INFECTION AND IMMUNITY, Aug. 2003, p. 4333–4340 Vol. 71, No. 8
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.8.4333–4340.2003

Mechanism of Fluconazole Resistance in *Candida albicans* Biofilms: Phase-Specific Role of Efflux Pumps and Membrane Sterols

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Received 12 February 2003/Returned for modification 12 March 2003/Accepted 3 May 2003

Candida albicans **biofilms are formed through three distinct developmental phases and are associated with high fluconazole (FLU) resistance. In the present study, we used a set of isogenic *Candida* strains lacking one or more of the drug efflux pumps Cdr1p, Cdr2p, and Mdr1p to determine their role in FLU resistance of biofilms. Additionally, variation in sterol profile as a possible mechanism of drug resistance was investigated. Our results indicate that parent and mutant strains formed similar biofilms. However, biofilms formed by double and triple mutants were more susceptible to FLU at 6 h (MIC = 64 and 16 μg/ml, respectively) than the wild-type strain (MIC > 256 μg/ml). At later time points (12 and 48 h), all the strains became resistant to this azole (MIC ≥ 256 μg/ml), indicating lack of involvement of efflux pumps in resistance at late stages of biofilm formation. Northern blot analyses revealed that *Candida* biofilms expressed *CDR* and *MDR1* genes in all the developmental phases, while planktonic cells expressed these genes only at the 12- and 48-h time points. Functionality of efflux pumps was assayed by rhodamine (Rh123) efflux assays, which revealed significant differences in Rh123 retention between biofilm and planktonic cells at the early phase ($P = 0.0006$) but not at later stages (12 and 48 h). Sterol analyses showed that ergosterol levels were significantly decreased ($P < 0.001$) at intermediate and mature phases, compared to those in early-phase biofilms. These studies suggest that multicomponent, phase-specific mechanisms are operative in antifungal resistance of fungal biofilms.**

Microorganisms form biofilms on a variety of implanted medical devices, resulting in biofilm-associated infections that constitute a significant public health problem (13, 38). Both bacterial and fungal biofilms have been associated with significantly high antimicrobial resistance compared to their planktonically grown forms (4, 9, 51). Although bacterial biofilms have been studied in great detail (39, 40), the study of medically relevant fungal biofilms has only recently come to the forefront. *Candida* species, especially *Candida albicans*, are the most common fungi associated with biofilm-related infections (7, 38). Forty percent of patients with *Candida* isolated from intravenous catheters develop occult fungemia (2, 37), and the mortality rate for patients with catheter-related candidemia can be as high as 41% (37). Biofilm formation is also critical in the development of denture stomatitis, a superficial form of candidiasis that affects 65% of edentulous individuals (6–8).

We have recently developed denture and catheter models of fungal biofilms using physiological substrates and clinically relevant *C. albicans* strains (9). Using this model, we showed that *C. albicans* biofilms are highly resistant to the action of clinically important antifungal and antimicrobial agents including amphotericin B, chlorhexidine, nystatin, and fluconazole (9, 10, 29, 30). We also demonstrated that *C. albicans* biofilm formation proceeds in three developmental phases: (i) early phase (0 to 11 h), involving adhesion of fungal cells to the substrate, (ii) intermediate phase (~12 to 30 h), during which the blastospores coaggregate and proliferate, forming communities while producing a carbohydrate-rich extracellular matrix (ECM), and (iii) maturation phase (~31 to 72 h), in which the fungal cells are completely encased in a thick ECM (9). Additionally, our studies demonstrated that acquisition of antifungal resistance by *C. albicans* biofilms correlates with the developmental phases of these biofilms (9).

The multidrug resistance phenotype in planktonic *C. albicans* has previously been shown to be linked to proteins encoded by *CDR1*, *CDR2*, and *MDR1* genes (41, 48). These proteins act as membrane-localized efflux pumps that pump drugs from the fungal cells. Rhodamine 123 (Rh123) is a known fluorescent substrate of efflux pumps responsible for multidrug resistance in mammalian cells, bacteria, and yeasts (11, 12, 17, 21). It has been suggested that altered membrane sterol composition, which affects membrane permeability, is a possible mechanism of azole resistance among *C. albicans* cells grown in suspension (23). Thus, even though the mechanisms involved in drug resistance associated with these fungal cells are well characterized, their roles in biofilm-associated resistance remain to be elucidated.

