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# Detailed comparison of *Candida albicans* and *Candida glabrata* biofilms under different conditions and their susceptibility to caspofungin and anidulafungin

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*Candida* biofilm development can be influenced by diverse factors such as substrate, culture medium, carbohydrate source and pH. We have analysed biofilm formation of *Candida albicans* SC5314 and *Candida glabrata* ATCC 2001 wild-type strains in the presence of different media (RPMI 1640 versus YNB) and using different pH values (pH 5.6 or 7.0). We determined adhesion and biofilm formation on polystyrene, changes in the expression of adhesin genes during these processes and the susceptibility of mature biofilms to echinocandins. Biofilms formed on polystyrene by both *Candida* species proved to be influenced strongly by the composition of the medium rather than pH. *C. albicans* and *C. glabrata* formed thicker biofilms in RPMI 1640 medium, whereas in YNB medium, both species manifested adhesion rather than characteristic multilayer biofilm architecture. The stimulated biofilm formation in RPMI 1640 medium at pH 7.0 corroborated positively with increased expression of adhesin genes, essential to biofilm formation *in vitro*, including *ALS3* and *EAP1* in *C. albicans* and *EPA6* in *C. glabrata*. The thicker biofilms grown in RPMI 1640 medium were more tolerant to caspofungin and anidulafungin than YNB-grown biofilms. We also observed that mature *C. glabrata* biofilms were less susceptible in RPMI 1640 medium to echinocandins than *C. albicans* biofilms. Environmental conditions, i.e. medium and pH, can significantly affect not only biofilm architecture, but also the expression profile of several genes involved during the different stages of biofilm development. In addition, growth conditions may also influence the antifungal-susceptibility profile of fungal populations within biofilm structures. Therefore, before designing any experimental biofilm set-up, it is important to consider the potential influence of external environmental factors on *Candida* biofilm development.

## INTRODUCTION

*Candida albicans* is considered the most commonly isolated human fungal species, followed by *Candida glabrata* (Segireddy *et al.*, 2011). The importance of both species has increased dramatically because of their ability to form biofilms on different types of implant (Ramage *et al.*, 2006). In comparison with the biofilm architecture of *C. albicans*, which is composed of yeast cells, hyphae and pseudohyphae, *C. glabrata* biofilms exist as a multilayer structure of yeast cells; in both cases, the biofilms are embedded in extracellular matrix. Despite their structural diversity, biofilm formation by both *Candida* species is influenced by the choice of biomaterial, medium and carbohydrate source and concentration. Additionally, pH plays an important role, as *Candida* spp. may colonize different niches with respect to the environmental pH (Biswas & Chaffin, 2005; Schmidt *et al.*, 2008). To date, there is no strict recommendation for a specific medium

Abbreviations: 3-D, three-dimensional; CSLM, confocal scanning laser microscopy; FM, fluorescence microscopy.

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suitable for *in vitro* biofilm experiments that would strictly resemble the composition of the human fluids surrounding the device *in vivo*. Recently, Uppuluri *et al.* (2009) characterized *C. albicans* biofilms in synthetic urine medium that mimics the physiological conditions during biofilm development on urinary catheters.

The development of *C. albicans* and *C. glabrata* biofilms involves the regulation of specific genes, expression of which can be affected by the environment. For instance, *C. albicans* adhesins Als1, Als3 and Eap1 are highly expressed in biofilms *in vitro* (Li *et al.*, 2007; Nailis *et al.*, 2010) and *in vivo* (Nett *et al.*, 2009; Řičicová *et al.*, 2010). In *C. glabrata*, Epa6 has been shown to be involved in mature biofilm development (Iraqui *et al.*, 2005).

The major problem in treating biofilm-associated infections is the resistance of the fungal population to most classes of antifungals. However, caspofungin and anidulafungin were shown to have activity on mature *C. albicans* biofilms *in vitro* (Katragkou *et al.*, 2008; Pemán *et al.*, 2008) and *in vivo* (Shuford *et al.*, 2006; Kucharíková *et al.*, 2010a). Caspofungin was also shown to be active against mature *C. glabrata* biofilms formed *in vitro* (Choi *et al.*, 2007).

This study provides a detailed characterization of the influence of two different culture media (RPMI 1640 and YNB) at two different pHs (pH 5.6 and 7.0) on *C. albicans* SC5314 and *C. glabrata* ATCC 2001 adhesion, biofilm development and susceptibility to echinocandins.

## METHODS

**Strains.** For this study, *C. albicans* SC5314 (Gillum *et al.*, 1984) and *C. glabrata* ATCC 2001 (American Type Culture Collection) were used. The strains were routinely cultivated on YPD medium (1 % yeast extract, 2 % Bacto-peptone and 2 % D-glucose, supplemented with 2 % agar) at 37 °C.

