Characterization of Biofilm Formation and the Role of BCR1 in Clinical Isolates of Candida parapsilosis

Srisuda Pannanusorn,a,b Bernardo Ramírez-Zavala,c Heinrich Lünsdorf,d Birgitta Agerberth,a Joachim Morschhäuser,c Ute Römlinga

Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Swedena; Department of Parasitology, Mycology and Environmental Microbiology, Swedish Institute for Infectious Disease Control, Solna, Swedenb; Institute for Molecular Infection Biology, University of Würzburg, Würzburg, Gundeme; The Helmholtz Centre for Infection Research, Braunschweig, Germanyd; Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Swedena

In Candida parapsilosis, biofilm formation is considered to be a major virulence factor. Previously, we determined the ability of 33 clinical isolates causing bloodstream infection to form biofilms and identified three distinct groups of biofilm-forming strains (negative, low, and high). Here, we establish two different biofilm structures among strains forming large amounts of biofilm in which strains with complex spider-like structures formed robust biofilms on different surface materials with increased resistance to fluconazole. Surprisingly, the transcription factor Bcr1, required for biofilm formation in Candida albicans and C. parapsilosis, has an essential role only in strains with low capacity for biofilm formation. Although BCR1 leads to the formation of more and longer pseudohyphae, it was not required for initial adhesion and formation of mature biofilms in strains with a high level of biofilm formation. Furthermore, an additional phenotype affected by BCR1 was the switch in colony morphology from rough to crepe, but only in strains forming high levels of biofilm. All bcr1Δ/Δ mutants showed increased proteolytic activity and increased susceptibility to the antimicrobial peptides protamine and RP-1 compared to corresponding wild-type and complemented strains. Taken together, our results demonstrate that biofilm formation in clinical isolates of C. parapsilosis is both dependent and independent of BCR1, but even in strains which showed a BCR1-independent biofilm phenotype, BCR1 has alternative physiological functions.

Candida species are ranked as the fourth leading cause of nosocomial bloodstream infection (1). Although Candida albicans remains the most common Candida species, infections caused by species other than C. albicans are increasing (2). Currently, Candida parapsilosis has been recognized as the second or third most frequently isolated Candida species that causes bloodstream infection (2, 3, 4). C. parapsilosis infections particularly affect neonates (5, 6) and surgical intensive care unit (ICU) patients (7). C. parapsilosis is often found on the hands of health care workers, which is associated with outbreak infections (7, 8). In addition, the ability of C. parapsilosis to form biofilms on medical devices and the resulting phenotypic resistance to antifungal agents can be important in this context.

Biofilm formation is a developmental process (8). The accepted sequence of events elaborated for C. albicans is that first Candida cells have to adhere to a surface. This adhesion is influenced by surface parameters such as hydrophobicity and roughness, the culture medium, and/or the expression of adhesion molecules by Candida cells. For example, it has been shown that an increase of the hydrophobicity and roughness of surface materials leads to elevated biofilm formation by Candida spp. (9, 10). Adhesion molecules involved in surface adhesion have been well characterized in C. albicans. For example, the Eap1 and Als1 surface proteins are involved in adhesion of C. albicans to various materials and epithelial cells.

Second, in the initiation phase, the yeast cells attached to the surface subsequently multiply into microcolonies and develop into hyphal or pseudohyphal forms. Hyphal morphogenesis is required to establish robust biofilms, as several adhesion molecules are expressed by hyphae, for example, Als3 and Hwp1 proteins, which promote cell-cell or cell-substrate binding. Mutants of these adhesins are defective in biofilm formation (11). The transcription factors Tec1 and Efg1 directly control hyphal development. C. albicans mutants defective in TEC1 (12) or EFG1 (13) are unable to form hyphae and consequently fail to form biofilm.

Third, mature biofilms produce an extracellular matrix composed of carbohydrate together with other constituents, such as proteins, hexosamine, phosphorus, uronic acid (14), and extracellular DNA (15). The presence of extracellular matrix strengthens the biofilm structure and protects biofilms from host immunity and antifungal treatment. For example, β-1,3 glucan is a major carbohydrate component of the matrix of Candida biofilms and has an important role in antifungal resistance (16) and host immune evasion (17).

A major biofilm regulator is the transcriptional factor Bcr1, first identified to be required for biofilm formation in C. albicans. Many studies showed the importance of BCR1 in adherence of yeast cells to the surface as the first step to build up a biofilm (12, 18, 19). However, the transcription factor Bcr1 is involved in more than just initial adhesion. BCR1 is upregulated in hyphae, although this protein is not involved in hyphal morphogenesis. Instead, Bcr1 protein controls expression of many adhesins on hyphal cells, such as the Hwp1 and Als3 proteins. Therefore, BCR1 mutants of C. albicans retain the ability of hyphal formation but fail to express adhesins on hypha, which causes a defect in biofilm formation.

Received 20 July 2013. Accepted 27 November 2013 Published ahead of print 2 December 2013
Supplemental material for this article may be found at http://dx.doi.org/10.1128/EC.00181-13. Copyright © 2014, American Society for Microbiology. All Rights Reserved. doi:10.1128/EC.00181-13
formation. Like in C. albicans, BCR1 in C. parapsilosis was revealed to be required for biofilm formation (20, 21).

In this study, we characterized biofilm formation of clinical isolates of C. parapsilosis in molecular detail. The biofilm formation assay was carried out on the clinically relevant material siliccone elastomer (SE). Biofilm formation was quantified by metabolic activity measurement assessing XTT reduction and simultaneously observed by microscopy. Previously, three distinct groups of biofilms of C. parapsilosis (no, low, and high capacity to produce biofilm) were found (22). Here, the high biofilm-forming strains could be further discriminated by structure as monolayer filamentous and complex spider-like biofilms. The biofilms with complex spider-like structure were robustly formed on all tested surface materials (SE, Thermaxon cell culture c overslip, and polystyrene) and showed high resistance to fluconazole.

To analyze biofilm formation on the molecular level, deletion mutants of the major biofilm regulator BCR1 of representative strains for all biofilm types were generated. Surprisingly, under standard conditions in our study, we found biofilm formation of C. parapsilosis is both dependent and independent of BCR1. Deletion of BCR1 affected colony and cell morphology, but only in strains forming high levels of biofilm. Finally, in all strains, deletion of BCR1 caused a pronounced alteration in antimicrobial peptide susceptibility and aspartyl proteinase secretion.

MATERIALS AND METHODS

Strains and growth conditions. A total of 33 epidemiologically unrelated strains of C. parapsilosis from bloodstream infection were investigated for biofilm formation (22). The isolates were maintained in cryopreservative solution (Protect; Technical Service Consultants Ltd., United Kingdom) at −150°C. The isolates were grown on Sabouraud dextrose agar (SDA) at 37°C for 24 to 48 h after recovery. Single colonies were suspended in yeast nitrogen base (YNB) medium containing 10% glucose for subsequent experimentation.