In this study, we investigated the mechanisms of *C. albicans* biofilm-associated fluconazole resistance at the genetic level as well as the functional level. A set of isogenic *C. albicans* strains lacking (i) the *CDR1* or *MDR1* gene (single-knockout mutants), (ii) both *CDR1* and *CDR2* genes (double-knockout mutant), and (iii) *CDR1*, *CDR2*, and *MDR1* genes (triple-knockout mutant) was employed. The metabolic activities, dry weights, and viable cell counts of biofilms formed by these isogenic strains were compared to determine any possible effect of deletion of the *CDR* and/or *MDR1* genes on biofilm formation. The contribution of efflux pumps to azole resistance of candidal biofilms was investigated by determining the flu-

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TABLE 1. Isogenic *C. albicans* strains used in this study

Strain	Parent	Genotype
CAF2-1	SC5314	Δ*ura3*::*imm434*/*URA3*
DSY448	CAF2-1	Δ*cdr1*::*hisG-URA3-hisG*/Δ*cdr1*::*hisG*
DSY465	CAF2-1	Δ*mdr1*::*hisG-URA3-hisG*/Δ*mdr1*::*hisG*
DSY654	CAF2-1	Δ*cdr1*::*hisG*/Δ*cdr1*::*hisG* Δ*cdr2*::*hisG-URA3-hisG*/Δ*cdr2*::*hisG*
DSY1050	CAF2-1	Δ*cdr1*::*hisG*/Δ*cdr1*::*hisG* Δ*cdr2*::*hisG*/Δ*cdr2*::*hisG* Δ*mdr1*::*hisG-URA3-hisG*/Δ*mdr1*::*hisG*

conazole susceptibilities of biofilms formed by *CDR* and/or *MDR1* deletion mutants. Expression of *CDR* and *MDR1* genes at the early (6 h), intermediate (12 h), and mature (48 h) phases of biofilm development and in similarly grown planktonic cells was examined. Furthermore, we assessed the functional activity of efflux pumps by measuring the levels of Rh123 retained in *C. albicans* biofilms and planktonic cells at different developmental phases. Additionally, we investigated variation in sterol profile as a possible mechanism of azole resistance by comparing the sterol composition of *C. albicans* biofilms to that of planktonic cells. Our results suggest that (i) disruption of efflux pumps does not affect the biofilm formation abilities of the resulting mutants, (ii) efflux pumps contribute to azole resistance in early-phase *C. albicans* biofilms but not in later phases, and (iii) changes in sterol composition are involved in the resistance phenotype in the intermediate and mature phases of biofilm development.

MATERIALS AND METHODS

Strains. Table 1 describes the *C. albicans* strains used in this study. Strains CAF2-1, DSY448, DSY465, DSY654, and DSY1050 were generous gifts from D. Sanglard (Lausanne, Switzerland), and strain GDH2346 was a gift from L. J. Douglas (Glasgow, United Kingdom). The wild-type strain CAF2-1 was used in Northern blotting, Rh123 efflux, and sterol analyses. Strains were grown overnight at 37°C in yeast nitrogen base (YNB) medium with amino acids (Difco Laboratories, Detroit, Mich.; catalog no. 0392-15-9) supplemented with 50 mM glucose.

Biofilm formation and quantitation. Biofilms were formed on 1.5-cm^2 denture acrylic (polymethylmethacrylate [PMA]) strips (Makki Dental Prosthetics, Inc., Middleburg Heights, Ohio) as described previously (9, 10). Briefly, a standard inoculum of 10^7 cells/ml from an overnight culture of the fungal strains was applied to the surface of PMA strips placed in a 12-well tissue culture plate. The cells were allowed to adhere for 90 min at 37°C. Nonadherent cells were removed from the strips by gentle washing with 5 ml of phosphate-buffered saline (PBS). Strips were then submerged in 4 ml of YNB medium supplemented with 50 mM glucose and incubated for various durations at 37°C on a rocker. Strips with no *Candida* cells served as negative controls. Control and experimental strips were then incubated at 37°C for various time periods. Planktonic cultures were grown in the same way as biofilms, in 12-well tissue culture plates, except that denture acrylic strips were not added to the wells. Biofilm and planktonic cultures were grown for 6, 12, and 48 h, corresponding to early, intermediate, and mature phases of development, respectively. Biofilms formed on PMA strips were quantified by (i) a tetrazolium XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] reduction assay and (ii) dry weight measurement, as described previously (9, 10). Assays were carried out in triplicate and were repeated on different days.

Cell viability assessment. For assessing the viability of *C. albicans* cells, biofilms were scraped from denture strips and sonicated for 10 min in a water bath sonicator (Fisher Scientific Co., Pittsburgh, Pa.) to obtain a suspension of fungal cells. This suspension was incubated with 0.01% (vol/vol) trypan blue, and the viable candidal cells (not staining blue) were counted microscopically with a hemacytometer.

Antifungal susceptibility testing. Antifungal susceptibilities of biofilm and planktonic *C. albicans* were determined as described previously (9). Briefly, biofilms were grown on PMA strips for 6, 12, or 48 h and the acrylic strips were transferred to wells containing different concentrations (0.5 to 256 μg/ml) of fluconazole (Pfizer Pharmaceuticals Group, New York, N.Y.). Strips were further incubated for 48 h, and metabolic activities of biofilms were measured by the XTT reduction assay as described previously (9, 10, 44). MIC was defined as the antifungal concentration which caused 50% reduction in metabolic activity of a *C. albicans* biofilm compared with control (incubated in the absence of drug). For each drug concentration, separate strips were used in triplicate and experiments were performed on different days. The antifungal susceptibility of planktonic cells was determined similarly (9).

