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Adherence of *Candida albicans* to Silicone Induces Immediate Enhanced Tolerance to Fluconazole

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Wild-type and efflux pump-deficient cells of *Candida albicans* adhering to silicone were compared with planktonic cells by flow cytometry for their relative resistance to fluconazole (FCZ). Flow cytometry data on cells carrying a fusion of green fluorescent protein to efflux pump promoters confirmed that enhanced tolerance of attached cells to FCZ was due in part to increased expression of *CaMDR1* and *CDR1* promoters. Within 2 h of their attachment to silicone, the adherent cells demonstrated levels of FCZ tolerance shown by cells from 24-h biofilms. Following their mechanical detachment, this subset of cells retained a four- to eightfold increase in tolerance compared with the tolerance of planktonic cells for at least two generations. Enhanced efflux pump tolerance to FCZ appeared to be induced within the initial 15 min of attachment in a subset of cells that were firmly attached to the substrata.

Candida albicans is an adventitious pathogen commonly found as a commensal in healthy individuals (29). *C. albicans* produces a broad range of serious illnesses in immunocompromised hosts (21), being the fourth most common cause of nosocomial infections, usually arising from indwelling devices (3, 27). Medical implants provide adequate substrata for yeast attachment and biofilm formation and are associated with a high incidence of systemic candidiasis (3, 5, 7, 14).

Chandra et al. (3) reported that biofilms of *C. albicans* formed on polymethylmethacrylate and silicone elastomer disks had a highly heterogeneous architecture composed of cellular and noncellular elements. Antifungal resistance of the biofilm compared to that of planktonic cells increased in conjunction with three stages of biofilm formation, 0 to 11 h, 12 to 30 h, and ~38 to 72 h. The progression in drug resistance was associated with a concomitant increase in metabolic activity (tetrazolium reduction) of the developing biofilm. Biofilm structure, particularly on polymethylmethacrylate, was biphasic, being composed of an adherent blastospore layer covered by more sparse hyphal elements. In the later biofilm stages on silicone elastomer disks, the hyphal elements pervaded the matrix.

Hawser and Douglas (12) reported that the MIC of amphotericin B was up to 200 times higher for biofilms of *C. albicans* than for those of planktonic cells. Similarly, Kalya and Ahearn (15) reported higher MICs and minimal fungicidal concentrations of amphotericin B, miconazole, ketoconazole, fluconazole (FCZ), and itraconazole for cells of *C. albicans* adhering to silicone for 2 h than for planktonic cells. The protective nature of the biofilm exopolymeric matrix has been proposed as a mechanism for biofilm resistance (2); nevertheless, cells of *Candida* spp. adhering to silicone or polyethylene for as little as 2 h show less susceptibility to antimicrobial agents than do planktonic cells (15, 17).

FCZ has been the antifungal agent of choice for the treatment of mucosal and systemic *C. albicans* infections. The wide use of FCZ for the treatment of *C. albicans* infections and other mycoses is now being hindered by the appearance of resistant strains. Approximately 15 to 30% of oral candidiasis infections in patients with advanced human immunodeficiency virus infections are produced by clinically resistant strains of *C. albicans* (31). The most prevalent group of resistance mechanisms found in *C. albicans* targets the reduction of FCZ accumulation through the increased transcription of the multiple drug resistance transport protein-encoding genes *CDR1*, *CDR2*, and *CaMDR1* (4, 31).

Most studies of FCZ resistance have involved planktonic cells. Studies of fungal biofilms usually involve mature biofilms, and sparse information is available on the physiology and phenotype of fungal cells in the initial stages of biofilm formation. Both bacterial and fungal studies have revealed that attachment to a surface can either stimulate or repress the expression of genes directly involved in the attachment process (30). The goals here were to examine the initial stages of biofilm formation, i.e., firmly adherent cells of *C. albicans* (prior to cell division or pseudohypha formation), and to determine whether adhesion to a surface induces changes in the expression of genes that enhance survival in the presence of FCZ. Enhanced expression of multidrug efflux pumps has been reported for *Pseudomonas aeruginosa* biofilms (1, 6) and for *Magnaporthe grisea* adhering to plant tissue (28). Recently, Mukherjee et al. (20) extensively reviewed the mechanisms of FCZ resistance and reported that *C. albicans* expresses *CDR* and *MDRI* genes in all three stages of biofilm development, including analyses performed within 6 h.

The present study, with FCZ-susceptible strains of *C. albicans*, confirms that the capacity for increased FCZ tolerance in adherent cells is at least partially efflux pump related. Evidence is presented that this increased activity is induced upon attachment and is not necessarily a consequence of mature biofilm formation.

MATERIALS AND METHODS

Strains, cell growth, and maintenance. *C. albicans* CaI4 (Δ*ura3*::*imm434*/Δ*ura3*::*imm434*), courtesy of B. Lasker, Centers for Disease Control and Prevention, Atlanta, Ga., served as the parental strain for the generation of strains containing yEGFP (yeast-optimized fluorescence-activated cell sorting-

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TABLE 1. Genotypes of *C. albicans* strains

Strain	Genotype	Reference or source
Ca30	Wild type	14
CaI4	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	9
DSY449	Δ*cdr1*::*hisG*/Δ*cdr1*::*hisG*	25
DSY465	Δ*camdr1*::*hisG-URA3-hisG*/Δ*camdr1*::*hisG* Δ*cdr1*::*hisG*/Δ*cdr1*::*hisG*	25
DSY468	Δ*camdr1*::*hisG-URA3-hisG*/Δ*camdr1*::*hisG*	25
DSY653	Δ*cdr2*::*hisG-URA3-hisG*/Δ*cdr2*::*hisG*	26
DSY654	Δ*cdr1*::*hisG*/Δ*cdr1*::*hisG* Δ*cdr2*::*hisG-URA3-hisG*/Δ*cdr2*::*hisG*	26
CaCMR4	Δ*ura3*::*imm434*/Δ*ura3*::*imm434 CDR4*/Δ*cdr4*::*CaMDR1prom-GFP-ACT1t-URA3*	This study
CaCMR5	Δ*ura3*::*imm434*/Δ*ura3*::*imm434 CDR4*/Δ*cdr4*::*ACT1prom-GFP-ACT1t-URA3*	This study
CaCMR7	Δ*ura3*::*imm434*/Δ*ura3*::*imm434 CDR4*/Δ*cdr4*::*CDR1prom-GFP-ACT1t-URA3*	This study
CaCMR8	Δ*ura3*::*imm434*/Δ*ura3*::*imm434 CDR4*/Δ*cdr4*::*CDR2prom-GFP-ACT1t-URA3*	This study

enhanced green fluorescent protein [GFP]). In addition, *CaMDR1*, *CDR1*, and *CDR2* knockout strains were donated by D. Sanglard (Institut de Microbiologie, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland). *C. albicans* wild-type strain Ca30 (15) and clinical isolate JK21 (courtesy of A. Karlowsky, Department of Clinical Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada) were used as FCZ-susceptible and FCZ-resistant controls, respectively. Table 1 shows a description of the strains used in this study.

