

# *Candida albicans* forms biofilms on the vaginal mucosa

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Current understanding of resistance and susceptibility to vulvovaginal candidiasis challenges existing paradigms of host defence against fungal infection. While abiotic biofilm formation has a clearly established role during systemic *Candida* infections, it is not known whether *C. albicans* forms biofilms on the vaginal mucosa and the possible role of biofilms in disease. *In vivo* and *ex vivo* murine vaginitis models were employed to examine biofilm formation by scanning electron and confocal microscopy. *C. albicans* strains included 3153A (lab strain), DAY185 (parental control strain), and mutants defective in morphogenesis and/or biofilm formation *in vitro* (*efg1/efg1* and *bcr1/bcr1*). Both 3153A and DAY815 formed biofilms on the vaginal mucosa *in vivo* and *ex vivo* as indicated by high fungal burden and microscopic analysis demonstrating typical biofilm architecture and presence of extracellular matrix (ECM) co-localized with the presence of fungi. In contrast, *efg1/efg1* and *bcr1/bcr1* mutant strains exhibited weak or no biofilm formation/ECM production in both models compared to wild-type strains and complemented mutants despite comparable colonization levels. These data show for the first time that *C. albicans* forms biofilms *in vivo* on vaginal epithelium, and that *in vivo* biotic biofilm formation requires regulators of biofilm formation (*BCR1*) and morphogenesis (*EFG1*).

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## INTRODUCTION

Vulvovaginal candidiasis (VVC) affects 75 % of all women at least once in their lifetime (Sobel *et al.*, 1998), most often during childbearing years (Sobel, 1992). Another smaller group of women (5–8 %) experience recurrent vulvovaginal candidiasis (RVVC), defined as having three or more episodes per year (Sobel, 1992). Although several *Candida* species have been implicated in VVC and RVVC, *Candida albicans* is the predominant aetiological agent, causing 85–95 % of these infections (Sobel *et al.*, 1998).

Current understanding of resistance and susceptibility to VVC challenges existing paradigms of host defence against fungal infection. Although the adaptive immune response plays a major role in protection against oral and gastrointestinal mucosal *Candida* infections, the innate immune response plays a more prominent role in VVC. In resistant women, vaginal epithelial cells are important for

protection against infection (Fidel *et al.*, 2004). In susceptible women, symptomatic VVC is associated with an aggressive neutrophil migration into the vagina and subsequent inflammatory response initiated by the interaction of *C. albicans* with vaginal epithelial cells (Barousse *et al.*, 2001, 2005). This inflammatory response is not protective, but instead appears to be a major cause of the symptoms associated with infection.

Recently there has been tremendous interest in the role of biofilms in infectious diseases. It is estimated that 80 % of human infections result from pathogenic biofilms (NIH, 1999). Biofilms are communities of micro-organisms that are embedded in an extracellular matrix (ECM), forming a complex three-dimensional architecture. *Candida* biofilm development on abiotic surfaces can be divided into several growth stages, including early, intermediate and mature (Chandra *et al.*, 2001). During early biofilm formation, yeast cells adhere to an appropriate surface and initiate germ tube formation. The intermediate phase is characterized by continued hyphal elongation and production of ECM, which consists of cell wall polysaccharides and protein (Baillie & Douglas, 2000). Mature biofilms consist

Abbreviations: CM, confocal microscopy; ConA-TR, concanavalin A-Texas red conjugate; ECM, extracellular matrix; (RVVC), (recurrent) vulvovaginal candidiasis; SEM, scanning electron microscopy.

of a yeast base, with hyphal elements encased in ECM extending away from the surface. Newly formed daughter yeast cells grow out of hyphal elements and are released, seeding new niches for biofilm formation or infection. Moreover, biofilm formation is dependent on the ability to undergo morphogenesis; mutants defective in hyphal formation *in vitro* are also defective in biofilm formation (Nobile *et al.*, 2006b; Ramage *et al.*, 2002).

*Candida* biofilms have been studied primarily on abiotic surfaces (reviewed by Blankenship & Mitchell, 2006; Ramage *et al.*, 2006). Significant attention has been given to *Candida* biofilm formation of indwelling catheters, which can lead to life-threatening systemic infections (Crump & Collignon, 2000; Dominic *et al.*, 2007; Kojic & Darouiche, 2004). More recently, biofilm formation on biotic surfaces has received some attention; *C. albicans* was shown to form biofilms on oral mucosa in an immunosuppressed murine model of oropharyngeal candidiasis (Dongari-Bagtzoglou *et al.*, 2009). However, in VCC and RVCC it is unknown whether *C. albicans* exist as biofilms on the vaginal mucosa and, if so, whether the biofilms play a role in the immunopathogenesis.

The purpose of this study was to determine whether *Candida* forms biofilm on vaginal mucosa, using both an *in vivo* and a novel *ex vivo* model. Biofilm-defective *C. albicans* mutants were also used to define specific parameters of biofilm formation associated with the tissue.

## METHODS

***Candida albicans* strains and handling.** *C. albicans* strains used in this study are listed in Table 1. Wild-type (WT) strains included 3153A (lab strain) and DAY185 (parent strain). Strain DAY185 is a complemented prototroph derived from a triple auxotrophic strain (BWP17; parent, SC5314), which is frequently used to produce knockout and complemented strains of *C. albicans*. Strains HLC52 (*efg1/efg1*) and HLC74 (*efg1/efg1 + pEFG1*) were a gift from G. R. Fink (Whitehead Institute, Cambridge, MA, USA). Strains CJN702 (*bcr1/bcr1*) and CJN698 (*bcr1/bcr1 + pBCR1*) were a gift from A. P. Mitchell (Carnegie Mellon University, Pittsburgh, PA, USA). For each experiment, strains were subcultured from freezer stocks onto Sabouraud dextrose agar (SDA) plates and incubated at 30 °C

overnight. Cultures were washed in 1 × PBS, counted, and diluted in 1 × PBS for inoculation.