PCR amplification of *C. albicans CDR1* and *-2* and *MDR1* genes. *CDR1* and *-2* and *MDR1* genes were amplified by PCR using specific oligonucleotide primers. For *CDR1* and *CDR2* the forward primer was 5′-TATGTCAGATTCTAAGAT GTC-3′ and the reverse primer was 5′-TCGATACCTTCACCTCTG-3′; for *MDR1* the forward primer was 5′-AGGTGAACCCAATTCAAGTC-3′ and the reverse primer was 5′-ACAACTGGTTCCATAACGGT-3′. PCR conditions were as follows: denaturation (95°C, 2 min) followed by 33 cycles of denaturation (94°C, 1 min), annealing (50°C, 1 min), and extension (72°C, 3 min), ending with a 10-min extension at 72°C. PCR-amplified fragments were labeled with digoxigenin (DIG)-dUTP (Roche Molecular Biochemicals, Indianapolis, Ind.) according to the manufacturer's instructions and used as probes for Northern blot analyses.

Northern blot analysis. To determine whether expression of *CDR* and *MDR1* genes is altered during the developmental phases of biofilm formation, Northern analyses were performed on *C. albicans* (strain CAF2-1) biofilms and planktonic cells grown for 6, 12, or 48 h. Biofilm material was scraped from the surface of PMA strips, resuspended in PBS, and centrifuged (3,000 × *g*) to obtain a pellet. Planktonic cells were similarly collected. Total RNA was extracted from biofilm and planktonic cells and analyzed by Northern blotting as described previously (9, 25). UV-cross-linked RNA blots were prehybridized for 1 to 2 h at 50°C and then hybridized overnight with a DIG-labeled *CDR* or *MDR1* probe (30 ng/ml) at 50°C. Hybridizing gene transcripts were detected with the DIG High Prime detection kit (Roche Molecular Biochemicals) according to manufacturer's instructions. 25S rRNA was used as a control for RNA loading.

Rh123 efflux assay. To determine whether efflux pump activity varied with different developmental phases, the functional activity of these pumps was assayed as described previously (12). Briefly, *C. albicans* CAF2-1 was grown as a biofilm to 6, 12, or 48 h representing early, intermediate, and mature phases. At these time points, biofilms were scraped, resuspended in PBS, and sonicated for 10 min in a water bath sonicator (Fisher Scientific Co.). The number of fungal cells in the resulting suspension was determined with a hemacytometer. Planktonic cells were collected by aspiration, washed, and resuspended in PBS. Suspensions of *C. albicans* biofilms or planktonic cells (10^7 cells/ml for each) were incubated with 10 μM Rh123 at 37°C for 20 min and centrifuged at 12,000 × *g* in a microcentrifuge. The resulting pellet was washed twice, resuspended in 200 μl of PBS, and transferred to a 96-well plate. The fluorescence of the reaction mixture was recorded with a spectrofluorimeter (excitation and emission wavelengths of 485 and 538 nm, respectively). To determine whether cells assayed for Rh123 retention assay were metabolically active, we measured their metabolic activities using the Live/Dead kit, based on FUN-1 (2-chloro-4-[2,3-dihydro-3-methyl-{benzo-1,3-thiazol-2-yl}-methylidene]-1-phenylquinolinium iodide; Molecular Probes Inc., Eugene, Oreg.) by following the manufacturer's instructions. FUN-1 is a membrane-permeant nucleic acid-binding asymmetric halogenated cyanine dye that gives rise to cylindrical intravacuolar structures in metabolically active yeast cells (35). A biofilm or planktonic cell suspension (10^7 cells/ml) was incubated with FUN-1 for 45 min at 37°C, and fluorescence was estimated with a spectrofluorimeter (excitation and emission wavelengths, 485 and 585 nm, respectively). Rh123 retention by the cells was expressed as fluorescence accumulated per unit of metabolic activity.

Sterol extraction and analysis. Sterols were extracted from *C. albicans* biofilms and planktonic cells and analyzed by gas-liquid chromatography (GLC) as described previously (26, 52). Briefly, *Candida* biofilms and planktonic cells grown to different time points were harvested and washed twice with PBS. The harvested cells were refluxed for 3 h with ethanolic KOH, filtered, and mixed with

TABLE 2. MICs of fluconazole against different *C. albicans* strains grown to different time points as biofilms or planktonic cells

Strain	Genotype	MIC (μg/ml) for strain grown as:					
		Biofilm at:			Planktonic cells at:		
		6 h	12 h	48 h	6 h	12 h	48 h
CAF2-1	Wild type	>256	>256	>256	2	2	2
DSY448	Δ*cdr1*	256	>256	>256	1	1	1
DSY465	Δ*mdr1*	256	>256	>256	2	2	2
DSY654	Δ*cdr1* Δ*cdr2*	64	>256	>256	1	1	1
DSY1050	Δ*cdr1* Δ*cdr2* Δ*mdr1*	16	256	>256	0.13	0.13	0.13

equal volumes of double-distilled water. Sterols were extracted from this mixture with 4 volumes of heptane. The extracted sterols were derivatized with hexamethyldisilazane and trimethlylchlorosilane and analyzed by GLC using an HP-6890 series gas-liquid chromatography system (Hewlett-Packard) with an OV-1 column (26, 52). Authentic sterol standards (Sigma) were used to identify various sterol intermediates based on their retention times relative to that of ergosterol. The level of each sterol was determined from the corresponding peak areas as a percentage of the total.

