Nailis, H., Vandenbosch, D., Deforce, D., Nelis, H. & Coenye, T. (2010). Transcriptional response to fluconazole and amphotericin B in *Candida albicans* biofilms. *Res Microbiol* **161**:284-292.

Transcriptional response to fluconazole and amphotericin B in *Candida albicans* biofilms

Heleen Nailis[a], Davy Vandenbosch[a], Dieter Deforce[b], Hans J. Nelis[a], Tom Coenye[a*]

[a]Laboratory of Pharmaceutical Microbiology, Ghent University, Harelbekestraat 72, B-9000, Gent, Belgium

[b]Laboratory of Pharmaceutical Biotechnology, Ghent University, Harelbekestraat 72, B-9000, Gent, Belgium

E-mail adresses: heleen.nailis@ugent.be, davy.vandenbosch@ugent.be, dieter.deforce@ugent.be, hans.nelis@ugent.be, Tom.Coenye@ugent.be

*"Correspondence and reprints"; Laboratory of Pharmaceutical Microbiology, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium. Tel.: +32 9 264 81 41; Fax: +32 9 264 81 95.

Abstract

Biofilm formation is often associated with persistent *Candida albicans* infections. Treatment of these infections is difficult since sessile *C. albicans* cells show an increased resistance towards antifungal agents. The molecular mechanisms behind biofilm resistance in *C. albicans* are not yet understood. In the present study, we investigated the transcriptional response in young and mature in vitro grown biofilms after a short and longer exposure time to high doses of fluconazole or amphotericin B. Treatment of biofilms with high doses of antifungal agents resulted in a drug-specific transcriptional response. Exposure of biofilms to fluconazole induced an upregulation of genes encoding enzymes involved in the ergosterol biosynthesis (*ERG1, ERG3, ERG11* and *ERG25*). Treatment of biofilms with amphotericin B resulted in an overexpression of *KRE1* and *SKN1*, two genes encoding proteins involved in the β-1,6-glucan biosynthesis. Our data indicate that sessile *C. albicans* cells show a controlled regulation of gene expression as they quickly mount a drug-specific transcriptional response in the presence of high doses of antifungal agents. These transcriptional changes suggest an upregulation of the ergosterol biosynthesis (fluconazole) and an upregulation of the β-1,6-glucan biosynthesis (amphotericin B) in sessile *C. albicans* cells, that might contribute to a resistant biofilm phenotype.

Key-words: Candida albicans; biofilms; resistance; fluconazole; amphotericin B; gene expression; RT-quantitative PCR

1. Introduction

Candida albicans is a commensal fungus of healthy human individuals that can cause superficial and systemic infections when the immune defences are repressed or when the normal microbial flora is disturbed (Odds, 1988). Various antifungal agents are used to treat these infections, including azoles and polyenes (Pappas et al., 2004). Fluconazole (FLC) belongs to the azoles and interferes with the ergosterol biosynthesis by binding to the *ERG11* gene product, lanosterol 14-α-demethylase (Richardson, 1990). The latter enzyme is crucial for ergosterol production, and inhibition of its activity results in disruption of the cell membrane leading to growth inhibition of the fungus (Kelly et al., 1993). It is known that long-term, low level use of FLC results in the development of azole resistance in *C. albicans* (Franz et al., 1998). Amphotericin B (AMB) is a member of the polyene family (Warnock, 1991). This molecule binds to ergosterol and forms pores resulting in a disorganized membrane with increased permeability (Brajtburg et al., 1990). In addition, AMB induces cell damage by generating lethal reactive oxygen species (Brajtburg et al., 1990). Development of resistance against this antifungal agent has also been reported, but is less frequent (Ellis, 2002).

The molecular mechanisms of antifungal resistance in *C. albicans* have been well studied and changes in gene expression have frequently been reported in resistant clinical isolates (Sanglard, 2002; White, 1997; White et al., 1998). FLC resistance in particular is associated with overexpression of *ERG11* (Franz et al., 1998; Lopez-Ribot et al., 1998). Changes in the expression of other *ERG* genes such as *ERG3* and *ERG25* have also been observed (Henry et al., 2000). In addition, genes encoding efflux pumps (*MDR1*, *CDR1* and *CDR2*) are often upregulated in FLC-resistant isolates (Lopez-Ribot et al., 1998; Rogers and Barker, 2003; White et al.,

2002). Upregulation of *ERG* genes and of genes encoding efflux pumps were also detected in planktonic cells after exposure to FLC in vitro (Hernáez et al., 1998; Lepak et al., 2006). In vitro exposure of planktonic cells to AMB is correlated with underexpression of *ERG3* and *ERG11*, indicating that changes in the sterol composition are important for AMB resistance in *C. albicans* (Liu et al., 2005). Furthermore, changes in the β-1,6-glucan biosynthesis have also been proposed as a resistance mechanism against AMB (Gale, 1986). *SKN1* and *KRE1*, two genes involved in the β-1,6-glucan biosynthesis (Mio et al., 1997) were found to be differentially expressed after in vitro exposure of *C. albicans* planktonic cells to caspofungin, but not to AMB (Liu et al., 2005).

C. albicans infections are often associated with the formation of biofilms (Douglas, 2003); biofilms are three-dimensional structures of yeast cells and filaments that are embedded in an extracellular matrix (Kumamoto, 2002). Sessile *C. albicans* cells (cells in a biofilm) are phenotypically different from planktonic cells (cells that are not attached to a surface), with one of the most prominent features being the increased resistance towards antifungal agents (Chandra et al., 2001; Hawser and Douglas, 1995). Therefore, eradication of biofilms and treatment of the associated infections are very difficult (Kuhn and Ghannoum, 2004). In order to develop novel and effective anti-biofilm strategies it is essential to understand the molecular mechanisms of biofilm resistance. However, antifungal resistance in *C. albicans* biofilms is a complex phenomenon, and multiple mechanisms are likely to be involved (Kuhn and Ghannoum, 2004). It has been reported that genes encoding efflux pumps are highly expressed in young biofilms (Mateus et al., 2004; Mukherjee et al., 2003; Ramage et al., 2002), while reduced ergosterol levels provide a possible resistance mechanism in mature biofilms (Mukherjee et al., 2003). Furthermore, the presence of resistant

subpopulations in biofilms in the absence of antifungal agents has also been proposed as a cause of intrinsic biofilm resistance (Khot et al., 2006). Recently, it was demonstrated that the majority of the biofilm population was killed after exposure to high doses of AMB, and that only a small fraction of cells (persisters) survives the treatment (LaFleur et al., 2006). However, at present it is not known which mechanisms sessile cells use to survive in the presence of high doses of antifungal agents. Investigating the molecular mechanisms of resistance in persisters will help to understand resistance in *C. albicans* biofilms.