Cultures were grown at 30°C and 130 rpm in glucose yeast nitrogen base medium (GYNB; Bio 101, Carlsbad, Calif.) without amino acids but supplemented with complete supplement mixture lacking uracil (Bio 101) and 2% glucose (Fisher Scientific, Pittsburg, Pa.), except for *C. albicans* CaI4, which required supplementation with 40 μg of uridine (Sigma Chemical Co., St. Louis, Mo.)/ml. Starter cultures were subcultured to mid-logarithmic phase (optical density at 600 nm [OD_{600}], 1.0; equivalent to $\sim 10^7$ cells/ml), and the subcultures in turn served as inocula for all experiments.

Strain design. Primer sequences and annealing sites are shown in Table 2. yEGFP was used as a reporter of transcription from the *CaMDR1*, *CDR1*, and *CDR2* promoters. A segment from plasmid pGFP41 (18) containing yEGFP, a 0.4-kb fragment of the 3′ untranslated region of the *C. albicans* actin gene (*ACT1*), and the regulatory and coding regions of the orotidine 5′-monophosphate decarboxylase gene (*URA3*) was PCR amplified with primers GFPf and URA3r. Plasmid pCMR1 was constructed by cloning this segment into pBluescript II KS(−) (Stratagene, La Jolla, Calif.) by using the restriction enzymes ClaI and PstI. Two 2-kb segments of the *CDR4* gene (bases −339 to +1778 and +2366 to +4471 from the origin) were PCR amplified with primers 5′CDR4f and 5′CDR4r, primers 3′CDR4f and 3′CDR4r, and *C. albicans* CaI4 genomic DNA as the template. The enzymes KpnI and XhoI were used to clone the 5′ CDR4 fragment (2.1 kb) upstream of the GFP gene in pCMR1, resulting in the generation of plasmid pCMR2 (7.3 kb). The enzymes HpaI and SacI were used to clone the 3′ CDR4 fragment (2.1 kb) downstream of the 3′ end of *URA3* in pCMR2, resulting in plasmid pCMR3. The latter plasmid served as the vector backbone for cloning the promoter regions of the *CaMDR1* (−980 to −1), *CDR1* (−1200 to −1), *CDR2* (−883 to −1), and *ACT1* (−1027 to −1) genes. Each PCR product was digested with XhoI and ClaI and ligated to similarly digested plasmid pCMR3, resulting in the generation of plasmids pCMR4 (*CaMDR1* promoter), pCMR5 (*ACT1* promoter), pCMR7 (*CDR1* promoter), and pCMR8 (*CDR2* promoter).

$CaCl_2$-competent *Escherichia coli* DH5α was plated on Luria-Bertani medium supplemented with ampicillin (Sigma) to 100 μg/ml after the transformation procedure. The presence of the correct ligation product was confirmed through restriction enzyme analysis.

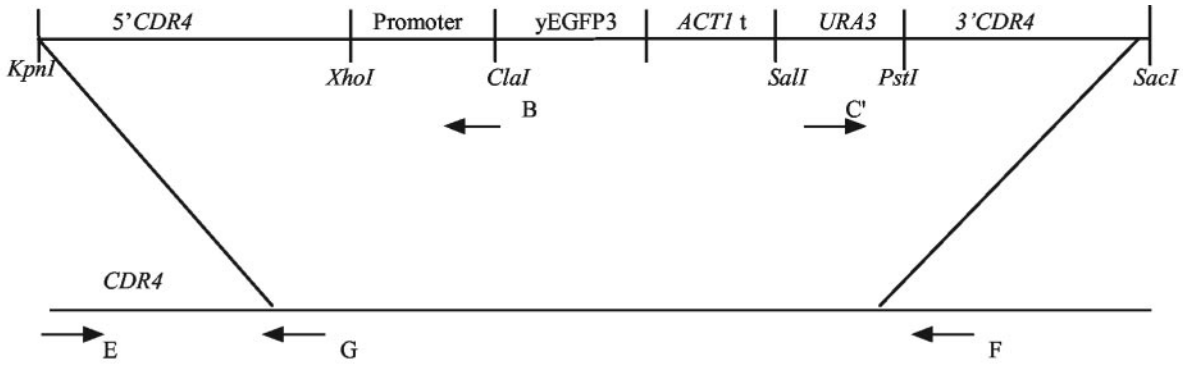
The promoter constructs present in pCMR4, pCMR5, pCMR7, and pCMR8 were PCR amplified with primers 5′CDR4for and 3′CDR4rev (Table 2) and inserted in one of the *CDR4* alleles of *C. albicans* CaI4 cells through double homologous recombination. Cells were transformed by a modification (25) of the lithium acetate procedure described by Gietz and Woods (10) for *Saccharomyces cerevisiae*, plated on GYNB, and incubated for 4 days at 30°C. Accurate integration of promoter fusion constructs in only one allele of the *CDR4* gene was accomplished by PCR (Fig. 1).