Strain construction. Representative strains of different biofilm production phenotypes (low, SM 449 and SM 706; high [filamentous], SM 424 and SM 526; high [spider-like], SM 588, SM 596, and SM 828) were selected for BCR1 mutant construction. Two independent mutants each were constructed from their respective wild-type strains. The entire BCR1 gene was deleted using the SAT1 flipping strategy (23). The upstream and downstream regions of BCR1 were amplified with primers BCR1Cp_01-BCR1Cp_02 and BCR1Cp_03-BCR1Cp_04, respectively, derived from C. parapsilosis ATCC 22019 as a template and cloned on both sides of the SAT1 flipper cassette of plasmid pSFS4 (24), which resulted in plasmid pBCR1CpM2. To delete both BCR1 alleles, two rounds of transformation and cassette recycling were performed. C. parapsilosis strains were transformed with the deletion cassette from pBCR1CpM2, and colonies were grown on yeast extract-peptone-dextrose (YPD) agar containing 200 µg/mL of nourseothricin (Nou) at 30°C for 48 h to select for transformants. The transformants were screened by PCR with primers BCR1Cp-checkF and BCR1Cp-checkR for correct insertion of the SAT1 flipper cassette into the first BCR1 allele. To recycle the SAT1 flipper cassette, the mutants were cultured in 3 ml of 0.7% YNB medium containing 2% maltose and incubated at 30°C with shaking. Overnight cultures were diluted 10−4, and 200 µL was spread on YPD agar containing 5 µg/mL of Nou. Small colonies grown on the plate after 48 h of incubation at 30°C were streaked on YPD agar and YPD agar containing 100 µg/mL of Nou. Nou-sensitive derivatives of the primary transformants grew on YPD agar but not on agar containing 100 µg/mL of Nou. Transformation with the deletion cassette from pBCR1CpM2 was carried out a second time to inactivate the remaining intact allele of BCR1. Transformants from the second round were screened with the same primers and with primer BCR1Cp_05-BCR1Cp_06 (negative PCR) for complete deletion of BCR1. Recycling of the cassette was carried out as described above. All mutations were verified by Southern blotting hybridization using the BCR1 upstream fragment as the probe.

To reintroduce an intact BCR1 copy into the mutants, the entire BCR1 gene, including 450 bp of upstream sequences and 347 bp of downstream sequences, was amplified from strain SM 596, which formed a high spider-like biofilm, with primers BCR1Cp01-BCR1Cp08. The PCR product was substituted for the BCR1 upstream fragment in pBCR1CpM2 to generate pBCR1CpM1K1. The complementation cassette from this plasmid was integrated into one of the bcr1Δ alleles of the homozygous mutants, followed by recycling of the SAT1 flipper cassette as described above.

Biofilm formation and adhesion assay. Biofilm formation on silicone elastomer (SE) squares was performed as previously described (22), and biofilm formation on Thermaxon cell culture coverslips and polystyrene 96-well plates was performed accordingly. All surfaces were pretreated with 10% human serum in phosphate-buffered saline (PBS) for 1 h at 37°C and washed. Cells grown overnight were added at a concentration of 1 × 106 cells/mL in YNB medium containing 10% glucose as the growth medium and allowed to adhere for 3 h. The SE square was washed with PBS to remove nonadherent cells and put in a new well with fresh medium. After 48 h, biofilm formation was quantified by assessing the metabolic activity of the biofilm cells with the reduction of XTT [2,3-bis(2-methyloxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide inner salt] (Sigma-Aldrich). The amount of biofilm was categorized as high (optical density at 490 nm [OD490] ≥ 0.1), low (0.025 ≤ OD490 < 0.1), and no (OD490 < 0.025) biofilm formation. The assay was carried out with three technical replicates, and at least two independent experiments were performed. C. parapsilosis ATCC 22019 served as a negative control, while C. parapsilosis outbreak isolate P 2:2 was arbitrarily chosen as a positive control.

Initial adhesion to the SE square assessed after 15 min, 30 min, and 3 h was carried out as described above. The standardized yeast inoculum was allowed to adhere to the pretreated SE square, and the SE square was washed twice to remove nonadherent cells. Finally, the SE square was placed in a new well containing 1.5 mL of PBS for assessment of metabolic activity by the XTT reduction assay.

SEM. Biofilm formed on SE was fixed in 1.5 mL fixative solution (0.5% glutaraldehyde, 2.5% paraformaldehyde in 10 mM HEPES, pH 7.0) for 2 h at 4°C. The fixed sample of biofilm was further processed for scanning electron microscopy (SEM) observation.

CLSM. Biofilm on SE was incubated with Calcofluor white solution (100 µg/mL) for 30 min at 37°C in the dark. The biofilm was observed with a Zeiss LSM 510 META confocal laser scanning microscope (CLSM). Biofilm images were visualized with a ×20 magnification objective and Xydo 405 laser at 405-nm excitation. Images were acquired using Zeiss LSM Image Browser software.

Antifungal susceptibility of C. parapsilosis biofilm. Susceptibility of biofilm-grown C. parapsilosis to fluconazole, amphotericin B, and caspofungin (Sigma-Aldrich) was tested. Fluconazole was solubilized in dimethylsulfoxide (DMSO), and amphotericin B and caspofungin were solubilized in water. Five hundred µL of YNB medium containing different concentrations (2-fold dilution) of antifungal drugs were prepared in 24-well plates. An SE square with a 48-h biofilm developed as described above was washed with PBS and transferred to a well containing new medium with antifungal drug. The biofilm was incubated with the drug for 48 h at 37°C. The SE square was washed with PBS, and viability of cells in the biofilm was quantified using the XTT reduction assay. MIC50 is defined as the minimum concentration of antifungal that reduced the metabolic activity of biofilm cells to 50% compared to the metabolic activity of the antifungal-free control. The assay was carried out in at least two independent experiments with three technical replicates each.

Assessment of colony morphology. Yeast cells were grown overnight in YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 30°C with shaking at 120 rpm. Cells were harvested by centrifugation at 4,000 rpm for 3 min and washed twice with PBS. The pellet was resuspended in PBS.
and adjusted to 10^3 cells/ml. A 100-µl suspension containing 100 cells was plated on YPD agar. After 5 days of incubation at 30°C, colony morphology was observed under a stereo microscope (Leica MZ6).

**Assessment of cell morphology.** A 5-day-old colony grown on YPD agar at 30°C was assessed for cellular morphology. Ten µg/ml Calcofluor white solution was prepared in water and adjusted with NaOH. Cells from a single colony were resuspended in 50 µl of Calcofluor white solution. Five µl of sample was applied on the slide, and the cellular morphology was observed under a fluorescence microscope at ×100 magnification.

**Secreted proteinase assay.** Assessment of the ability to secrete aspartyl proteinases was performed as previously described, with slight modifications (25). Briefly, 5 µl of cell suspension from a YPD overnight culture adjusted to 10^2 cells/ml was spotted on yeast carbon base agar containing 0.2% bovine serum albumin (BSA). After 5 days of incubation at 37°C, the plate was stained for 15 min with 0.1% amido black solution in 1% acetic acid and 40% methanol. A clear zone around the colony indicated proteolytic activity after overnight destaining with 50% methanol.

