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Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms

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A main characteristic associated with microbial biofilms is their increased resistance to antimicrobial chemotherapies. However, at present very little is known about the phenotypic changes that occur during the transition from the planktonic to the biofilm mode of growth. *Candida albicans* biofilms displayed an organized three-dimensional structure, and consisted of a dense network of yeasts and filamentous cells deeply embedded in exopolymeric matrix. These biofilms were intrinsically resistant to fluconazole. Moreover, the resistance phenotype was maintained by sessile cells when resuspended as free-floating cells, thus demonstrating that biofilm integrity and the presence of exopolymeric material are not the sole determinants of biofilm resistance. Under planktonic conditions, one of the main mechanisms of azole resistance in *C. albicans* is through active efflux of these drugs mediated by ATP-binding cassette (ABC) transporters and major facilitators. In this study we used northern hybridization to monitor expression of genes belonging to two different types of efflux pump, the ABC transporters and major facilitators (encoded by CDR and MDR genes, respectively), in *C. albicans* populations under both planktonic and biofilm growth. It was demonstrated that expression of genes encoding both types of efflux pump were up-regulated during the course of biofilm formation and development. Moreover, antifungal susceptibilities of biofilms formed by a set of *C. albicans* mutant strains deficient in efflux pumps were investigated to determine their contribution to biofilm resistance. Remarkably, mutants carrying single and double deletion mutations in Δ*cdr1*, Δ*cdr2*, Δ*mdr1*, Δ*cdr1*/Δ*cdr2* and Δ*mdr1*/Δ*cdr1* were hypersusceptible to fluconazole when planktonic, but still retained the resistant phenotype during biofilm growth. These analyses demonstrate that *C. albicans* biofilm resistance is a complex phenomenon that cannot be explained by one mechanism alone, instead it is multifactorial and may involve different molecular mechanisms of resistance compared with those displayed by planktonic cells.

Keywords: *Candida albicans*, biofilm, efflux pumps, resistance

Introduction

Candida albicans is a pleiomorphic fungus that can exist either as a commensal or opportunistic pathogen with the ability to cause a variety of infections, ranging from superficial to life threatening. Predisposing factors for *C. albicans* infections include immunosuppressive therapy, antibiotic therapy, use of indwelling devices such as intravenous catheters, HIV infection, diabetes and old age. The most recent surveys have shown *Candida* to be the third or fourth most commonly isolated bloodstream pathogen from US hospitals, now surpassing Gram-negative bacilli in frequency.[1,2] Importantly, yeasts (mainly *C. albicans*) are the third leading cause of catheter-related infections, with the

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second highest colonization to infection rate and the overall highest crude mortality.[3] Biomaterials such as stents, shunts, prostheses (voice, heart valve, knee, etc.), implants (lens, breast, denture, etc.), endotracheal tubes, pacemakers and various types of catheter, to name a few, have all been shown to support colonization and biofilm formation by *Candida.*[4] Indeed, it has been speculated that biofilms account for as much as 65% of all microbial infections.[5] Biofilms are spatially organized heterogeneous communities of cells embedded within an extra-polymeric matrix that are interspersed with ramifying fluid channels. Sessile (biofilm) cells display unique phenotypic traits in comparison with planktonic cells.[6] The most notable of these is that sessile cells are notoriously resistant to both antimicrobial agents and host immune factors. Biofilm-associated infections are therefore difficult to treat because of their decreased susceptibility to antimicrobial therapy.[7] In fact, resistance has been reported to increase 1000-fold under some conditions.[7,8] In the case of *C. albicans* biofilms, our group and others have reported that they are up to 4000 times more resistant to fluconazole when compared with planktonic, free-floating cells.[9–14]

The precise mechanisms responsible for the intrinsic drug resistance associated with microbial biofilms remain unclear. It has been suggested that biofilm resistance may be related to contributions from the extracellular matrix that prevent active drug diffusion,[15] the physiological state of the cell,[7,16] efflux pumps on the cell membrane that pump antifungal drugs out of the cell[17] or differential gene expression patterns by sessile cells.[6,18] However, none of these proposed mechanisms alone can account for the general observation of resistance associated with microbial biofilms.