**Mouse strains.** Female C57BL/6 or CBA/J mice, 6–8 weeks of age (Charles River and NIH) were used in this study. All mice were maintained at an American Association for the Accreditation of Laboratory Animal Care-accredited animal facility at Wayne State University (WSU) or Louisiana State University Health Sciences Center (LSUHSC) and housed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals under an animal study proposal approved by the WSU animal investigation committee and the LSUHSC Institutional Animal Care and Use Committee (IACUC).

***In vivo* model.** Mice were administered 0.1 mg 17- $\beta$ -oestradiol (Sigma-Aldrich) in 0.1 ml sesame seed oil (Sigma-Aldrich) subcutaneously 3 days prior to inoculation. They were inoculated intravaginally with 20  $\mu$ l *C. albicans* 3153A ( $2.5 \times 10^6$  ml<sup>-1</sup>), DAY185 ( $2.5 \times 10^8$  ml<sup>-1</sup>) or biofilm mutants ( $2.5 \times 10^8$  ml<sup>-1</sup>). Higher inocula were used for DAY185 and mutants because initial experiments demonstrated that 3153A colonizes mucosal tissue better than DAY185 and biofilm mutants, as previously reported (Naglik *et al.*, 2008; Taylor *et al.*, 2000). At specified time points, mice were euthanized and their vaginae were excised and cut longitudinally to expose the mucosal surface. The vaginae were then bisected; half the tissue was used to determine fungal load by quantitative plate count and the other half was used for confocal microscopy or scanning electron microscopy.

***Ex vivo* model.** Mice were treated with oestrogen as described above 3 days prior to vaginal harvest. On experimental days, mice were euthanized and their vaginae were excised and cut longitudinally to expose the mucosal surface. The vaginae were placed with the mucosal side facing up in 6-well tissue culture treated plates with 500  $\mu$ l PBS (1 ×), which results in the vaginal mucosal surface being exposed and unsubmerged. Penicillin-streptomycin (1 ×, MP Bio-medicals) was added to the PBS to prevent growth of normal vaginal bacteria. *C. albicans* isolates ( $1 \times 10^6$  blastoconidia) were added to the mucosal surface in 0.1 ml PBS and incubated at 37 °C with CO<sub>2</sub>. The inoculum was based on preliminary studies that revealed optimal colonization for microscopic evaluations. Tissues were removed from the incubator at specified time points and bisected but were not washed prior to analysis. Half the tissue was used to determine fungal load and the other half was used for microscopy.

**C.f.u. counts.** Following harvest, vaginae were placed in 5 ml sterile tubes with 1 ml sterile H<sub>2</sub>O. Tissues were weighed and homogenized, and 10-fold dilutions were prepared and plated to SDA with ampicillin and erythromycin to prevent the growth of normal vaginal

**Table 1.** Strains used in this study

Strain	Genotype	Source
3153A	Wild-type	Slutsky <i>et al.</i> (1985)
DAY185	<i>ura3<math>\Delta</math>::imm434/ura3<math>\Delta</math>::imm434 HIS1::his1::hisG/his1::hisG ARG4::URA3::arg4::hisG/arg4::hisG</i>	Norice <i>et al.</i> (2007)
HLC52	<i>ura3<math>\Delta</math>::imm434/ura3<math>\Delta</math>::imm434 efg1::hisG/efg1::hisG-URA3-hisG</i>	Lo <i>et al.</i> (1997)
HLC74	<i>ura3<math>\Delta</math>::imm434/ura3<math>\Delta</math>::imm434 efg1::hisG/efg1::hisG LEU2/LEU2:pEFG1-URA3-LEU2</i>	Lo <i>et al.</i> (1997)
CJN702	<i>ura3<math>\Delta</math>::imm434/ura3<math>\Delta</math>::imm434 arg4::hisG/arg4::hisG his1::hisG::pHIS1/his1::hisG bcr1::ARG4/bcr1::URA3</i>	Nobile & Mitchell (2005)
CJN698	<i>ura3<math>\Delta</math>::imm434/ura3<math>\Delta</math>::imm434 arg4::hisG/arg4::hisG his1::hisG::pHIS1-BCR1/his1::hisG bcr1::ARG4/bcr1::URA3</i>	Nobile & Mitchell (2005)

bacteria. Following an overnight incubation at 37 °C, colonies were counted and log c.f.u. ml<sup>-1</sup> and log c.f.u. (g tissue)<sup>-1</sup> were calculated.

**Confocal microscopy (CM).** Excised vaginal tissue was placed in 12-well dishes and stained with 1 mg ml<sup>-1</sup> Calcofluor White (Fluka) to visualize yeast and hyphae and 50 µg ml<sup>-1</sup> concanavalin A–Texas red conjugate (ConA-TR, Molecular Probes) to stain the biofilm ECM for 20 min at room temperature. No prior washing or sectioning of tissue was performed. Vaginal tissues were removed from staining wells, gently rinsed with 1 × PBS to remove exogenous stain, and placed epithelial side up onto glass microscope slides. Vaginal tissues were covered with a glass coverslip and slides were examined under oil immersion with a Nikon Eclipse E800 microscope with Metamorph software.

**Scanning electron microscopy (SEM).** Vaginal tissue was placed in 10% formalin. Tissues were dehydrated in 20% ethanol for 1 min, followed by three successive 1 min incubations in 40%, 60%, 80%, 95% and 100% ethanol, and finally by three successive 1 min incubations in acetone. Dried samples were affixed to SEM stubs and placed into a gold/palladium sputter coater. Specimens were viewed at 300–2000 × magnification in a Hitachi S-2700 scanning electron microscope (LSUHSC Imaging Core) with the voltage set to 15 kV.