The goal of the present study was to investigate the expression of genes, known to be regulated in response to antifungal agents in planktonic cells or known to be expressed in resistant strains, in *C. albicans* biofilms. For this, young (0.5 h and 1 h) and mature (24 h and 72 h) biofilms were grown after a short (1 h) and longer (22 h) exposure time to high doses of FLC or AMB, and also after removal of the antifungal drugs. The expression of genes encoding efflux pumps (*MDR1*, *CDR1* and *CDR2*), genes encoding enzymes involved in the ergosterol biosynthesis (*ERG1*, *ERG3*, *ERG11* and *ERG25*) and genes encoding proteins involved in the β-1,6-glucan biosynthesis (*KRE1* and *SKN1*) were quantified, using RT-qPCR (Reverse Transcriptase quantitative PCR).

2. Materials and Methods

2.1. Strains

C. albicans strain SC5314 (American Type Culture Collection MYA-2876) was used throughout the study. Cells were stored at –80 °C in Microbank tubes (Prolab Diagnostics, Ontario, Canada) and routinely transferred to Sabouraud Dextrose Agar plates (SDA; Oxoid, Hampshire, UK). These were incubated at 37 °C for 24 h.

2.2. Biofilm growth

C. albicans SC5314 biofilms were grown on silicone disks in 24-well microtiter plates (TPP, Trasadingen, Switzerland). Silicone sheets were prepared from a medical grade silicone rubber kit (Q7-4735; Dow Corning Corp., Midland, MN, USA) according to the manufacturer's instructions. Thirteen-millimetre diameter disks were punched from the sheets, subsequently washed with a detergent (2% RBS35 solution; Sigma, St. Louis, MO, USA), rinsed with MilliQ water (Millipore, Billerica, MA, USA) and heat-sterilized. Start cultures were prepared by incubating *C. albicans* cells for 16 h in Sabouraud Dextrose Broth (SDB; Oxoid, Hampshire, UK) at 37 °C in a water bath with shaking; cells were subsequently washed three times with and finally resuspended in 1 ml 0.9% (w/v) NaCl. A 0.4% cell suspension was prepared in Yeast Nitrogen Base (YNB; BD, Franklin Lakes, NJ, USA) supplemented with 50 mM glucose (Sigma, St. Louis, MO, USA); this cell suspension contains approx. 5% filaments (Nailis et al., 2009). One ml of this suspension was added to each well and plates were incubated for 30 min (0.5 h old biofilms) or 1 h at 37 °C. Silicone disks were then washed three times with 1 ml 0.9% (w/v) NaCl to remove non-adherent cells. Disks were placed in new plates, 1 ml diluted YNB (1:5; final glucose concentration: 10 mM) (YNB 0.2x) was added to each well and plates were further

incubated for 24 h or 72 h at 37 °C. Biofilm structures were visualized using confocal laser scanning microscopy (CLSM), as described previously (Nailis et al., 2009).

2.3. Antifungal agents

Diflucan® (Pfizer, New York, NY, USA) and Fungizone® (Bristol-Myers Squibb, New York, NY, USA) were the pharmaceutical formulations used. Diflucan contains 2000 mg/l of FLC. Fungizone contains AMB powder that was dissolved in MilliQ water and further diluted to obtain stock solutions of 64 mg/l. Aliquots of both antifungal agents were stored at -80 °C. The minimal inhibitory concentrations (MICs) of FLC and AMB were determined according to the European Committee for Antimicrobial Susceptibility Testing (EUCAST) procedure (Cuenca-Estrella et al., 2003).

2.4.Treatment of biofilms with antifungal agents

Biofilms were grown as described above. Silicone disks were subsequently transferred to new 24-well microtiter plates, and antifungal agents were added (final concentration of 1000 mg/l for FLC and 32 mg/l for AMB, in buffered antifungal-free medium). RPMI-1640 2x supplemented with 200 mM glucose and buffered to pH 7.0 with 330 mM MOPS (Sigma, St. Louis, MO, USA) was used for this purpose. Control biofilms were grown in buffered RPMI without antifungal agents. Plates were incubated at 37 °C for 1 h or 22 h, after which cells were washed three times with 0.9% (w/v) NaCl. For the regrowth experiments, biofilms were treated for 22 h after which they were washed five times with 1 ml of 0.9% (w/v) NaCl, and disks were transferred to new 24-well microtiter plates. One ml of buffered RPMI was added to the wells and plates were further incubated up to 24 h at 37 °C.

2.5. Cell quantification

To enumerate the number of culturable sessile cells, plating was used. Silicone disks were transferred to 10 ml SDB and cells were subjected to three cycles of 30 s sonication (Branson 3510, 42 kHz, 100 W; Branson Ultrasonics Corp., Danbury, CT, USA) and 30 s vortex mixing. Using this procedure, all cells were removed from the silicone and clumps of cells were broken apart, while viability and culturability of cells were not affected (data not shown). Serial tenfold dilutions of the resulting cell suspensions were plated on SDA and plates were incubated for 24 h at 37 °C, after which colonies were counted. The experiments were performed at least in triplicate (independent biofilm experiments; biological replicates) with 4 disks per biofilm experiment (technical replicates) ($n \geq 12$). Independent sample t-tests were carried out using SPSS 15.0 software to determine whether differences were statistically significant ($p<0.05$). Reductions were considered biologically relevant when a statistically significant reduction ($p<0.05$) of at least 1 $\log_{10}$ unit was observed.