**Susceptibility to antimicrobial peptides.** The susceptibility of yeast cells to antimicrobial peptides, LL-37 (Innovagen), protease sulfate (Sigma-Aldrich), and rational peptide-1 (RP-1) (Innovagen) was assessed using an inhibition zone assay. Cells of overnight culture in 7.5 ml YPD medium were harvested and washed twice with PBS. The concentration of yeast cells was adjusted to an OD_{590} of 0.3 in YPD medium. Thirty µl of sample was mixed with 6 ml YPD medium containing 1% agarose type-I (Sigma-Aldrich) and medium E, a salt solution of 0.8 mM MgSO_4, 9.5 mM citric acid, 57.5 mM KHPO_4, 16.7 mM NaH_2PO_4. This agarose solution was poured into a petri dish, and a 3-mm-diameter well was punched into the agarose, in which 10 µg of the respective antimicrobial peptide was added. The plate was incubated at 30°C for 24 h. Nystatin was used as a positive control in the test. *C. albicans* DAY 286 (wild type) and *C. albicans* strain 702 (bcr1Δ/Δ) were included as control strains. At least two independent experiments were performed.

**RNA isolation.** RNA was isolated from overnight cultures and from biofilms formed on SE and grown for 48 h in YNB medium using the hot sodium dodecyl sulfate (SDS)-phenol method (26). Cells from overnight culture were harvested, washed twice with PBS, and resuspended in 400 µl of 50 mM Na-acetate, pH 5.3, 10 mM EDTA (AE buffer), 40 µl of 10% SDS, 400 µl of acid phenol-chloroform-isoamyl alcohol (125:24:1), pH 4.3 (Ambion). The suspension was incubated at 65°C for 15 min and vortexed every minute, cooled on ice, and centrifuged. The aqueous phase was mixed with the same volume of chloroform-isoamyl alcohol (24:1) and centrifuged, and RNA was precipitated with 2.5 volumes of cold isopropanol. After incubation at −20°C for 1 h, RNA was collected by centrifugation, washed with 70% ethanol, dried, and dissolved in 0.1% diethylpyrocarbonate (DEPC)-treated water. RNA was treated with 10 U of DNase (RNases free DNase; Promega) for 2 h at 37°C and treated with phenol-chloroform extraction. RNA was gently collected by isopropanol precipitation, dried, and resuspended as described above. The quality of the RNA was assessed by gel electrophoresis and by PCR amplification of *BCR1* with primers BCR1cp05 and BCR1cp06. Only DNA-free, nondegraded RNA was used in subsequent experiments. To isolate RNA from biofilm, five SE squares with biofilm were washed twice with PBS, and cells were removed from the SE squares by vortexing with glass beads before RNA isolation.

**qRT-PCR for BCR1 expression analysis.** cDNA was synthesized from 1 µg of RNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer’s instructions. A quantitative real-time PCR (qRT-PCR) was performed in triplicate using the iQa Universal SYBR green supermix (Bio-Rad) on the ABI Prism 7000 apparatus (Applied Biosystems). The *BCR1* expression signal was normalized to *ACT1* (27). Analysis in triplicate was performed from at least two independent RNA isolations. Primers used in the qRT-PCR study are listed in Table S2 in the supplemental material.

**RESULTS**

Biofilm formation on SE is highly variable among *C. parapsilosis* isolates. As reported previously on *C. parapsilosis* biofilm formation (22), the capacity to form a biofilm after 48 h of incubation was highly variable among the 33 clinical isolates of *C. parapsilosis*. The isolates were categorized as having high, low, or no capacity to form biofilms. Among the isolates, 15/33 formed large amounts of biofilm, 7/33 formed small amounts of biofilm, and 11/33 were not able to form biofilm on SE (Fig. 1). The difference in the amount of biofilm formed among groups of strains was statistically significant (*P < 0.0001* by analysis of variance [ANOVA]). In combination with the quantitative XTT measurement, biofilm formation was simultaneously observed by inverted light mi-
Distinct biofilm structures could be observed among the groups of strains with different biofilm formation capacities. *C. parapsilosis* isolates incapable of forming a biofilm presented only a few yeast cells on the surface (Fig. 2A). *C. parapsilosis* isolates with a low capacity to form a biofilm displayed aggregates of yeast cells on the surface (Fig. 2B). In contrast, isolates with high biofilm formation, as indicated by the XTT reduction assay, displayed two different biofilm structures. Four of 15 isolates (SMI 424, SMI 526, SMI 785, and SMI 886) displayed a biofilm morphology consisting of monolayers of pseudohyphae (Fig. 1 and 2C), while 11 of 15 isolates, including one isolate (SMI 828) from an outbreak infection (28), formed a biofilm structure composed of macrocolonies with a spider-like appearance consisting of pseudohyphae and yeast cells (Fig. 1 and 2D) (22). The different biofilm structure of high biofilm formers was confirmed by scanning electron microscopy (Fig. 2E and F) and confocal microscopy (see Fig. S1 in the supplemental material).

Colonies of the isolates on YPD agar plates correlated with the capability of the strains to form a biofilm (Fig. 1). A rough or rough/faint colony morphotype was exhibited by 13 of 15 isolates which formed high levels of biofilm. The exception was two isolates, SMI 526 and SMI 886, which formed a large amount of biofilm with monolayers of filaments which showed a smooth phenotype. Rough/faint is a novel colony morphotype found in isolates derived from an outbreak infection (Srisuda Pannasorn, Thomas Schön, Joachim Morschhäuser, and Ute Römling, unpublished data). Only the smooth morphotype was displayed by 11 isolates with no capability of forming a biofilm. The smooth morphotype was also expressed by 5 of 7 isolates forming low levels of biofilm, while the remaining two isolates had a rough/faint morphotype.

**Biofilm formation by *C. parapsilosis* isolates on three different materials.** The material surface is one determinative factor for formation of a biofilm. To investigate whether biofilm formation of clinical isolates of *C. parapsilosis* is significantly affected by surface characteristics, we tested biofilm formation on two other materials, a Theranova plastic coverslip and a polystyrene 96-well plate. The results were compared to biofilm formation on SE. SE is a clinically relevant material and model surface for biofilm formation on intravascular catheters and indwelling bioprosthetic devices (29). The polystyrene 96-well microtiter plate is a widely used material to assess biofilm formation that allows the perfor-

---

**FIG 2** Characteristic biofilm architecture of representative *C. parapsilosis* isolates formed on silicone elastomer (SE) after 48 h at 37°C in YNB medium containing 10% glucose. (A) Single yeast cells are attached on SE in strains which showed no biofilm formation (SMI 416). (B) An isolate with low biofilm formation displaying a monolayer of aggregated yeast cell (SMI 706). (C) An isolate with a high capacity to form a biofilm consisting of monolayers of pseudohyphae (SMI 424). (D) Isolate with a high capacity to form a biofilm consisting of complex spider-like macrocolonies with aggregated yeast cells and pseudohyphae (SMI 588). For panels A to D, magnification is ×200. (E and F) Scanning electron microscopy of isolates with high biofilm formation consisting of monolayer filaments SMI 424 (E) and complex spider-like macrocolonies SMI 588 (F).
mance of high-throughput assays for biofilm formation studies (30). Thermanox coverslip is a common material used in tissue culture, and it is occasionally used to assess biofilm formation (31).