**Statistical analysis.** Statistical analysis of the data was conducted using Prism software (GraphPad). Data were analysed by ANOVA (analysis of variance) followed by Dunnett's multiple comparison test. Significance was defined as  $P < 0.05$ .

## RESULTS

### *C. albicans* forms *in vivo* and *ex vivo* biofilms on vaginal mucosae

To initially determine whether *C. albicans* forms biofilms on vaginal mucosae, we used both *in vivo* and *ex vivo* colonization models using WT strain 3153A. For the *in vivo* model, vaginal tissue was analysed 48 h post-inoculation for biofilm formation by scanning electron microscopy (SEM) and confocal microscopy (CM). This is a commonly used early time point to monitor colonization during experimental vaginitis. SEM analysis revealed fungal colonization and the presence of a complex network of both yeast and hyphae, and ECM accumulation both *in vivo* and *ex vivo* (Fig. 1a, b). CM analysis confirmed that biofilm ECM was present, as demonstrated by the bright red cell-associated staining and the hazy diffuse red material covering the hyphae (Fig. 1d).

We also examined *ex vivo* biofilm formation on vaginal explants at 48 h post-inoculation, which is a time point correlating with development of mature biofilm *in vitro* (Chandra *et al.*, 2001). *Ex vivo* model biofilms were phenotypically similar to *in vivo* biofilms. At 48 h, a characteristic biofilm was visible by SEM, consisting of yeast and hyphae surrounded by ECM (Fig. 1b). Biofilm ECM was also evident by CM, both cell associated and interspersed between the hyphae (Fig. 1e). No *Candida* colonization was observed in control uninoculated vaginal epithelium, either *in vivo* or *ex vivo* (Fig. 1c, f and data not shown). It is important to note that although ConA-TR stains epithelial cells, the fluorescence intensity was weaker and exposure

time was increased to visualize epithelial cells compared to ConA-TR-stained ECM material (Fig. 1d, e). The presence of c.f.u. following the plating of tissue homogenates under *in vivo* or *ex vivo* conditions confirmed the presence of viable *C. albicans*.

### Kinetics of vaginal mucosal biofilm formation

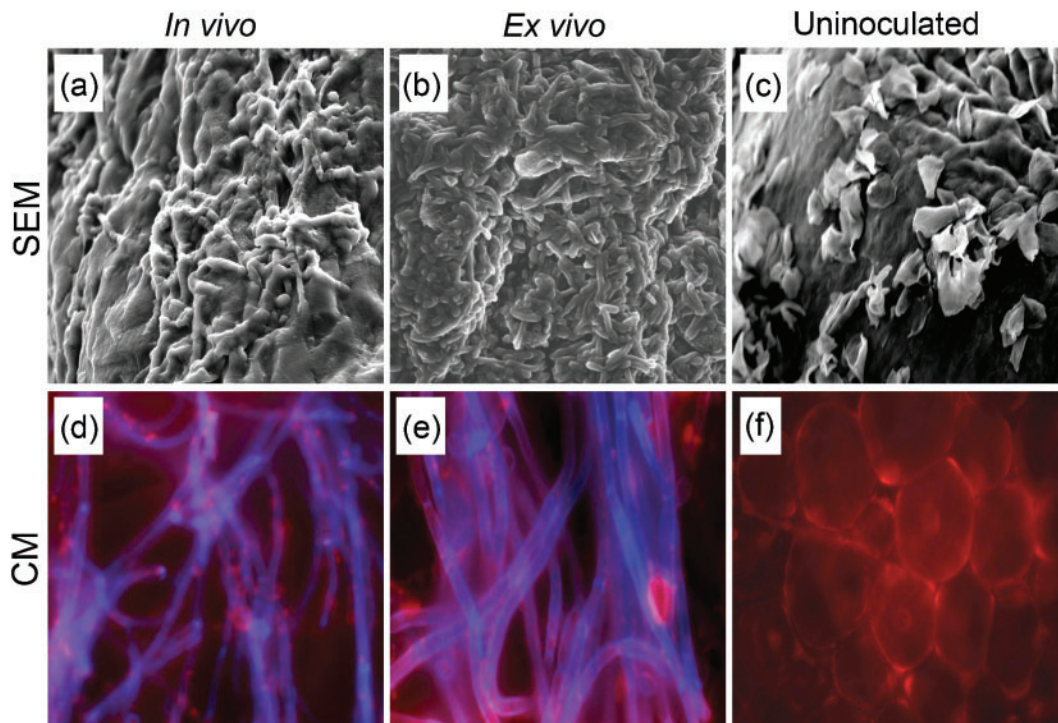
We next examined the kinetics of biofilm development in both models by SEM, CM and quantitative plate count analysis. Vaginae were harvested at 8, 24, 48 and 72 h post-inoculation with *C. albicans* 3153A. Half the tissue was used to determine fungal load (c.f.u.). For the *in vivo* model, yeast had initiated germination on the vaginal epithelium (SEM) by 8 h post-inoculation, with little or no ECM (CM) (Fig. 2). At 24 h the initial layer of yeast was evident under the elongating hyphae. Also, a small amount of ECM material was visible, as demonstrated by the diffuse red staining in the CM. At 48 h, the biofilm was more extensive and ECM appeared thicker and covered some of the cellular portion of the biofilms revealed by SEM and CM (Fig. 2, red staining). By 72 h, ECM accumulation was more uniform by SEM, appearing as a smooth coat on top of the fungal biofilm. By CM, ECM staining appeared cell-associated and also more diffuse, lying above the cellular portion of the biofilm. The pattern of colonization with the biofilm was not confluent over all of the vaginal tissue, but did form patches of thicker and thinner biofilms spread over the tissue (data not shown). Fungal burden also increased steadily with time, starting at  $\approx 10^3$  c.f.u. (g tissue)<sup>-1</sup> at 2 h and increasing to  $\approx 10^5$  c.f.u. (g tissue)<sup>-1</sup> by 72 h (Fig. 3a). Maximal fungal burden was observed by 72 h; however, very little change was observed between 48 and 72 h.