The mean fluorescence intensity emitted by yEGFP (FL1) was analyzed for each strain by flow cytometry after chemical induction of the expression of each promoter. Cells grown overnight in GYNB to a final density of 10^7 cells/ml were

TABLE 2. Primer sequences

Name	Sequence[a]	Annealing site	Restriction enzyme
GFPf	5′-GGG *ATCGAT* ATGAGTAAAGGA-3′	+1 (GFP)	ClaI
URA3r	5′-AAA *CTGCAG* AAGGACCACCTTT-3′	+924 (*URA3*)	PstI
5′CDR4f	5′-CCC *GGTACC* GGCTAGCAGTTTGAG-3′	−325 (*CDR4*)	KpnI
5′CDR4r	5′-GG *CTCGAG* CGCATCTGCTGCAGG-3′	+1764 (*CDR4*)	XhoI
3′CDR4f	5′-CACCTGCAGGGGTGCAATGCA-3′	+2366 (*CDR4*)	None
3′CDR4r	5′-CCC*G AGCTC* CAACGTTTACGTCT-3′	+4458 (*CDR4*)	SacI
MDR1pf	5′-CAA *CTCGAG* CACACAGCCGTGAATCTTA-3′	−5421 (*CaMDR1*)	XhoI
MDR1pr	5′-CCC *ATCGAT* GTGAAGTTCTATGTAAGTAGATG-3′	−1 (*CaMDR1*)	ClaI
CDR1pf	5′-CC *CTCGAG* GTTACTCAATAAGTATTAA-3′	−1182 (*CDR1*)	XhoI
CDR1pr	5′-CC *ATCGAT* AATTTTTTTCTTTTTGACC-3′	−1 (*CDR1*)	ClaI
CDR2pf	5′-CC *CTCGAG* ACTAGAAGGTTATCAAGA-3′	−866 (*CDR2*)	XhoI
CDR2pr	5′-CCC *ATCGAT* ATGTTTTTATTGTATGTG-3′	−1 (*CDR2*)	ClaI
ACT1pf	5′-CC *CTCGAG* AGAGCTATTAAGATCACCAG-3′	−1017 (*ACT1*)	XhoI
ACT1pr	5′-CC *ATCGAT* TTTGAATGATTATATTTTTTT-3′	−1 (*ACT1*)	ClaI
E	5′CCACATCAGGAGATTATTCGAGCT-3′	−399 (*CDR4*)	None
B	5′-ACATCACCATCTAATTCAACAAG-3′	+42 (GFP)	None
C′	5′-TTTCCTATGAATCCACTATTGAACC-3′	+208 (*URA3*)	None
F	5′-TCCCTGATAGCACTTGGCATTTAT-3′	+4630 (*CDR4*)	None
G	5′-GGAGTTCTTCGGAAATTGACCATAAAA-3′	+1847 (*CDR4*)	None

[a] Recognition sequences used for the ligation of PCR products are shown in italic type. Sequences correspond to genes of *C. albicans*, except for the yEGFP gene from *Aqueroea victoria* (19) and *hisG* for *Salmonella enterica* serovar Typhimurium ATP phosphoribosyltransferase (9, 26).



E (422 to - 399 of *CDR4*) B (+64 to +42 of yEGFP3) = 3.3 Kb in mutants
no product in WT

C' (+208 to +231 of *URA3*) + F (+4653 to +4630 of *CDR4*) = 2.9Kb in mutants
no product in WT

E 422 to -399 of *CDR4*) G (+1874 to +1847 of *CDR4*) = 2.3Kb in WT and in mutants from the
intact allele

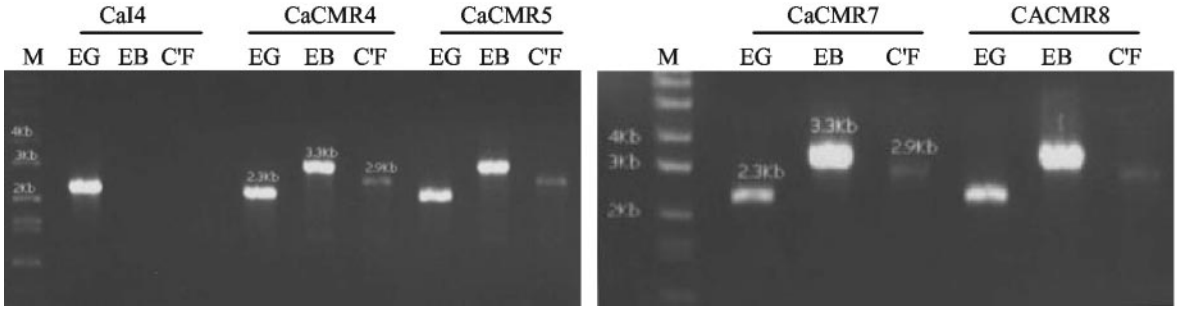


FIG. 1. Confirmation of chromosomal integration by PCR. Primers E, B, C′, and F were used to confirm the integration of the promoter fusion constructs into one of the *CDR4* alleles of CaI4. A 10-μl volume of PCR mixture and a 10-μl volume of Hi-Lo DNA molecular weight marker (Minnesota Molecular Inc., Minneapolis, Minn.) (lanes M) were loaded on a 2% agarose gel and run at 80 V for 2 h. Strains, primer sets, and band sizes are indicated. WT, wild type.

incubated for 1 h at 30°C and 130 rpm in the presence of 10 μg of benomyl/ml, 1 mM β-estradiol, or 1 mg of fluphenazine (Sigma)/ml.

Adherence. Cells were allowed to adhere to substrata with modifications of the "primary adhesion" test described by Ahearn et al. (1). Cells for inocula were grown in GYNB to mid-exponential phase and harvested by centrifugation at 3,000 × *g* for 10 min. The pellets were washed once in sterile 0.9% NaCl and suspended in sterile phosphate-buffered saline (PBS) to a final density of 0.5×10^8 to 1×10^8 cells/ml. Flat sterile medical-grade silicone disks, with a total surface area of 312.5 mm^2, were incubated in 3.0 ml of cell suspension for 2 h at 30°C and 100 rpm. After the prescribed adherence period, each silicone disk was rinsed by dipping five times in each of three changes of 180 ml of sterile saline (0.9% NaCl). Rinsed disks with adherent yeast cells then were used for subsequent experiments. The cells retained on the disks were singular, some with attached buds, but without germ tubes or hyphal forms, and always were present at <10% the initial density of cells per milliliter in the inoculum (per square millimeter).

The number of adherent cells per disk was determined by a modified radiolabeled-cell procedure (1, 18). In separate experiments with each strain, cell inocula prepared as described above and washed in PBS were inoculated into GYNB at a final concentration of 1×10^8 to 2×10^8 cells/ml and incubated for 1 h at 30°C and 130 rpm. Following incubation, the cells were radiolabeled with a final concentration of 2.5 μCi of L-[3,4,5-^{3}H]leucine (NEN Research Products, Dupont Company, Wilmington, Del.)/ml for 30 min. Radiolabeled cells were washed three times in sterile saline, suspended in PBS to a final density of 10^8 cells/ml, and allowed to adhere for 2 h. Disks rinsed as described above and with firmly adherent cells were transferred to glass scintillation vials containing 10 ml of OptiFluor scintillation cocktail (Packard Instrument Co., Downers Grove, Ill.), and the disintegrations per minute were read in a liquid scintillation counter (LS-7500; Beckman Instruments, Inc., Fullerton, Calif.) for 1 min per disk. The number of adherent cells per disk was determined by extrapolation of the disintegrations per minute obtained from each silicone disk to a nomograph correlating disintegrations per minute produced by known cell numbers. The data were normalized for any nonspecific background radiation from the disks.

