Transcriptional regulation of drug-resistance genes in *Candida albicans* biofilms in response to antifungals

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Biofilm formation is a major virulence attribute of *Candida albicans* and is directly associated with therapeutic failure. One method by which *Candida* acquires antifungal resistance is the expression of drug-resistance genes. This study aimed to evaluate the transcriptional regulation of several genes associated with antifungal resistance of *C. albicans* under planktonic, recently adhered and biofilm growth modes and in *C. albicans* biofilms in response to antifungal agents. Initially, the antifungal susceptibility of *C. albicans* cultures in different growth modes was evaluated by standard antifungal susceptibility testing. Next, to assess *CDR1*, *CDR2*, *MDR1*, *ERG11*, *FKS1* and *PIL1* expression, RNA was harvested from cells in each growth mode, and from biofilms after drug treatment, and subjected to quantitative real-time RT-PCR (qRT-PCR). Biofilm *C. albicans* was more resistant to antifungals than recently adhered cells and stationary-phase planktonic cultures. Transcriptional expression of *CDR1*, *CDR2*, *MDR1*, *ERG11* and *FKS1* was lower in recently adhered *C. albicans* than in the stationary-phase planktonic cultures. In contrast, *PIL1* levels were significantly increased in recently adhered and biofilm modes of growth. The expression of *MDR1* in biofilms greatly increased on challenge with amphotericin B but not with the other drugs tested ($P<0.01$). *ERG11* was significantly upregulated by ketoconazole ($P<0.01$). Caspofungin and amphotericin B significantly upregulated *FKS1* expression, whereas they significantly downregulated *PIL1* expression ($P<0.01$). These results indicate that the expression of drug-resistance genes is associated with higher drug resistance of *Candida* biofilms, and lay a foundation for future large-scale genome-wide expression analysis.

Received 25 January 2011

Accepted 2 April 2011

INTRODUCTION

Candida albicans is the major fungal pathogen causing infections in humans, ranging from superficial mucosal infection to systemic mycoses (Samaranayake & MacFarlane, 1990). Candidal infections rank as the third or fourth leading cause of hospital-acquired infection in the USA and are associated with high morbidity and mortality rates (Pfaller & Diekema, 2007). The ability of free-floating (planktonic) cells to exhibit an attached biofilm mode of growth is a major virulence attribute of *Candida* species and a direct cause of therapeutic failure (Tumbarello *et al.*, 2007). As with other microbial biofilms, a key phenotypic feature of *Candida* biofilms is their high degree of drug resistance (Seneviratne *et al.*, 2008a). Therefore, a thorough understanding of *Candida* biofilm properties is of great clinical importance.

In general, four classes of antifungal drugs are used to manage fungal infections, namely polyenes, azoles, fluoropyrimidines

and echinocandins (Ruhnke *et al.*, 2008). One mechanism by which *C. albicans* acquires increased antifungal resistance is the expression of drug-resistance genes. For instance, the expression of genes regulating drug efflux pumps, which expel a wide range of toxic metabolites and drugs out of cells, is associated with increased drug resistance against azole antifungals in the planktonic mode of *C. albicans* (Prasad & Kapoor, 2004). Acquired drug resistance in *Candida* is related to ATP-binding cassette transporters encoded by the *Candida* drug-resistance genes *CDR1* and *CDR2*, as well as major facilitator transporters encoded by the multidrug-resistance gene *MDR1* (Nakamura *et al.*, 2001). Resistance of *C. albicans* against azole antifungals is associated with overexpression or mutation of *ERG11*, which encodes 14- α -demethylase in the ergosterol biosynthesis pathway. In contrast, echinocandins are poor substrates for multidrug efflux transporters, and resistance is commonly associated with point mutations or overexpression of the *FKS1* gene (Nett *et al.*, 2010).

Most of the published studies examining the transcriptional expression of drug-resistance genes in *C. albicans* have been

Abbreviation: qRT-PCR, quantitative real-time RT-PCR.

confined to the planktonic mode of growth, and few data are available for the biofilm mode (Franz *et al.*, 1998, 1999; Holmes *et al.*, 2008; Lopez-Ribot *et al.*, 1998; Perea *et al.*, 2001; Sanglard *et al.*, 1995; White, 1997). Therefore, we aimed to study the influence of mode of growth (planktonic, adhesion and biofilm) on the transcriptional expression of several genes that are associated with drug resistance in *C. albicans*. In addition, we included the *PIL1* gene, which encodes the hyphal-specific echinocandin-binding protein (Pil1p), as we found previously that Pil1p is significantly upregulated in *Candida* biofilms and have proposed it as a possible drug target against *Candida* biofilms (Seneviratne *et al.*, 2008c). In the present study, we also tested the response of *C. albicans* biofilms to three classes of antifungals, namely azoles, polyenes and echinocandins.