For the *ex vivo* model, considerable yeast had germinated on the vaginal mucosa by 8 h, with little ECM present (CM) and the mucosal surface could be seen under the hyphae (SEM) (Fig. 2). At 24 h, a hyphal network was formed (SEM) and biofilm ECM was evident (CM, red staining). By 48 h, the biofilm was significantly denser than at 24 h and ECM appeared more prevalent (SEM). At 72 h, yeast forms (CM) were evident at the apical surface of the biofilm in conjunction with the residential hyphae, which coincided with the predicted dispersal phase of biofilm growth (Blankenship & Mitchell, 2006). As with *in vivo* biofilms, *ex vivo* biofilm fungal burden steadily increased over time, starting at  $\approx 10^4$  c.f.u. (g tissue)<sup>-1</sup> at 2 h and increasing to  $\approx 10^8$  c.f.u. (g tissue)<sup>-1</sup> by 48–72 h (Fig. 3b). Maximal fungal burden was observed by 48 h and c.f.u. remained relatively unchanged up to 72 h.

### Role of morphogenesis regulator EFG1 and biofilm regulator BCR1

To determine requirements for biofilm formation on vaginal tissue and whether biofilm formation was required for vaginal colonization, we evaluated *C. albicans* mutant



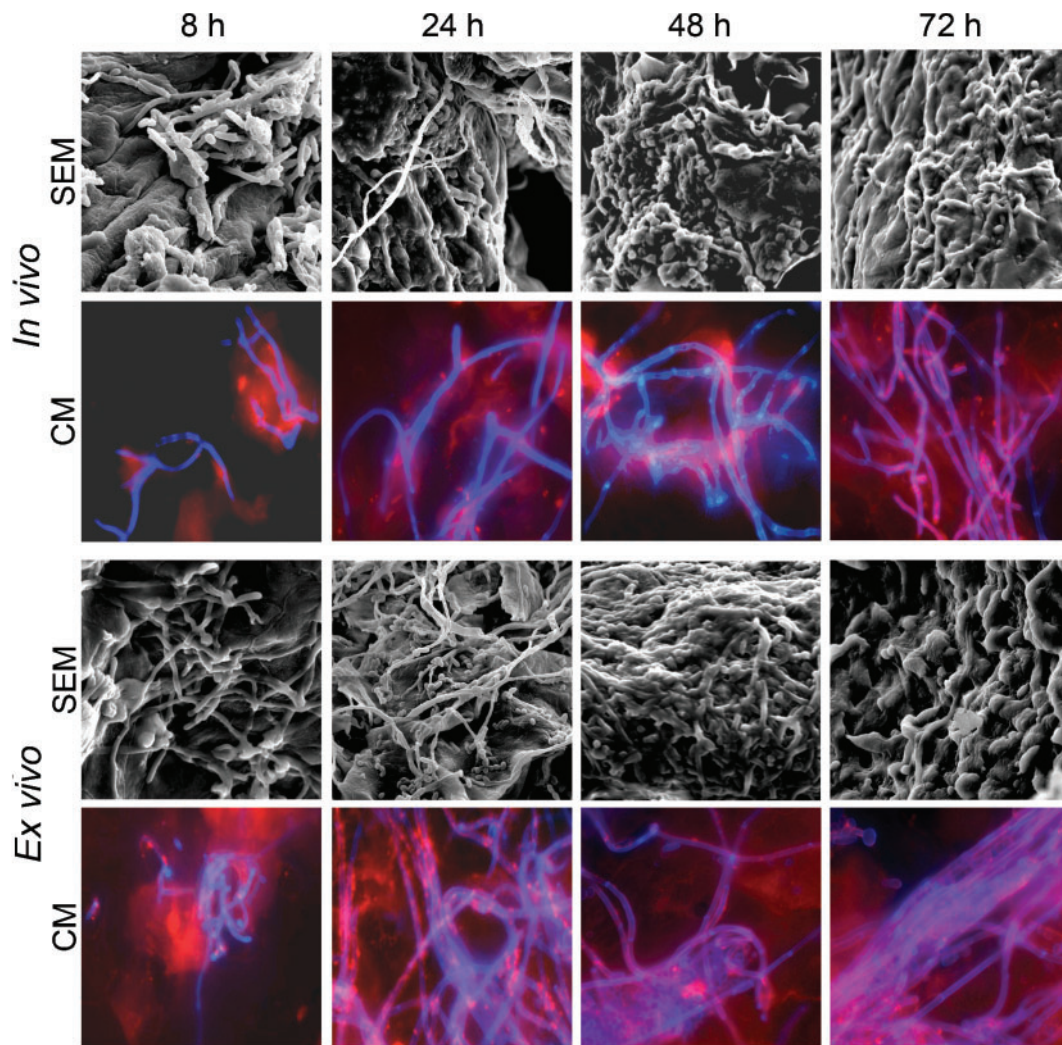


**Fig. 1.** Establishment of vaginal mucosal biofilm. (a, d) For the *in vivo* model, mice were administered oestrogen and intravaginally inoculated with *C. albicans* 3153A ( $5 \times 10^4$  blastoconidia). At 48 h post-inoculation, vaginae were excised and the tissue was processed for SEM or CM. For CM, vaginae were stained with calcofluor white (blue) to visualize yeast/hyphae, and with ConA-TR to visualize biofilm ECM. (b, e) For the *ex vivo* model, mice were administered oestrogen and vaginae were harvested at 48 h post-inoculation. Tissues were inoculated with *C. albicans* 3153A ( $10^6$  blastoconidia). At 48 h post-inoculation, vaginae were bisected and processed for SEM or CM and confirmation of fungal burden. (c, f) *In vivo* control tissues were uninoculated. SEM images were taken at  $1000\times$  magnification, and CM images at  $600\times$  magnification. The figure shows representative images of areas of biofilm growth from three independent repeats;  $n=4$  mice or vaginal explants per time point.