Biofilm formation. Nonradiolabeled cells firmly adhering to silicone were transferred to fresh GYNB and incubated for an additional 24 to 48 h at 30°C and 100 rpm. A biofilm was observed on the silicone disks even when growth in the surrounding medium was sparse. Cells released from the biofilm and growing in surrounding GYNB are henceforth referred to as daughter cells, and cells

retained on the disks are referred to as biofilm cells. Disks with biofilms were rinsed as described above for the preparation of adherent cells.

Detachment. Silicone disks with adherent cells, rinsed in PBS for the removal of loosely associated cells as described above, were placed in 1.0 ml of sterile PBS and subjected to five cycles of a two-step process consisting of 1 min of sonication at 75 Hz (Bransonic Ultrasonic Cleaner 2210; Branson Ultrasonic Co., Danbury, Conn.) followed by 30 s of vigorous vortexing. Detachment efficiency was determined from the reduction in radioactivity from the disks after the detachment process.

FCZ susceptibility. Mid-exponential-phase planktonic cells (10^6 cells/ml), adherent cells (about 10^6 cells/312.5 mm^2), 24-h biofilm cells (1×10^6 to 2×10^6 cells/312.5 mm^2), detached cells after adherence for 2 h (0.8×10^6 cells/ml), and biofilm daughter cells from 24- and 48-h biofilms were incubated in fresh medium at 30°C under static conditions for 4 h in the presence of FCZ. The FCZ concentrations ranged from 0.125 to 128 μg/ml in twofold increments. After FCZ challenge, the cells were harvested, suspended in sterile PBS, and stained for 1 min with 0.025 mg of propidium iodide (PI)/ml and 25 mM sodium deoxycholate. Exposure of cells to FCZ results in an increase in membrane permeability, which in turn allows for labeling of the cells with PI. Representative experiments by Ramani et al. (23) and Ramani and Chaturvedi (24) showed that cellular permeability to PI, as determined by flow cytometry, correlated with recoverable cell numbers and with the results of the NCCLS broth microdilution method. Based on similar experiments, we selected the lowest FCZ concentration at which at least 90% of the cells in the FCZ-exposed population fluoresced following exposure to PI (mean fluorescence intensity at 620 nm [FL2] above the 1,000 electronic peak channel) as the minimal permeation concentration (MPC). The MPCs coincided with the MICs of FCZ established for the wild-type strain by standard procedures. Different cell populations were compared on the basis of their MPCs. The FL2 emitted by 10,000 cells was recorded and analyzed with CELLQuest software on a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany). Electronic gates were set up based on live and heat-killed cells, excluding cell debris. The instrument was calibrated before each experiment according to the manufacturer's instructions. Viable cells, heat-killed cells, cells after the detachment procedure, cells exposed to sodium deoxycholate, and cells exposed to both PI and sodium deoxycholate were used as controls.

In separate experiments, mid-exponential-phase planktonic cells (10^6 cells/ml) and adherent cells (10^6 cells/disk) of CaI4 were incubated at 30°C and 130 rpm for 30 min in fresh GYNB supplemented with 10.24 μM rhodamine 123 (Rh123; Sigma). After the incubation period, cells were washed twice in PBS, and the mean fluorescence emitted at 530 nm by intracellular Rh123 was read by flow cytometry.

Adhesion and yEGFP expression. yEGFP expression in planktonic cultures of CaI4 (parental strain), CaCMR4, CaCMR7, and CaCMR8 was confirmed. Cells (10^6/ml) were incubated in the presence of 10 μg of benomyl/ml, 1 mM β-estradiol, or 1 mg of fluphenazine/ml for 1 h at 30°C and 130 rpm. The mean fluorescence intensity (FL1) emitted by 10,000 cells per sample was quantified by flow cytometry with CELLQuest software. Cultures incubated under the same conditions but without the drug served as a control.

The effect of adhesion on the expression of GFP from the different promoters was analyzed by flow cytometry and epifluorescence microscopy. For flow cytometric analysis, disks with adherent cells were incubated for 4 and 24 h at 30°C and 100 rpm and, after detachment, the mean fluorescence intensity emitted by 10,000 cells per sample was quantified by flow cytometry. Fluorescence was determined for planktonic cells prior to attachment and for daughter cells from 24- and 48-h biofilms.

For epifluorescence microscopy analysis, the cells were allowed to adhere to one side of glass coverslips (diameter, 12 mm; thickness, 0.17 mm), and the fluorescence emitted by cells at different time points after the onset of adherence (15 min, 30 min, 1 h, 2 h, 4 h, and 6 h) was analyzed and compared to that emitted by planktonic cells. The laser scanning confocal microscope (Zeiss LSM510) was equipped with a ×40 F Fluor oil immersion objective, a 100-W Hg vapor arc lamp as an excitation source, a B-2E/C fluorescein isothiocyanate filter block (excitation wavelength, 465 to 495 nm; barrier filter wavelength, 515 to 555 nm), and Zeiss LSM software.

Effect of growth stage on yEGFP expression. Cells were grown in GYNB to late lag phase (OD_{600}, 0.1); until stationary phase was reached, 3 samples of 1.0 ml each were taken from each culture, washed once with saline, suspended in PBS, and analyzed by flow cytometry. Progression through the different growth stages was determined by measuring changes in the OD_{600} with a Turner SP-830 spectrophotometer (Barnstead/Thermolyne, Dubuque, Iowa).

Statistical analyses. All assays were performed at least in triplicate, and the standard error (SE) for each set of data was determined. Means were compared by using the Student *t* test (Microsoft Excel 2000) ($P < 0.01$).