Recent advances in our understanding of the molecular mechanisms leading to azole resistance in *C. albicans* indicate that increased efflux of drug, mediated mostly by the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) transporters, confers resistance to azole antifungal agents.[19–22] The ABC transporters in *C. albicans* constitute a multigene family, and include several CDR genes with a demonstrated role in resistance.[23,24] These ABC transporters, which have also been associated with drug resistance in a variety of eukaryotic cells, include a membrane pore composed of transmembrane segments and two ABCs on the cytosolic side of the membrane, which provide the energy source for the pump.[25,26] Importantly, multiple antifungal agents can be substrates for these transporters and thus their overexpression can lead to cross-resistance among different drugs. Among members of the MFS, the *MDR1* gene[27] encodes a major facilitator that has been implicated in *C. albicans* azole resistance, and its overexpression leads to fluconazole resistance exclusively.[28]

In the present study, it was our aim to analyse whether *C. albicans* efflux pumps contribute to sessile recalcitrance to antifungal agents. We have investigated the expression of *C. albicans MDR1*, *CDR1* and *CDR2* genes during both planktonic and biofilm modes of growth. We have also studied their contribution to fluconazole resistance in *C. albicans* biofilms by analysing the susceptibility profiles of a set of mutants carrying single and double deletion mutations in the corresponding genes.

Materials and methods

C. albicans strains and culture conditions

The *C. albicans* strains used throughout this study are listed in Table 1. Initial experiments used *C. albicans* laboratory strains 3153A and SC5314. All deletion mutant strains were generously provided by Dominique Sanglard (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland). Strains were stored on Sabouraud dextrose slopes (BBL, Cockeysville, MD, USA) at –70°C. Isolates were propagated in yeast peptone dextrose (YPD) medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose (US Biological, Swampscott, MA, USA)]. Batches of medium (20 mL) were inoculated from YPD agar plates containing freshly grown *C. albicans*, and incubated overnight in an orbital shaker at 30°C. All *C. albicans* strains grew in the budding-yeast phase under these conditions. Cells were harvested and washed in sterile phosphate-buffered saline [PBS: 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride,

Table 1. *C. albicans* strains used in this work

Strain name	Knockout	Genotype
3153A	wild type	
SC5314	wild type	
DSY448	Δ*cdr1*	Δ*cdr1::hisG-URA3-hisG/*Δ*cdr1::hisG*
DSY653	Δ*cdr2*	Δ*cdr2::hisG-URA3-hisG/*Δ*cdr2::hisG*
DSY654	Δ*cdr1*/Δ*cdr2*	Δ*cdr1::hisG/*Δ*cdr1::hisG* Δ*cdr2::hisG-URA3-hisG/*Δ*cdr2::hisG*
DSY465	Δ*mdr1*	Δ*mdr1::hisG-URA3-hisG/*Δ*mdr1::hisG*
DSY468	Δ*cdr1*/Δ*mdr1*	Δ*cdr1::hisG/*Δ*cdr1::hisG* Δ*mdr1::hisG-URA3-hisG/*Δ*mdr1::hisG*

pH 7.4 (Sigma, St Louis, MO, USA)]. Cells were then suspended in RPMI-1640 supplemented with L-glutamine and buffered with morpholine-propanesulphonic acid (MOPS: Angus Buffers and Chemicals, Niagara Falls, NY, USA), counted in a haemocytometer and adjusted to the desired cell density (1.0×10^6 cells/mL).

XTT colorimetric assay for monitoring biofilm formation in 96-well microtitre plates