Statistical analyses. All experiments were repeated at least three times on separate days. Comparison of multiple sets of data was performed by analysis of variance, and paired comparisons were performed by Student's *t* test using StatView software (version 4.5; Abacus Concepts).

RESULTS

Deletion of *CDR1* and *-2* and *MDR1* genes in *C. albicans* mutants does not affect the abilities of these strains to form a biofilm. To evaluate whether disruption of the *CDR1*, *CDR2*, or *MDR1* gene leads to altered abilities to form biofilms, we determined the metabolic activities, dry weights, and viable-cell counts of biofilms formed by mutant strains with these genes disrupted. Biofilms were formed on denture acrylic strips as described previously (10). Our data showed that strains with the efflux pump(s) deleted (efflux pump-deleted strains) as well as the parent strain formed thick ECM-encased biofilms, showing similar morphologies when stained with Calcofluor white, a carbohydrate-binding dye (data not shown). Biofilms formed by all the strains predominantly contained blastospores, although some hyphae were also visible. These features were similar to those observed in biofilms formed by strain GDH2346, which was previously used to standardize our biofilm model (9). Moreover, biofilms formed by mutant and parent strains did not differ significantly in their metabolic activities ($P > 0.05$; data not shown), as determined by using the XTT tetrazolium dye-based assay, or dry weights ($P > 0.05$; data not shown). To examine the correlation between cell density and metabolic activity, we determined the numbers of viable cells in biofilms formed by the wild-type and mutant strains. Our data revealed no difference between the viable-cell counts in 48-h biofilms formed by mutant and parent strains ($P > 0.05$; data not shown). Moreover, there were no differences in viable-cell counts at the early and intermediate phases of biofilm development between wild-type and mutant strains (data not shown). Additionally, to ascertain that the disruption of genes does not lead to altered growth, we monitored the growth of these strains and found no differences in their growth curves (data not shown). Thus, our data demonstrate that biofilms formed by efflux pump-deleted strains were similar to those formed by the wild-type strain, indicating that any effects of these pumps on biofilm-associated drug resistance are not due to gross changes in biofilm structure or morphology.

Antifungal susceptibility of *C. albicans* biofilms is affected by drug efflux pumps in a developmental phase-specific manner. In our previous studies, we have shown that *C. albicans* biofilms exhibit high resistance to fluconazole and that this resistance phenotype correlates with the corresponding developmental phase (early, intermediate, or maturation) of biofilms (9). Since efflux pumps have previously been implicated in azole resistance in planktonic *C. albicans* cells (48), we investigated whether the *CDR1* and *-2* and *MDR1* genes play a role in biofilm-associated fluconazole resistance. The fluconazole susceptibilities of planktonic *C. albicans* and biofilms formed by efflux pump-deleted mutants were determined and compared to that of the parental strain. Antifungal susceptibility assays with planktonically grown isogenic *C. albicans* strains revealed drastically reduced fluconazole MICs compared to those for biofilms (Table 2). Our results showed that mature biofilms formed by mutant and parental strains were highly resistant to fluconazole (MIC > 256 μg/ml).

To examine whether the observed fluconazole resistance varies with developmental phases of biofilm formation, MICs of fluconazole for biofilms grown to early (6 h), intermediate (12 h), and mature (48 h) phases were determined. As shown in Table 2, early-phase biofilms formed by all mutants were more susceptible to fluconazole than intermediate- and mature-phase biofilms (Table 2). Additionally, at the 6-h time point, MICs of fluconazole against single (DSY448 and DSY465), double (DSY654), and triple (DSY1050) mutants were 256, 64, and 16 μg/ml, respectively. Moreover, biofilms formed by mutant strains exposed to a high concentration of fluconazole (256 μg/ml) exhibited a time-dependent decrease in growth inhibition, while this drug had no effect on a biofilm formed by the wild-type strain (Fig. 1). These results demonstrated that the azole resistance of biofilms formed by efflux pump-deleted mutant strains varies with the developmental phase and that efflux pumps play an additive role in contributing to antifungal resistance in early-phase biofilms.

