters are dormant cells that are tolerant of multiple antibiotics. We wanted to learn whether yeast persisters were similarly multidrug tolerant. Mature, 48-h biofilms were challenged with 100  $\mu\text{g/ml}$  amphotericin B, 100  $\mu\text{g/ml}$  chlorhexidine, or a combination of the two drugs. No additional killing was detected when biofilms were treated with both amphotericin B and chlorhexidine compared to cells treated with individual antimicrobials (Fig. 2). Similarly, the numbers of persisters were essentially the same (1 to 3%) when biofilms were treated sequentially for 24 h with amphotericin B and then chlorhexidine or vice versa (data not shown). These experiments indicated the presence of a single uniform persister population.

To examine the possible roles of drug efflux transporters in resistance to these two antimicrobials, the susceptibilities of the wild-type strain and a mutant lacking Cdr1p, Cdr2p, Mdr1p, and Flu1p were tested. The MICs for the wild type and the quadruple mutant DSY1024 (*cdr1Δ::hisG/cdr1Δ::hisG cdr2Δ::hisG/cdr2Δ::hisG camdr1Δ::hisG/camdr1Δ::hisG flu1Δ::hisG/flu1Δ::hisG-URA3-hisG*) (30) were the same, 1  $\mu\text{g/ml}$  for amphotericin B and 8  $\mu\text{g/ml}$  for chlorhexidine. This suggests that resistance to amphotericin B and chlorhexidine is not influenced by *C. albicans* efflux transporters, and therefore, that efflux does not contribute to persister survival.

**Persisters are phenotypic variants of the wild type.** We next wanted to learn whether cells that resisted killing by drugs were phenotypic variants of the wild type or mutants. Biofilms were treated with amphotericin B or chlorhexidine (100  $\mu\text{g/ml}$ ), after which they were disrupted by being vortexed, washed, and reinoculated. The new biofilm, derived from persisters that survived drug treatment, was again treated with antifungals,

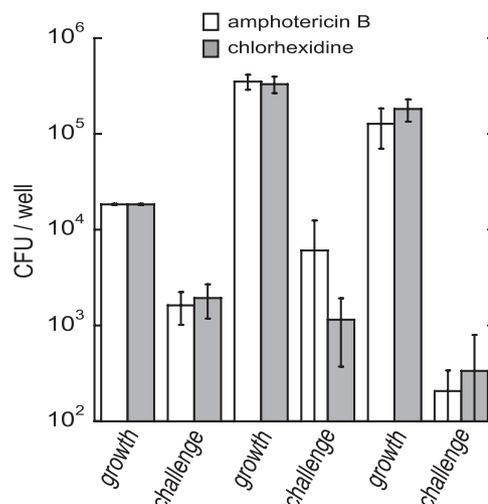


FIG. 3. Heritability of persister formation. Biofilms were treated with 100  $\mu\text{g/ml}$  amphotericin B or 100  $\mu\text{g/ml}$  chlorhexidine for 24 h, after which they were disrupted by being vortexed, washed, and reinoculated in order to form new biofilms. The biofilms were sampled for CFU determination before and after antibiotic treatment. The procedure was repeated a total of three times. The experiment was performed in triplicate, and the error bars indicate standard deviations.

and the procedure was repeated a total of three times (Fig. 3). It is apparent that the population produced by surviving persisters is not more resistant to drugs, but rather gives rise to a new persister subpopulation. If the surviving cells were mutants, we would have seen either complete resistance upon reapplication of the antifungal or a progressive increase in the numbers of surviving cells with each treatment cycle. Thus, we conclude that *C. albicans* persisters, similar to their bacterial counterparts, are phenotypic variants of the wild type that arise in a clonal population of genetically identical cells.