2.6. RT-qPCR

Biofilms were grown and treated with antifungal agents as described above. Biofilms were then washed with 1 ml 0.9% (w/v) NaCl, and subjected to sonication and vortex mixing as described above. RNA extraction, cDNA synthesis and RT-qPCR were performed as described previously (Nailis et al., 2006). Experiments were carried out in triplicate using starting material from independent biofilm experiments (biological replicates). Full-length gene sequences were obtained from the *C. albicans* database (http://www-sequence.stanford.edu/group/candida/search.html). Primers and MGB Taqman probes were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). Sequences of the primers and MGB Taqman probes

designed in the present study are listed in Table 1. The sequences of the primers and probes for the reference genes have been reported previously (Nailis et al., 2006). RT-qPCR data were normalized by geometric averaging of five internal reference genes. *ACT1*, *RIP*, *RPP2B*, *PMA1* and *LSC2* were used for this purpose as they have previously shown to be stably expressed in *C. albicans* biofilms (Nailis et al., 2006). Following normalization, normalized relative quantities were used to calculate relative gene expression levels as described previously (Nailis et al., 2006). Gene expression levels (fold over- or underexpression) in biofilms in the absence of antifungal agents were calculated as the expression of a gene in biofilms, relative to its expression in start cultures (planktonic cells grown for 16 h in SDB at 37°C). Gene expression levels (fold up- or downregulation) in biofilms treated with antifungal agents were calculated as the expression of a gene in biofilms treated with FLC or AMB, relative to its expression in untreated biofilms. Gene expression levels (fold up- or downregulation) in biofilms after removal of the antifungal agent were calculated as the expression of a gene in biofilms after regrowth, relative to its expression in biofilms after treatment. Confidence intervals were calculated to determine whether differences in gene expression were statistically significant between two biological conditions ($p<0.05$).

3. Results and Discussion

3.1. Biofilm formation on silicone disks in microtiter plates

Young biofilms contained $2.5x10^4$ CFU/disk after 0.5 h and $5.0x10^4$ CFU/disk after 1 h, whereas mature biofilms contained $6.3x10^6$ CFU/disk (Table 2). CLSM analysis of a 1 h old biofilm showed mainly yeast cells that were adhered to the silicone surface (Fig. 1A). After 24 h (Fig. 1B) and 72 h (data not shown) biofilms showed a three-dimensional structure of mainly yeast cells and few filaments, embedded in an extracellular matrix. Similar structures were obtained in previous studies (Chandra et al., 2001; Zhao et al., 2006), although we observed less filaments. This is likely due to differences in growth conditions as we used YNB for biofilm growth in our model system. Previous studies frequently use abiotic surfaces that are incubated with fetal bovine serum prior to biofilm formation; serum is known to induce filamentation in *C. albicans* (Ponton et al., 1994).

3.2. Effect of antifungal agents on pre-grown biofilms

In the present study, we wanted to determine whether the age of the biofilm affects the activity of antifungal agents and whether this anti-biofilm activity is dependent on the exposure time. For this, we used very high doses of FLC (1000 mg/l; 8000 times the MIC) and AMB (32 mg/l: 256 times the MIC). These concentrations were selected because they resulted in the survival of only a small fraction of cells (AMB) or because they resulted in tolerance (FLC) for mature biofilms. No antifungal effect was observed after treatment of mature biofilms with FLC for 1 h or 22 h, indicating that mature biofilms are tolerant to high doses of FLC (Table 2); this confirms previous observations (Chandra et al., 2001; Hawser and Douglas, 1995; Mukherjee et al., 2003; Ramage et al., 2002). Prolonged incubation with FLC (up to 144 h) did

not result in a reduction in cell number (data not shown). However, when young biofilms (0.5 h and 1 h) were treated for 22 h with FLC, only approx. $2.5x10^4$ CFU were recovered from the silicone disks (Table 2). Prolonged incubation of young biofilms with high doses of FLC resulted in a reduction in cell number as approx. $3.2x10^3$ CFU per disk were recovered after 144 h. These findings indicate that high doses of FLC have an antifungal effect on young biofilms. Previous studies demonstrated that adherent *C. albicans* cells are as tolerant to FLC as mature biofilms, but in these studies lower FLC concentrations (≤ 256 mg/l) were used (Mateus et al., 2004; Mukherjee et al., 2003).

Treatment of mature biofilms for 22 h with 32 mg/l of AMB resulted in a 99.5 % reduction in cell number, indicating that only a small fraction of cells, likely "persisters", survived the treatment (Table 2) ($p<0.05$). AMB was already found to show activity against mature biofilms at a concentration of ≥ 1 mg/l (Chandra et al., 2001; Hawser and Douglas, 1995; Kuhn et al., 2002), and exposure of mature biofilms to high doses of this antifungal agent is known to result in the survival of persisters (LaFleur et al., 2006). Recently, it was demonstrated that the presence of persisters in *C. albicans* biofilms is strain-dependent, and that biofilms formed by *C. albicans* strain SC5314 contain a very low fraction of persisters only after exposure to 50 mg/l of AMB (Al-Dhaheri and Douglas, 2008). However, different methods for biofilm growth were used, and this could explain why we were able to detect persisters in *C. albicans* SC5314 biofilms after treatment with 32 mg/l of AMB. Combined, these findings indicate that the presence of persisters in *C. albicans* biofilms is not only strain-dependent, but also seems to be dependent on the biofilm model system used. A short exposure of mature biofilms to AMB also resulted in the presence of persisters, as similar reductions in cell number were observed after 1 h

and after 22 h of treatment (Table 2). Longer treatment (up to 144 h) did not result in more eradication (data not shown). When young biofilms (1 h old) were treated with AMB for 1 h or 22 h, approx. 99.5 and 96.8 % of the population was killed, respectively (Table 2). Similar results were obtained for young biofilms grown for 0.5 h (Table 2). Thus, irrespective of the age of the biofilm, a similar fraction of the cell population survives treatment with high doses of AMB. Chandra et al. (2001) found that resistance to AMB develops at later stages of biofilm formation. However, these authors used different biofilm growth conditions, different susceptibility methods and lower concentrations of AMB (Chandra et al., 2001), which likely explain the observed discrepancies. Taken together, our data show that a small fraction (1 to 4 %) of persisters are present in both young and mature biofilms, even after a short exposure to AMB.