Expression of genes encoding efflux pumps is temporally regulated in *C. albicans* biofilms. Since early-, intermediate-, and mature-phase biofilms showed differences in fluconazole resistance pattern, it is logical to assume that the *CDR1* and *-2* and *MDR1* genes may be differentially expressed at the transcriptional level in these developmental phases. To determine whether developmental-phase-dependent biofilm-associated azole resistance is correlated with *CDR1* and *-2* and *MDR1* expression at the mRNA level, we investigated the temporal

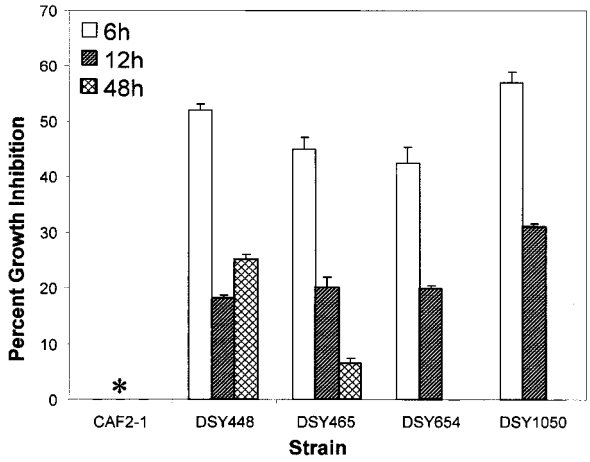


FIG. 1. Percent growth inhibition of *C. albicans* biofilms exposed to high concentration of fluconazole. Percentages of inhibition for biofilms grown to the early (6 h), intermediate (12 h), or late (48 h) phase of development and exposed to 256 μg of fluconazole/ml were determined. Strains used were: CAF2-1 (wild type), DSY448 (Δ*cdr1*), DSY465 (Δ*mdr1*), DSY654 (Δ*cdr1* Δ*cdr2*), and DSY1050 (Δ*cdr1* Δ*cdr2* Δ*mdr1*). For each strain, drug susceptibility decreased from the early to late phase of biofilm development. Additionally, deletion of two and three efflux pumps led to progressively decreasing susceptibility to fluconazole. Metabolic activity was normalized to the control without fluconazole, which was taken as 100%. Data (means ± standard deviations) are representative of three separate experiments. *, the wild-type (CAF2-1) strain showed 0% inhibition at all the time points.

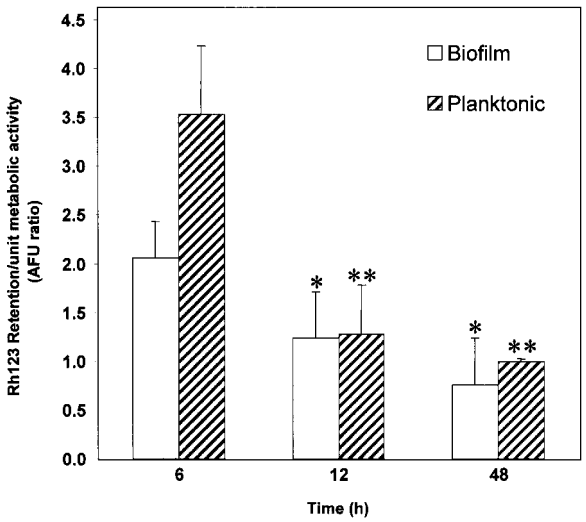


FIG. 3. Rh123 accumulation by early-, intermediate-, and mature-phase biofilms and planktonic cells of *C. albicans.* Data were analyzed by two-way analysis of variance, and a value of $P < 0.05$ was considered significant. *, $P < 0.05$ versus 6-h biofilms; **, $P < 0.0001$ versus 6-h planktonic cells. AFU, arbitrary fluorescence units.

expression of these genes in *C. albicans* biofilms at the transcriptional level by Northern blot analyses as described previously (9). The expression profile of the *CDR1* and *-2* and *MDR1* genes in 6-, 12-, and 48-h biofilms formed by the wild-type strain was compared with that of planktonic *C. albicans*. As shown in Fig. 2, the *CDR* transcript was detected in the early-, intermediate-, and mature-phase biofilms of *C. albicans*. In contrast, expression of these genes in planktonic cells was detected only at 12 and 48 h (Fig. 2). The *MDR1* transcript was detected in early-phase biofilms at 6 h but not in planktonic cells at the same time point (Fig. 2). Moreover, the levels of the *MDR1* transcript for both planktonic cells and biofilms were highest at 12 h but minimal at 48 h (Fig. 2). The levels of *MDR1* transcript at the 6-h time point did not differ from those at the 48-h time point. Therefore, our data showed that the expression of genes encoding efflux pumps in biofilm and planktonic cells is temporally regulated at different developmental phases.