METHODS

Organisms and culture conditions. *C. albicans* strain SC5314, which is well characterized and has been used in our previous studies (Jayatilake *et al.*, 2007; Lu *et al.*, 2006), was subcultured on Sabouraud dextrose agar (SDA; Gibco) and maintained at 4 °C during the experimental period. The purity of the cultures was confirmed periodically by Gram staining and the use of commercially available carbohydrate assimilation tests (API 32C identification system; bioMérieux).

Preparation of *Candida* cultures in recently adhered, biofilm and planktonic modes. *Candida* cultures in the different modes of growth were prepared according to a previously published protocol (Jin *et al.*, 2004). In brief, *Candida* cells were grown in SDA medium at 37 °C for 18 h. A loopful of yeast was inoculated into a flask of yeast nitrogen base (YNB; Difco) medium supplemented with 50 mM glucose and incubated in a rotary shaker at 75 r.p.m. overnight. Cells were washed twice with 20 ml PBS (0.1 M, pH 7.2) and resuspended in YNB supplemented with 100 mM glucose until it reached an OD₅₂₀ of 0.38 (1×10^7 cells ml⁻¹). This standard cell suspension was used to obtain recently adhered, biofilm and stationary-phase planktonic cultures. First, 100 µl suspension was placed into each well of a 96-well microtitre plate and incubated for 90 min at 37 °C in a shaker at 75 r.p.m. to allow yeast adherence to the well surface. The growth medium was aspirated and each well was washed with PBS to remove loosely adherent cells, leaving cells in the 'recently adhered mode' of growth, to which YNB containing 100 mM glucose was added. Plates were then incubated at 37 °C in a shaker at 75 r.p.m. for 24 h to obtain cultures in biofilm mode. A portion of the standard cell suspension was incubated under identical conditions for 24 h to obtain stationary-phase planktonic cultures. Stationary-phase planktonic cultures reached a concentration of approximately 10^8 cells ml⁻¹ by 24 h and were considered to be high-density planktonic cultures. For each growth phase, microscopic examination of the cultures was performed to rule out contamination.

Antifungal susceptibility testing of stationary-phase planktonic, recently adhered and biofilm cultures. The commonly used antifungal agents amphotericin B, caspofungin and ketoconazole, belonging to the polyene, echinocandin and azole classes of antifungals, respectively, were used in this study. The antifungal agents were prepared as described previously (Ellepola & Samaranayake, 2001). In brief, stock solutions of each drug were serially diluted with RPMI 1640 (Life Technologies) supplemented with 2% glucose to obtain drug concentrations ranging from 480 to 0.225 µg ml⁻¹ for amphotericin B, 100 to 0.1 µg ml⁻¹ for caspofungin and 64 to 0.125 µg ml⁻¹ for

ketoconazole. Antifungal susceptibility testing was performed using an XTT reduction assay, as described previously (Seneviratne *et al.*, 2008b). In brief, 24 h *Candida* biofilms were washed with 100 µl PBS to remove non-adherent cells. Each drug solution (100 µl) was added to the microtitre plate containing *Candida* biofilms. Biofilms were then incubated at 37 °C for 24 h with the antifungals and the metabolic activity of the fungal cells was determined by XTT assay. XTT (Sigma) solution (1 mg ml⁻¹ in PBS) was prepared, filter-sterilized through a 0.22 µm pore size filter and stored at -70 °C. Menadione (Sigma) solution (0.4 mM) was prepared and filter-sterilized immediately before each assay. Prior to each assay, XTT solution was thawed and mixed with the menadione solution at a ratio of 5:1 by volume. The biofilms were first washed with 200 µl PBS, and then 200 µl XTT solution (PBS:XTT:menadione at 158:40:2) was added to each of the pre-washed wells and the control wells. High-density planktonic cultures exposed to antifungals in microtitre plates were mixed directly with 200 µl XTT solution, as described in previous studies (Seneviratne *et al.*, 2008b). Microtitre plates with biofilm and high-density planktonic cultures were then incubated in the dark for 3 h at 37 °C. Following incubation, 100 µl solution was transferred to new wells and the colour change in the solution was measured with a microtitre plate reader (SpectraMAX 340 Tunable Microplate Reader; Molecular Devices) at 490 nm. The MIC value was defined as the lowest drug concentration with a 50% reduction in opacity (MIC₅₀) compared with the drug-free control. Each experiment was repeated three times.