Biofilm formation of 33 clinical isolates on the three materials is summarized in Table 1. Ten of the 11 isolates which formed a high level of complex biofilm structure with a spider-like appearance on SE also formed a high level of biofilm with a similar structure on Thermanox and polystyrene (data not shown). Interestingly, the four isolates which formed a high level of biofilm consisting of monolayers of pseudohyphae on SE did not form a biofilm on the Thermanox coverslip, and only one isolate displayed a low level of biofilm formation on polystyrene. Eleven isolates were negative for biofilm formation on SE and on the other materials. These results showed that only complex biofilm structure formation is a stable characteristic on all surfaces. The choice of material is critical for the assessment of biofilm formation in the case of biofilm producers displaying a less complex structure. These findings might have implications for the epidemiology of biofilm-forming strains of *C. parapsilosis*.

**Initial adhesion of *C. parapsilosis* clinical isolates to SE.** To investigate biofilm formation of clinical *C. parapsilosis* isolates in more detail, we tested whether adhesion after 3 h is correlated with the amount of biofilm formed after 48 h. Representative isolates of the different biofilm phenotypes were tested for their 3-h adhesion capability (Fig. 3). Estimation of adhesion by the XTT metabolic activity test revealed that the largest amount of adhesion was displayed by *C. parapsilosis* isolates with the capacity to form complex spider-like biofilm structures, while the remaining isolates with no, low, or high levels of biofilm with monolayer filament structure showed low-level, indifferent adhesion to SE (Fig. 3A). Interestingly, strain SMI 706 consistently exhibited high adhesion, although it formed a small amount of biofilm after 48 h. Microscopic examination established the correlation between the XTT result and the number of cells adherent to SE squares after 3 h (Fig. 3B and data not shown).

We observed flocculation (clumping) of isolates which formed high complex biofilm throughout the growth phase. In order to investigate whether *C. parapsilosis* cells initially adhere to SE as a single cell or a clump of yeast cells, initial adhesion of flocculating isolates was compared to that of isolates which did not flocculate. Adhesion to substrate was observed after 15 and 30 min to ensure that cell division has not occurred. Representative isolates which flocculated (SMI 576 and SMI 828) and isolates with low (SMI 449) and no biofilm (SMI 416) formation were chosen. In isolates with high biofilm formation, more cells adhered to the SE surface than did cells with low or no biofilm formation (Fig. 4A). All isolates irrespective of flocculation adhered to SE as single cells (Fig. 4B).

**Antifungal susceptibility of *C. parapsilosis* biofilms.** Biofilm formation is considered a major virulence factor of non-*albicans Candida* spp. and is required to overcome the colonization barrier even in environments with high antimicrobial presence. Biofilms of representative isolates with different biofilm formation capability were tested for susceptibility to amphotericin B, caspofungin, and fluconazole, three commonly used antifungal drugs (Fig. 5). Planktonic cells of all isolates were sensitive to amphotericin B (0.25 to 1 μg/ml), caspofungin (0.5 to 1 μg/ml), and fluconazole (0.125 to 4 μg/ml). Although in general high biofilm-forming isolates showed 2-fold-less susceptibility to amphotericin B and caspofungin than low biofilm-forming isolates, the biofilms of all isolates were considered to be sensitive to amphotericin B (MIC<sub>50</sub> 0.125 to 0.5 μg/ml) and caspofungin (MIC<sub>50</sub> 1 to 2 μg/ml). In contrast, isolates forming high levels of complex biofilm (SMI 388, SMI 596, and SMI 828) exhibited resistance to fluconazole (MIC<sub>50</sub> ≥128 μg/ml), while the remaining isolates forming a high level of biofilm consisting of a monolayer of filament (SMI 424 and SMI 526) and low biofilm formers (SMI 449 and SMI 706) were susceptible to the tested fluconazole concentrations (64 to 256 μg/ml).

**Effect of BCR1 deletion on biofilm formation.** It has been shown that *BCR1* is required for biofilm formation in *C. parapsilosis* (20). To investigate the role of *BCR1* in biofilm formation of our clinical isolates, *BCR1* was deleted from representative strains forming high levels of biofilm with spider-like structures (SMI 388, SMI 596, and SMI 828), high levels of biofilm with filament (SMI 424 and SMI 526), and low levels of biofilm (SMI 449 and SMI 706). The *BCR1* deletion strains were subsequently complemented with *BCRI* from SMI 596, an isolate which formed high levels of biofilm with spider-like structure. The sequence of *BCRI* of SMI 596 was 100% identical to *BCRI* of *C. parapsilosis* CDC 317 from the *Candida* genome database (data not shown). *bcr1Δ*Δ mutants of strains SMI 424 and SMI 526 could not be complemented.

Surprisingly, deletion of *BCRI* had a variable effect on biofilm formation in strains with different biofilm-forming abilities (Fig. 6). *bcr1Δ*Δ mutants of SMI 449 and SMI 706, forming small amounts of biofilm, showed a decrease in biofilm formation after

### Table 1 Biofilm formation by 33 clinical isolates of *C. parapsilosis* on three materials

| Isolate no. | Biofilm-forming capability on: |
|-------------|-------------------------------|
|             | SE | Thermanox | Polystyrene |
| SMI 416, SMI 501, SMI 534, SMI 546, SMI 630, SMI 661, SMI 681, SMI 694, SMI 798, SMI 902, SMI 944 | Negative | Negative | Negative |
| SMI 774, SMI 928 | Positive (low) | Negative | Negative |
| SMI 449, SMI 559, SMI 624 | Positive (low) | Negative | Positive (low) |
| SMI 596, SMI 886 | Positive (low) | Positive (high) | Positive (low) |
| SMI 563, SMI 576, SMI 588, SMI 596, SMI 768, SMI 775, SMI 794, SMI 812, SMI 828, SMI 948 | Positive (low) | Negative | Positive (low) |
| SMI 802 | Positive (high) | Positive (high) | Positive (low) |

*a* High capacity to produce biofilm with monolayers of pseudohyphae.
48 h compared to the wild type as assessed by the XTT assay (Fig. 6A). The bcr1Δ/Δ mutant of SMI 424, a strain with high biofilm formation capability consisting of monolayer filament, was also defective in biofilm formation. However, the bcr1Δ/Δ mutants of SMI 526 (high biofilm former, filament type) and SMI 588, SMI 596, and SMI 828 (high biofilm former, spider type) did not show an altered biofilm formation capability compared to the wild type.