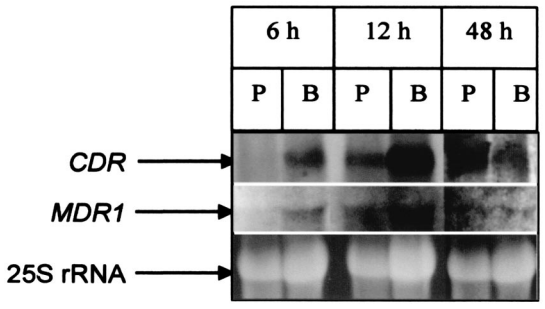
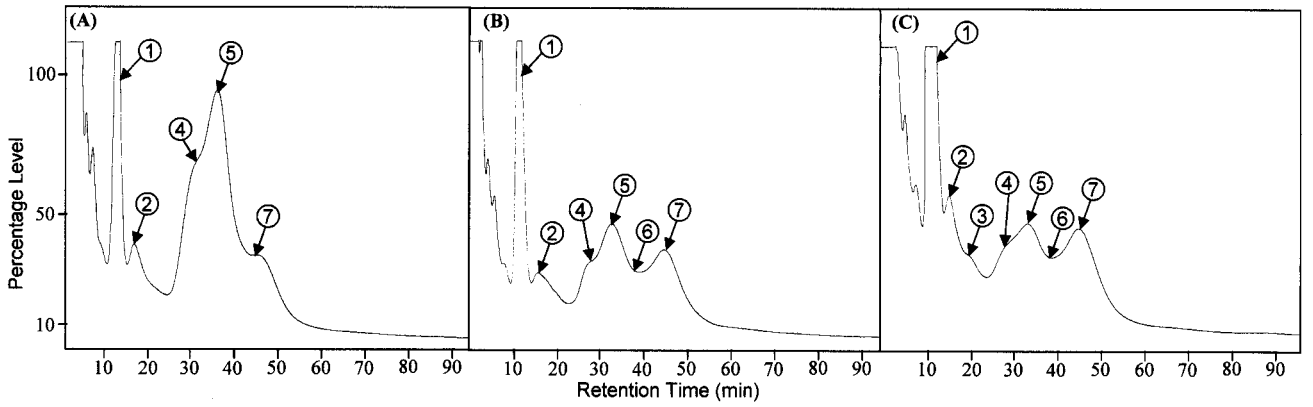


FIG. 2. Expression of *CDR* and *MDR1* genes in (P) planktonic (P) and biofilm (B) forms of *C. albicans*. Total RNA was isolated from biofilms and planktonic cells grown for 6, 12, and 48 h. Sixty micrograms of total RNA was analyzed by Northern blotting using a *CDR*- or *MDR1*-specific probe as described in Materials and Methods. Top, *CDR* transcript; middle, *MDR1* transcript; bottom, 25S rRNA (loading control). Results are representative of three separate experiments.

Functional analysis of drug efflux activity at the protein level. Functional activities of efflux pumps have traditionally been assayed with Rh123, a fluorescent substrate for these proteins which is retained in cells lacking functional efflux pumps (11, 12). We employed this Rh123-based assay to determine whether the levels of *CDR* and *MDR1* mRNA are correlated with the efflux pump activity of biofilms and planktonic *C. albicans* at different developmental phases. Our results showed that, at 6 h, the level of Rh123 retained in biofilms was significantly lower than the corresponding levels in planktonic cells ($P = 0.0006$), indicating higher efflux pump activity in early-phase biofilms (Fig. 3). Moreover, compared to that of the early-phase biofilm, the levels of Rh123 at the intermediate and mature phases were significantly reduced ($P < 0.05$; Fig. 3). In contrast to results for the 6-h time point, at 12 and 48 h, no significant differences in Rh123 levels between biofilm and planktonic cells were found ($P > 0.05$). These results further confirmed our data obtained from Northern blot analyses and suggested that efflux pumps are important in biofilm-associated resistance only at the early phase of development.

***C. albicans* biofilms have altered sterol composition at intermediate and mature phases compared to that at the early phase.** The cellular target of fluconazole in *C. albicans* is a cytochrome P-450 hemoprotein involved in the ergosterol biosynthetic pathway (19). Alterations in sterol composition have previously been linked to antifungal resistance in planktonic cells (18, 24, 47). Since our data showed no role of efflux pumps in the fluconazole resistance of intermediate- and mature-phase biofilms, we investigated whether this phenotype can be attributed to changes in sterol composition. Total membrane



(D) Percentage levels (mean ± SD) of sterols in biofilm and planktonic *C. albicans* grown to 6, 12, and 48h

Peak No.	Sterol	Biofilm			Planktonic		
		6 h	12 h	48 h	6 h	12 h	48 h
1	Squalene	11.40 ± 0.006	33.84 ± 0.064	20.09 ± 0.064	22.44 ± 0.006	9.23 ± 0.006	31.25 ± 0.010
2	Breakdown product 1	8.35 ± 0.012	8.48 ± 0.036	13.32 ± 0.110	10.03 ± 0.006	14.57 ± 0.032	14.26 ± 0.006
3	Breakdown product 2	n.d.*	n.d.*	6.13 ± 0.070	n.d.*	n.d.*	2.03 ± 0.017
4	Zymosterol	27.50 ± 0.422	12.63 ± 0.006	10.53 ± 0.330	n.d.*	n.d.*	4.87 ± 0.012
5	Ergosterol	42.55 ± 0.582	25.10 ± 0.012	21.42 ± 0.789	41.75 ± 0.023	34.00 ± 0.577	31.00 ± 0.029
6	4,14-dimethylzymosterol	n.d.*	10.99 ± 0.047	7.90 ± 0.040	10.13 ± 0.015	16.18 ± 0.017	4.85 ± 0.023
7	Obtusifoliol	10.20 ± 0.056	8.96 ± 0.015	19.86 ± 0.050	15.64 ± 0.012	25.99 ± 0.006	11.33 ± 0.167