**Isolation of persisters.** Several dyes have been reported to discriminate between live and dead fungal cells, including fluorescein, which was recently reported to specifically stain dead *S. cerevisiae* cells (52). Exponentially growing *C. albicans* cells, untreated or killed with amphotericin B, stained with fluorescein diacetate in the expected manner (Fig. 4 A and B). We then stained a biofilm with fluorescein diacetate (Fig. 4C to E). After addition of amphotericin B, there was a visible decrease in the number of live (dark) cells, and their morphology became aberrant (Fig. 4D). After 48 h of amphotericin B treatment, there were only a small number of unstained cells, and importantly, they appeared to have normal morphology. These cells looked like regular pseudohyphae or yeast and were indistinguishable from morphologically normal untreated cells. This finding is similar to that for bacterial persisters, which look like regular cells (47).

Are the dim *C. albicans* cells in the amphotericin B-treated biofilm live persisters or rather dead cells that for some reason happened to be unstained? The excellent difference observed between live and dead fluorescein diacetate-stained cells suggested that putative persisters could be sorted physically. In order to minimize size heterogeneity in the sorting process, we took advantage of the *flo8* (*riv1*) null mutant strain that is defective in both hyphal and higher-order biofilm formation.

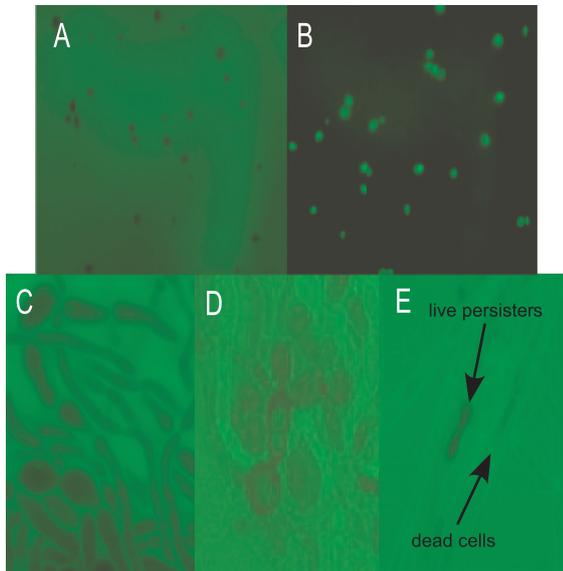


FIG. 4. Live-dead staining of *C. albicans* with fluorescein diacetate. Planktonic or biofilm cells were stained with 100 µg/ml fluorescein diacetate and examined by fluorescence microscopy. (A) Live planktonic cells. (B) Dead planktonic cells after treatment with 100 µg/ml amphotericin B (×400 magnification). (C, D, and E) Biofilms (×1,000 magnification) of untreated control and after 18 and 48 h of amphotericin B treatment (100 µg/ml), respectively.

This strain produces a sparse layer of attached yeast cells and formed a normal level of persisters (Table 1). The absence of hyphae in the strain made it a good choice for sorting. Mature, 48-h biofilms were stained with fluorescein diacetate, disrupted, and sorted with a MoFlo instrument. The sample contained debris from disrupted biofilms, and light scattering was used to determine the predominant particle group, which represented yeast cells. This group was then isolated with a scatter gate and analyzed for fluorescent properties (Fig. 5A). Cells representing the mean in the fluorescence distribution (Fig. 5B) from a biofilm stained with fluorescein diacetate but not treated with an antibiotic were sorted directly onto a plate with YPD medium, and after a 48-h incubation, produced colonies. The sorting worked well under these conditions, with eight cells producing eight colonies (Fig. 5D). Next, we sorted cells from a biofilm treated with amphotericin B. There was a strong shift of the bulk of the population to higher fluorescence (Fig. 5C), reflecting the staining of dead cells with fluorescein diacetate. Two subpopulations, a bright (R2) and a dim (R4) one, were then chosen for physical sorting onto YPD plates. Ninety-six of the sorted dim particles produced 21 colonies. This shows that a considerable part of the dim cells observed after treatment with amphotericin B were indeed live persisters. The imperfect correlation between sorted events and the colonies they produced in this case may have been caused by a considerable background of debris. Indeed, the concentration of live cells was low, and debris is not likely to exhibit bright-green fluorescence. Therefore, debris would be sorted and appear disproportionately represented in the dim subpopulation. Al-