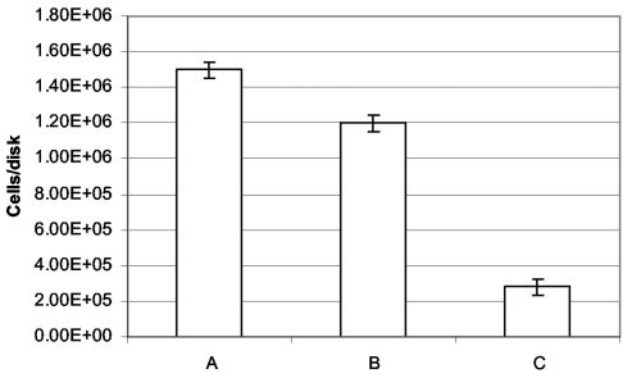


FIG. 2. Quantification of *C. albicans* adherence and detachment. *C. albicans* Ca30 was allowed to adhere to medical-grade silicone disks (312.5 mm^2) for 2 h at 30°C with shaking at 100 rpm. Bars denote the total numbers of adherent cells (A), detached cells (B), and cells retained on disks after detachment (C). Data are the average of three independent experiments. Error bars represent variations between those experiments (~4.5×10^4 cells/disk).

RESULTS

Adherence of *C. albicans* to medical-grade silicone disks. Maximal and consistent adherence of *C. albicans* to medical-grade silicone disks was obtained with 3 ml of cell suspension (10^8 cells/ml) after 2 h at 30°C with shaking at 100 rpm. The average number of adherent Ca30 cells was 1.5×10^6 cells/disk (4.8×10^3 cells/mm^2) (Fig. 2). Mutant strains (CaI4, transport knockout mutants, and yEGFP mutants) (Table 1), except for JK21, which showed negligible adherence, achieved comparable adherence levels.

More than 80% of the wild-type and mutant cells (i.e., ~1.0×10^6 cells) were recovered after the detachment procedure. Microscopic examination confirmed that a sparse cell population was retained on the silicone disks after the detachment process. The average percentage of cells showing an FL2 value (permeation by PI) above the 1,000 electronic peak channel after the detachment procedure was 8.5% ($n = 3$, SE = 0.49); the corresponding FL2 value for nonsonicated planktonic cells in mid-exponential cultures was 8.9% ($n = 3$, SE = 0.58). Thus, the effect of sonication on cell membrane integrity was considered nonsignificant ($P > 0.1$).

FCZ susceptibility testing. Over 90% of total planktonic cells of *C. albicans* Ca30 and CaI4 were susceptible to FCZ at 1 μg/ml. In contrast, only 11% of cells of the heterologous resistant control JK21 were permeated when incubated with FCZ at 128 μg/ml (Table 3). In only 6 h (i.e., 2 h of adherence and 4 h of FCZ challenge in an adherent state), Ca30 and CaI4 cells showed a 100-fold increase in tolerance to FCZ compared to planktonic cells. This increase in tolerance was not related to a decrease in drug diffusion into the cell. The difference between the intracellular levels of Rh123 in planktonic cells (2,216 ± 118) and adherent cells (2,434 ± 242), measured as the average fluorescence (arbitrary units; logarithmic scale) emitted at 530 nm by 10,000 cells in three independent experiments, was statistically nonsignificant ($P > 0.1$). *C. albicans* Ca30 biofilms (after 24 h of incubation) as well as CaI4 biofilms of the same age had the same relative levels of FCZ tolerance ($P > 0.1$) as adherent cells of the same strains (Table 3). After

TABLE 3. Effect of FCZ on permeation of *C. albicans* cells

Population[a]	MPC (μg of FCZ/ml)	% Permeated cells[b]
Planktonic JK21 cells	128	11
Planktonic Ca30 cells[c]	1	95
Planktonic CaI4 cells[c]	1	94
Adherent Ca30 cells[c]	128	50
Adherent CaI4 cells[c]	128	48
24-h Ca30 biofilms[c]	128	43
24-h CaI4 biofilms[c]	128	39
Detached Ca30 cells	4	91
Detached CaI4 cells	4–8	92
24-h daughter Ca30 cells	2	98
24-h daughter CaI4 cells	2	96
24-h planktonic Ca30 cells	1–2	94
24-h planktonic CaI4 cells	2	96
48-h daughter Ca30 cells	1–2	96
48-h daughter CaI4 cells	2–4	91
48-h planktonic Ca30 cells	1–2	90
48-h planktonic CaI4 cells	4	94

[a] The permeation of *C. albicans* cells by FCZ was evaluated with planktonic cells, attached cells, mature biofilms, and biofilm daughter cells.
[b] Percentage of cells with a mean fluorescence intensity at 620 nm (FL2) of greater than 1,000 at the indicated MPC.
[c] Unlike planktonic cells, adherent and biofilm cells were subjected to mild sonication prior to flow cytometric analysis; nonetheless, adherent and biofilm cells showed less than 50% the permeation shown by plantonic cells.

being allowed to adhere for 2 h, detached, and then kept in a planktonic state for 4 h, Ca30 and CaI4 cells showed significantly decreased tolerance to FCZ, but the tolerance was still four- to eightfold greater than that of the initial planktonic population (Table 3). Retained tolerance to FCZ was also observed by Ramage et al. (22) for cells mechanically detached from a mature (24-h) biofilm. The FCZ tolerance of daughter cells from 24- and 48-h biofilms was similar to that of planktonic cells of the same age and significantly less than that of cells firmly adhering to silicone disks for less than 6 h (Table 3).

Planktonic and adherent null mutant cells, except for those of DSY653, were significantly more susceptible to FCZ than wild-type cells (Ca30) (Table 4). Single and double mutations of the *cdr1*, *cdr2*, and *mdr1* genes do not seem to affect the resistance to FCZ of mature biofilms (22). In contrast, a 16-fold decrease in the susceptibility of the Δ*camdr1* strain and a greater than 500-fold decrease in the susceptibility of the Δ*cdr1* strain were observed for adherent cells versus planktonic cells.

TABLE 4. Concentration of FCZ necessary for permeation of 90% of adherent *C. albicans* cells

Strain	FCZ concn, μg/ml, required for permeation of the following cells[a]:	
	Planktonic	Primary adherent
Ca30 (wild type)	1	128 (59)
DSY465 (Δ*camdr1*)	0.5	8
DSY449 (Δ*cdr1*)	0.125	64
DSY468 (Δ*camdr1* Δ*cdr1*)	0.0625	1
DSY653 (Δ*cdr2*)	1	128 (79)
DSY654 (Δ*cdr1* Δ*cdr2*)	0.125	64

[a] When permeation of 90% of the cells was not achieved at the maximum FCZ concentration tested, the percentage of permeated cells achieved at that concentration is shown in parentheses.