CLSM analysis confirmed our findings as treatment of 72 h old biofilms with FLC for 22 h has no effect (Fig. 1C), while exposure to AMB for 22 h resulted in a disruption of the three-dimensional structure and a reduction in cell number (Fig. 1D). For AMB, similar results were obtained in a previous study (Hawser and Douglas, 1995).

3.3. Gene expression in biofilms in the absence of antifungal agents

In a next set of experiments, gene expression was quantified in biofilms relative to gene expression in start cultures (prior to treatment with antifungal agents). By doing so, we observed a marked overexpression of *SKN1* in young biofilms; this gene was 27.0 ± 13.5 and 22.1 ± 13.8 (mean ± SEM) fold upregulated after 0.5 h and 1 h of biofilm growth, respectively. Upregulation of *SKN1* suggests a reorganization of the β-1,6-glucan content in the cell wall, a mechanism which is known to be implicated in transient AMB resistance in *C. albicans* (Gale, 1986; Liu et al., 2005). Khot et al.

(2006) recently found that the expression of *SKN1* was induced in a subpopulation of cells which are most closely in contact with the silicone surface. The majority of cells in a young biofilm grown in our model system are also in intimate contact with the silicone surface (see Fig. 1A). Combined, these data suggest that contact sensing with the silicone surface induces the expression of *SKN1*, which may render sessile *C. albicans* cells resistant to AMB even in the absence of antifungal drugs. On the other hand, overexpression of *SKN1* could be a sign of biofilm growth as this gene is highly upregulated only in young biofilms.

No statistical significant changes in the expression of genes encoding efflux pumps, nor in *KRE1* or *ERG* gene expression were observed between biofilms and start cultures (data not shown). However, previous studies showed that *MDR1, CDR1, CDR2* are overexpressed in young biofilms (Mateus et al., 2004; Mukherjee et al., 2003; Ramage et al., 2002). Discrepancies between these studies and our results are likely due to the fact that we detected a high number of transcripts for *MDR1, CDR1, CDR2* (evidenced by low cycle threshold values) not only in young biofilms but also in start cultures (data not shown). Similarly to Mukherjee et al (2003), we detected lower gene expression levels for *CDR1* and *MDR1* after 24 h of biofilm growth than after 1 h ($p<0.05$), confirming that the expression of genes encoding efflux pumps is higher in young biofilms.

3.4. Transcriptional response to FLC in biofilms

We also investigated gene expression in biofilms in the presence of high doses of FLC. Gene expression in biofilms treated with high doses of FLC was compared to gene expression in untreated biofilms, and results are listed in Table 3. After 1 h of treatment with high doses of FLC, young biofilms showed an upregulation of *ERG25*,

while in mature biofilms an overexpression of *ERG1, ERG3*, *ERG11* and *ERG25* was observed. After longer treatment, upregulation of *ERG* genes was more pronounced in young biofilms, but not in mature biofilms (Table 3). It is possible that the response of *ERG* gene expression in 24 h old biofilms is attenuated because mature biofilms are tolerant to high doses of FLC. However, we observed a high upregulation of *ERG* genes in tolerant 72 h old biofilms after 22 h of treatment with high doses of FLC. In order to better understand the role of *ERG* gene expression in tolerance and sensitivity in biofilms, we examined gene expression in young biofilms treated with 0.5 mg/l of FLC. Young biofilms were tolerant to low doses of FLC as a mature biofilm with approx. $2.0x10^6$ CFU per disk was obtained after 22 h of treatment. As can be seen in Fig. 2, *ERG* genes were overexpressed in tolerant young biofilms, although the fold upregulations were lower than those observed in young biofilms after exposure to high doses of FLC. Upregulation of *ERG* genes was previously observed in planktonic cells after in vitro exposure to FLC (Lepak et al., 2006) and also after exposure to another azole, itraconazole (De Backer et al., 2001). Combined, these data suggest a general response of the expression of *ERG* genes to FLC sensing in biofilms and planktonic cells. The overexpression of *ERG* genes is probably related to biofilm resistance, but is not sufficient as sensitive biofilms show a similar gene expression pattern as tolerant biofilms.

Surprisingly, no statistical significant overexpression of genes encoding efflux pumps were observed in biofilms after exposure to high doses of FLC (Table 3). Similar results were obtained after treatment of planktonic cells with itraconazole (De Backer et al., 2001). However, when planktonic cells were exposed to FLC an upregulation of *CDR1* was observed (Hernáez et al., 1998). In the current study, an overexpression of *CDR2* was found in tolerant young biofilms (Fig. 2), suggesting

that efflux pumps play a role in biofilm resistance in a dose-dependent manner. Furthermore, we observed an overexpression of *KRE1* and *SKN1* in young biofilms after exposure to low doses of FLC (Fig. 2). This could be a sign of biofilm growth as *SKN1* was also induced in young biofilms in the absence of antifungal agents (see above).

Together, the current study showed an upregulation of genes encoding enzymes involved in the ergosterol biosynthesis in sessile cells in the presence of FLC. This transcriptional response may contribute to biofilm resistance in *C. albicans*.

3.5. Transcriptional response to AMB in biofilms

We also investigated gene expression in biofilms in the presence of high doses of AMB. Gene expression in biofilms treated with AMB was compared to gene expression in untreated biofilms, and results are listed in Table 4. No statistical significant differences in the expression of *ERG3, ERG11* and *ERG25* were observed between biofilms exposed to AMB and untreated biofilms. A marked overexpression of *SKN1* was seen in young and mature biofilms already after 1 h of exposure (Table 4), and its expression was even more induced after 22 h. Furthermore, *KRE1* was significantly upregulated in young biofilms (1 h old) after 1 h of treatment, and in mature biofilms (24 h old) both after 1 h and 22 h ($p<0.05$). In addition, *ERG1* was significantly overexpressed in biofilms after 22 h of treatment ($p<0.05$). As mentioned above, exposure of biofilms to high doses of AMB resulted in the survival of a small fraction of cells, and these persisters seem to exhibit high expression levels of *SKN1* (and to a lesser extent *KRE1* and *ERG1*). Khot et al. (2006) demonstrated that *SKN1 and KRE1* were highly upregulated in biofilms in the absence of AMB. Overexpression of *SKN1* and *KRE1* suggest changes in cell wall maintenance, which

in turn could explain the high resistance of biofilms towards AMB (Gale, 1986; Liu et al., 2005). Consequently, overexpression of both genes could be responsible for the resistant phenotype of persisters in the presence of high doses of AMB on the one hand, and also for the intrinsic resistance of biofilms in the absence of AMB on the other hand (our data and those from Khot et al. [2006]). It has already been demonstrated that planktonic cells show a downregulation *of ERG3, ERG11* and *ERG25* after exposure to much lower concentrations of AMB (Liu et al., 2005), indicating that biofilms show a specific transcriptional response to AMB. Based on our findings, changes in cell wall maintenance could be an important resistance mechanism in sessile *C. albicans* cells towards AMB.