C. albicans biofilms were formed on the surface of wells of microtitre plates as described previously by our group.[13,14] Briefly, biofilms were formed by pipetting standardized cell suspensions (100 µL of a suspension containing 1.0×10^6 cells/mL in RPMI-1640) into selected wells of microtitre plates and incubating over 48 h at 37°C. A semi-quantitative measure of biofilm formation was calculated using a 2,3-bis(2-methoxy-4-nitro-5-sulpho-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay, essentially as described previously.[13,29,30] Briefly, XTT (Sigma) was prepared as a saturated solution at 0.5 g/L in Ringer's lactate. This solution was filter sterilized through a 0.22 µm pore size filter, aliquoted and then stored at –70°C. Before each assay, an aliquot of stock XTT was thawed and menadione (Sigma; 10 mM prepared in acetone) dispensed to a final concentration of 1 µM. A 100 µL aliquot of XTT/menadione was then added to each pre-washed biofilm and to control wells to measure background XTT levels. The plates were then incubated in the dark for 2 h at 37°C and the colorimetric change (a direct reflection of the metabolic activity of the biofilm) was measured in a microtitre plate reader at 490 nm (Benchmark Microplate Reader; Bio-Rad, Hercules, CA, USA). Microscopic examinations of biofilms formed in microtitre plates were also carried out in parallel.

Antifungal susceptibility testing

The antifungal agent fluconazole (Pfizer, Inc., New York, NJ, USA) was used in this study. It was prepared at stock concentrations of 1024 mg/L in RPMI-1640 (Angus Buffers and Chemicals). Antifungal susceptibility testing to determine MICs for planktonic cells was carried out using the National Committee for Clinical Laboratory Standards (NCCLS) M-27A broth microdilution method with reading of endpoints at 48 h.[31] In additional experiments, in order to assess the antifungal susceptibility profiles of *C. albicans* sessile cells resuspended from preformed biofilms, the biofilms were formed in tissue culture flasks. Briefly, cells were washed, resuspended in RPMI and counted. *C. albicans* cells were then added to 25 mL of RPMI in a vent cap tissue culture flask (Corning, New York, NY, USA) at a cell density of 1.0×10^6 cells/mL. The flasks were incubated statically for 4 h to allow initial adherence of the cells, after which the medium was decanted and replaced with 50 mL of pre-warmed RPMI. The flasks were then gently rocked to promote biofilm formation at 37°C. After 24 h biofilm formation, sessile cells were washed, scraped and suspended in RPMI. Cell numbers were estimated by cell counting using a haemocytometer. A standardized cell suspension was prepared to an equal density to that used for determination of planktonic antifungal susceptibility testing described by NCCLS.[31] The sessile MICs were then determined using the NCCLS broth microdilution method, as above. For antifungal susceptibility testing of cells within the biofilm, biofilms were formed in selected wells of microtitre plates and incubated for selected time intervals at 37°C. The biofilms were then washed thoroughly three times with sterile PBS before the addition of fluconazole in serially double diluted concentrations and incubated for a further 48 h at 37°C. A series of antifungal-free wells was also included to serve as controls. Sessile MICs were determined at 50% inhibition (SMIC50) compared with drug-free control wells using the XTT reduction assay.[13]

Scanning electron microscopy

For scanning electron microscopy (SEM), *C. albicans* strain 3153A biofilms were formed on Thermanox plastic coverslips (Nunc, Denmark) within 6-well cell culture plates (Corning) by dispensing standardized cell suspensions (4 mL of a suspension containing 1.0×10^6 cells/mL in RPMI-1640) on to appropriate discs and incubating at 37°C for 24 h. The biofilms were washed and placed in fixative [4% (v/v) formaldehyde and 1% (v/v) glutaraldehyde in PBS] overnight. The samples were rinsed in 0.1 M phosphate buffer (2×3 min) and then placed in 1% Zetterquist's osmium for 30 min. The samples were subsequently dehydrated in a series of ethanol washes (70% for 10 min, 95% for 10 min, 100% for 20 min), then treated (2×5 min) with hexamethyldisilizane (HMDS; Polysciences Inc., Warrington, PA, USA), and finally air dried in a desiccator. The specimens were coated with gold/palladium (40%/60%). After processing, samples were observed in an SEM (Leo 435 VP) in high vacuum mode at 15 kV. The images were processed for display using Photoshop software (Adobe, Mountain View, CA, USA).