strains with deletions in transcriptional regulators of morphogenesis (*efg1/efg1*) and biofilm formation (*bcr1/bcr1*) and complemented mutants in the *in vivo* and *ex vivo* models. These mutants have previously been shown to form poor or no biofilms on abiotic surfaces (Nobile *et al.*, 2006a, b; Nobile & Mitchell, 2005; Ramage *et al.*, 2002). For the *in vivo* model, we chose to examine biofilm mutants at 72 h, the time at which mature biofilms are evident with wild-type strains. Microscopic evaluation demonstrated that *in vivo* biofilms of strain DAY185 (parental control strain) were similar in appearance to 3153A biofilms at 72 h (Fig. 4a). Similar results were observed with the wild-type strain SC5314, which is the parent of control strain DAY185 (data not shown). This also confirms that the *URA3* marker did not affect vaginal colonization levels, as SC5314 contains *URA3* at the native locus and DAY185 contains *URA3* at a non-native locus. Yeast and hyphae were present and ECM was evident, as indicated by the smooth covering overlying the cells in the SEM image and diffuse red (ConA-TR) staining overlying the cells in the CM image. In contrast, the *efg1/efg1* mutant produced only yeast and pseudohyphal forms, and no ECM was detected by CM, as indicated by the lack of diffuse ConA-TR

staining, although staining of underlying epithelial cells was evident. The *efg1/efg1* mutant was not detectable by SEM, possibly due to the inability of this strain to remain attached to the epithelium during the tissue-processing procedure. Biofilm formation was restored in the complemented strain *efg1/efg1+pEFG1* to levels similar to those of the WT strain. Despite the lack of biofilm formation *in vivo*, the *efg1/efg1* mutant strain exhibited colonization levels that were not significantly different from the parental control strain or the complemented mutant (Fig. 4b).

*BCR1* is a transcriptional regulator that is required for biofilm formation on abiotic surfaces *in vitro* and *in vivo*, forming thin weakly adherent biofilms composed of yeast and hyphae, but with little ECM production (Nobile *et al.*, 2006a; Nobile & Mitchell, 2005). During vaginal *in vivo* biofilm formation the *bcr1/bcr1* mutant was also able to produce some hyphae but very little ECM was visible by SEM, which is evident by the lack of smoothness and the clarity of the cell shape. The ability to form a vigorous biofilm with substantial ECM production was restored in the complemented mutant (*bcr1/bcr1+pBCR1*). In fact



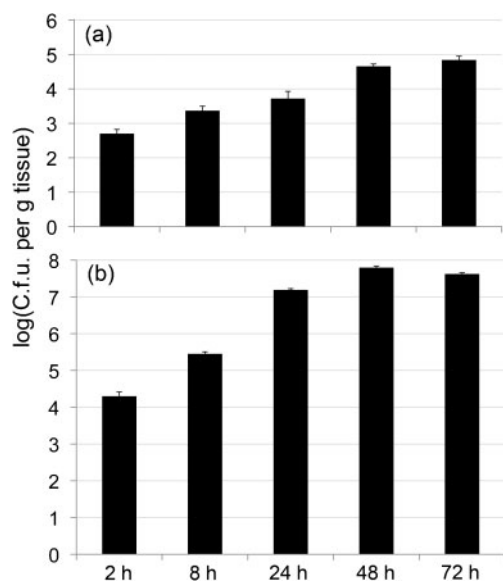
**Fig. 2.** Microscopic analysis of vaginal mucosal biofilm formation *in vivo* and *ex vivo*. For the *in vivo* model, mice were administered oestrogen and intravaginally inoculated with *C. albicans* 3153A ( $5 \times 10^4$  blastoconidia), and vaginæ were harvested at 8, 24, 48 and 72 h. For the *ex vivo* model, mice were administered oestrogen and vaginæ were harvested and inoculated with *C. albicans* 3153A ( $10^6$  blastoconidia) and incubated for 8, 24, 48, or 72 h. Tissues were bisected and processed for SEM, CM or fungal burden (Fig. 3). SEM images were taken at  $1000\times$  magnification, and CM images at  $600\times$  magnification. The figure shows representative images of areas of biofilm growth from three independent repeats,  $n=4$  mice or vaginal explants per time point.

both CM and SEM showed apparently increased ECM production by the complemented strain. Again, there was no significant difference in colonization levels with the *bcr1/bcr1* mutant strain compared with the parental and complemented strains (Fig. 4b).

For the *ex vivo* model, oestrogenized vaginal explants were inoculated with the same isolates and incubated for 48 h, the earliest time point for mature biofilm formation by wild-type strains. Microscopic examination demonstrated that the WT parent strain DAY185 formed biofilms *ex vivo* similar to 3153A (Fig. 5a). Similar results were observed with the wild-type strain SC5314, which is the parent of control strain DAY185 (data not shown). An extensive

hyphal network was present with significant ECM, as indicated by the smooth covering overlying the cells in the SEM image and diffuse red staining (ConA-TR) overlying the calcofluor-white-stained fungal cells in the CM image. The *efg1/efg1* mutant colonized and produced yeast and pseudohyphal forms at 48 h with no ECM present (SEM). In contrast to DAY185, there was a lack of diffuse ConA-TR staining with the *efg1/efg1* mutant by CM, indicating a lack of ECM. Similar to the *in vivo* model, very few organisms were visible by SEM. Interestingly, the complemented mutant was unable to produce significant amounts of hyphae *ex vivo*, but still was able to produce ECM visible by CM and SEM. The *bcr1/bcr1* mutant produced both yeast and a few hyphae, but no ECM was