Adherent cells lacking the *CaMDR1* gene were susceptible to FCZ at levels eightfold lower than those to which cells lacking the *CDR1* gene were susceptible (64 and 8 μg/ml, respectively). The effect of the mutations was so extensive that cells of adherent double mutants (Δ*camdr1* Δ*cdr1*) showed susceptibility to FCZ similar to that of planktonic cells (CaI4). However, cells lacking the *CDR2* gene were not affected in their ability to respond to FCZ challenge. The FCZ susceptibility of DSY653 and DSY654 cells resembled that of wild-type and DSY449 cells, respectively, in both the planktonic and the adherent states (Table 4).

Promoter expression. Basal expression of the *CaMDR1* promoter (CaCMR4 cells) was only 1.5 times higher than that in CaI4 cells (parental strain). After promoter expression was induced with benomyl, CaCMR4 cells emitted a mean fluorescence intensity (FL1) 11 times higher than that emitted by parental cells and 7 times higher than that emitted in the absence of benomyl induction. The difference in expression from the *CaMDR1* promoter in the presence and in the absence of FCZ was statistically significant ($P < 0.001$). Basal *CDR1* expression (CaCMR7 cells) was also 1.5-fold higher than the autofluorescence emitted by parental cells. After induction with β-estradiol, *CDR1* expression increased up to 16-fold from its basal state and was 21-fold higher than that of parental cells. This increase was time dependent and, for up to 60 min, represented a linear increase in fluorescence yield per unit of time. The basal expression of *CDR2* was 1.65-fold higher than the autofluorescence emitted by parental cells. After promoter induction with fluphenazine, the relative fluorescence emitted by CaCMR8 cells was 5.2-fold higher than that emitted in the absence of the inducing agent. This difference in expression was statistically significant ($P < 0.001$) (Table 5).

Growth stage and yEGFP expression. As cells progressed through the different growth stages, the activity of the *CaMDR1*, *ACT1*, and *CDR2* promoters remained stable (Fig. 3). Expression from the *CDR1* promoter showed a higher degree of variation, decreasing as the culture progressed from lag to logarithmic phases and steeply increasing as the culture left logarithmic phase and entered stationary phase (Fig. 3). These results were consistent with those found by Harry et al. (11) and Krishnamurthy et al. (16) with RNA slot blots.

Effect of adhesion on the expression of genes coding for efflux transport proteins. Expression from the *CaMDR1*, *CDR1*, and *CDR2* promoters was greater in adherent cells than in planktonic cells (Table 6). *CaMDR1* and *CDR2* expression was approximately twofold higher and *CDR1* expression was fivefold higher in adherent cells versus planktonic cells. The fluorescence emitted by CaI4 (parental strain) and that emitted by CaCMR5 were similar for adherent cells and planktonic cells.

Additionally, epifluorescence microscopy analysis of *C. albicans* strains CaCMR4 and CaCMR7 adhering to glass microscope slides showed that upregulation of these efflux pump promoters occurred as early as 15 to 30 min after adhesion (Fig. 4). Planktonic cells of these strains and both planktonic cells and adherent cells of CaI4 did not fluoresce (Fig. 4).

The expression from the *CaMDR1*, *CDR1*, and *CDR2* promoters in daughter cells of 24-h biofilms and in cells that were detached after an initial attachment period of 2 h and allowed

TABLE 5. Relative fluorescence of *C. albicans* cells before and after induction of efflux pump expression

Strain	Mean ± SD fluorescence intensity (FL1)[a] (arbitrary units; logarithmic scale) for cells given the following treatment:			
	None (control)	Benomyl (10 μg/ml)	β-Estradiol (1 mM)	Fluphenazine (1 mg/ml)
CaI4	30.27 ± 0.3	50.18 ± 7.35	35.09 ± 1.42	47.32 ± 2.22
CaCMR4	76.94 ± 10.41	554.57 ± 22.73		
CaCMR7	44.27 ± 3.96		727.61 ± 52.05	
CaCMR8	50.07 ± 0.44			260.61 ± 17.37

[a] Emitted by 10,000 cells per sample from three independent cultures.

to grow in a planktonic state for 24 h was statistically indistinguishable from the expression of these promoters in 24-h planktonic cultures (Table 7). After 48 h, the expression of *CaMDR1* was twofold lower in daughter cells than in planktonic cells or detached cells. *CDR1* expression in 48-h cultures (CaCMR7 cells) was up to 9.5-fold higher in planktonic cells and detached cells than in daughter cells (Table 7).

DISCUSSION

Both wild-type cells of *C. albicans* (Ca30) and CaI4 cells that adhered firmly to silicone disks within 2 h of attachment showed a 100-fold increase in tolerance to FCZ exposure over that of logarithmic-phase planktonic cells. Under microscopic examination, these cells were singular, without extracellular capsular material. Increased FCZ tolerance of adherent cells of Ca30 and CaI4 developed rapidly and was inherent to the bound cells.

Chandra et al. (3) found that *C. albicans* M-61 (clinical isolate) grown adherent to silicone disks did not develop a matrix (extracellular material) until after 12 to 24 h of incubation. We observed that adherent Ca30 and CaI4 cells achieved levels of FCZ tolerance similar to that of 24-h Ca30 and CaI4 biofilms, in which exopolymeric material and hyphal forms were visible upon microscopic observation. The similar percentages of permeation of firmly adherent and 24-h biofilm cells upon FCZ exposure suggest that the degree of adhesion selects for a resistance phenotype. All adherent cells, however, may not exhibit equivalent higher efflux pump activities. The numbers of cells with high efflux pump activities varied with the strain but always seemed to be much less than 10% the initial inoculum. We recorded comparable levels of uptake of Rh123 (a compound that is noninhibitory but is structurally similar to FCZ) in both adherent CaI4 cells and planktonic log-phase CaI4 cells, thus further excluding the presence of any physical barrier to FCZ surrounding adherent cells.