RNA extraction from sessile and planktonic cell populations

C. albicans biofilms were formed in tissue culture flasks as described above. Total RNA was extracted from biofilms of *C. albicans* 3153A and SC5314, at both 24 and 48 h. Also, RNA was obtained from planktonic cultures of both strains during the logarithmic phase (*c.* 5 h) and during stationary phase (24 h). For all phases of growth, cells were first washed in ice-cold sterile PBS and then resuspended in TRI reagent (Molecular Research Centre Inc., Cincinnati, OH, USA). For biofilms, the cells were washed in the flasks and then removed from the flask surface with a sterile scraper. The cells were

then mechanically disrupted using 0.5 mm glass beads in a mini-beadbeater (Biospec Products, Bartlesville, OK, USA). RNA was separated from other cellular debris with bromochloropropane and precipitated with isopropanol following the manufacturer's instructions.

Northern blot analysis

Equal quantities (*c.* 5 μg) of total RNA, as determined by A_{260} measurements, were separated by electrophoresis and subsequently transferred to Nylon membranes (Nytran; Schleicher & Schuell, Keene, NH, USA) using the Turboblotter apparatus (Schleicher & Schuell). A probe for *MDR1* was purified from plasmids containing inserts of the respective genes as described before.[21,28] Probes specific for *CDR1* and *CDR2* genes were prepared as described previously[32] by PCR amplification from plasmids containing these sequences (pDS243 and pDS246 for *CDR1* and *CDR2*, respectively), with the following primers: 5′-GAGATCTACCCTTTAAGATA (forward) and 5′-TCTGAATCGGGATTCAATTG (reverse) for *CDR1*, and 5′-GGTATATAAACTGGACAACA (forward) and 5′-CGGAATCTGGGTCTAATTGT (reverse) for *CDR2*. The identity between the two resulting probes at the level of nucleotide sequence is <50%. All probes were labelled by random priming (Random Primers DNA Labelling System; Gibco-BRL, Gaithersburg, MD, USA), and hybridizations carried out using Rapid-Hyb buffer (Amersham Life Science Inc., Arlington Heights, IL, USA) following the manufacturer's instructions. After hybridization, blots were washed and exposed to autoradiography film (Kodak, Rochester, NY, USA). Nylon membranes were probed sequentially with the different probes following stripping of the previously bound probe with 95°C-heated 1× SSC buffer (0.15 M sodium chloride, 15 mM sodium citrate, pH 7.0) with 0.5% SDS twice for 15 min. For preparation of figures, digital images were processed using the Adobe Photoshop program.

Results

Antifungal susceptibility profiles of resuspended sessile C. albicans

Biofilms formed by *C. albicans* 3153A and SC5314 displayed intrinsic resistance to fluconazole (SMIC50 > 1024 mg/L; Table 2). Corroborating previous observations by our group and others,[14,33] SEM experiments carried out in parallel demonstrated that *C. albicans* biofilms formed on the surface of plastic coverslips displayed an organized three-dimensional structure, and consisted of a dense network of yeasts and filamentous cells that were deeply embedded in matrix consisting of exopolymeric material (Figure 1). Interestingly, the presence of this exopolymeric matrix was demonstrated even after the destructive processes involved in sample preparation for SEM. In order to examine whether innate resistance was due to the observed biofilm architecture, including the presence of exopolymeric material encasing the cells, sessile *C. albicans* cells grown within a biofilm were resuspended as free-floating cells and tested using standardized NCCLS testing methods and compared with planktonically grown *C. albicans* cells. It was demonstrated that resuspended sessile *C. albicans* strains used in this study were resistant to fluconazole (MIC 256 mg/L) at initial cell densities equivalent to those used under NCCLS guidelines. Parallel examination of these strains under planktonic conditions using NCCLS methodology demonstrated that these strains were sensitive to fluconazole challenge (0.25–4 mg/L) (Table 2). Also, the resistance characteristics of resuspended sessile cells were lost when the recovered cells were plated on to solid media and cells from these plates used as initial inoculum for susceptibility testing (data not shown).