**Biofilm formation.** Biofilms were formed in 96-well polystyrene plates according to Ramage *et al.* (2001). Briefly, *C. albicans* and *C. glabrata* were incubated at 37 °C overnight on YPD plates. Cell suspensions of $1\times10^6$ cells $ml^{-1}$ were prepared for both species by counting in RPMI 1640 medium (with glutamine and phenol red, without bicarbonate) buffered with MOPS (Sigma), or in YNB (Yeast Nitrogen Base with amino acids and ammonium sulfate; Difco) buffered with 0.05 M $KH_2PO_4/Na_2HPO_4$. Both media were supplemented with D-glucose (2 % final). Of note, RPMI 1640 medium itself contains 0.2 % D-glucose and therefore this medium was supplemented with D-glucose up to 2 % as a final concentration. A supplementary table showing detailed composition of RPMI 1640 and YNB media used in this study is available in JMM Online. The pH was adjusted to 5.6 or 7.0. In RPMI 1640 medium, pH 7.0 was achieved with 1 M NaOH, according to the NCCLS M27-A3 protocol (NCCLS, 2008). In the same medium, the pH was adjusted to 5.6 with a few drops of 1 M HCl. Inoculum of each strain (100 µl) was added to the flat-bottom 96-well polystyrene plate in quadruplicate. After a period of adhesion (90 min) at 37 °C, non-attached *Candida* cells were removed by two rounds of washing with 1× PBS. One set of plates was analysed immediately after the period of adhesion. A second set of plates was incubated at 37 °C for an additional 48 h in fresh medium. Mature biofilms (48 h) were washed twice with 1× PBS. Remaining cells were evaluated for their metabolic activity by the reduction of XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide], as described by Ramage *et al.* (2001). Each assay in quadruplicate was reproduced five times.

The mature (48 h) *C. albicans* and *C. glabrata* biofilms used for fluorescence microscopy (FM) and confocal scanning laser microscopy (CSLM) were developed on highly adhesive, round tissue-culture coverslips (diameter 13 mm; Sarstedt). One millilitre of cell suspension ($1\times10^6$ cells $ml^{-1}$) prepared in RPMI 1640 or YNB medium at pH 5.6 or 7.0 was added to the wells, each containing a coverslip, and incubated at 37 °C for 90 min. Then, the coverslips were gently washed twice with 1 ml 1× PBS, placed into a clean 24-well tissue-culture plate and covered with fresh medium for an additional 48 h. The mature biofilms were further washed twice with 1× PBS before visualization.

**FM.** Visualization of the attached biofilms and of the morphological composition of the biofilm top layer was carried out by FM. Intact coverslips with attached biofilms were transferred to clean 24-well tissue-culture plates. Each well contained 500 µl 1 × PBS with 20 µl Calcofluor White (0.05 %, v/v) (Sigma). Biofilm structures were observed with a Zeiss Axioplan 2 fluorescence microscope. Images were acquired with a Zeiss Axiocam HRm camera using Axiovision 3.0 software (Carl Zeiss). FM was performed three times independently. Each time, two coverslips were used per condition.

**CSLM.** The three-dimensional (3-D) structure of mature *C. albicans* and *C. glabrata* biofilms grown under different conditions was determined by CSLM. The biofilms formed on coverslips were transferred gently to a clean 24-well tissue-culture plate and stained with 50 µg Concanavalin A–Alexa Fluor 488 conjugate $ml^{-1}$ (C-11252; Molecular Probes) in PBS. The samples were incubated for 1 h at 37 °C in the dark with gentle agitation. Confocal images were acquired according to Řičicová *et al.* (2010). The vertical (*xz*) sections or side views of the 3-D reconstructed images were used to determine biofilm thickness and architecture. The thickness was estimated from the outer edges of the area where fluorescent signal gain intensity was above half of its maximum. CSLM was performed twice independently. Each time, two coverslips were used per condition.

**Quantitative real-time PCR.** Mature (48 h) *Candida* biofilms were formed in six-well tissue-culture plates to obtain the necessary amount of biofilm biomass. The biofilms were scraped off the bottom of the wells by using sterile cell scrapers and washed in DEPC-treated water. Total RNA from biofilms was extracted by using a RiboPure-Yeast kit (AM1926; Ambion). Then, 1 µg RNA was treated with amplification-grade DNase I and used for cDNA synthesis using a Reverse Transcription kit (A3500; Promega). Quantitative PCR was performed on a StepOnePlus real-time PCR system (Applied Biosystems) using a Kapa SYBR Fast kit (Kapabiosystems) according to the manufacturer's instructions. Data for each target gene were calculated and expressed as fold regulation in comparison to the reference gene *ACT1* for each condition tested, using the standard curve quantification method (Livak & Schmittgen, 2001). The following primers were used for *C. albicans* analyses: those for *ACT1* and *EAP1* were described previously (Řičicová *et al.*, 2010). *ALS1* and *ALS3* primers were designed as described by Green *et al.* (2005). The sequence of the *C. glabrata ACT1* forward primer was 5′-CGCTTTGGACTTCGAACAAGAA-3′ and that of the reverse primer was 5′-GTTACCGATAGTGATGACTTGAC-3′. For *C. glabrata EPA6*, the sequence of the forward primer was 5′-CAAAATCTAAACTGGC-AGACAATAATG-3′ and that of the reverse primer was 5′-AGAT-CAGAACCAGAACCTTGGATAATA-3′ (K. De Brucker, J. Serneels, E. Boons, T. Schwarzmüller, J. Quintin, M. Henrigues, K. Kuchler, P. Van Dijck & H. Tournu, unpublished data).