Moreover, cells detached from silicone after 2 h of adherence retained tolerance levels that were four- to eightfold higher than those seen in planktonic cells for up to 4 h after detachment. This retained tolerance was lower than that of adherent cells and could be attributed to the residual activity of transport proteins induced when the cells were firmly adherent to the substratum. These data further support earlier reports

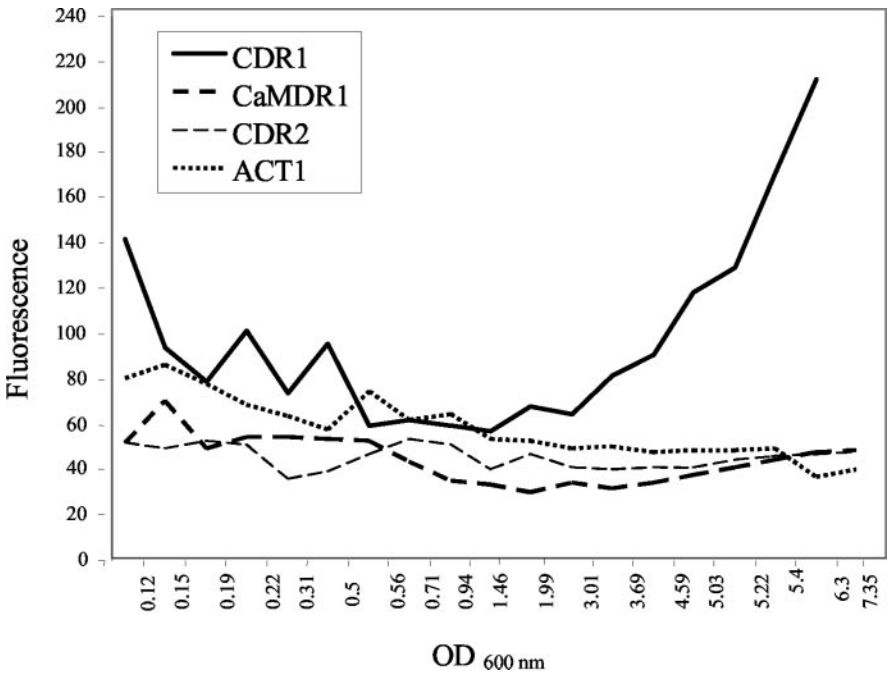


FIG. 3. yEGFP expression during different growth stages. Relative fluorescence (FL1) emitted by CaCMR4 (*CaMDR1* promoter), CaCMR5 (*ACT1* promoter), CaCMR7 (*CDR1* promoter), and CaCMR8 (*CDR2* promoter) during late lag to logarithmic and stationary phases is shown.

TABLE 6. Effect of early adherence on gene expression

Strain	Construct	Mean ± SD relative fluorescence (FL1)[a] (arbitrary units; logarithmic scale) of the following cells:		
		Planktonic	Adherent	Adherent cell/planktonic cell ratio
CaI4	Parental	47.71 ± 0.62	51.89 ± 0.92	1
CaCMR5	*CaMDR1* promoter-yEGFP	98.89 ± 17	103.43 ± 11.3	0.95
CaCMR4	*ACT1* promoter-yEGFP	43.34 ± 0.33	93.56 ± 6.62	2.2
CaCMR7	*CDR1* promoter-yEGFP	42.29 ± 1.04	220.03 ± 3.34	5.2
CaCMR8	*CDR2* promoter-yEGFP	44.63 ± 0.45	73.9 ± 3.35	1.65

[a] From three independent experiments. The Student *t* test reflected *P* values of 0.005 for *CaCMR1*, 0.0001 for *CDR1*, and 0.005 for *CDR2*, indicating 99% confidence.

that biofilm structure is not required for the increased resistance to FCZ of surface-attached *C. albicans* (12, 13, 20, 31).

The significance of the CaMDR1p, Cdr1p, and Cdr2p transport proteins for FCZ resistance in both planktonic and adherent cells was evident in the detrimental effect that knockout of their genes had on the extent of FCZ tolerance acquired upon attachment. Strains lacking the *CaMDR1* and *CDR1* genes, both in the planktonic and in the adherent states, were significantly more susceptible to FCZ than parental strain CaI4. However, the contribution of *CDR2* to the FCZ tolerance of planktonic and adherent *C. albicans* cells was minimal. These observations are in accordance with the results obtained by other investigators (4, 11, 20) studying the extent of the contribution of different genes to FCZ resistance in planktonic cultures.

From the study of knockout mutants, we were able to conclude that the impact of adhesion on FCZ tolerance correlated with the expression of *CaMDR1* and particularly with that of *CDR1* but not with the expression of *CDR2*. The expression of yEGFP from the *CDR1* and *CaMDR1* promoters was detected as early as 15 and 30 min after adhesion by epifluorescence microscopy, respectively, prior to cell replication on the substratum. We propose that either physical (i.e., direct contact with the substratum) or environmental signals encountered by