BCR1 mutants reconstituted with the BCR1 wild-type allele recovered biofilm formation to a level similar to that of the wild type (Fig. 6A). As expected, the biofilm formation of complemented mutants where deletion of BCR1 had no effect did not show an altered biofilm level. The two independently constructed BCR1 mutants of each strain and respective complemented strains showed the same phenotype (data not shown). Also, when selected strains with the Bcr1-dependent and -independent phenotype were grown in synthetic defined (SD) medium (20), a medium commonly used to assess biofilm formation, a similar effect of Bcr1 on biofilm formation was observed (data not shown). These results indicate that biofilm formation in C. parapsilosis clinical isolates can be both dependent and independent of BCR1.

Assessment of biofilm formation of the isolates, their bcr1Δ/Δ mutants, and reconstituted strains was complemented by scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) (Fig. 7). Biofilm formation of the isolates as observed by microscopy was consistent with the amount of biofilm as estimated by metabolic activity. In addition, CLSM analysis showed that bcr1Δ/Δ mutants of strains forming a Bcr1-independent biofilm (SMI 588, SMI 596, and SMI 828 [spider-like biofilm structure]) formed more and longer pseudohyphae and had an extended biofilm structure compared to the wild types and the
complemented mutants. Furthermore, strain SMI 526 (monolayer filament structure) showed extended biofilm formation and flocculation upon deletion of Bcr1 (see Fig. S2 in the supplemental material). In contrast, strains with Bcr1-dependent biofilm formation (SMI 449, SMI 706, and SMI 424) did not show elongation of pseudohyphae. These results demonstrate that BCR1 suppresses pseudohyphal formation in strains with Bcr1-independent biofilm formation, in contrast to results in the literature, which reported a positive role of Bcr1 in pseudohyphal formation (20, 32).

**BCR1 expression in biofilms.** The BCR1 expression level of representative *C. parapsilosis* strains with Bcr1-dependent and -independent biofilm formation was investigated. BCR1 was expressed in overnight cultures and biofilms of *C. parapsilosis* grown for 48 h, although expression of BCR1 was reduced in Bcr1-independent biofilm (Fig. 8). These results, in combination with CLSM observations, demonstrate that Bcr1 is expressed and has a structural role in strains with Bcr1-independent biofilm formation.

**Effect of BCR1 deletion on cell surface adhesion.** Adherence of cells to surfaces is the first step of biofilm development. Besides controlling cell-cell adhesion, BCR1 is also required for cell-substrate adhesion (18). To determine the role of BCR1 in early biofilm formation, we assessed the adherence to SE at 3 h for the bcr1Δ/Δ mutants compared to the wild type. In agreement with the results for the biofilm grown for 48 h, we found a reduction in adhesion upon deletion of BCR1 only in strains where BCR1 significantly reduced biofilm formation after 48 h (SMI 449 and SMI 706) (Fig. 6B). bcr1Δ/Δ mutants from strains with spider-like biofilm structure and SMI 526 showed no difference in adherence compared to the wild type, consistent with the role of BCR1 after 48 h of biofilm formation.

When cells are grown in YNB medium containing 10% glucose, flocculation is constantly observed in strains forming high levels of spider-like biofilm (SMI 588, SMI 596, and SMI 828). We observed that flocculation was reduced when those strains were cultured in YPD medium. To investigate the relevance of preexperimenal culture conditions on BCR1-dependent biofilm formation, strains were cultured overnight in YPD medium prior to the experiment, and adherence after 3 h and biofilm formation after 48 h was assessed. We found that adherence after 3 h was reduced in all tested bcr1Δ/Δ mutant strains compared to the wild type. The complementation with BCR1 recovered adherence after 3 h to wild-type levels (Fig. 6C). Although adhesion at 3 h was influenced by BCR1, biofilm formation at 48 h was still independent of BCR1 in strains forming high levels of spider-like biofilms (Fig. 6D). These results indicated that BCR1 is required for initial cell surface adhesion in *C. parapsilosis*, similar to *C. albicans* under conditions where a biofilm phenotype needs to be established.

**Effect of BCR1 deletion on colony and cell morphology.** Biofilm formation in *C. parapsilosis* was shown to be influenced by phenotypic switching (33). Therefore, we next tested whether deletion of BCR1 affected the colony and cell morphology of these strains. Out of seven strains with BCR1 deletion, we found a pronounced effect of BCR1 on colony morphology in three strains (SMI 526, SMI 588, and SMI 596) with BCR1-independent biofilm formation (Fig. 9 and Table 2). Upon BCR1 deletion, the colony morphology changed from rough or rough/faint to crepe in these isolates. Importantly, upon reintroduction of the wild-type BCR1 gene, the colony morphotype switched back to the wild type. Microscopic observation of the cell morphology of the crepe morphotype showed that it is mainly composed of elongated yeast cells and pseudohyphae (Fig. 9). This is in contrast to the rough or rough/faint wild type that is composed of round yeast cells.

**Effect of BCR1 deletion on antifungal susceptibility.** SMI 828 and its mutants were used to determine whether the BCR1 deletion has an effect on antifungal susceptibility of a BCR1-independent biofilm. The susceptibility of the bcr1 deletion mutant and complemented strain to the tested drugs (amphotericin B, caspofungin, and fluconazole) was similar to that of the wild-type strain (see Fig. S3 in the supplemental material). These results suggested that the deletion of BCR1 had no effect on antifungal susceptibility of *C. parapsilosis* forming complex spider-like biofilm.

**bcr1Δ/Δ mutants show an increase in proteolytic activity.** We sought to investigate additional phenotypes affected by BCR1 unrelated to biofilm formation but related to virulence. Secreted aspartyl proteinases have been identified as a major virulence factor in several *Candida* spp., including *C. parapsilosis* (25, 34). To investigate the role of BCR1 on the secretion of proteinases, we used the spot assay. Interestingly, we found that bcr1Δ/Δ mutants of all strains independent of its biofilm phenotype showed increased secretion of proteinases compared to wild-type strains (Fig. 10A). Complementation of mutants with BCR1 reduced the level of proteinase secretion to almost wild-type levels. This finding indicates that the transcription factor Bcr1 is a direct or indirect negative regulator of proteinase secretion.

---

**FIG 4** Initial adhesion of *C. parapsilosis* isolates to SE. (A) Adhesion to an SE square by *C. parapsilosis* after 15 and 30 min. Adhesion of *C. parapsilosis* to SE was assessed by measuring the metabolic activity using the XTT reduction assay. Metabolic activity of SMI 828 was arbitrarily set to 1. (B) *C. parapsilosis* SMI 576 forming flocs adhered to the silicone surface as single cells after 15 min. Black arrows indicate single cells of *C. parapsilosis* adherent on the SE square. Three independent experiments with two technical replicates were performed.
Antifungal susceptibility of *C. parapsilosis* biofilms. Biofilms formed on SE for 48 h were exposed to amphotericin B, fluconazole, and caspofungin for 48 h at 37°C. The metabolic activity of biofilm cells treated with antifungals was determined by the XTT assay. The positive control is antimycotic-free biofilm, and its activity was arbitrarily set to 1.
**DISCUSSION**

Previously, we screened 33 clinical isolates of *C. parapsilosis* causing bloodstream infection for their biofilm-forming ability (22). In this work, we characterize biofilm formation of 33 isolates in more detail, focusing on biofilm structure and development, the role of BCR1 in biofilm formation, and its effect on alternative phenotypes.