Analysis of MDR1 and CDR genes in biofilms

Differential patterns of gene expression in sessile cells as compared with their planktonic counterparts may represent

Table 2. Antifungal susceptibility testing of *C. albicans* wild type and multidrug transporter mutants under planktonic and biofilm growing conditions

Strain	Planktonic MIC at 48 h (NCCLS)	Sessile MIC at 48 h (NCCLS)	SMIC50 at 48 h (1 h adherence) (XTT assay)	SMIC50 at 48 h (24 h biofilm) (XTT assay)
3153A	4	256	>1024	>1024
SC5314	2	256	>1024	>1024
Δ*cdr1*	0.25	128	>1024	>1024
Δ*cdr2*	0.25	128	>1024	>1024
Δ*cdr1*/Δ*cdr2*	0.25	256	>1024	>1024
Δ*mdr1*	0.25	64	>1024	>1024
Δ*cdr1*/Δ*mdr1*	0.25	256	>1024	>1024

Values are in mg/L.

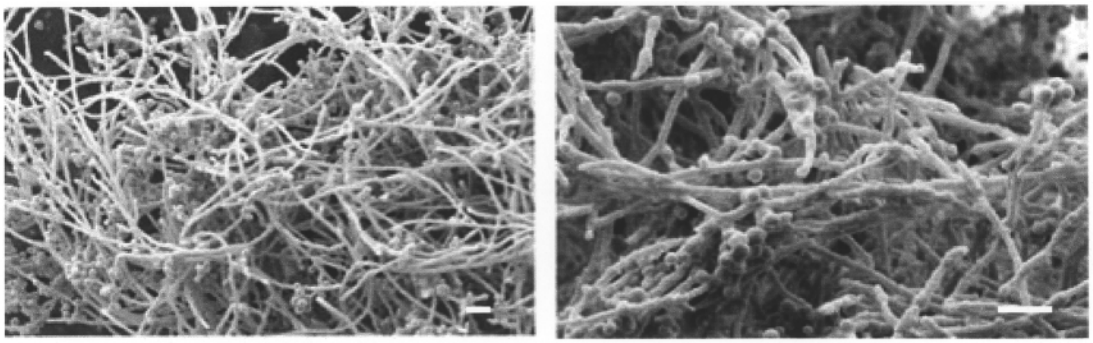

Figure 1. SEM images of 24 h *C. albicans* biofilms formed on plastic coverslips. Note the extensive amount of exopolymeric material still visible despite the destructive nature of SEM procedures. Bars, 10 µm for both panels.

an alternative mechanism responsible for the resistance properties associated with biofilm formation in *C. albicans*. At the molecular level, our group and others have recently demonstrated that one of the main mechanisms responsible for azole resistance in *C. albicans* planktonic populations is the increased efflux of drug mediated by ABC and MFS transporters.[19,28,32] These observations, combined with the fact that resuspended sessile cells still display high levels of azole resistance, led us to investigate whether cells growing in a biofilm overexpress genes encoding members of these two families of transporters. Total RNA extracted from the *C. albicans* 3153A and SC5314 strains growing in RPMI medium in the absence of antifungal drug was analysed using probes specific for *MDR1*, *CDR1* and *CDR2* genes. *MDR1* was transiently overexpressed in biofilm cells at 24 h, and was particularly noticeable in the case of strain 3153A. There was negligible expression of *MDR1* in both planktonically grown cells and late (48 h) sessile *C. albicans* populations (Figure 2). Messenger RNA levels for both *CDR1* and *CDR2* transcripts were higher in sessile *C. albicans* populations (both at 24 and 48 h), whereas planktonically grown cells showed minimal expression of these mRNA transcripts.

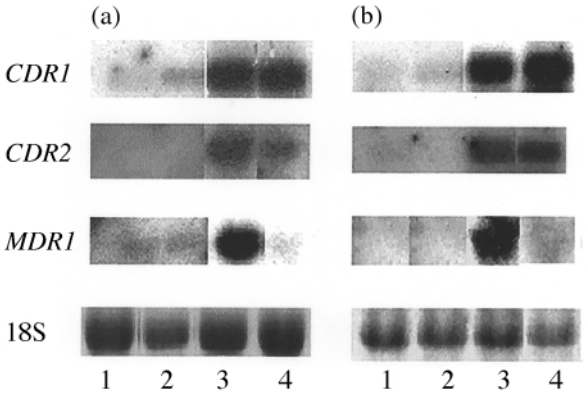

Figure 2. Northern blots of total RNA from *C. albicans* strains 3153A (a) and SC5314 (b) under planktonic and biofilm growing conditions, probed with *MDR1*, *CDR1* and *CDR2* radiolabelled probes (1, exponential phase planktonic cells; 2, stationary phase planktonic cells; 3, 24 h sessile phase cells; 4, 48 h sessile phase cells). Hybridizations were carried out as described in Materials and methods. The bottom panels show amounts of 18S rRNA used to standardize signal levels according to lane loading parameters.