**Fig. 3.** *In vivo* and *ex vivo* kinetics of vaginal fungal burden. For the *in vivo* model (a), mice were administered oestrogen and intravaginally inoculated with *C. albicans* 3153A ( $5 \times 10^4$  blastoconidia), and vaginae were harvested at 8, 24, 48 and 74 h. For the *ex vivo* model (b), mice were administered oestrogen and vaginae were harvested and inoculated with *C. albicans* 3153A ( $10^6$  blastoconidia) and incubated for 8, 24, 48, or 72 h. Tissues were bisected and processed for quantitative plate counts. The same mice were used for microscopy (Fig. 2) and c.f.u. analysis. Results represent means ( $\pm$ SE) of three independent repeats;  $n=4$  mice or vaginal explants per time point.

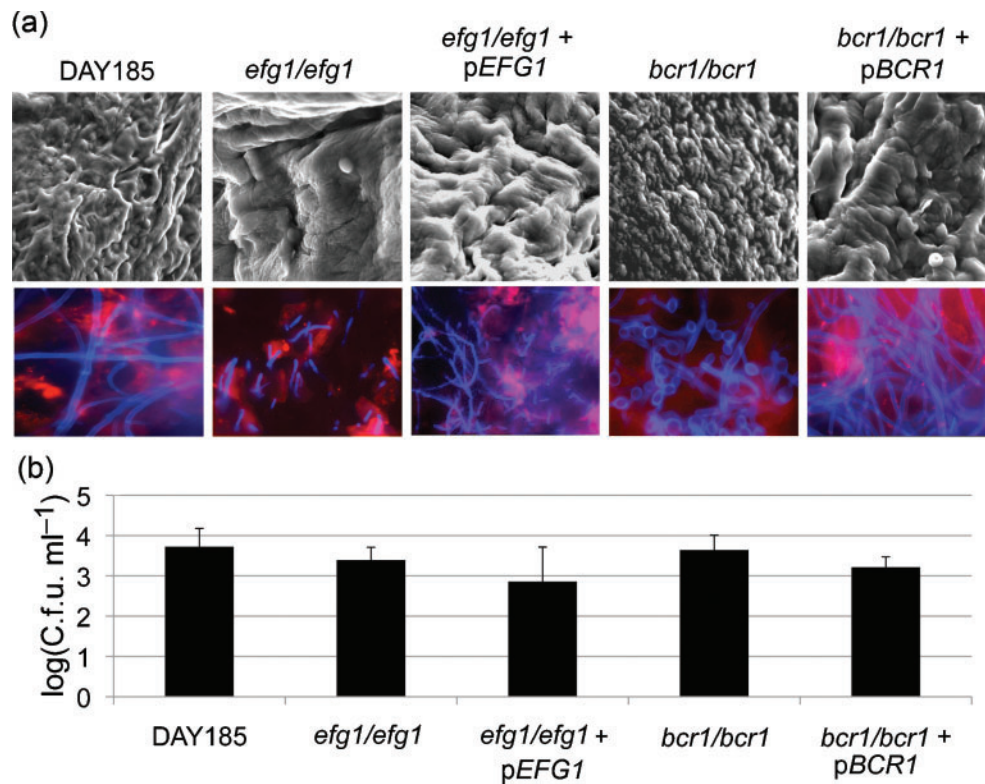
visible by SEM. The same result was also evident by CM, which showed a lack of diffuse ConA-TR staining, although underlying lightly stained epithelial cells could be seen (Fig. 5a). In contrast, ECM production was restored in the complemented mutant. However, no differences in colonization were observed between WT strain DAY185, mutants, or complemented mutants (Fig. 5b).

## DISCUSSION

Biofilms are present ubiquitously and have established a unique niche on abiotic and biotic surfaces. To our knowledge this is the first report of an *in vivo* fungal mucosal biofilm in an immunocompetent animal model of vaginitis. Prior to this study, the only *in vivo* vaginal biofilm characterized was bacterial, consisting of *Gardnerella vaginalis* (Scott *et al.*, 1989; Swidsinski *et al.*, 2008; van der Meijden *et al.*, 1988). In this study, we demonstrate by microscopic analysis that *C. albicans* biofilms are present on the vaginal mucosa. *C. albicans* vaginal biofilms have biofilm architecture typical of *in vitro*-grown *C. albicans* biofilms, consisting of yeast and hyphae forming a complex network surrounded by ECM (Chandra *et al.*, 2001; Ramage *et al.*, 2001). ECM was

evident by SEM, appearing as a thick film covering the cellular portion of the biofilm. For CM analysis, we used ConA-TR staining to visualize ECM. ConA is a lectin that binds to *C. albicans* polysaccharides secreted as part of the matrix.  $\beta$ -Glucan is a cell wall polysaccharide that has been detected in *Candida* ECM by a *Limulus* amoebocyte lysate clotting enzyme based assay (Nett *et al.*, 2007a, b). However, a monoclonal  $\beta$ -glucan antibody, which stains the cell wall or cell-associated matrix, was unable to stain the non-cell-associated ECM that is interspersed between the cells (Dongari-Bagtzoglou *et al.*, 2009). In this same study, ConA staining effectively stained the interspersed ECM, and it has been used by many investigators to visualize *Candida* biofilm matrix (Chandra *et al.*, 2001; Dongari-Bagtzoglou *et al.*, 2009; Nobile & Mitchell, 2005). Therefore, our observations satisfy the definition of a biofilm and provide evidence for the presence of biofilm on the vaginal mucosa. In addition to the *in vivo* model, we developed an *ex vivo* model using vaginal explants, which were kept in a moist controlled environment. Although no exogenous nutrients were provided, *C. albicans* was able to adhere, and to form hyphae and subsequent biofilms encased in ECM on vaginal explants. The ability to form a biofilm in the absence of exogenous growth media demonstrates that growth occurs most likely by scavenging host nutrients.