3.6. Transcriptional response to removal of the antifungal agent

To test whether the transcriptional response in biofilms after exposure to antifungal agents was transient or permanent, gene expression was investigated in biofilms after removal of FLC and AMB. For this, biofilms were grown for 72 h, subsequently treated with antifungal drugs for 22 h, and further incubated in antifungal-free medium. As can be seen in Table 5, biofilms showed a marked overexpression of *SKN1* after removal of FLC. This gene was 49.6 ± 9.2 and 31.7 ± 6.6 (mean ± SEM) fold upregulated after 1 h and 24 h of regrowth, respectively. Overexpression of *SKN1* could be a sign of biofilm regrowth as this gene was also upregulated during biofilm formation (see above). Furthermore, the expression of *ERG* genes remained unchanged (Table 5), demonstrating that these genes were still upregulated 24 h after removal of FLC. A transient molecular response with upregulation of *ERG11*, *CDR1 and CDR2* in the presence of FLC and a downregulation of *ERG11* in the post-exposure period has been observed in planktonic cells (Henry et al., 2000;

Lepak et al., 2006). After removal of AMB, gene expression remained unchanged in biofilms, except for *SKN1*; this gene was 4.6 ± 1.2 (mean ± SEM) fold upregulated 1 h after removal of the antifungal agent (Table 5). Our data indicate that the expression of *SKN1* is highly induced in *C. albicans* persisters after exposure to AMB, and that this gene remains upregulated in biofilms after removal of the antifungal agent.

3.7. Concluding remarks

In the present study, it was demonstrated that high doses of FLC have an antifungal effect on young biofilms, while mature biofilms were tolerant to this antifungal agent. Treatment of young and mature biofilms with high doses of AMB resulted in the survival of a small fraction (0.5 to 4 %) of persisters. Exposure of biofilms to FLC resulted in an overexpression of genes encoding enzymes involved in the ergosterol biosynthesis. However, genes encoding efflux pumps were not regulated in biofilms in response to high doses of FLC. Treatment of biofilms with high doses of AMB resulted in an upregulation of genes encoding proteins involved in the β-1,6-glucan biosynthesis. Transcriptional changes in sessile *C. albicans* cells in the presence of antifungal agents suggest an upregulation of the associated biosynthetic pathways, thereby contributing to a resistant biofilm phenotype. Future research will provide a direct elucidation of these resistance mechanisms in sessile *C. albicans* cells. Taken together, our data indicate that young and mature biofilms show a rapid and antifungal-specific transcriptional response to exposure with FLC and AMB. This molecular adaptation could help to explain the high resistance of *C. albicans* biofilms towards antifungal agents.

Acknowledgements

We are grateful to Alistair Brown (Aberdeen University, UK) for sharing *C. albicans* SC5314 with us. We would like to acknowledge Leen Molderez and Marlies De Kezel for technical assistance. We are grateful to Jo Vandesompele for providing useful advice concerning qPCR data analysis. We kindly acknowledge Ine Lentacker, Kevin Braeckmans, Stefaan Desmedt and Jo Demeester for the use of the CLSM facilities. This work was funded by the Belgian Federation against Cancer and the FWO (Fonds voor Wetenschappelijk Onderzoek).

References

1. Al-Dhaheri, R.S., Douglas, L.J., 2008. Absence of amphotericin B-tolerant persister cells in biofilms of some *Candida* species. Antimicrob. Agents Chemother. 52, 1884-1887.

2. Brajtburg, J., Powderly, W.G., Kobayashi, G.S., Medoff, G., 1990. Amphotericin B: Current understanding of mechanisms of action. Antimicrob. Agents Chemother. 34, 183-188.

3. Chandra, J., Kuhn, D.M., Mukherjee, P.K., Hoyer, L.L., McCormick, T., Ghannoum, M.A., 2001. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. J. Bacteriol. 183, 5385-5394.

4. Cuenca-Estrella, M., Moore, C.B., Barchiesi, F., Bille, J., Chryssanthou, E., Denning, D.W., Donnelly, J.P., Dromer, F., Dupont, B., Rex, J.H., Richardson, M.D., Sancak, B., Verweij, P.E., Rodriguez-Tudela, J.L.; AFST Subcommittee of the European Committee on Antimicrobial Susceptibility Testing, 2003. Multicenter evaluation of the reproducibility of the proposed antifungal susceptibility testing method for fermentative yeasts of the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antimicrobial Susceptibility Testing (AFST-EUCAST). Clin. Microbiol. Infect. 9, 467-474.

5. De Backer, M.D., Ilyina, T., Ma, X.J., Vandonink, S., Luyten, W.H., Vanden Bossche, H., 2001. Genomic profiling of the response of *Candida albicans* to itraconazole treatment using a DNA microarray. Antimicrob. Agents Chemother. 45, 1660-1670.

6. Douglas, L.J., 2003. *Candida* biofilms and their role in infection. Trends Microbiol. 11, 30-36.

7. Ellis, D., 2002. Amphotericin B: spectrum and resistance. J. Antimicrob. Chemother. 49, 7-10.

8. Franz, R., Kelly, S.L., Lamb, D.C., Kelly, D.E., Ruhnke, M., Morschhäuser, J., 1998. Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains. Antimicrob. Agents Chemother. 42, 3065-3072.

9. Gale, E.F., 1986. Nature and development of phenotypic resistance to amphotericin B in *Candida albicans*. Adv. Microb. Physiol. 27, 277-320.

10. Hawser, S.P., Douglas, L.J., 1995. Resistance of *Candida albicans* biofilms to antifungal agents *in vitro*. Antimicrob. Agents Chemother. 39, 2128-2131.