Transcriptional regulation of drug-resistance genes. To study the transcriptional regulation of genes associated with drug resistance in *C. albicans* (*CDR1*, *CDR2*, *MDR1*, *ERG11*, *FKS1* and *PIL1*), we cultured cells in recently adhered and biofilm modes as described above but in polystyrene six-well plates instead of in 96-well plates (Seneviratne *et al.*, 2008c). Adhered *Candida* cells were collected from six-well plates after 1.5 h (recently adhered mode) and 24 h (biofilm mode), washed with PBS and processed for RNA extraction. In parallel, 24 h stationary-phase planktonic cultures of *C. albicans* were harvested and total RNA was obtained. We also examined transcriptional regulation in 24 h *C. albicans* biofilms in response to antifungal treatment. *Candida* biofilms were incubated for 24 h with subMIC concentrations of antifungals (1.88 µg amphotericin B ml⁻¹, 20 µg caspofungin ml⁻¹ and 32 µg ketoconazole ml⁻¹). Cells were then washed with PBS and processed for RNA extraction.

RNA extraction, cDNA synthesis and quantitative real-time PCR. Total RNA was extracted from *Candida* cultures using the SV Total RNA isolation system (Promega) according to the manufacturer's instructions (Samaranayake *et al.*, 2005). RNA purity and integrity were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Additionally, gel electrophoresis was performed to verify that the RNA was intact. cDNA was synthesized from 2 µg total RNA using oligo(dT) primers and Superscript III reverse transcriptase (Invitrogen), as described previously (Seneviratne *et al.*, 2008c). For quantitative analysis of gene expression, the mRNA level was measured using quantitative real-time RT-PCR (qRT-PCR). The primers (Sigma-Aldrich) used in this assay are listed in Table 1. qRT-PCR was carried out using an ABI PRISM 7900HT (Applied Biosystems) sequence detection system using SYBR Green incorporation (SYBR Green PCR Master Mix; Applied Biosystems) in duplicate for at least three separate experiments. Twenty microlitres of PCR mix (10 µl SYBR PCR Master Mix, 1 µl cDNA, 2 µl primer mix and 7 µl double-distilled water) was used for each gene and qRT-PCR was performed using the following cycling conditions: 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Fluorescence intensities were quantified using StepOne software (Applied Biosystems). The relative quantities of the target genes were normalized against *ACT1* housekeeping gene expression and analysed using the comparative $\Delta\Delta C_t$ method, taking the amplification efficiency into

Table 1. Gene-specific primers used for qRT-PCR

F, Forward; R, reverse.

Target gene	Sequence (5'→3')	Amplicon size (bp)
<i>ACT1</i>	F: GCTTTTGGTGTGTTGACGAGTTTCT R: GTGAGCCGGGAAATCTGTATAGTC	72
<i>CDR1</i>	F: GTACTATCCATCAACCATCAGCACTT R: GCCGTTCTTCCACCTTTTTGTA	79
<i>CDR2</i>	F: TGCTGAACCGACAGACTCAGTT R: AAGAGATTGCCAATTGTCCATA	104
<i>MDR1</i>	F: TCAGTCCGATGTCAGAAAATGC R: GCAGTGGGAATTTGTAGTATGACAA	91
<i>FKS1</i>	F: CGTGAAATTGATCATGCCTGTAC R: AACCTTCTGGGCTCCAAA	71
<i>ERG11</i>	F: GGTGGTGATTTGAATGATTTGACTTAT R: GGCATATGCATTCTAAGAGTTTCCT	92
<i>PIL1</i>	F: TAAGCAATTGAGTGCTTGGG R: GGTTGGACAGAACCTTCGAT	174

consideration (Pfaffl, 2001). Groups were compared by analysis of variance with the significance level set at $P < 0.01$.

RESULTS

Antifungal susceptibility testing

Biofilm *C. albicans* cultures were generally more resistant to antifungals than recently adhered cells and stationary-phase, high-density planktonic cultures (Table 2), as reported in previous studies (Baillie & Douglas, 1999; Chandra *et al.*, 2001; Seneviratne *et al.*, 2008b). However, all cultures, including stationary-phase, high-density planktonic cultures, were resistant to ketoconazole. This is in agreement with previous studies on the dose-dependent response of fungistatic drugs (Seneviratne *et al.*, 2008b; Beggs, 1989).