Kinetics studies showed that *in vivo* and *ex vivo* biofilm formation followed closely with increases in fungal burden. Although the c.f.u. assay may under-represent the actual cell numbers, due to the presence of hyphae in the biofilm, we obtained consistent measurements using this method. One caveat is that testing mutants with defects in morphogenesis could affect the c.f.u. levels relative to a wild-type strain. However, systemic infection models routinely use the c.f.u. assay to assess tissue fungal burden *in vivo*. One way to address this is to use an alternative assay for fungal burden such as a chitin assay to confirm results from the c.f.u. assay. Our results are also consistent with the kinetics of *in vitro* biofilms grown on silicone discs, which show steady increases in dry weight measurements until 36–48 h and then remain stationary (Chandra *et al.*, 2001; Hawser & Douglas, 1994). In addition, the SEM and CM results for both models progressively showed evidence of ECM. The *in vivo* and *ex vivo* vaginal biofilm development phases closely resemble those of *in vitro* biofilms (Chandra *et al.*, 2001; Ramage *et al.*, 2001). *In vitro* biofilms on abiotic surfaces have distinct developmental phases: the early phase ( $\approx 0$ –11 h), in which adhesion and morphogenesis occurs; the intermediate phase ( $\approx 12$ –30 h), in which the hyphae continue growth and ECM begins to emerge; and the maturation phase ( $\approx 38$ –72 h), by which ECM completely surrounds the biofilm. Interestingly, the *ex vivo* model had an accelerated pace compared to the *in vivo* model, which could be due to several factors. First, the ability of the whole intact tissue *in vivo* to mount an antifungal response could play a role in limiting fungal growth. Second, *in vivo* vaginal tissue

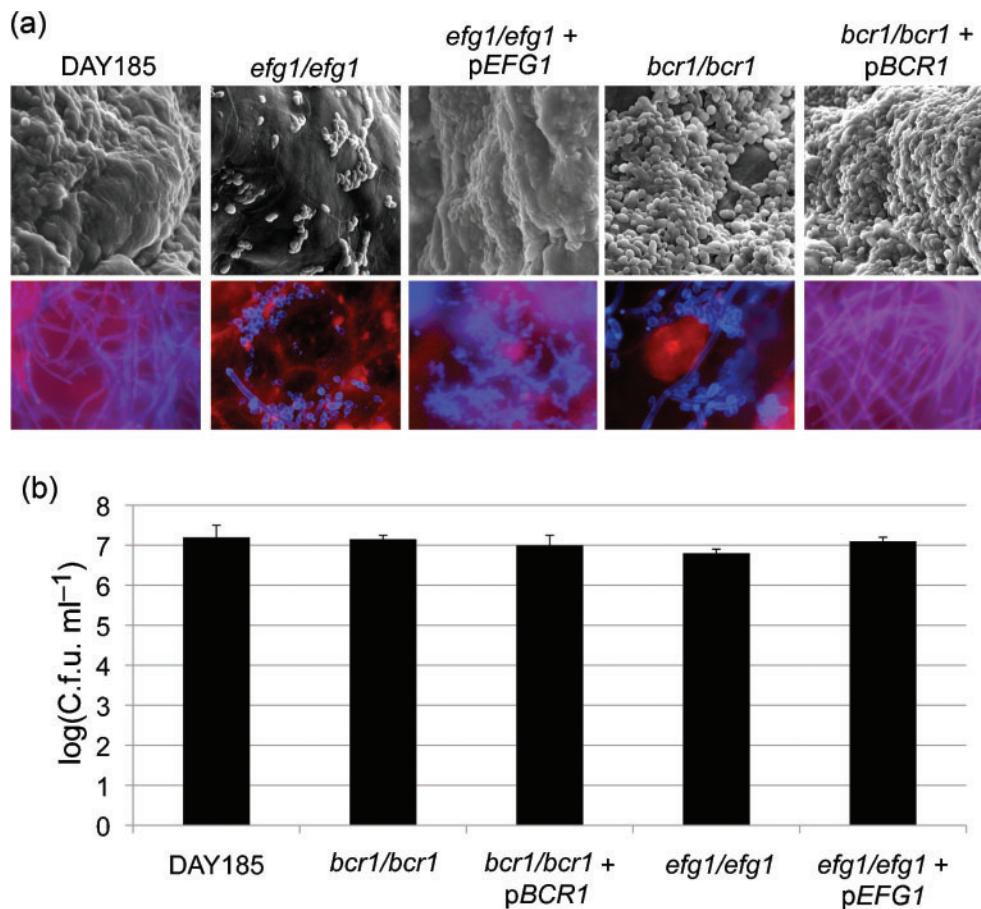


**Fig. 4.** Role of *EFG1* and *BCR1* during *in vivo* vaginal biofilm formation. Mice were administered oestrogen and intravaginally inoculated with *C. albicans* strain DAY185, *efg1/efg1*, *efg1/efg1 + pEFG1*, *bcr1/bcr1* or *bcr1/bcr1 + pBCR1* ( $5 \times 10^6$  blastoconidia), and vaginae were harvested at 72 h. Tissues were bisected and processed for (a) SEM or CM, or (b) quantitative plate counts. The figure shows representative images or means ( $\pm$  SE) of three independent repeats;  $n=4$  mice or vaginal explants per time point.

contains many secretions, composed of electrolytes, organic acids and proteins, which maintain properties such as pH and osmolarity (Owen & Katz, 1999). Finally, *ex vivo* in a tissue-culture setting, the vaginal tissue is completely exposed and unsubmerged in PBS containing antibiotics, whereas intact tissue has a degree of protection by bacterial microbiota.