Contribution of drug efflux to biofilm resistance: antifungal susceptibility profiles of biofilms formed by C. albicans multidrug transporter mutants

Overexpression of genes encoding multidrug transporters suggested a role for drug efflux in the intrinsic resistance to azoles displayed by *C. albicans* biofilms. To assess further the contribution of MDR and CDR genes to biofilm antifungal drug resistance, a set of isogenic strains carrying single or double deletion mutations of genes encoding efflux pumps (Δ*cdr1*, Δ*cdr2*, Δ*mdr1*, Δ*cdr1*/*cdr2*, Δ*mdr1*/*cdr1*) was analysed. When grown as planktonic populations, all mutant strains were hypersusceptible to fluconazole as compared with their parental strains (Table 2). The ability of the different strains to form biofilms was assessed using a microtitre plate biofilm model. Biofilm formation was also monitored by microscopy techniques. These experiments indicated that all mutant strains were able to form biofilms comparable (both metabolically and microscopically) to those formed by the parental strains from which they were derived. To determine the antifungal susceptibility profile of biofilms formed by each mutant, the strains were grown as mature biofilms (24 h) on flat-bottomed microtitre plates or adhered to the surface of a flat-bottomed microtitre plate for 1 h and then challenged with serial dilutions of fluconazole (1024–1 mg/L). Antifungal susceptibility testing of resuspended sessile cells from biofilms formed by each mutant strain was also determined. As shown in Table 2, it was revealed that preformed biofilms from all isolates were highly resistant to fluconazole challenge (>1024 mg/L). The non-destructive XTT assay clearly demonstrated that the biofilms were metabolically active at equivalent levels to unchallenged control biofilms. All strains that were allowed to adhere for 1 h and then

challenged with serial dilutions of fluconazole were also able to form biofilms in high concentrations of fluconazole (>1024 mg/L). The biofilms formed under these conditions were less dense than unchallenged controls but demonstrated metabolic activity comparable to the controls (Table 2). Furthermore, resuspended sessile cells also displayed high-level fluconazole resistance (64–256 mg/L).

Discussion

Biofilms, both fungal and bacterial, are notoriously recalcitrant to antimicrobial challenge. Multiple explanations have been proposed to account for resistance.[6,7,9,15,16,18] Here, we describe a series of experiments that focuses on sessile resistance factors with the express aim of elucidating mechanisms of resistance within *C. albicans* biofilms.

Our group and others have demonstrated that intact *C. albicans* biofilms, grown on different substrates, display variable levels of resistance to fluconazole and other azole derivatives.[9,11–14] The glycocalyx and general structural integrity of biofilms has long been considered to be an important factor in conferring resistance to sessile cells.[15,34] In a first series of experiments, we evaluated whether the matrix and structural integrity of the biofilm were linked to sessile *C. albicans* resistance. First, we showed that preformed biofilms were not affected at high concentrations of fluconazole (>1024 mg/L). We also demonstrated that sessile cells (1 h adherence) could grow, proliferate and form biofilms in high concentrations of fluconazole (1024 mg/L). These results confirmed that sessile *C. albicans* were highly resistant to azoles but indicated that sessile resistance was perhaps not entirely related to the biofilm ultrastructure and matrix material. In fact, Baillie & Douglas[10] reported that in relation to *C. albicans* matrix polymers, drug resistance was unrelated to the extent of matrix formation. Therefore, we destroyed the biofilm integrity of *C. albicans* biofilms and resuspended sessile cells in RPMI, then processed them as described by NCCLS guidelines. This assay provided a means of direct correlation with planktonic MIC determination techniques. Our results listed in Table 2 demonstrated that free-floating sessile cells were also resistant to fluconazole challenge (with MICs increased 64–1000 times compared with planktonic cells). Still, these sessile MICs were up to eight times less resistant to fluconazole challenge than intact biofilms, which indicated that the glycocalyx and its integrity may potentially contribute to the overall observed resistance.