Transcriptional expression of drug resistance-associated genes in different growth modes

Transcriptional expression of *CDR1*, *CDR2*, *MDR1*, *ERG11* and *FKS1* was lower in recently adhered *C. albicans* cultures than in stationary-phase planktonic cultures (Fig. 1a–e). In contrast, expression of *PIL1* was significantly increased in recently adhered cells (Fig. 1f; $P < 0.01$). There was no significant difference between stationary-phase

cultures and biofilm cultures in terms of expression of *CDR1*, *CDR2*, *MDR1*, *FKS1* and *ERG11*. Interestingly, expression of *PIL1* was significantly higher in the biofilm cultures than in the planktonic cultures ($P < 0.01$).

Transcriptional regulation of drug resistance-associated genes in *Candida* biofilms in response to antifungals

Expression of *CDR1* was not significantly altered when biofilms were challenged with antifungal agents (Fig. 2a). *CDR2* expression increased significantly on caspofungin challenge (Fig. 2b). Interestingly, *MDR1* expression was greatly increased on amphotericin B challenge but not with other drugs (Fig. 2c). Caspofungin and amphotericin B significantly increased the expression of *FKS1* (Fig. 2d; $P < 0.01$). Caspofungin upregulated *FKS1* expression to a greater extent than ketoconazole and amphotericin B, but the difference was not statistically significant ($P > 0.05$). Of all the antifungals tested, only ketoconazole significantly upregulated *ERG11* expression (Fig. 2e; $P < 0.01$). In contrast, amphotericin B and caspofungin significantly downregulated *PIL1* expression (Fig. 2f; $P < 0.01$).

DISCUSSION

C. albicans is the major fungal pathogen of humans (Navarro-García *et al.*, 2001). In recent years, *Candida* infections have increased disproportionately as a result of the increased number of compromised host populations, such as patients with AIDS, diabetes and various cancers, and organ-transplant recipients (Beck-Sagué *et al.*, 1993; Wisplinghoff *et al.*, 2004). The number of drug-resistant *Candida* strains has also increased dramatically owing to the increased use of antifungal agents. The expression of drug-resistance genes as a mechanism by which *Candida* acquires drug resistance is a well-known phenomenon in the planktonic mode of growth of this fungus. In the present study, we examined the transcriptional regulation of a subset of drug-resistance genes associated with drug resistance in *C. albicans* under various growth modes and when biofilms were exposed to antifungals.

We found that adhered cells of *C. albicans* were more resistant to antifungals than stationary-phase, high-density planktonic cultures, and resistance increased further after the formation of biofilms. This phenomenon has been

Table 2. Antifungal susceptibility testing of *C. albicans* planktonic, recently adhered and biofilm growth modes

Antifungal agent	MIC ($\mu\text{g ml}^{-1}$)		
	Stationary-phase, planktonic culture	Recently adhered mode	Biofilm mode
Amphotericin B	1.8	7.5	30
Caspofungin	0.25	0.5	>100
Ketoconazole	>128	>128	>128

Fig. 1. Relative transcriptional expression of drug-resistance genes in different growth modes of *C. albicans*. Results were normalized against *ACT1* housekeeping gene expression, which was assigned a value of 1, and are shown as means $\pm$ SD of three independent experiments. *, $P < 0.01$.

observed by various research groups, including ourselves (Mateus *et al.*, 2004; Seneviratne *et al.*, 2008b). However, levels of expression of the drug efflux pump-related genes

CDR1, *CDR2* and *MDR1* were significantly lower in adhered cells than in planktonic cultures. Therefore, it is unlikely that efflux pump-related genes play a major role in

Fig. 2. Relative transcriptional expression of drug-resistance genes in *C. albicans* biofilms in response to antifungal agents. Results were normalized against *ACT1* housekeeping gene expression, which was assigned a value of 1, and are shown as means $\pm$ SD of three independent experiments. AMB, Amphotericin B; KTC, ketoconazole; CAS, caspofungin. *, $P < 0.01$.

adhesion-conferred drug resistance of *C. albicans*. Our results are in agreement with those of other studies, which found that multidrug efflux pumps have a relatively minor role in the mechanism of resistance of *C. albicans* biofilms against fluconazole, an azole drug (Mukherjee *et al.*, 2003; Ramage *et al.*, 2002). However, our study demonstrates the minor involvement of efflux pumps in resistance to polyene and echinocandin classes of drugs.