Biofilm formation of *C. parapsilosis* is initiated with the adherence of single yeast cells to the surface even under growth conditions where already extensive cell clumping is observed. This indicates that specific yeast cell-expressed surface adhesins initiate biofilm formation. In general, biofilm formation and the genetic control of biofilm formation has been resolved mainly in *C. albicans* (8). These analyses demonstrated that biofilm formation starts with the attachment of single yeast cells to the surface, which is followed by initiation, where microcolonies and germ tubes are formed. During maturation, the biomass expands and extracellular matrix accumulates. Upon dispersion, yeast cells are released to the medium. The initial adherence step in *C. albicans* is controlled by the transcription factor Efg1, a positive regulator of expression of the Eap1 adhesin (36, 37, 38). Although Efg1 has an effect on biofilm formation in *C. parapsilosis* (39), the function of Eap1 needs to be investigated.

We observed that different clinical isolates have different biofilm structures. In strains classified as not forming a biofilm, very few single cells adhered to the surface. Strains with a low capability to form a biofilm displayed adherent yeast cells which formed clusters (Fig. 2) (22). In addition, strains with biofilms consisting of monolayers of pseudohyphae do not display a mature biofilm structure (Fig. 2) (8). It will be interesting to investigate whether common regulators are nonfunctional in clinical isolates with similar biofilm structures.

Hyphal formation and biofilm development are tightly coupled in *C. albicans*, as the transcriptional regulator Efg1 is also involved in hyphal growth (36, 37, 38). Recently, a master circuit consisting of six transcriptional regulators (including Efg1) has been demonstrated to control biofilm formation by affecting the expression of more than 1,000 genes (19). The transcription factor Bcr1, a positive regulator of adherence that activates a number of cell wall protein and adhesin genes (12, 18, 19), also belongs to this network. Although BCR1 is not required for hyphal development, the expression of BCR1 is upregulated in hyphae during biofilm formation in order to activate expression of hyphal surface protein genes (32). Deletion of BCR1 in *C. parapsilosis* showed the requirement of Bcr1 for biofilm formation in this species (20). This finding indicated a similar function of Bcr1 in regulation of biofilm between *C. albicans* and *C. parapsilosis*. Surprisingly, in our study, we demonstrated that biofilm formation in *C. parapsilosis* is both BCR1 dependent and BCR1 independent. Only strains with low levels of biofilm formation and one isolate with monolayer pseudohyphal biofilm had a BCR1-dependent biofilm. Other strains with a high capacity to form a biofilm were BCR1 independent. However, although not significantly influencing the amount of biofilm formation, we observed an extended biofilm structure and increased cell filamentation in those strains. In addition, initial adhesion was BCR1 dependent when pregrown in a medium not stimulating biofilm formation (data not shown). These results...

**FIG 6** Effect of deletion of BCR1 on *C. parapsilosis* biofilm formation on an SE square. (A) Biofilm formation on an SE square after 48 h. (B) Initial biofilm formation of cells to an SE surface after 3 h. (C and D) Initial biofilm formation after 3 h of adhesion (C) and 48 h of biofilm formation (D) with strains precultured in YPD medium overnight. Assays were performed at least two times independently with three technical replicates.
Microscopy analysis of the effect of BCR1 on biofilm formation after 48 h. Scanning electron microscopy (A) and confocal laser scanning microscopy (B) of SMI 706, its bcr1 deletion mutant, and a complemented strain with BCR1-dependent biofilm formation. Scanning electron microscopy (C) and confocal laser scanning microscopy (D) of SMI 588, its bcr1 deletion mutant, and complemented strain with BCR1-independent biofilm.
indicate that BCR1 can also have a role in the temporal development of biofilm formation.

It is puzzling that deletion of BCR1 in strains with BCR1-independent biofilm formation resulted in robust pseudohyphal production and increased filamentation. A recent report observed a similar differential effect of Bcr1 in C. albicans. Deletion of BCR1 resulted in filamentation in cells from an opaque, but not white, colony (40). This finding indicates that BCR1 is a specific repressor of filamentation in a certain strain background. Indeed, besides BCR1, five additional transcription factors (TEC1, EFG1, NDT80, ROB1, and BRG1) build up the regulatory network controlling biofilm formation in C. albicans (19). In C. parapsilosis, two transcription factors (BCR1 and EFG1) are known to regulate biofilm formation (20, 21, 39). Whether any of the above-mentioned factors determines the role of Bcr1 in different strain backgrounds needs to be investigated.

Environmental factors, such as medium (41), surface material (9), surface pretreatment (9), and medium renewal (42), influence biofilm formation in vitro. In our study, SE was used to assess biofilm formation, since it most closely resembles clinical material related to catheter infections. Biofilm formation of C. parapsilosis on this substrate was highly variable among the 33 tested isolates. In contrast, biofilm on Thermanox coverslips and polystyrene was less pronounced, in agreement with previous reports (29) that SE facilitates biofilm formation more than other materials. Hydrophobicity and roughness are two characteristics of surface material which are positively correlated with biofilm formation. Yoshijima et al. (10) reported decreased yeast cell adhesion to an acrylic surface coated with hydrophilic materials. The study by Frade et al. (9) also showed that C. albicans biofilms displayed less metabolic activity on hydrophilic surfaces than on hydrophobic surfaces.

Phenotypic resistance of Candida biofilms to antifungal agents is a common phenomenon, especially resistance to fluconazole (41, 43). In agreement with results in the literature, we show here that strains forming a complex spider-like biofilm displayed significantly enhanced resistance to fluconazole (128 μg/ml), whereas the planktonic counterparts were susceptible. In contrast, all biofilms remain susceptible to amphotericin B and caspofungin, which is in agreement with other studies (41, 44, 45). However, although effective against biofilm cells, use of amphotericin B has drawbacks due to its toxicity against mam-

![FIG 8 Expression of BCR1 in C. parapsilosis strains with different biofilm capabilities. Relative expression of Bcr1 was arbitrarily set to 1 for the SMI 449 wild type. qRT-PCRs of at least two independent RNA isolations were analyzed with three technical replicates each.](image)

![FIG 9 Effect of BCR1 deletion on colony and cell morphology. (Upper) C. parapsilosis SMI 588, its bcr1 deletion mutant, and a complemented strain were cultured on YPD agar at 30°C, and the colony morphology was imaged after 5 days. (Lower) Cells were then resuspended in 10μg/ml Calcofluor white and observed under a fluorescence microscope at ×100 magnification.](image)

**TABLE 2 Effect of BCR1 deletion on colony morphology in C. parapsilosis isolates**

| Isolate no. | Biofilm-forming capability | Colony morphology |
|-------------|----------------------------|-------------------|
|             | Wild type                  | bcr1Δ/Δ | pBCR1  |
| SMI 449     | Low                        | Smooth | Smooth | Smooth |
| SMI 706     | Low                        | Rough/faint | Smooth | Rough/faint |
| SMI 424     | High (filament)            | Rough/faint | Rough/faint | NA* |
| SMI 526     | High (filament)            | Smooth | Crepe | NA |
| SMI 588     | High (spider)              | Rough/faint | Crepe | Rough/faint |
| SMI 596     | High (spider)              | Rough | Crepe | Rough |
| SMI 828     | High (spider)              | Rough/faint | Rough/faint | Rough/faint |

*NA, not applicable.*
malian cells and is a cause of kidney damage in patients (46). Caspofungin is a representative of the echinocandin class of antifungal agents, which targets proteins involved in β-1,3 glucan synthesis. The effectiveness of caspofungin against Candida biofilms elevates this drug as a proper candidate for fungal biofilm treatment (43).