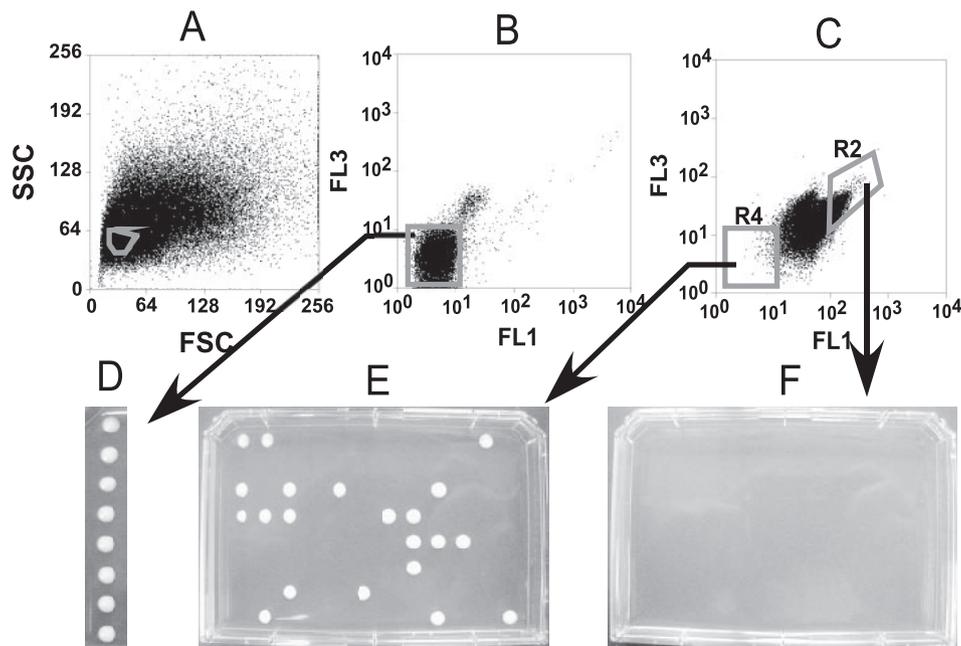


FIG. 5. Isolation of persister cells from a biofilm. *C. albicans* MC191 was grown as a biofilm for 48 h in RPMI 1640 medium in microtiter plate wells. A homogenous population of cells from disrupted biofilms was obtained by applying a forward scatter and a side scatter gate, as shown in panel A, for all subsequent analyses. (B) A biofilm was stained with 100 µg/ml fluorescein diacetate for 24 h, disrupted, washed three times with PBS, and analyzed with a MoFlo cell sorter. Single events representing individual cells were physically sorted directly on YPD medium and incubated for 48 h (D). (C) A biofilm was treated with 100 µg/ml amphotericin B, stained with fluorescein diacetate, and similarly analyzed with the cell sorter. Two distinct populations were separated based on green fluorescence intensity, as shown. (E) Particles representing 96 events from the dim population, R4, were sorted onto YPD agar and incubated for 48 h. (F) Particles representing over 6,000 events from R2 were sorted onto YPD agar and incubated for 48 h.

ternatively, it is possible that some of the dim cells were actually dead. There was no growth from over 6,000 sorted bright cells (Fig. 5F).