The quantitative RT-PCR assay was performed twice independently. RNA isolation and cDNA synthesis were done twice from different biofilms.


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**Biofilm antifungal-susceptibility testing.** Caspofungin (Merck) and anidulafungin (Pfizer) were used in this study. The stock solution of caspofungin was prepared in sterile water. Anidulafungin was prepared in 100 % DMSO and kept at −20 °C before use. Prior to the experiment, both antifungals were dissolved in corresponding sterile medium according to the NCCLS M27-A3 protocol (NCCLS, 2008).

Susceptibility testing of planktonic cells to caspofungin and anidulafungin was performed according to the NCCLS M27-A3 protocol (NCCLS, 2008). Data were determined as minimal inhibitory concentrations of the drug that inhibit fungal growth by 50 or 90 % ($MIC_{50}$ or $MIC_{90}$, respectively).

Mature *Candida* biofilms (24 h) were formed in 96-well polystyrene plates in RPMI 1640 medium, pH 5.6 or 7.0, or YNB medium, pH 5.6 or 7.0, as described above. Solutions of caspofungin (8–0.01562 μg $ml^{-1}$) and anidulafungin (16–0.03125 μg $ml^{-1}$) were added to preformed mature biofilms and incubated for an additional 24 h. Biofilms treated only with fresh medium served as untreated controls. Measurement of biofilm cell metabolic activity using the XTT-reduction assay was performed as described above. Antifungal effect was determined by comparing the reduction in the mean $A_{490}$ of the antifungal-challenged biofilm condition to the unchallenged controls. The results are shown as sessile $MIC_{50}$ and $MIC_{90}$ values ($SMIC_{50}$ and $SMIC_{90}$, respectively). These values determine the minimal inhibitory concentration of the drug that inhibits the growth of sessile cells by 50 and 90 %, respectively. Each assay was performed in quadruplicate each time and repeated three times.

**Statistical analyses.** For the statistical analyses, Student's *t*-test was used. Results were considered to be statistically significant when $P<0.05$.

## RESULTS

### Medium composition modulates adherence and biofilm formation more than pH in *C. glabrata* and *C. albicans*

*Candida* biofilm development in the host is affected by variable and sometimes changing environmental conditions, depending on the infection site. Many studies *in vitro* have reported the use of a multitude of media, substrates and techniques to study biofilms (Biswas & Chaffin, 2005; Schmidt *et al.*, 2008; Uppuluri *et al.*, 2009). Here, we compared the effect of two commonly used media, namely RPMI 1640 and YNB, at two different pH values (pH 5.6 and 7.0) on *C. glabrata* and *C. albicans* adhesion and mature biofilm development. Biofilms were formed on polystyrene and assessed by the XTT-reduction assay measured at 490 nm. The results are shown in Fig. 1 and are determined as the mean ± SD of the metabolic activity of adhering or biofilm-forming cells. *C. glabrata* adhesion properties were independent of the choice of medium or pH ($P>0.05$) (Fig. 1a), whereas the effect of growth conditions on the ability of *C. albicans* to adhere was more pronounced (Fig. 1c). *C. albicans* manifested significantly better adhesion properties when grown in RPMI 1640 medium, compared with its adhesion in YNB medium ($P<0.05$). Moreover, in RPMI 1640 medium at pH 7.0, *C. albicans* had significantly enhanced adhesion capabilities compared with those at pH 5.6 ($A_{490}=0.566\pm0.108$ and $0.924\pm0.209$, respectively) ($P<0.05$). However, the pH did not have a significant effect on *C. albicans* adhesion in YNB ($P>0.05$). The metabolic activity of *C. albicans* mature biofilm-forming cells was significantly reduced in YNB medium ($A_{490}=0.530\pm0.072$ and $0.660\pm0.031$, respectively) compared with RPMI 1640 medium ($A_{490}=1.240\pm0.188$ and $1.410\pm0.086$, respectively) ($P<0.05$) (Fig. 1d). Despite the fact that pH 7.0 enhanced the yeast-to-hyphae transition, it was not shown to have a significant effect on a mature *C. albicans* biofilm ($P>0.05$). Considering *C. glabrata* biofilm formation (Fig. 1b), the medium is a crucial factor during its maturation. *C. glabrata* biofilm-forming cells demonstrated significantly less metabolic activity when grown in YNB medium ($A_{490}=0.360\pm0.075$ and $0.453\pm0.044$, respectively) compared with the results obtained after growth in RPMI 1640 medium ($A_{490}=1.310\pm0.272$ and $1.483\pm0.274$, respectively) ($P<0.05$). Similar to adhesion, *C. glabrata* biofilm development was not influenced by the pH tested ($P>0.05$).