Recently, it was shown that BCR1 not only affects biofilm formation but also increases susceptibility to antimicrobial peptides in C. albicans (35). We identify here three novel physiological traits of C. parapsilosis regulated by BCR1, namely, colony morphology, asparatyl proteinase secretion, and antimicrobial susceptibility but also increased susceptibility to antimicrobial peptides (SAP). The function of BCR1 in C. parapsilosis with respect to susceptibility against antimicrobial peptides (protamine, RP-1, and LL-37) is in agreement with a recent study (35) where bcr1Δ/Δ mutants of C. albicans were shown to display an increased susceptibility to the antimicrobial peptides rational peptide 1 (RP-1), human β-defensin 2 (hBD-2), and protamine but not LL-37. The mechanism of action of these antimicrobial peptides in fungi is still unknown. Secretd aspartyl proteases, however, are not involved in susceptibility to antimicrobial peptides, as their secretion and resistance to antimicrobial peptides is inversely regulated by BCR1.

In conclusion, we demonstrated in this study that biofilm formation in C. parapsilosis can be dependent and independent of BCR1. BCR1 has, however, additional physiological roles in colony and cell morphology switch, asparatyl proteinase secretion, and susceptibility against antimicrobial peptides.

ACKNOWLEDGMENTS

We are grateful to Victor Fernandez for the idea to initiate the study. We thank Geraldine Butler for providing C. albicans strains and Monica Lindh for assistance with antimicrobial peptide experiments.

S. Pannanusorn received a personal scholarship from Thammasat University, Thailand, and the Karolinska Institutet/Department of Microbiology, Tumor and Cell Biology. Travel funding of S. Pannanusorn to the University of Würzburg was supported by The Swedish Society of Medicine (SLS).

REFERENCES

1. Wisplinghoff H, Bischoff T, Tallent M, Seiffert H, Wenzel RP, Edmond MB. 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin. Infect. Dis. 39:309–317. http://dx.doi.org/10.1086/421946.
2. Tortorano AM, Peman J, Bernhardt H, Klingspor L, Kibbler CC, Faure O, Biraghi E, Canton E, Zimmermann K, Seaton S, Grillot R, ECMM Working Group on Candidaemia. 2004. Epidemiology of candidaemia in Europe: results of 28-month European Confederation of Medical Mycology (ECMM) hospital-based surveillance study. Eur. J. Clin. Microbiol. Infect. Dis. 23:317–322. http://dx.doi.org/10.1007/s10096-004-1103-y.
3. Klingspor L, Tornqvist E, Johansson A, Petrinß B, Forsum U, Hedin G. 2008. A prospective epidemiological survey of candidaemia in Sweden. Scand. J. Infect. Dis. 36:52–55. http://dx.doi.org/10.1080/00365540.310017447.
4. Maganti H, Yamamura D, Xu J. 2011. Prevalent nosocomial clusters among causative agents for candidaemia in Hamilton, Canada. Med. Mycol. 49:530–538. http://dx.doi.org/10.3109/13693786.2010.547880.
5. Spiliopoulos A, Dimitriou G, Jelastopulu E, Giannakopoulos I, Anastassiou E, Christofidou M. 2012. Neonatal intensive care unit candidaemia: epidemiology, risk factors, outcome, and critical review of published case series. Mycopathologia 412:19–22. http://dx.doi.org/10.1007/s11046-011-9498-3.
6. Trofa D, Gacsor A, Nosanchuk J. 2008. Candida parapsilosis, an emerging fungal pathogen. Clin. Microbiol. Rev. 21:606–625. http://dx.doi.org/10.1128/CMR.00013-08.
7. Pfaffer MA, Messer SA, Moet GJ, Jones RN, Castanheira M. 2011.

FIG 10 Phenotypes affected by BCR1. (A) Effect of BCR1 deletion on asparatyl proteinase secretion. Proteinases secreted by C. parapsilosis were analyzed using the spot assay on YCB agar containing 0.2% BSA. The plate was incubated at 37°C for 5 days. Clear zones of proteolytic activity were observed after staining with 0.1% amido black and destaining. (B) Effect of BCR1 deletion on antimicrobial peptide susceptibility of C. parapsilosis. The susceptibility to LL-37, protamine, and RP-1 was investigated using the inhibition zone assay. The zones of inhibition were photographed after incubation for 24 h at 30°C.
Candida bloodstream infections: comparison of species distribution and resistance to echinocandin and azole antifungal agents in intensive care unit (ICU) and non-ICU settings in the SENTRY Antimicrobial Surveillance Program (2008–2009). Int. J. Antimicrob. Agents. 38:85–9. http://dx.doi.org/10.1016/j.ijantimicag.2011.02.016.

Finkel JS, Mitchell AP. 2011. Gene control of Candida albicans biofilm development. Nat. Rev. Microbiol. 9:109–118. http://dx.doi.org/10.1038/nrmicro2475.

Frade JP, Arrhington-Skagg BA. 2011. Effect of serum and surface characteristic on Candida albicans biofilm formation. Mycoses 54:e154–e162. http://dx.doi.org/10.1111/j.1365-3141.2010.01862.x.

Yoshijima Y, Murakami K, Kayama S, Liu D, Hirota K, Ichikawa T, Miyake Y. 2010. Effect of substrate surface hydrophobicity on the adherence of yeast and hyphal Candida. Mycoses 53:221–226. http://dx.doi.org/10.1111/j.1365-3141.2009.01694.x.

Nobile CJ, Schneider HA, Nett JE, Sheppard DC, Filler SG, Andes DR, Mitchell AP. 2008. Complementary adhesion function in C. albicans biofilm formation. Curr. Biol. 18:1017–1024. http://dx.doi.org/10.1016/j.cub.2008.06.034.

Nobile CJ, Andes DR, Nett JE, Smith FJ, Jr, Yue F, Phan Q-T, Edwards MC, Uppuluri P, Thomas D, Cleary I, Henriques M, Lopez-Monp抬t P, Frade JP, Arthington-Skaggs BA. 2012. Identification of gene duplication and the role of secreted aspartyl protease 1 in Candida albicans biofilm virulence. J. Infect. Dis. 205:923–933. http://dx.doi.org/10.1093/infdis/jir783.