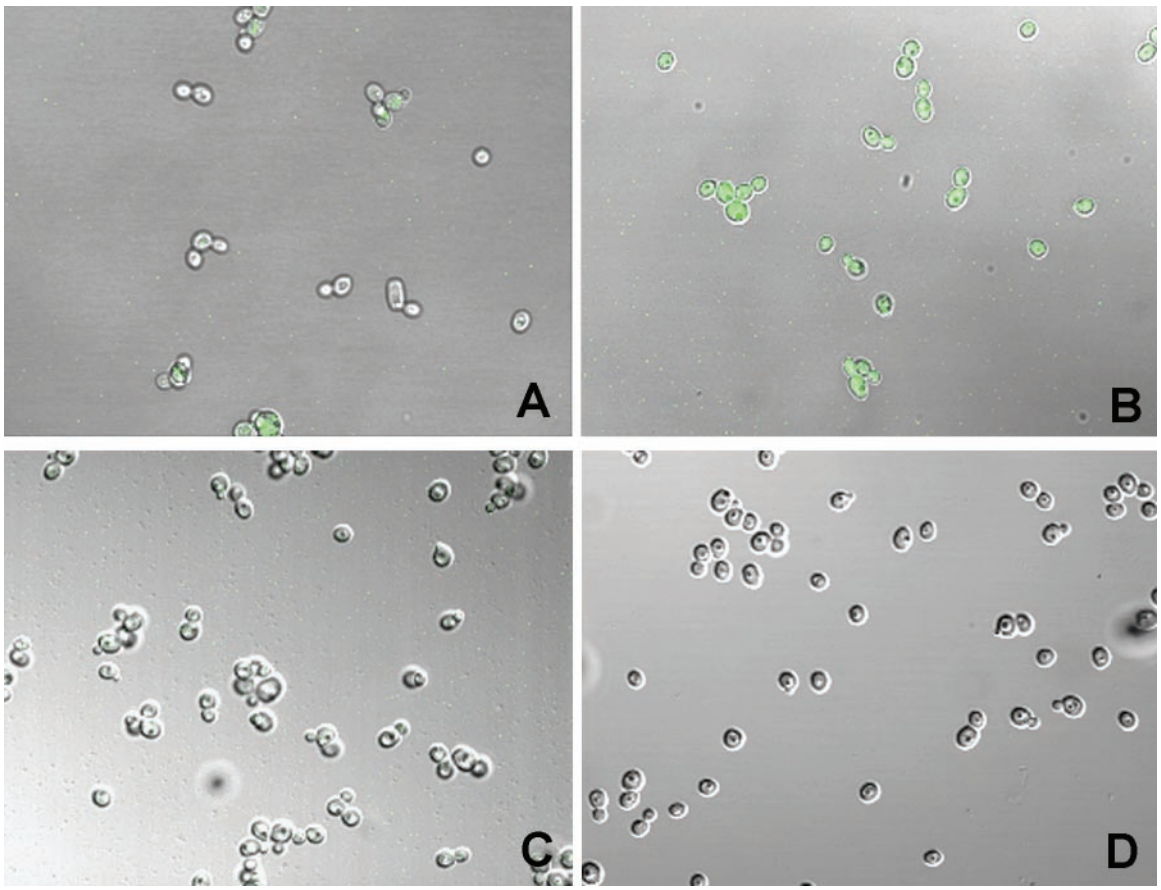


FIG. 4. Epifluorescence micrographs of *C. albicans* CaCMR7 in the planktonic state (A) and 15 min after adherence (B) to glass slides, demonstrating increased expression from the *CDR1* promoter after adherence. In contrast, CaI4 parental control cells in the planktonic (C) and adherent (D) states showed a lack of fluorescence.

TABLE 7. yEGFP expression in daughter cells

Cell population[a]	Mean ± SD relative fluorescence (FL1)[b] (arbitrary units; logarithmic scale) at:	
	24 h	48 h
CaCMR4 planktonic	46 ± 7	83 ± 8[c]
CaCMR4 daughter	50 ± 4.1	48 ± 8.8[c]
CaCMR4 detached	49 ± 3.7	91.5 ± 13.7[c]
CaCMR7 planktonic	141 ± 28	1,509 ± 93.2[c]
CaCMR7 daughter	118 ± 9.8	160 ± 34.3[c]
CaCMR7 detached	162.4 ± 6.6	1,765 ± 301[c]
CaCMR8 planktonic	53 ± 6.5	94 ± 12
CaCMR8 daughter	50 ± 13.6	96 ± 5.2
CaCMR8 detached	50 ± 4.8	91.5 ± 14

[a] Daughter, planktonic cells that were born from mature biofilms; detached, planktonic cells that were allowed to adhere to medical-grade silicone disks for 2 h prior to detachment.
[b] From four independent experiments.
[c] Results were statistically significantly different ($P = 0.031$ for CaCMR4 cells and $P = 0.004$ for CaCMR7 cells) between daughter cells and detached cells or planktonic cells of the same strain.

adherent organisms induce the expression of at least two genes that contribute to the increased tolerance of adherent cells to FCZ.

Mukherjee et al. (20) reported that *C. albicans* biofilms expressed the *CDR* and *MDRI* genes in all developmental stages, as determined after 6, 12, and 48 h, and that biofilm sterol levels at 6 h were similar to those of planktonic cells but were decreased after 12 and 48 h. The authors observed that changes in sterol profiles coincided with apparent reduced pump activity and suggested that they may be a critical component in the azole resistance of biofilms. Similarly, we determined that the expression of the *CaMDR1* and *CDR1* genes was significantly lower in daughter cells from 48-h biofilms than in firmly adherent cells (2 h after attachment), suggesting that efflux pump expression in adherent cultures is transient.

From observations with knockout mutant cells, it appeared that CaMdr1p played a major role in the tolerance to FCZ of adherent cells. The absence of functional CaMdr1p in adherent DSY465 cells (Δ*camdr1 CDR1*) resulted in a lower resistance to FCZ, despite the existence of a functional *CDR1* gene, than that recorded for adherent DSY449 cells (Δ*cdr1 CaMDR1*). Nevertheless, it was expression from the *CDR1* promoter that showed the greatest degree of upregulation after adhesion. A possible explanation for the higher level of expression of *CDR1* than of *CaMDR1* lies in the specificity of the two proteins. CaMdr1p is reportedly highly specific for FCZ transport (8), whereas Cdr1p has less substrate specificity (16). Consequently, *CDR1* upregulation could provide the cell with a general defense mechanism.

In addition, the differences in yEGFP fluorescence levels were not due to the growth stage of the cells at the time of adhesion, since the expression of *CaMDR1*, *CDR1*, and *CDR2* declined during mid-logarithmic phase, the time during which cells were adhering to the silicone disks. Also, all yEGFP fluorescence readings were taken from cells grown for 16 h from a very small inoculum (10^4 cells in 100 ml of medium). It is unlikely that pump expression was residual from any stationary-phase cells or a cellular response to fresh medium.

Our heterologous FCZ-resistant control strain produced negligible adhesion and biofilm formation under our test conditions and was unsuitable for direct comparisons with regard to adherence-induced tolerance. Nevertheless, cells with heterologous resistance could develop within a mature biofilm, thus adding to the complexity of mature biofilms. In summary, adherent cells deficient in drug efflux pump expression, were unable to achieve as high a level of tolerance to FCZ as adherent cells of wild-type strain Ca30 and parental strain CaI4. Expression from the *CaMDR1* and *CDR1* promoters was higher in adherent cells than in planktonic cells. These findings were generally similar to those of Mukherjee et al. (20). However, we were able to demonstrate that this change in FCZ tolerance as cells went from a planktonic state to a firmly adherent state was rapid, statistically significant, and remained for up to two generations after detachment from the initial firmly adherent state. The overall data indicated that the difference in FCZ susceptibility between planktonic and firmly adherent cells of the strains studied here was a consequence of the attachment process involving the expression of the CaMdr1p and Cdr1p efflux pumps.

We have performed extensive investigations to establish that the FCZ-susceptible strains of *C. albicans* studied here behave in a manner similar to that of well-studied strains in the literature with regard to FCZ resistance (13, 14, 20, 31). In general, FCZ resistance noted for cells in biofilms appears to be partially associated with surface-induced upregulation of drug efflux pumps (13). Our data from adhesion assays, flow cytometry, and epifluorescence microscopy suggest that this upregulation may be immediate upon attachment of a subset of cells with the capacity for firm adhesion to a substratum.

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