### RPMI 1640 medium promotes the development of mature biofilms and induces the yeast-to-hyphae transition in *C. albicans*

*C. albicans* biofilms are known to be composed of yeast and hyphal cells, building a layered, attached structure (Ramage *et al.*, 2006). Therefore, we examined the morphology of the cells within a mature biofilm (48 h) by FM and the biofilm thickness with CSLM for the four conditions tested. The biofilm architecture of *C. albicans* and *C. glabrata* is illustrated in Fig. 2. The measurements of biofilm thickness are displayed in Table 1. The choice of the different media influenced the biofilm thickness and, additionally, the pH contributed to the yeast-to-hyphae transition in *C. albicans*.

In comparison with the biofilms formed in RPMI 1640 medium (Fig. 2c, d), *C. glabrata* cells manifested adhesion rather than multilayer biofilm architecture in YNB medium, regardless of the pH (Fig. 2a, b). Therefore, in this particular case, the thickness could not be estimated reliably. On the other hand, in RPMI 1640 medium at pH 5.6, *C. glabrata* cells formed scattered biofilm layers, with some areas of the substrate remaining uncovered (Fig. 2c). The thickness of the mature biofilms on covered places was approximately 20 μm. Despite this, in the same medium, but at pH 7.0, the polystyrene surface was fully covered with yeast cells and the thickness of the mature biofilm increased to approximately 25 μm (Fig. 2d). Similarly to *C. glabrata*, *C. albicans* manifested adhesion rather than biofilm architecture when grown in YNB medium at both pHs (Fig. 2e). Although pH 7.0 enhanced the yeast-to-hyphae transition, which is an important factor for *C. albicans* multilayer biofilm development, the polystyrene remained unevenly covered (Fig. 2f). These patches of *Candida* cells were measured and their thickness was revealed to be approximately 50 μm. On the other hand, *C. albicans* biofilms formed in RPMI 1640 medium at pH 5.6 or 7.0 were composed mainly of hyphae (Fig. 2g, h). During biofilm development in RPMI 1640


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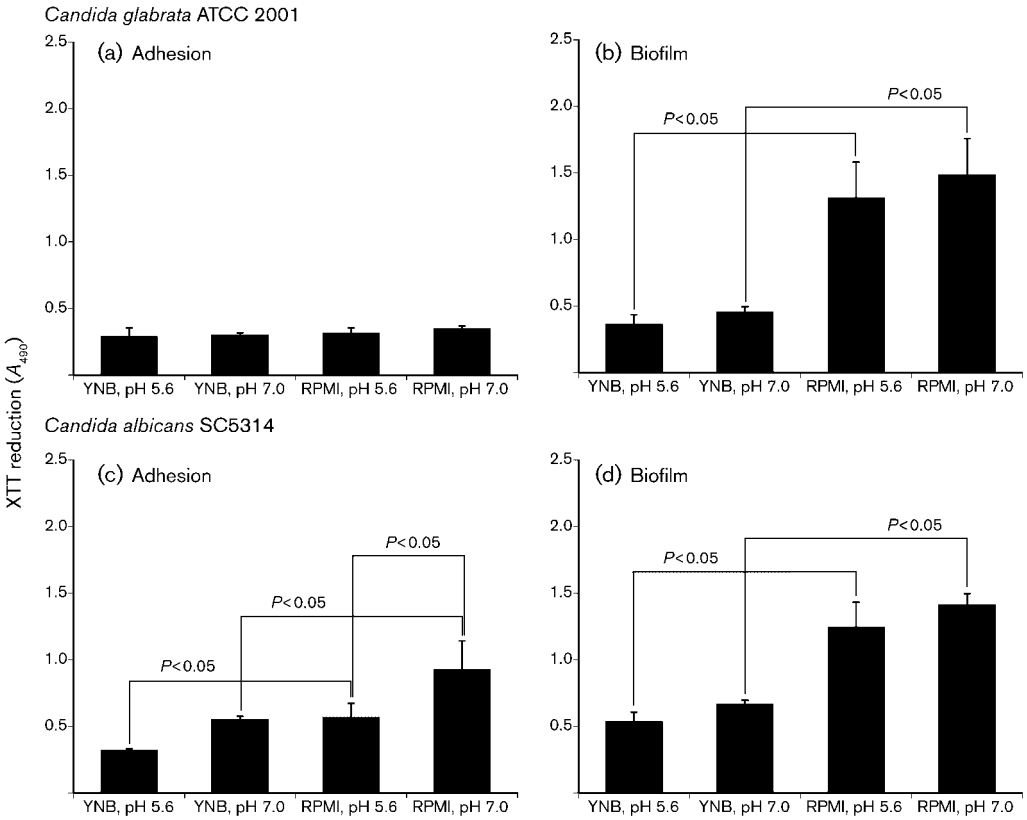


**Fig. 1.** RPMI 1640 medium significantly promotes biofilm formation in *C. glabrata* ATCC 2001 and *C. albicans* SC5314. An XTT-reduction assay, determined at $A_{490}$, was used as a measure of metabolic activity of *C. glabrata* ATCC 2001 (a, b) and *C. albicans* SC5314 (c, d) cells during adhesion and biofilm formation on 96-well polystyrene plates in RPMI 1640–MOPS and YNB media, at pH 5.6 or 7.0. The XTT measurements were performed after a period of adhesion (90 min at 37 °C), whilst biofilms were quantified after 48 h at 37 °C. The results were considered as statistically significant when $P<0.05$. SD was calculated from five independent experiments. Each condition was tested in quadruplicate.