11. Henry, K.W., Nickels, J.T., Edlind, T.D., 2000. Upregulation of *ERG* genes in *Candida* species by azoles and other sterol biosynthesis inhibitors. Antimicrob. Agents Chemother. 44, 2693-2700.

12. Hernáez, M.L., Gil, C., Pla, J., Nombela, C., 1998. Induced expression of the *Candida albicans* multidrug resistance gene *CDR1* in response to fluconazole and other antifungals. Yeast. 14, 517-526.

13. Kelly, S.L., Arnoldi, A., Kelly, D.E., 1993. Molecular genetic analysis of azole antifungal mode of action. Biochem. Soc. Trans. 21, 1034-1038.

14. Khot, P.D., Suci, P.A., Miller, R.L., Nelson, R.D., Tyler, B.J., 2006. A small subpopulation of blastospores in *Candida albicans* biofilms exhibit resistance to amphotericin B associated with differential regulation of ergosterol and bèta-1,6-glucan pathway genes. Antimicrob. Agents Chemother. 50, 3708-3716.

15. Kuhn, D.M, George, T., Chandra, J., Mukherjee, P.K., Ghannoum, M.A., 2002. Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. Antimicrob. Agents Chemother. 46, 1773-1780.

16. Kuhn, D.M., Ghannoum, M.A., 2004. *Candida* biofilm: antifungal resistance and emerging therapeutic options. Curr. Opin. Investig. Drugs. 5, 186-197.

17. Kumamoto, C.A., 2002. *Candida* biofilms. Curr. Opin. Microbiol. 5, 608-611.

18. LaFleur, M.D., Kumamoto, C.A., Lewis, K., 2006. *Candida albicans* biofilms produce antifungal-tolerant persister cells. Antimicrob. Agents Chemother. 50, 3839-3846.

19. Lepak, A., Nett., J., Lincoln, L., Marchillo, K., Andes, D., 2006. Time course of microbiologic outcome and gene expression in *Candida albicans* during and following *in vitro* and *in vivo* exposure to fluconazole. Antimicrob. Agents Chemother. 50, 1311-1319.

20. Liu, T.T., Lee, R.E., Barker, K.S., Lee, R.E., Wei, L., Homayouni, R., Rogers, P.D., 2005. Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in *Candida albicans*. Antimicrob. Agents Chemother. 49, 2226-2236.

21. Lopez-Ribot, J.L., McAtee, R.K., Lee, L.N., Kirkpatrick, W.R., White, T.C., Sanglard, D., Patterson, T.F., 1998. Distinct patterns of gene expression associated with development of fluconazole resistance in serial *Candida albicans* isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. Antimicrob. Agents Chemother. 42, 2932-2937.

22. Mateus, C., Crow, S.A. Jr., Ahearn, D.G., 2004. Adherence of *Candida albicans* to silicone induces immediate enhanced tolerance to fluconazole. Antimicrob. Agents Chemother. 48, 3358-3366.

23. Mio, T., Yamada-Okabe, T., Yabe, T., Nakajima,T., Arisawa, M., and Yamada-Okabe, H., 1997. Isolation of the *Candida albicans* homologs of *Saccharomyces cerevisiae KRE6* and *SKN1*: expression and physiological function. J. Bacteriol. 179, 2363-2372.

24. Mukherjee, P.K., Chandra, J., Kuhn, D.M., Ghannoum, M.A., 2003. Mechanisms of fluconazole resistance in *Candida albicans* biofilms: phase-specific role of efflux pumps and membrane sterols. Infect. Immun. 71, 4333-4340.

25. Nailis, H., Coenye, T., Van Nieuwerburgh, F., Deforce, D., Nelis, H.J., 2006. Development and evaluation of different normalization strategies for gene expression studies in *Candida albicans* biofilms by real-time PCR. BMC Mol. Biol. 7, 25.

26. Nailis, H., Vandenbroucke, R., Tilleman, K., Deforce, D., Nelis, H., Coenye, T., 2009. Monitoring *ALS1* and *ALS3* gene expression during *in vitro Candida albicans* biofilm formation under continuous flow conditions. Mycopathologia 167, 9-17.

27. Odds, F.C., 1988. Meeting *Candida* and Candidiosis, 2nd ed. Bailliere Tindall, London, UK.

28. Pappas, P.G., Rex, J.H., Sobel J.D., Filler, S.G., Dismukes, W.E., Walsh, T.J., Edwards, J.E., 2004. Infectious Disease Society of Amerika; Guidelines for treatment of candidiasis. Clin. Infect. Dis. 38, 161-189.

29. Ponton, J., Quindos, G., Arilla, M.C., Mackenzie, D.W., 1994. Simplified adsorption method for detection of antibodies to *Candida albicans* germ tubes. J. Clin. Microbiol. 32, 217-219.

30. Ramage, G., Bachmann, S., Patterson, T.F., Wickes, B.L., López-Ribot, J.L., 2002. Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. J. Antimicrob. Chemother. 49, 973-980.

31. Richardson, K., 1990. The discovery and profile of fluconazole. J. Chemother. 2, 51-54.

32. Rogers, P.D., Barker, K.S., 2003. Genome-wide expression profile analysis reveals coordinately regulated genes associated with stepwise acquisition of azole resistance in *Candida albicans* clinical isolates. Antimicrob. Agents Chemother. 47, 1220-1227.

33. Sanglard, D., 2002. Resistance of human fungal pathogens to antifungal drugs. Curr. Opin. Microbiol. 5, 379-385.

34. Warnock, D.W., 1991. Amphotericin B: an introduction. J. Antimicrob. Chemother. 28, 27-38.

35. White, T.C., 1997. Increased mRNA levels of *ERG16, CDR*, and *MDR1* correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. Antimicrob. Agents Chemother. 41, 1482-1487.

36. White, T.C., Marr, K.A., Bowden, R.A., 1998. Clinical, cellular and molecular factors that contribute to antifungal drug resistance. Clin. Microbiol. Rev. 11, 382-402.

37. White, T.C., Holleman, S., Dy, F., Mirels, L.F., Stevens D.A., 2002. Resistance mechanisms in clinical isolates of *Candida albicans*. Antimicrob. Agents Chemother. 46, 1704-1713.