*n.d. – not detected

FIG. 4. Variations in sterol profiles of *C. albicans* biofilms at different developmental phases. Sterol patterns for biofilms grown to the early (A), intermediate (B), or mature (C) phase were determined by GLC. (D) Percentages of sterols identified in *C. albicans* biofilms and planktonic cells (chromatograph not shown), determined from the corresponding peak areas and retention times relative to ergosterol. Peaks 1 to 7 (A to C) represent sterols described in panel D. SD, standard deviation.

sterols were isolated from biofilms and planktonic cells and analyzed by GLC (26, 52). Representative chromatographs showing the patterns of sterols present in biofilms grown to different developmental phases are shown in Fig. 4A to C. The levels of individual sterols were calculated from their relative retention times compared to that of ergosterol and are tabulated in Fig. 4D. As can be seen in Fig. 4D, the level of ergosterol decreased by 41% between early- (6 h) and intermediate (12 h)-phase biofilms (Fig. 4D; $P < 0.001$). Moreover, ergosterol level was reduced by 50% at mature phase, compared to that for early-phase biofilms (Fig. 4D; $P < 0.001$). In contrast, planktonically grown cells showed only 18% reduction in ergosterol level between the 6- and 12-h time points (Fig. 4D; $P = 0.0021$). Furthermore, the levels of other ergosterol intermediates fluctuated with no apparently consistent pattern (Fig. 4D). These results show that the sterol composition of *C. albicans* biofilms is modulated during different developmental phases, which likely contributes to candidal biofilm resistance at intermediate and mature phases.

DISCUSSION

Bacterial as well as fungal biofilms are characterized by significantly enhanced resistance to antimicrobial agents (4, 10, 13, 15, 45). Multiple mechanisms including drug efflux pumps, ECM, metabolic quiescence, and unique architecture have been proposed to explain bacterial biofilm-associated drug resistance (3, 14, 16, 31, 33, 50). Efflux pumps are critically involved in the antimicrobial resistance of planktonically grown bacteria, but their role in biofilm-associated resistance varies. Thus, while efflux pumps have been implicated in the resistance of *Pseudomonas aeruginosa* biofilms to low doses of ofloxacin, these proteins are not involved in resistance to other antimicrobials including ciprofloxacin, chloramphenicol, and tobramycin (5, 14). Similarly, biofilms formed by *Escherichia coli* demonstrate the involvement of an efflux pump in resistance to a low concentration of ciprofloxacin (0.004 mg/liter) but not to a higher concentration (0.1 mg/liter) (34). These studies indicate a dose- and drug-dependent role for efflux pumps in antimicrobial resistance of bacterial biofilms.

Antifungal resistance of planktonically grown *C. albicans* has been linked to the expression of efflux pumps such as Cdr1p, Cdr2p, and Mdr1p (1, 32, 42, 48, 49, 53). Therefore, in this study, we investigated the role of efflux pumps in the antifungal resistance of *C. albicans* biofilms. We determined the antifungal susceptibilities of biofilms formed by mutants carrying single, double, or triple deletion mutations of the *CDR* and *MDR1* genes. Our results showed that, at the early phase of develop-

ment, biofilms formed by these mutants were more susceptible to fluconazole than those formed by the wild-type strain. However, among the mutants, the triple-knockout strain was the most susceptible (MIC = 16 μg/ml), indicating the involvement of efflux pumps in the azole resistance of early-phase biofilms. Interestingly, at later developmental phases (12 and 48 h), biofilms formed by the mutants displayed complete resistance to fluconazole (MIC ≥ 256 μg/ml), similar to those formed by the wild-type parent strain. These results indicate that efflux pumps contribute to azole resistance in the early phase of biofilm formation but not in the later phases. To further investigate the role of efflux pumps in azole resistance, we determined the levels of *CDR* and *MDR1* gene mRNA in biofilms formed by the wild-type strain. Our results clearly showed that expression of *CDR* genes is temporally regulated during *C. albicans* biofilm formation, with higher levels of gene transcripts detected in early- and intermediate-phase biofilms than in planktonic cells. Interestingly, the *CDR* and *MDR1* genes were expressed at all developmental phases in *C. albicans* biofilms but only after 12 h in planktonic cells.

Using a different model of biofilm formation, Ramage et al. (43) recently reported that efflux pumps including Cdr1p, Cdr2p, and Mdr1p are not involved in *C. albicans* biofilm-associated drug resistance. Our results are in agreement with those of Ramage et al. (43) regarding the role of efflux pumps in the drug resistance of mature *C. albicans* biofilms. However, these investigators examined the role of efflux pumps only at 24 and 48 h, at which times the biofilms were already completely formed. We have previously shown that *C. albicans* biofilms pass through three distinct developmental phases: early (6 h), intermediate (12 h), and mature (48 h) (9). In the present study, we investigated the phase-dependent expression of the *CDR* and *MDR1* genes during biofilm formation and demonstrated that efflux pumps contribute to candidal resistance only at the early phase. Our data demonstrate that it is prudent to examine biofilm-related processes, including resistance, at all three phases of biofilm development. Another difference between our study and that reported previously (43) is that we employed triple-knockout mutants in addition to single and double mutants, which were more susceptible to fluconazole than the wild type at the 6-h growth phase. These studies further confirmed the role of efflux pumps in azole resistance during the early phase of biofilm development.