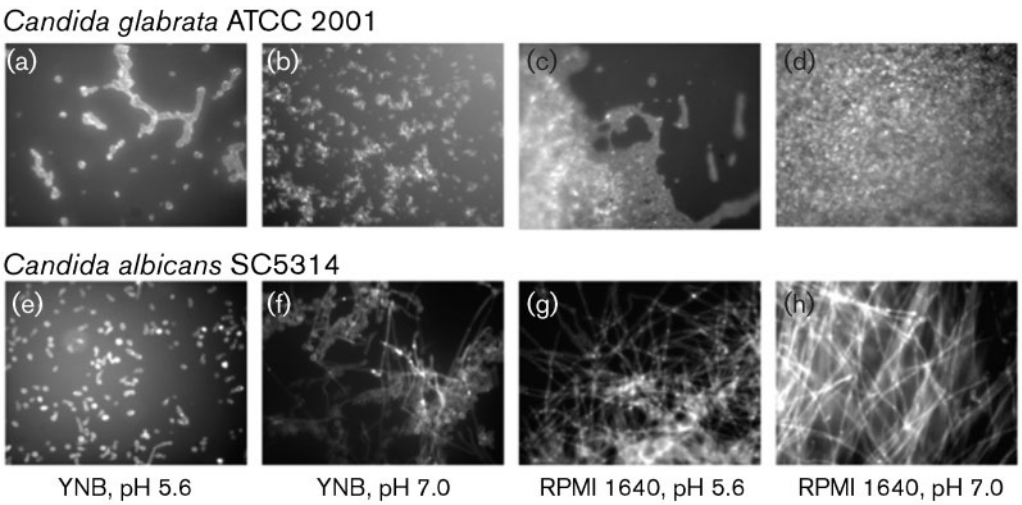


**Fig. 2.** Growth medium and pH influence cell morphology and biofilm thickness. FM images display the top layers of 48 h-old biofilms developed on polystyrene coverslips. Mature *C. glabrata* ATCC 2001 (a–d) and mature *C. albicans* SC5314 (e–h) biofilms were stained with Calcofluor White prior to visualization. Magnification, ×40. FM was performed three times on two coverslips used per condition.


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**Table 1.** Thickness of mature *C. glabrata* ATCC 2001 and *C. albicans* SC5314 biofilms as determined by CSLM

Approximate values of biofilm thickness, represented in μm, were obtained from vertical sections through 3-D confocal images.

| Growth conditions | *C. glabrata* ATCC 2001 | *C. albicans* SC5314 |
|---|---|---|
| YNB, pH 5.6 | 10–15 | 29–34 |
| YNB, pH 7.0 | 10–15 | 51 |
| RPMI 1640, pH 5.6 | 20 | 60 |
| RPMI 1640, pH 7.0 | 25 | 100 |

medium at pH 5.6, the biofilm thickness was approximately 60 μm (Fig. 2g), whereas in the same medium, but at pH 7.0, the thickness reached approximately 100 μm (Fig. 2h). It is noteworthy to mention that the observations of hazy material covering *C. albicans* or *C. glabrata* biofilms formed in RPMI 1640–MOPS medium (Fig. 2c, d, g, f) suggest the presence of extracellular matrix.

These results demonstrate that RPMI 1640 medium induces the yeast-to-hyphae transition in *C. albicans*, resulting in thicker biofilms compared with growth in YNB medium. In addition, we showed that *C. glabrata* forms multilayer biofilms on polystyrene only in the presence of RPMI 1640 medium at pH 7.0.

## Transcriptional expression of adhesin genes correlates positively with biofilm formation

We anticipated that the expression profile of *Candida* biofilm-associated genes may change due to exposure to different environmental conditions. We therefore analysed the changes in expression of genes participating significantly in adhesion and biofilm formation, such as *C. glabrata EPA6* (Iraqui *et al.*, 2005) and *C. albicans ALS1*, *ALS3* and *EAP1* (Nailis *et al.*, 2010; Nobile *et al.*, 2006). The results are displayed in Figs 3 and 4(a, b, c), respectively. The transcript profile of *EPA6* was induced significantly in cells growing in RPMI 1640–MOPS medium compared with YNB medium, regardless of the pH used ($P<0.05$) (Fig. 3). Additionally, significant upregulation was observed in the same medium at pH 7.0 ($P<0.05$) (Fig. 3). For comparison, we examined the *EPA6* expression profile from 48 h-old planktonic cultures (stationary phase) in RPMI 1640 or YNB medium at pH 5.6 or 7.0. Neither RPMI 1640 medium nor the pH promoted *EPA6* expression, compared with YNB medium when cells were grown in liquid cultures (data not shown), indicating that the results obtained from sessile populations are biofilm-specific.