38. Zhao, X., Daniels, K.J., Oh, S.H., Green, C.B., Yeater, K.M., Soll, D.R., Hoyer, L.L., 2006. *Candida albicans* Als3p is required for wild-type biofilm formation on silicone elastomer surfaces. Microbiology 152, 2287-2299.

Legends to figures

Fig. 1: CLSM images of *C. albicans* SC5314 biofilms grown for 1 h (Fig. 1 A) and 24 h (Fig. 1 B) on silicone disks in 24-well microtiter plates. CLSM images of mature *C. albicans* SC5314 biofilms (72 h) treated for 22 h with 1000 mg/l of FLC (Fig. 1C) or with 32 mg/l of AMB (Fig. 1D). Sessile *C. albicans* cells were stained with Concanavalin A, resulting in red labelled cells and red stained extracellular matrix. Scale bar: 10 µm.

Fig. 2: Average gene expression levels in young (1 h) *C. albicans* SC5314 biofilms after exposure to low doses of FLC (0.5 mg/l) for 22 h. Gene expression levels (fold up- or downregulation) were defined as the expression of a gene in biofilms treated with FLC, relative to its expression in untreated biofilms. * represents statistically significant difference in gene expression between biofilms treated with FLC and untreated biofilms ($p<0.05$).

Tables

Table 1: Sequences of primers and MGB Taqman probes for *C. albicans* genes used in the real-time PCR assays.

555

Gene	Protein product and/or function	Orientation	Sequence (5' to 3')
CDR1	ABC membrane transporter involved in active efflux of a variety of components including antifungal agents	Forward	CAGCAACCATGGGTCAATTATG
		Reverse	GTAGCCAAATTGGCAGCATTATC
		Probe	ATGTCTTTCAGTGAATTAG
CDR2	ABC membrane transporter involved in active efflux of a variety of components including antifungal agents	Forward	GATTCAAGCCATTCTTTCTACTGGAT
		Reverse	AGTAACCAATTCTCTAGGTGCACAAG
		Probe	AGCTAATGCAAAAGTC
MDR1	MF membrane transporter involved in active efflux of a variety of components including antifungal agents	Forward	TTCCGTGTTGGGTTTCATCA
		Reverse	TGGTCCGTTCAAGTAAAACAAAACT
		Probe	CCTTGTTATGATTGCTATTC
ERG1	Squalene epoxidase, catalyzes epoxidation of squalene to 2,3(S)-oxidosqualene	Forward	AGAATGTGTTAACGGGCCAATT
		Reverse	ATGGTTGAATAACAACATTGGGAAT
		Probe	ATTATTGAGTGGCATGTTAC
ERG3	C-5 sterol desaturase; introduction of C-5(6) double bond into episterol.	Forward	GGCCATCTGTTTACAAAGTGTTACA
		Reverse	TGAGAAGCAAATGGAGTACAAACAA
		Probe	AAACCTCATCACAAGTGG
ERG11	Lanosterol 14-α-demethylase, member of the cytochrome P450 family; catalyzes the conversion of lanosterol	Forward	GCTAATTCTGTTTCATTTAACTCTTCTGAT
		Reverse	GGTGAAGAAACCCCTTTAGAAACTT
		Probe	AAGTTGATTATGGGTTTGGG
ERG25	Putative C-4 methyl sterol oxidase, important for C4 demethylation of ergosterol biosynthesis intermediates	Forward	TATTTCATTGGTGGATACTCTTCATCTT
		Reverse	GGACCAGCTTCGGTATCCAAA
		Probe	TAGATGGTGGGATTTCA
KRE1	Predicted Glycosyl Phosphatidyl Inositol anchor protein with a role in β-1,6-glucan biosynthesis.	Forward	CCTTGCGGCAGATAAAACGT
		Reverse	GCATCAGTACCTGTGACCCATACT
		Probe	CAGTTCAGTATCACCTACTT
SKN1	N-glycosylated type II membrane protein with a predicted role in β-1,6-glucan biosynthesis	Forward	CCCTGAAATTGATGCATTGGA
		Reverse	CATAAGGAGCAACTTGTAATGATTGAG
		Probe	TTCATGGTGTTATAGGTAGAGTT

Table 2: Average number of sessile *C. albicans* SC5314 cells (CFU/disk) in untreated biofilms and in biofilms treated for 1 h or 22 h with FLC (1000 mg/l) or AMB (32 mg/l).

Average number of sessile *C. albicans* cells (Log_{10} CFU/disk)									
Untreated		Treated with FLC (1 h)		Treated with FLC (22 h)		Treated with AMB (1 h)		Treated with AMB (22 h)	
Age (h)	CFU[a]	CFU[a]	Reduction[b]	CFU[a]	Reduction[b]	CFU[a]	Reduction[b]	CFU[a]	Reduction[b]
0.5	4.4 ± 0.4	ND	ND	4.5 ± 0.6	-0.1	ND	ND	3.0 ± 0.9	1.4[c]
1	4.7 ± 0.4	4.3 ± 0.4	0.4	4.4 ± 0.7	0.3	2.4 ± 0.7	2.3[c]	3.2 ± 0.7	1.5[c]
24	6.8 ± 0.3	6.4 ± 0.4	0.4	6.8 ± 0.4	0	4.6 ± 0.3	2.2[c]	4.5 ± 0.6	2.3[c]
72	6.8 ± 0.3	ND	ND	6.5 ± 0.4	0.3	ND	ND	4.5 ± 0.4	2.3[c]

[a] Log_{10} average number of CFU/disk ± SD (n ≥ 12)

[b] Log_{10} reduction of cells after treatment, compared to the cell number of untreated biofilms at the same stage of growth.

[c] Reductions were considered biologically relevant when a statistically significant reduction ($p<0.05$) of at least 1 log_{10} unit was observed; ND = Not determined.

Table 3: Average gene expression levels (with corresponding SEM; n=3) in young (0.5 h and 1 h) and mature (24 h and 72 h) *C. albicans* SC5314 biofilms after exposure to high doses of FLC (1000 mg/l). Gene expression levels (fold up- or downregulation) were defined as the expression of a gene in biofilms treated with FLC for 1 h or 22 h, relative to its expression in untreated biofilms.