**Persisters in biofilm-deficient strains.** Given that persisters were produced only in the biofilm, it was interesting to test biofilm-defective mutants for persister production. Several mutants, e.g., strains lacking the transcription factors Efg1p and Cph1p or strains lacking the MAP kinase Mkc1p, have been reported to produce defective biofilms. A collection of such mutants was tested for persister formation. All strains tested produced biofilms under the conditions of these experiments, though their biomass in many cases appeared to be distinctly less than that of the wild type. After challenge with 100  $\mu\text{g/ml}$  amphotericin B, the biofilms were disrupted and plated for colony counting. Somewhat unexpectedly, all tested strains produced normal levels of persisters (Table 1). Of particular interest was strain CKY138, lacking Efg1p and Cph1p, positive transcriptional regulators of filamentation. This strain is severely defective for biofilm formation, showing reduced adherence and a failure to produce the elaborate three-dimensional structure that characterizes wild-type biofilms. Nevertheless, an *efg1* $\Delta$ /*cph1* $\Delta$  double-mutant strain gave rise to persisters. Therefore, persister formation is not dependent on the formation of a complex biofilm structure. Apparently, the ability to attach to a surface, which all of these strains exhibit, is sufficient to promote persister production. This is consistent with previously published work, which suggested that attachment alone is sufficient to confer resistance to fluconazole on a young biofilm (32) and that resistances to fluconazole and amphotericin B were similar in sessile wild-type and *efg1* $\Delta$ /*cph1* $\Delta$  cells, as measured by XTT reduction (43).

Given that attachment rather than biofilm architecture appeared to be important for persister formation, it was especially interesting to test a strain deficient in Mkc1p, a recently described contact-dependent kinase that affects biofilm formation and invasiveness (26). However, a strain lacking Mkc1p was able to produce normal levels of persisters (Table 1). Therefore, our analysis of biofilm mutants suggests that known genes are not involved in persister formation. The isolation of persisters, as described in this study, opens the possibility of obtaining their transcription profile and thus identifying genes involved in the persister phenotype.

## DISCUSSION

The discovery of persisters in bacterial biofilms helped explain the puzzling resistance of biofilms to killing (19). A similar paradox exists in the field of fungal biofilms—planktonic cells may be highly susceptible to a range of antimicrobials, while mature biofilms appear to be resistant to killing by “everything” compared to planktonic populations. A biphasic pattern of killing is a defining feature demonstrating the presence of persisters in bacterial populations. In this study, we found that *C. albicans* biofilms exhibit this striking pattern of killing, with the majority of cells being fairly susceptible and a small subpopulation,  $10^{-3}$  to  $10^{-2}$  cells, highly tolerant of antimicrobials. Our analysis was limited by necessity to highly microbicidal antifungals, amphotericin B and chlorhexidine, which can effectively kill slowly growing or nongrowing cells; both of these compounds are membrane acting. Persisters appeared to be

completely invulnerable to amphotericin B, which was rather unexpected. It has been reported that biofilm cells have a decreased level of ergosterol, to which amphotericin B binds prior to forming a pore in the membrane (34). It is possible that persisters have an even lower level of ergosterol than the bulk of biofilm cells and that other factors, such as dormancy, contribute to their survival against amphotericin B. Importantly, an experiment with the nonspecific antiseptic chlorhexidine suggests that *C. albicans* persisters are not merely cells with diminished ergosterol levels. A prominent subpopulation of cells tolerant of chlorhexidine was observed, as the majority of cells were killed at a low concentration of 10  $\mu\text{g/ml}$  and additional killing did not occur until the concentration reached a very high dose of 500  $\mu\text{g/ml}$ . At even higher concentrations, chlorhexidine was able to completely eradicate the biofilm. Interestingly, this biofilm eradication concentration of chlorhexidine, 1 mg/ml, is comparable to the 2-mg/ml concentration found in mouthwash used to treat *C. albicans* oral thrush infections. It is important to note that neither amphotericin B nor chlorhexidine is a substrate of Cdr1p, Cdr2p, Mdr1p, or any known drug efflux pump. Our experiments showed that the MIC of the wild type to these antimicrobials was the same as that of a strain with multiple deletions in the MDRs.

The biphasic killing produces an impression of the presence of resistant mutants in a population, and we examined the possible heritability of resistance in the surviving cells. Repeated reinoculation of persisters after amphotericin B treatment restored the original biofilm with a new persister subpopulation. This is similar to the nonheritable nature of bacterial persisters (18).

In order to observe persisters, we used fluorescein diacetate, which specifically stains dead yeast cells. After treatment with amphotericin B, most of the cells became morphologically aberrant (bloated) and then stained with fluorescein diacetate, while a small fraction of cells retained normal morphology and were dark. We then used cell sorting to isolate the dark cells. The dark cells produced colonies, while the bright ones did not. This experiment demonstrates the existence of unique survivor cells within the antifungal-treated biofilm.