Further, we examined the changes in the expression profile of *C. albicans* adhesion-associated genes such as *ALS1*, *ALS3* and *EAP1*. The results are displayed in Fig. 4(a, b, c), respectively. None of the three genes analysed was regulated in YNB-grown biofilms, regardless of the pH. On the contrary, RPMI 1640 medium influenced the expression of these adhesins in a pH-dependent manner. The *ALS3* gene

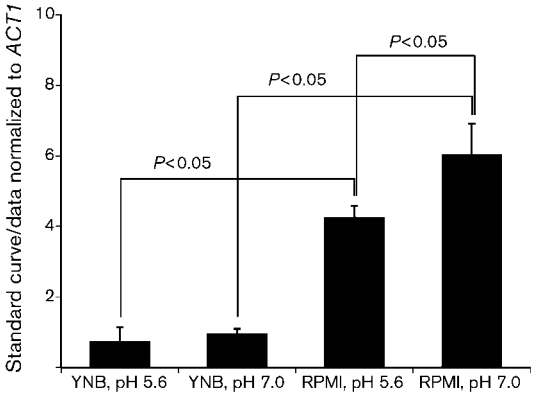


**Fig. 3.** *EPA6* in *C. glabrata* ATCC 2001 is upregulated significantly in RPMI 1640 medium. Expression analyses of the adhesion-encoding gene in mature (48 h) *C. glabrata* biofilms developed in RMPI 1640 or YNB medium at pH 5.6 or 7.0 are shown. The data were normalized to *ACT1* using the standard curve quantification method. The increased expression levels of *EPA6* were considered as statistically significant when $P<0.05$. SD was calculated from two independent experiments on different occasions.

was upregulated significantly at pH 7.0 compared with pH 5.6 and in YNB-grown biofilms ($P<0.05$) (Fig. 4b). A similar pattern of expression was observed for the *EAP1* gene ($P<0.05$) (Fig. 4c). In contrast, *ALS1* expression was not promoted by the conditions tested (Fig. 4a). It is noteworthy to mention that the data demonstrated above were normalized to *ACT1*; it is known that the expression of this gene may change during morphogenesis (Delbrück & Ernst, 1993; Michán & Pueyo, 2009), which is characteristic for *C. albicans* during biofilm development. Because of this, in addition, the data were also normalized to *TEF1*. Regardless of the housekeeping gene used, the expression levels of *ALS1*, *ALS3* and *EAP1* were identical to those observed above (data not shown). This observation excludes the potential influence of housekeeping-gene expression on the evaluation of the expression of tested genes.

## RPMI 1640-grown biofilms are less susceptible to echinocandins than YNB-grown biofilms in *C. albicans*, but not in *C. glabrata*

The results mentioned above led us to the assumption that the increased biofilm thickness observed for *C. albicans* and *C. glabrata* grown in RPMI 1640 medium at pH 5.6 and 7.0 might have an influence on the efficiency of antifungal drugs against biofilms. Hence, we tested the effect of two echinocandins, caspofungin and anidulafungin, against biofilms formed under the different environmental conditions. *C. albicans* and *C. glabrata* planktonic cell cultures were susceptible to both echinocandins (Table 2). Following antifungal treatment, biofilms were assessed by XTT measurements, and the data are displayed in Table 3. Both caspofungin and anidulafungin were shown to be very


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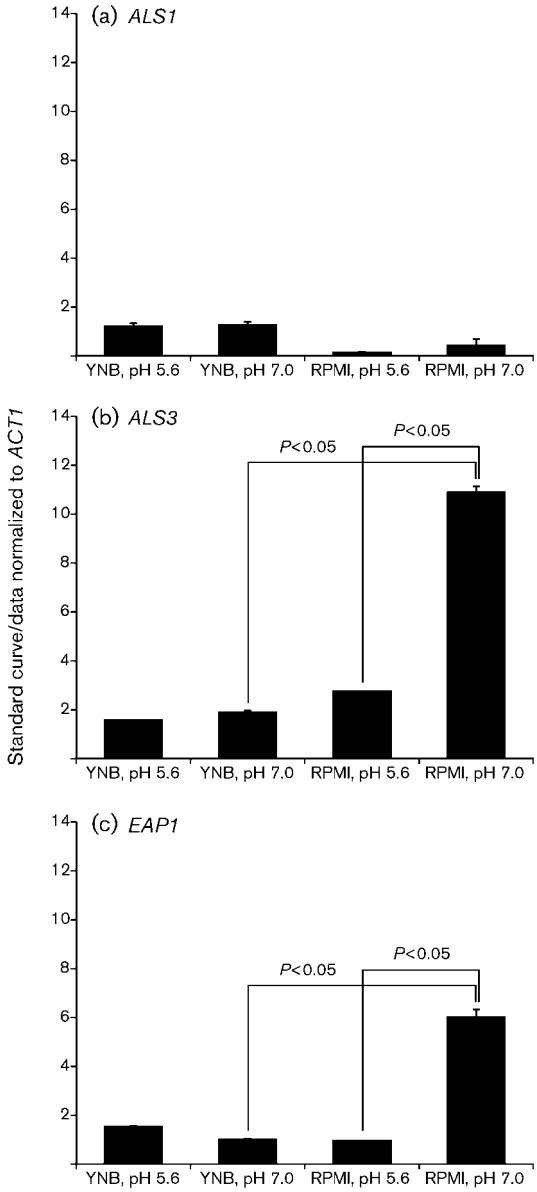