Under selective pressure from treatment with fluconazole, yeast cells can generate resistance through a variety of mechanisms (reviewed by White *et al.*[22]). Factors contributing to resistance under planktonic growth conditions include alterations in the target enzyme, including overexpression and point mutations, and increased efflux of drug mediated by ABC and MFS transporters.[22] Because of the susceptibility profiles of *C. albicans* biofilms, it was our hypothesis that efflux pumps may contribute to the innate resistance they exhibited. Northern blot analysis, using probes for *CDR1* and *CDR2* genes, showed that mRNA levels for these genes were up-regulated when the *C. albicans* cells were in a sessile mode of growth compared with planktonic cells (Figure 2). Moreover, mRNA levels for the *MDR1* gene were transiently increased in 24 h biofilms (Figure 2). Expression of *ERG11*, encoding the target enzyme for azoles, remained similar under biofilm growing conditions (not shown). These results clearly indicate that efflux pumps are up-regulated in cells within a biofilm and thus suggested a possible role in conferring azole resistance. However, when we analysed a set of isogenic *C. albicans* mutant strains defective in multidrug transporters it was shown that intact biofilms formed on microtitre plates from all efflux pump knockout strains retained their capacity to resist fluconazole at high concentrations (>1024 mg/L). Furthermore, *C. albicans* efflux pump mutant cells, adherent for 1 h, were able to resist the action of high concentrations of azole drug (up to 1024 mg/L), and could also grow and proliferate in the presence of the drug to form organized biofilm architecture similar to that of unchallenged control biofilms. In these studies we have been able to show that specific efflux pumps play a critical role within *C. albicans* biofilms, as indicated from the northern blot analysis; however, we have not been able to demonstrate a defined role in sessile resistance. Nevertheless, because the complete inventory of ABC and MFS transporters in yeast includes a large network of efflux pumps with overlapping specificities,[26,35] we cannot disregard the fact that other members of these families are overexpressed in the absence of functional copies of *CDR1*, *CDR2* and *MDR1* genes. Recently, a similar study by De Kievit *et al.*[17] described a series of studies examining the multidrug efflux pumps of *Pseudomonas aeruginosa*. Contrary to our study, these workers did not detect overexpression of efflux pumps and concluded that the specific efflux pumps they examined did not play a role in sessile antibiotic resistance. Likewise, efflux systems did not seem to play a role in *Escherichia coli* biofilm resistance to ciprofloxacin.[6]

C. albicans efflux pumps are essential factors that have evolved independently of azole drugs. It has been postulated that the main role for these transport systems is to allow the uptake of essential nutrients, excretion of metabolic and deleterious products, as well as communication between cells and the environment.[25,26,35–37] Because of the highly structured but heterogeneous biofilm environment, the ability to display efficient transport systems may allow individual cells within the biofilm to maintain an appropriate, local physicochemical environment for growth and survival. A most intriguing feature of multidrug transporters is their ability to bind to a range of structurally unrelated drugs and, remarkably, these same transporter systems have also been reported

to play a role in resistance to antibiotics. Therefore, it is conceivable that, in a biofilm environment, the efflux pumps encoded by *CDR1*, *CDR2* and *MDR1* genes in *C. albicans* cells are employed primarily as a means of cellular detoxification. Thus, physiological overexpression of these genes originally associated with a sessile existence may coincidentally impact on antimicrobial susceptibility. Preliminary microarray analysis of *C. albicans* biofilms, compared with planktonically grown cells, shows clear differences in gene expression profiles associated with these two different modes of growth (G. Ramage, M. Lorenz, J. L. López-Ribot, T. F. Patterson, G. P. Fink & B. L. Wickes, unpublished results). In all probability, resistance of sessile cells is a multifactorial phenomenon. Our future goals are to identify these mechanisms of resistance as a means of understanding the complex and organized lifestyle of sessile cells and its consequences during infection.

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