To further evaluate *C. albicans* vaginal biofilms, we tested several biofilm-deficient mutants in the *in vivo* and *ex vivo* murine models. *EFG1* encodes a transcriptional factor that is required for morphogenesis under most hypha-inducing conditions *in vitro* (Lo *et al.*, 1997; Stoldt *et al.*, 1997). Accordingly, the *efg1/efg1* mutant is unable to form biofilms *in vitro* in RPMI or YNB + glucose; however, its biofilm formation ability *in vivo* has not been previously reported (Ramage *et al.*, 2002; Watamoto *et al.*, 2009). In both our *in vivo* and *ex vivo* vaginal biofilm models, the *efg1/efg1* mutant is also defective in morphogenesis and biofilm formation, lacking typical biofilm architecture and ECM production. This observation is in agreement with previous reports that *EFG1* is required for production of hyphae and subsequent biofilm formation *in vitro* (Ramage *et al.*, 2002). Interestingly, few or no organisms were

detectable by SEM, which requires significant sample processing with several dehydration procedures. This observation suggests that although colonization is detectable by c.f.u. analysis, these strains exhibit defects in adhesion, and are unable to remain adherent throughout the SEM processing procedure. The complemented mutant was able to form a normal biofilm consisting of yeast and hyphae and substantial ECM production *in vivo* similar to WT. However, *ex vivo*, this strain was unable to produce hyphae, forming a yeast-dominated biofilm with considerable ECM. One explanation for this observation is that morphogenesis-inducing signals could be stronger *in vivo* than *ex vivo*. Another possibility is that the differences in microenvironment that result from the way the explants were incubated (unsubmerged and exposed to  $\text{CO}_2$ ) could influence the growth of the mutants. This could lead to differential *EFG1* expression patterns in the WT strain compared with the complemented strain *ex vivo*. This is important when considering appropriate models for the future, as well as defining what the actual signals are *in vivo* which lead to biofilm formation. Although this remains to be proven, these results also imply that Efg1 acts on two (separable) levels during biofilm formation, i.e. ECM production and hyphal growth.



**Fig. 5.** Role of *EFG1* and *BCR1* during *ex vivo* vaginal biofilm formation. Mice were administered oestrogen and vaginae were harvested and inoculated with *C. albicans* strains DAY185, *efg1/efg1*, *efg1/efg1* + pEFG1, *bcr1/bcr1*, *bcr1/bcr1* + pBCR1 ( $10^6$  blastoconidia) and incubated for 48 h. Tissues were bisected and processed for (a) SEM or CM, or (b) quantitative plate counts. The figure shows representative images or means ( $\pm$  SE) of three independent repeats;  $n=4$  mice or vaginal explants per time point.

*BCR1* encodes a transcriptional regulator that is required for production of vigorous biofilms on abiotic surfaces *in vitro* and *in vivo* in a catheter model (Nobile *et al.*, 2006a; Nobile & Mitchell, 2005). The defect in biofilm formation is likely due to a lack of proper adhesin expression on the cell surface, but not due to a defect in morphogenesis (Nobile *et al.*, 2006a). In our vaginal models, the *bcr1/bcr1* mutant was defective in biofilm formation both *in vivo* and *ex vivo*, producing little or no ECM. However, the ability to form mature biofilms in both models was restored in the complemented strain. These results are in agreement with a previous study that demonstrated that *BCR1* is necessary for normal biofilm development *in vitro* and *in vivo* in a rat catheter model (Nobile *et al.*, 2006a, b). One notable difference is that the *bcr1/bcr1* mutant formed more hyphae and ECM under *in vivo* conditions compared with *ex vivo* conditions. This again indicates that there may be stronger morphogenesis-inducing signals *in vivo*, which could be due to differences in the microenvironment resulting from the incubation method for the explants.

Another issue is that while the *efg1/efg1* mutant was unable to remain attached for SEM visualization, the *bcr1/bcr1* mutant was more adherent. *Efg1* is upstream of *Bcr1* in relation to the regulation of genes involved in adherence and biofilm formation. Therefore, the *efg1/efg1* mutant has additional defects, including defects in morphogenesis. While the adhesion of these mutants has not been directly compared on vaginal tissues, it is possible that the *Efg1* regulates additional adhesins not regulated by *Bcr1*. Another possible explanation is that these mutants could result in differential induction of epithelial surface proteins that are adhesin targets. Overall, the fact that the mutants that are deficient in biofilm formation *in vitro* are also deficient in biofilm formation *in vivo* and *ex vivo* on vaginal mucosa supports the assertion that *C. albicans* forms true biofilms in our models.

This first report of *C. albicans* biofilm formation on vaginal mucosal tissue is extremely relevant as a newly characterized biotic surface *Candida* biofilm in an immunocompetent



host. It is unknown if *Candida* always exists as a mature biofilm on mucosal surfaces, or only during times of increased fungal burden (e.g. exacerbation of the symptomatic condition during infection). This raises a series of interesting questions: does the presence of a biofilm determine whether *C. albicans* behaves as a pathogen, and does the biofilm play a role in the host response and/or pathogenesis of disease? Another unknown is how commensal vaginal bacteria influence *C. albicans* mucosal biofilm formation. Finally, with *C. albicans* rarely exhibiting resistance to azoles during VVC (Bauters *et al.*, 2002; Mathema *et al.*, 2001), how does this relate to the high rates of biofilm resistance on abiotic surfaces? The *in vivo* and novel *ex vivo* murine models employing biofilm-defective mutants will be excellent to investigate these important questions.

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