Mukherjee *et al.* (2003) observed that sterol composition, particularly ergosterol, is significantly decreased in the late phase of *C. albicans* biofilm formation. Thus, they proposed that a reduction in membrane ergosterol composition or its replacement by other types of sterols, such as lanosterol, contributes to the reduced susceptibility to azoles and polyenes. Azole resistance of *C. albicans* has also been shown to be associated with increased expression of *ERG11* (Lupetti *et al.*, 2002; Morschhäuser, 2002). Some authors have also suggested that increased expression of *ERG11* in the presence of an azole could be a feedback mechanism to compensate for the reduced ergosterol content. Our study showed that *ERG11* expression in biofilms is significantly upregulated on challenge with ketoconazole but not with other antifungals. Therefore, we speculate that *ERG11* might play a role in the azole resistance of *C. albicans* biofilms but not with other classes of drugs.

The glucan synthase gene *FKS1*, which encodes 1,3- β -D-glucan, is associated with resistance to amphotericin B and anidulafungin in *Candida* biofilms (Nett *et al.*, 2010). We noted that *FKS1* expression was significantly upregulated after treatment of *C. albicans* biofilms with amphotericin B and caspofungin. Therefore, it is conceivable that these biofilms rapidly increase glucan synthesis in response to noxious environmental stress exerted by antifungal agents. It is also known that point mutations or hot spots in the *FKS1* gene may cause higher-order resistance, although some studies have presented conflicting evidence (Walker *et al.*, 2010). This is a novel area that could be explored further to understand the mechanism of higher-order drug resistance in *C. albicans* biofilms.

PIL1 expression was significantly reduced on challenge with caspofungin and amphotericin B. It has been proposed that *PIL1* is associated with β -1,3-glucan synthase and plays a role in its regulation (Edlind & Katiyar, 2004). A protein complex containing both Pil1p and Fks1p proteins has been identified (Gavin *et al.*, 2002). Therefore, our results suggest that Pil1p and Fks1p have opposing effects on the regulation of glucan synthesis, particularly in response to caspofungin and amphotericin B. At present, we are in the process of generating and characterizing *PIL1* mutants. Understanding the roles of *PIL1* and *FKS1* may have implications in developing and modifying echinocandin-based regimens to eliminate *C. albicans* biofilms more efficiently.

Finally, *MDR1* expression increased significantly when *Candida* biofilms were treated with amphotericin B. Because the amphotericin B molecule is too large for drug efflux pumps to expel it, increased *MDR1* expression is

unlikely to result in increased expulsion of this antifungal out of the cell. Amphotericin B kills fungi by acting on ergosterol in the fungal cell membrane to increase membrane permeability (Vanden Bossche *et al.*, 1994). In addition to this primary action, amphotericin B exerts oxidative stress on the fungal cells (Brajtburg *et al.*, 1990; Ellis, 2002; Vanden Bossche *et al.*, 1994). It is known that upregulation of the *MDR1* gene is influenced by two regulatory elements: the hydrogen peroxide-responsive element and the benomyl-responsive element (Rognon *et al.*, 2006). Therefore, it is possible that increased expression of *MDR1* is a consequence of oxidative damage induced by amphotericin B, perhaps as part of an oxidative defence system for protecting cells. This possible mechanism is supported by the fact that expression of *MDR1* was not significantly altered by the other two drugs, caspofungin and ketoconazole, which do not cause oxidative damage to fungal cells. *MDR1* overexpression has been linked to oxidative resistance in *C. albicans* (Kusch *et al.*, 2004). We propose that an increase in *MDR1* expression in *Candida* biofilms is a protective response to oxidative damage caused by amphotericin B but is not necessarily a resistance mechanism.

In conclusion, resistance of *C. albicans* biofilms to different classes of antifungals seems to be associated with transcriptional regulation of different drug-resistance genes. The data presented here shed some new light on drug resistance in *C. albicans* biofilms and lay a foundation for future large-scale genome-wide expression analysis.

ACKNOWLEDGEMENTS

This work was supported by the Hong Kong Research Grants Council, RGC no. HKU 7624/06M to L.P.S. and HKU internal funding 200907176121 to C.J.S. We thank Dr Trevor Lane for editorial assistance.

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