**Fig. 4.** *ALS3* and *EAP1* in *C. albicans* SC5314 are highly expressed under the most biofilm-favourable growth conditions. The expression level of adhesion-encoding genes *ALS1* (a), *ALS3* (b) and *EAP1* (c) in mature (48 h) *C. glabrata* and *C. albicans* biofilms developed in RMPI 1640 or YNB medium at pH 5.6 or 7.0 is shown. All expression levels are shown as relative fold expression compared with the *ACT1* level using the standard curve quantification method. SD was calculated from two independent experiments on different occasions.

potent against *C. albicans* mature biofilms under all conditions tested, as indicated by the low $SMIC_{50}$ and $SMIC_{90}$ values, whilst *C. glabrata* biofilms were slightly less susceptible. For both species, the highest $SMIC_{90}$ values were always observed in the condition promoting the thickest biofilms – RPMI 1640 medium at pH 7.0. This reduced susceptibility to the antifungal treatment was also observed on biofilms grown in RPMI 1640 medium at pH 5.6, but to a lower extent. In conclusion, YNB-grown biofilms seem more susceptible to echinocandins than RPMI 1640-grown biofilms.

## DISCUSSION

This study gives a detailed comparison between the biofilms formed by *C. albicans* SC5314 and *C. glabrata* ATCC 2001 grown in RPMI 1640 and YNB media. We also elucidated the effect of pH 5.6 and 7.0 during biofilm development, because adaptation to changes in pH during biofilm formation is crucial not only for its development, but also for survival of microbial communities at colonized host sites (Schmidt *et al.*, 2008) as well as on medical devices (Ramage *et al.*, 2006). The two media tested, YNB and RPMI 1640, have remained favourite choices for *in vitro* biofilm formation (Biswas & Chaffin, 2005; Cateau *et al.*, 2008; Chandra *et al.*, 2001). YNB medium is less nutrient-rich for growth of planktonic cells than RPMI 1640 medium, which mimics the composition of human fluids (Chandra *et al.*, 2005). This medium was used previously for *C. albicans* attachment on polyurethane prior to subcutaneous catheter implantation *in vivo* (Řičicová *et al.*, 2010). Moreover, RPMI 1640 is the standard medium for *in vitro* susceptibility testing of planktonic cells to antifungals according to the NCCLS M27-A3 protocol (NCCLS, 2008). However, there are no studies that have focused strictly on the testing of RPMI 1640 and YNB media on the different steps of biofilm development.

From our study, it is clear that the medium and pH tested did not have any effect on the *C. glabrata* adhesion process, whereas the ability of *C. albicans* to adhere was significantly higher in RPMI 1640 than in YNB medium. This observation could be explained by the induced *C. albicans* yeast-to-hyphae transition process, particularly in RPMI 1640 medium. The yeast-to-hyphae transition has been considered one of the crucial factors involved in *C. albicans* attachment to a surface (Blankenship & Mitchell, 2006). Neutral pH promotes the yeast-to-hyphae transformation and contributes to better *C. albicans* adhesion in our assay in RPMI 1640 medium. In contrast, *C. glabrata* is not able to switch from yeast to hyphae, but it was able to form a multilayer, highly potent biofilm, as described previously (Kucharíková *et al.*, 2010b; Nikawa *et al.*, 1997). In addition to cell morphology, the thickness of the biofilms also showed great variation. Both *C. glabrata* and *C. albicans* formed significantly thicker biofilms in RPMI 1640 medium, whereas in YNB medium, cells proliferated in an adherent manner rather than forming a multilayer biofilm. Strikingly, RPMI 1640 medium seems to be superior for biofilms formed by both species. In addition, pH 7.0 helped to promote a dense biofilm structure composed of a basal layer of yeast cells followed by hyphae in *C. albicans*, or a multilayer structure of the yeast cells in *C. glabrata*.

Because of the striking effect of the different media on biofilm formation and architecture in both *Candida*


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**Table 2.** Susceptibility testing of *C. glabrata* ATCC 2001 and *C. albicans* SC5314 planktonic cells to caspofungin and anidulafungin

$MIC_{50}$ and $MIC_{90}$ values are shown as μg $ml^{-1}$.

| Antifungal agent | *C. glabrata* ATCC 2001 | | *C. albicans* SC5314 | |
|---|---|---|---|---|
| | $MIC_{50}$ | $MIC_{90}$ | $MIC_{50}$ | $MIC_{90}$ |
| Caspofungin | 0.25 | 0.5 | 0.125 | 0.125 |
| Anidulafungin | 0.5 | 0.5 | 0.0625 | 0.0625 |

species, we investigated the expression profile of the *C. glabrata EPA6* gene and the *C. albicans ALS1*, *ALS3* and *EAP1* genes, all involved directly or indirectly in biofilm formation. It was demonstrated previously that *C. glabrata EPA6* is transcribed at the highest level during the stationary-growth phase, in contrast to other *EPA* genes, and that cells in the stationary phase adhered better than metabolically active cells (Iraqui *et al.*, 2005). In all of our assays, *C. glabrata* cultures were grown up to the stationary phase to ensure that the medium contained totally adherent cell populations. We observed an upregulation of *C. glabrata EPA6* during biofilm formation in RPMI 1640 medium at both pHs. This upregulation of *EPA6* correlates well with the increased thickness and viability of the biofilms developed in the same medium.