The production of fungal persisters by biofilm populations alone was unexpected. We did not find persisters in either exponential- or stationary-phase planktonic cultures of *C. albicans*. In principle, stimulation by fresh medium or regrowth could have accounted for the presence of persisters in biofilms, but not in planktonic stationary-phase cultures. In our experiments, both biofilms and stationary-phase cultures were washed, and then antibiotics were added in fresh growth medium. It is important to note that stationary-phase yeast cells do not grow when resuspended without dilution in the same volume of fresh medium. Nevertheless, stationary-phase cultures did not contain persisters; persisters were observed only in the biofilm. It seems that the inability of a planktonic stationary-phase culture to form persisters cannot be explained by their disappearance due to stimulation with fresh medium.

Stationary-phase cultures grown in RPMI 1640 medium produce cell aggregates but do not form persisters. Indeed, crowding does not seem to be important—addition of the quorum-sensing molecule farnesol or tyrosol did not affect the numbers of persisters in biofilms (M. LaFleur, unpublished data). These observations suggest that attachment to

a substratum is the important factor that determines persister formation in *Candida*.

In bacteria, the frequency of persisters increases sharply with the cell density of the population (18), and there are actually more persisters in a nongrowing stationary-phase culture than in a slowly growing biofilm (48). It was also surprising to find that persisters were present at normal levels in all of the presently described mutants that produce defective biofilms. These results point to attachment, rather than formation of a complex biofilm structure, as the stimulus for persister formation. At the same time, a mutant in the Mkc1p MAP kinase that is involved in contact sensing and control of the biofilm phenotype produced normal levels of persisters, as well. This suggests that additional, unidentified sensors of attachment are probably responsible for triggering a program that produces persisters.

A transcription profile of isolated persisters has recently been obtained in *E. coli*. Persisters overexpress several toxin-antitoxin modules. Induction of toxin expression from a controllable promoter caused the production of persisters, and deletion of a particular toxin-antitoxin module, *hipBA*, caused a decrease in persisters in a biofilm (19). Upon overexpression, these toxins block important targets in the cell, leading to dormancy. Bacterial persisters have characteristics of dormant cells in that they are slowly growing or nongrowing (4) and lack noticeable expression of degradable green fluorescent protein, indicating low levels of transcription (47). It is premature to speculate whether the drug tolerance of surviving *C. albicans* cells reported in this study is also caused by dormancy, but it is useful to note that dormancy has been documented in fungi. The ascospore of *Saccharomyces cerevisiae* is the best-studied dormant form of yeast. As a typical spore, it is highly tolerant of a wide range of noxious factors (36). *C. albicans* produces chlamydospores, a thick-walled asexual form derived from hyphae (31). However, chlamydospores are not known to confer long-term viability and do not appear to be present at sites of infection or in biofilms (17, 31). Another dormant form of yeast, known as quiescence, occurs after 5 to 7 days of incubation of *S. cerevisiae* at 30°C in complete media (13). Quiescent cells exhibit increased thermotolerance, osmotolerance, and tolerance of a variety of cell wall-acting agents compared to dividing yeasts (9, 13, 38). Several genes are known to be involved in the entry into, the exit from, or the maintenance of quiescence (13). However, it is unknown whether these cells are tolerant of antifungals.

Future studies will show whether yeast persisters are dormant cells that have any connection to the previously described dormant forms. For now, we may say that yeasts and bacteria have evolved analogous survival strategies through convergent evolution that assign the function of survival to a small part of the population. The fact that persisters in yeast are specific to biofilms suggests they are not the result of "mistakes," such as random overexpression of nonspecific proteins that may lead to stasis, as has recently been suggested for bacteria (50). The finding of a persister subpopulation in *C. albicans* will help to solve the puzzle of biofilm resistance to antifungals, and the method of isolating persisters reported in this study provides a powerful tool to search for persister genes.

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