Schmitt ME, Brown TA, Trumpower BL. 1990. A rapid and simple method for preparation of RNA from Saccharomyces cerevisiae. Nucleic Acids Res. 18:3091–3092. http://dx.doi.org/10.1093/nar/18.10.3091.

Rossignol T, Logue ME, Reynolds K, Grenon M, Lowndes NF, Butler G. 2007. Transcriptional response of Candida parapsilosis following exposure to echinocandins, anidulafungin, and amphotericin B. Antimicrob. Agents Chemother. 51:2304–2312. http://dx.doi.org/10.1128/AAC.01438-06.

Brilliowska-Dabrowska A, Schötz T, Pannanussorn S, Lönbro N, Bernhöff I, Bonnelod J, Hägglöf J, Wistedt A, Fernandez V, Arendrup MC. 2009. A nosocomial outbreak of Candida parapsilosis in southern Sweden verified by genotyping. Scand. J. Infect. Dis. 41:135–142. http://dx.doi.org/10.1080/00365540802358301.

Kuhn DM, Chandra J, Mukherjee PK, Ghannoum MA. 2002. Comparison of biofilms formed by Candida albicans and Candida parapsilosis on bioprothetic surfaces. Infect. Immun. 70:878–888. http://dx.doi.org/10.1128/IAI.70.2.878-888.2002.

Tobudic S, Lassnigg A, Kratzer C, Graninger W, Presterl E. 2010. Antifungal activity of amphotericin B, caspofungin and posaconazole on Candida albicans biofilms in intermediate and mature development phases. Mycoses 53:208–214. http://dx.doi.org/10.1111/j.1365-3141.2009.01690.x.

García-Sánchez S, Aubert S, Iqbal I, Janbon G, Ghigo J-M, d’Enfer C. 2004. Candida albicans biofilms: a developmental state associated with specific and stable gene expression patterns. Eukaryot. Cell. 3:536–545. http://dx.doi.org/10.1128/EC.3.2.536-545.2004.

Nobile CJ, Mitchell AP. 2005. Regulation of cell-surface genes and biofilm formation by the C. albicans transcription factor Bcr1p. Curr. Biol. 15:1150–1155. http://dx.doi.org/10.1016/j.cub.2005.05.047.

Laffey SF, Butler G. 2005. Phenotype switching affects biofilm formation by Candida albicans. Microbiology 151:1073–1081. http://dx.doi.org/10.1099/mi.0.27739-0.

Naglik JR, Challacombe SJ, Hube B. 2003. Candida albicans secreted aspartyl proteinases in virulence and pathogenesis. Microbiol. Mol. Biol. Rev. 67:400–428. http://dx.doi.org/10.1128/MMBR.67.3.400-428.2003.

Jung S-I, Finkel JS, Solis NV, Chaili S, Mitchell AP, Yeaman MR, Filler SG. 2013. Bcr1 functions downstream of Sod1 to mediate antifungal peptide resistance in Candida albicans. Eukaryot. Cell. 12:411–419. http://dx.doi.org/10.1128/EC.00285-12.

Lif F, Palecek SP. 2003. EAP1, a Candida albicans gene involved in binding to human epithelial cells. Eukaryot. Cell. 2:1266–1273. http://dx.doi.org/10.1128/EC.2.6.1266-1273.2003.

Lewis RE, Lo H-J, Raad II, Kontoyiannis DP. 2002. Lack of catheter infection by the efg1/efg1 cph1/cph1 double-null mutant, a Candida albicans strain that is defective in filamentous growth. Antimicrob. Agents Chemother. 46:1153–1155. http://dx.doi.org/10.1128/AAC.46.4.1153-1155.2002.

Li F, Svarovsky MJ, Karlsson AJ, Wagner JP, Marchillo K, Oshel P, Andes D, Palecek SP. 2007. Eap1p, an adhesin that mediates Candida albicans biofilm formation in vitro and in vivo. Eukaryot. Cell. 6:931–939. http://dx.doi.org/10.1128/EC.00049-07.

Connolly LA, Riccombeni A, Grozer Z, Holland LM, Lynch DB, Andes DR, Gacser A, Butler G. 2013. The APSES transcription factor Efg1 is a global regulator that controls morphogenesis and biofilm formation in Candida parapsilosis. Mol. Microbiol. 90:36–53. http://dx.doi.org/10.1111/mmi.12345.

Guo G, Xie J, Tao L, Nobile CJ, Sun Y, Cao C, Tong Y, Huang G. 2013. Bcr1 plays a central role in the regulation of opaque cell filamentation in Candida albicans. Curr. Microbiol. 66:1310–1319. http://dx.doi.org/10.1007/s00284-013-0607-0.

Sasse C, Schillig R, Dieroff F, Weyler M, Schneider S, Mogavero S, Rogers PD, Morschhäuser J. 2011. The transcription factor Nd100 does not contribute to biofilm – Efg1, -Tak1, and -Upc2-mediated filamentation resistance in Candida albicans. PLoS One 6:e28515. http://dx.doi.org/10.1371/journal.pone.0028515.

Horvath P, Nosanchuk JD, Hamari Z, Vagvolgyi G, Gacsar A. 2012. The identification of gene duplication and the role of secreted aspartyl protease I in Candida parapsilosis virulence. J. Infect. Dis. 205:923–933. http://dx.doi.org/10.1093/infdis/jir783.

Kuhn DM, George T, Chandra J, Mukherjee PK, Ghannoum MA. 2002. Antifungal susceptibility of Candida biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. Antimicrob. Agents Chemother.
Role of BCR1 in Clinical Isolates of C. parapsilosis

45. Melo AS, Bizerra FC, Freymüller E, Arthington-Skaggs BA, Colombo AL. 2011. Biofilm production and evaluation of antifungal susceptibility amongst clinical Candida spp. isolates, including strains of the Candida parapsilosis complex. Med. Mycol. 49:253–262. http://dx.doi.org/10.3109/13693786.2010.530032.

46. Odds FC, Brown AJP, Gow NAR. 2003. Antifungal agents: mechanisms of action. Trends Microbiol. 11:272–279. http://dx.doi.org/10.1016/S0966-842X(03)00117-3.

47. Lott T, Kuykendall R, Welbel S, Pramanik A, Lasker B. 1993. Genomic heterogeneity in the yeast Candida parapsilosis. Curr. Genet. 23:463–467. http://dx.doi.org/10.1007/BF00312635.

48. Enger L, Joly S, Pujol C, Simonson P, Pfaller M, Soll DR. 2001. Cloning and characterization of a complex DNA fingerprinting probe for Candida parapsilosis. J. Clin. Microbiol. 39:658–669. http://dx.doi.org/10.1128/JCM.39.2.658-669.2001.

49. Fanning S, Xu W, Solis N, Woolford CA, Filler SG, Mitchell AP. 2012. Divergent targets of Candida albicans biofilm regulator Bcr1 in vitro and in vivo. Eukaryot. Cell 11:896–904. http://dx.doi.org/10.1128/EC.00103-12.