During *C. albicans* biofilm development, *ALS1*, *ALS3* and *EAP1* play roles in different stages of biofilm formation. Yeater *et al.* (2007) demonstrated, by microarray analyses of biofilms, that the expression of *ALS1* increases during the early stages of biofilm development (6–12 h) and declines at the later stages. This documented profile of expression could explain why we did not observe any regulation of *ALS1* under any of the conditions tested, as biofilms were grown for 48 h before analyses. However, the data are controversial for that gene, as it is expressed differentially depending on growth *in vitro* (García-Sánchez *et al.*, 2004; Nailis *et al.*, 2009) or *in vivo* (Nett *et al.*, 2009; Řičicová *et al.*, 2010) and also depending on the growth conditions. In our assays, the *C. albicans ALS3* and *EAP1* genes were upregulated in an ascending manner from pH 5.6 to 7.0 in RPMI 1640 medium.

Both caspofungin and anidulafungin were effective against *C. albicans* mature biofilms, regardless of the environmental conditions used. Similarly to our study, the data of Jacobson *et al.* (2008) showed the $SMIC_{50}$ for anidulafungin to be $\leqslant$0.03125 μg $ml^{-1}$ in 28 of 30 *C. albicans* clinical isolates tested when the biofilm was formed in RPMI 1640 medium. In contrast to the work of Katragkou *et al.* (2008), we did not observe any indication of paradoxical growth during caspofungin activity against a mature *C. albicans* biofilm, even when high concentrations (4–8 μg $ml^{-1}$) of the drug were administered to biofilms (data not shown). *C. glabrata* cells forming mature biofilm were less susceptible than the diploid species to caspofungin and anidulafungin in both media tested, albeit with up to half the thickness of *C. albicans* biofilms. In a previous study, *C. glabrata* bloodstream clinical isolates displayed a median caspofungin $SMIC_{90}$ of 1 μg $ml^{-1}$ (Choi *et al.*, 2007), similar to data that we obtained. Both *C. albicans* and *C. glabrata* showed reduced susceptibility to echinocandins when biofilms were formed in RPMI 1640 medium compared with YNB medium, which corresponded with denser biofilm structures. The pH was not shown to have a significant effect on antifungal susceptibility of the cells within the biofilm.

In conclusion, *C. albicans* and *C. glabrata* biofilms displayed heterogeneous architecture, expression profiles and susceptibility to caspofungin and anidulafungin when formed under diverse environmental conditions. The medium and its pH influenced adhesion and biofilm development in different manners. Therefore, it is important to clearly define growth conditions prior to and during the biofilm experimental set-up. It is also essential to mimic physiological conditions when performing biofilm formation *in vitro*. In particular, RPMI 1640 medium contributed to the development of thick biofilm structures in both *C. albicans*

**Table 3.** Antifungal-susceptibility testing of *C. glabrata* ATCC 2001 and *C. albicans* SC5314 biofilms to caspofungin and anidulafungin

$SMIC_{50}$ and $SMIC_{90}$ values are shown as μg $ml^{-1}$.

| Antifungal agent | Growth conditions | *C. glabrata* ATCC 2001 | | *C. albicans* SC5314 | |
|---|---|---|---|---|---|
| | | $SMIC_{50}$ | $SMIC_{90}$ | $SMIC_{50}$ | $SMIC_{90}$ |
| Caspofungin | YNB, pH 5.6 | 0.5 | 0.5 | 0.0625 | 0.25 |
| | YNB, pH 7.0 | 1 | 1 | 0.25 | 0.25 |
| | RPMI 1640, pH 5.6 | 2 | 2 | 0.25 | 0.5 |
| | RPMI 1640, pH 7.0 | 1 | 2 | 0.5 | 1 |
| Anidulafungin | YNB, pH 5.6 | 0.5 | 0.5 | 0.03125 | 0.03125 |
| | YNB, pH 7.0 | 0.5 | 0.5 | 0.03125 | 0.03125 |
| | RPMI 1640, pH 5.6 | 2 | 2 | 0.0625 | 0.5 |
| | RPMI 1640, pH 7.0 | 1 | 2 | 0.0625 | 0.5 |



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and *C. glabrata*. Moreover, during *C. albicans* biofilm development, the choice of a pH of 7.0 contributed to a faster yeast-to-hyphae transition, which resulted in denser biofilm structures compared with those formed in YNB medium. It is important to take into consideration that different environmental conditions will affect not only biofilm architecture, but also its susceptibility to antifungal agents, which underscores the need for standardized methods.

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