Gene expression levels in biofilms after treatment with high doses of FLC (1000 mg/l)									
Biofilms treated with FLC	***CDR1***	***CDR2***	***MDR1***	***ERG1***	***ERG3***	***ERG11***	***ERG25***	***KRE1***	***SKN1***
Young biofilms (0.5h) + FLC (22h)	-2.0 ± 0.6	2.1 ± 0.6	-3.1 ± 1.4	9.8 ± 1.2[a]	6.3 ± 3.1[a]	4.4 ± 1.4[a]	5.7 ± 1.6[a]	-1.8 ± 0.5	2.1 ± 0.8
Young biofilms (1h) + FLC (1h)	1.0 ± 0.2	1.1 ± 0.2	-1.0 ± 0.2	1.7 ± 0.4	2.5 ± 0.7	2.7 ± 1.0	2.1 ± 0.3[a]	1.3 ± 0.4	1.1 ± 0.3
Young biofilms (1h) + FLC (22h)	1.0 ± 0.5	4.2 ± 1.4	-1.9 ± 0.9	16.2 ± 1.1[a]	11.6 ± 4.5[a]	7.4 ± 1.3[a]	8.6 ± 2.6[a]	-1.3 ± 0.5	2.2 ± 1.0
Mature biofilms (24h) + FLC (1h)	1.0 ± 0.3	-1.1 ± 0.1	1.2 ± 0.4	2.6 ± 0.3[a]	3.3 ± 0.7[a]	2.7 ± 0.3[a]	2.2 ± 0.4[a]	1.3 ± 0.2	1.2 ± 0.4
Mature biofilms (24h) + FLC (22h)	-2.3 ± 1.0	1.1 ± 0.6	-2.3 ± 1.1	5.0 ± 1.8[a]	1.5 ± 1.1	3.4 ± 0.8[a]	2.0 ± 0.3[a]	-2.4 ± 1.3	-1.8 ± 0.9
Mature biofilms (72h) + FLC (22h)	1.8 ± 0.5	2.0 ± 0.4	1.3 ± 0.5	39.3 ± 10.1[a]	11.3 ± 6.7[a]	23.5 ± 10.2[a]	20.5 ± 11.0[a]	1.7 ± 0.8	2.8 ± 1.3

[a] Statistically significant difference in gene expression between biofilms treated with FLC and untreated biofilms ($p<0.05$).

Table 4: Average gene expression levels (with corresponding SEM; n=3) in young (0.5 h and 1 h) and mature (24 h and 72 h) *C. albicans* SC5314 biofilms after exposure to high doses of AMB (32 mg/l). Gene expression levels (fold up- or downregulation) were defined as the expression of a gene in biofilms treated with AMB for 1 h or 22 h, relative to its expression in untreated biofilms.

Gene expression levels in biofilms after treatment with high doses of AMB (32 mg/l)						
Biofilms treated with AMB	***ERG1***	***ERG3***	***ERG11***	***ERG25***	***KRE1***	***SKN1***
Young biofilms (0.5h) + AMB (22h)	3.8 ± 2.0[a]	2.5 ± 1.7	1.2 ± 0.3	1.7 ± 0.9	1.9 ± 1.3	40.0 ± 32.1[a]
Young biofilms (1h) + AMB (1h)	-1.4 ± 0.3	1.3 ± 0.3	-1.3 ± 0.5	1.0 ± 0.3	6.6 ± 2.1[a]	16.0 ± 6.6[a]
Young biofilms (1h) + AMB (22h)	3.5 ± 1.9[a]	2.1 ± 1.5	1.2 ± 0.3	1.9 ± 1.0	1.1± 0.6	22.4 ± 12.9[a]
Mature biofilms (24h) + AMB (1h)	-2.2 ± 0.2	1.1 ± 0.1	-1.8 ± 0.2	-1.3 ± 0.3	4.9 ± 1.3[a]	13.0 ± 3.1[a]
Mature biofilms (24h) + AMB (22h)	4.1 ± 1.4[a]	1.3 ± 0.7	1.2 ± 0.4	2.3 ± 1.0	5.7 ± 2.4[a]	59.9 ± 17.5[a]
Mature biofilms (72h) + AMB (22h)	-1.6 ± 1.4	-4.2 ± 2.4	-2.4 ± 1.7	-2.0 ± 1.3	1.4 ± 0.5	21.8 ± 6.1[a]

[a] Statistically significant difference in gene expression between biofilms treated with AMB and untreated biofilms ($p<0.05$).

Table 5: Average gene expression levels (with corresponding SEM; n=3) in *C. albicans* SC5314 biofilms regrown in antifungal-free medium after exposure to high doses of FLC (1000 mg/l) or AMB (32 mg/l). Gene expression levels (fold up- or downregulation) were defined as the expression of a gene in biofilms after 1 h or 24 h of regrowth, relative to its expression in biofilms after treatment with antifungal agents.

Gene expression levels in biofilms after removal of the antifungal agent									
Biofilms after regrowth	***CDR1***	***CDR2***	***MDR1***	***ERG1***	***ERG3***	***ERG11***	***ERG25***	***KRE1***	***SKN1***
FLC + 1h regrowth	1.4 ± 0.4	2.7 ± 2.4	3.5 ± 2.4	-2.9 ± 0.6	-2.6 ± 0.6	-3.6 ± 0.7	-1.6 ± 0.4	3.1 ± 0.8	49.6 ± 9.2[a]
FLC + 24h regrowth	2.8 ± 0.4	2.2 ± 0.7	5.3 ± 2.0	1.2 ± 0.2	2.4 ± 0.4	1.0 ± 0.1	2.9 ± 0.6	4.6 ± 1.3	31.7 ± 6.6[a]
AMB + 1h regrowth	ND	ND	ND	-1.4 ± 0.7	-1.5 ± 0.5	-1.9 ± 0.1	-1.4 ± 0.4	2.2 ± 0.8	4.6 ± 1.2[a]
AMB + 24h regrowth	ND	ND	ND	2.5 ± 1.5	2.4 ± 1.5	1.1 ± 0.4	2.5 ± 1.2	3.7 ± 1.6	3.8 ± 1.6

[a] Statistically significant difference in gene expression between biofilms after regrowth and biofilms after treatment ($p<0.05$). ND: Not determined