Antifungal susceptibility assays revealed that biofilms formed by mutants lacking the *CDR* and *MDR1* genes were resistant to fluconazole (MIC ≥ 256 μg/ml) at intermediate and mature phases. However, Northern blot analyses showed that, during these phases, *C. albicans* biofilms expressed the *CDR* and *MDR1* genes at the mRNA level. One possible reason for this discordance could be that mRNA expression at these phases is not translated into corresponding functional proteins. In this regard, previous investigations have shown that gene expression at the mRNA level is not always correlated at the functional protein level and that it is not possible to deduce protein levels from transcript analyses (20). Therefore, we decided to perform functional analyses of efflux pump proteins using the previously described Rh123-based method (12). In this assay, lower retention of Rh123 by cells indicates higher pump activity, since Rh123 retention is a measure of drug resistance mediated by efflux pumps. Our results showed that, at 6 h, biofilms have less Rh123 retention than planktonic cells, indicating that the former is more resistant to the drug at this time point. This correlated well with our Northern blot analyses, which showed expression of *CDR* and *MDR1* genes at 6 h in biofilms but not in planktonic *C. albicans* cells. Subsequent reductions in Rh123 retention (indicating increased pump activity) at 12 and 48 h also correlated with the Northern blot analysis results. Therefore, these studies revealed that efflux pump proteins are functional in intermediate- and mature-phase biofilms, although they do not seem to play a role in azole resistance at these developmental phases.

Since efflux pumps did not appear to contribute to the azole resistance of *C. albicans* biofilms at intermediate and mature phases, we decided to investigate whether the observed resistance is due to changes in membrane integrity mediated by variations in sterol composition. The effect of sterols on the fluidity and asymmetry of the membrane has a significant effect on the sensitivity and resistance of *C. albicans* cells to antifungals (19, 28). Hitchcock et al. (22) earlier showed that a *C. albicans* strain which is resistant to both polyene and azole groups of antifungal antibiotics had a larger lipid content and lower polar lipid-to-neutral lipid ratio than other strains. The main distinctive feature of the lipid composition of this *C. albicans* strain was the absence of ergosterol, which was replaced by methylated sterols, mainly lanosterol, 24-methylene-24,25-dihydrolanosterol, and 4-methylergostadiene-3-ol. These investigators suggested that the altered membrane sterol pattern provided a common basis for antifungal resistance by preventing the binding of a drug and/or reducing its permeability (22). Recent reports also suggest that changes in the status of the membrane lipid phase and asymmetry could contribute to azole resistance in *C. albicans* (27, 36). Moreover, microarray analyses revealed that the *ERG2* (encoding the ergosterol biosynthesis pathway enzyme C-8 sterol isomerase) and *CDR1* genes are upregulated in a planktonically grown fluconazole-resistant strain of *C. albicans* (46). Therefore, we reasoned that changes in sterol patterns may also play a role in biofilm-associated drug resistance.

Sterol analyses of *C. albicans* biofilms and planktonic cells grown to different time points revealed that, at 6 h, biofilm and planktonic *C. albicans* had similar ergosterol levels. However, at later stages, the ergosterol level of the biofilm was significantly less than that of planktonic cells. During biofilm development, the ergosterol level was reduced by 41% at intermediate phase and by 50% at the mature phase, compared to that for early-phase (6-h) biofilms. Moreover, levels of other intermediate sterols, such as zymosterol, 4,14-dimethylzymosterol, obtusifoliol, in the biofilm and planktonic *C. albicans* were also significantly altered. Notably, differences in sterol profiles were more pronounced at 12 and 48 h, the same growth phases when efflux pumps do not seem to have any role on drug resistance. These results suggest that membrane sterol composition is a critical component of biofilm-associated azole resistance at the intermediate and mature phases. It is possible that there exists a threshold level of ergosterol that contributes to resistance. Changes in sterol profile may lead to altered membrane permeability and hence prevent or retard the entry of antifungal agents into candidal cells. Altered sterol levels can also influence fluconazole resistance indirectly i.e., mediated by a cell wall protein or lipid.

In conclusion, we present evidence that, while efflux pumps play a critical role in azole resistance in early-phase biofilms, alteration in sterol composition is an important mechanism of antifungal resistance at the intermediate and mature phases of biofilm formation. To the best of our knowledge, and based on the current literature available, these results demonstrate for the first time that antifungal resistance in *C. albicans* biofilms is a developmental-phase-specific multifactorial phenomenon.

ACKNOWLEDGMENTS

We thank Ryan Munyon, Eric Bugyris, Katie Krepkowich, and Maher Balkis for technical assistance.

This work was supported by the Center for AIDS Research at Case Western Reserve University/University Hospitals of Cleveland (AI-36219), and the National Institutes of Health (RO1-DE13992 and 1RO1DE13932-01A1) to M.A.G.

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Editor: T. R. Kozel