Critical Role of Bcr1-Dependent Adhesins in C. albicans Biofilm Formation In Vitro and In Vivo

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The fungal pathogen Candida albicans is frequently associated with catheter-based infections because of its ability to form resilient biofilms. Prior studies have shown that the transcription factor Bcr1 governs biofilm formation in an in vitro catheter model. However, the mechanistic role of the Bcr1 pathway and its relationship to biofilm formation in vivo are unknown. Our studies of biofilm formation in vitro indicate that the surface protein Als3, a known adhesin, is a key target under Bcr1 control. We show that an als3/als3 mutant is biofilm-defective in vitro, and that ALS3 overexpression rescues the biofilm defect of the bcr1/bcr1 mutant. We extend these findings with an in vivo venous catheter model. The bcr1/bcr1 mutant is unable to populate the catheter surface, though its virulence suggests that it has no growth defect in vivo. ALS3 overexpression rescues the bcr1/bcr1 biofilm defect in vivo, thus arguing that Als3 is a pivotal Bcr1 target in this setting. Surprisingly, the als3/als3 mutant forms a biofilm in vivo, and we suggest that additional Bcr1 targets compensate for the Als3 defect in vivo. Indeed, overexpression of Bcr1 targets ALS1, ECE1, and HWP1 partially restores biofilm formation in a bcr1/bcr1 mutant background in vitro, though these genes are not required for biofilm formation in vitro. Our findings demonstrate that the Bcr1 pathway functions in vivo to promote biofilm formation, and that Als3-mediated adherence is a fundamental property under Bcr1 control. Known adhesins Als1 and Hwp1 also contribute to biofilm formation, as does the novel protein Ece1.

Citation: Nobile CJ, Andes DR, Nett JE, Smith FJ Jr, Yue F, et al. (2006) Critical role of Bcr1-dependent adhesins in C. albicans biofilm formation in vitro and in vivo. PLoS Pathog 2(7): e63. DOI: 10.1371/journal.ppat.0020063

Introduction

Biofilms are microbial communities that are associated with solid surfaces. Most bacteria and fungi exist predominantly in such communities in nature, and they form the basis for numerous interactions that affect human health. Cells in a biofilm display phenotypes that are distinct from their free-living counterparts, including extreme resistance to many antimicrobial agents [1–4]. Their health impact is reflected in the fact that implanted medical devices, such as intravascular catheters, are major risk factors for bloodstream and deep tissue infection [5, 6]. These devices serve as substrates for biofilm development; the mass and intrinsic drug resistance of the biofilm limits efficacy of host defenses and antimicrobial therapy. These biofilm-based infections are estimated to cause about 50% of all nosocomial infections [5, 7].

The fungal pathogen Candida albicans is a major cause of device-associated infections [5, 8, 9]. It produces adherent biofilms on a variety of different surfaces in vitro [3, 4, 10, 11]. Biofilm formation begins with surface adherence of yeast-form cells, which grow to yield a basal layer. Basal layer cells include some hyphae, or long tubular chains of cells, which extend to yield an upper layer that is almost exclusively hyphae. As the biofilm matures, it produces an extracellular matrix containing predominantly carbohydrate and protein [1, 12, 13].

C. albicans Bcr1, a C2H2 zinc finger protein, has a significant role in biofilm formation: bcr1/bcr1 insertion and deletion mutants form only rudimentary biofilms on silicone catheter material in vitro [14]. Bcr1 is required for expression of several cell wall protein genes, and we have proposed that Bcr1 is a positive regulator of adherence. Many Bcr1 target genes had been identified initially as hyphal-specific genes, and BCR1 RNA accumulation depends upon the hyphal developmental activator Tec1 [14]. Bcr1 is not required for hyphal morphogenesis, and we believe that it acts downstream of Tec1 to activate the acquisition of hyphal adherence properties.

Biofilms are considerably more complex in vivo than in vitro. For example, in vivo, biofilms form on intravascular catheters under conditions of vascular flow, and are exposed to and incorporate many plasma constituents. The complex-
Synopsis

The formation of biofilms (surface-attached microbial communities) on implanted medical devices such as catheters is a major cause of fungal and bacterial infections. Prior studies of the fungal pathogen *Candida albicans* have shown that the regulator Bcr1 is required for biofilm formation in vitro, but the mechanism through which it promotes biofilm formation and its significance for biofilm formation in vivo was uncertain. The authors demonstrate that Bcr1 is required for biofilm formation in vivo in a rat model of catheter-based infection. Manipulation of Bcr1 target genes through mutation and gene overexpression shows that the known surface adhesins Als3 has a pivotal role in biofilm formation and that adhesins Als1 and Hwp1 also contribute to biofilm formation. The results thus indicate that adherence is the key property regulated by Bcr1 and highlight a group of adhesins as potential therapeutic targets.

Key Role of Bcr1 Target Gene ALS3 in Biofilm Formation In Vitro

To test the roles of Bcr1 target genes in biofilm formation, we carried out biofilm formation assays with mutants defective in each gene. We observed no significant biofilm defect in *HYR1* ($p = 0.463$), *ECHEL1* ($p = 0.850$), *CHS2/CHT2* ($p = 0.909$), or *RHT5/RHT5* ($p = 0.323$) mutant strains versus the reference strain (Figure 2, Strain Set B; Figure 4). The *ALS1/ALS1* and *HWP1/HWP1* mutants also produced substantial biofilms (Figure 4), although the biofilms often sloughed off the substrate. Biofilm biomass determinations further indicated that the *HWP1/HWP1* mutant has a partial biofilm defect compared to the reference strain (Figure 2, Strain Set B; $p = 0.092$). In contrast to the *ALS1/ALS1* and *HWP1/HWP1* strains, the *ALS3/ALS3* mutant displayed a severe defect in biofilm formation compared to the reference strain (Figure 2, Strain Set B; $p = 0.005$), and introduction of a single wild-type *ALS3* allele rescued the defect substantially (Figure 2, Strain Set B; $p = 0.005$). Confocal scanning laser microscopy (CSLM) imaging revealed that the *ALS3/ALS3* mutant formed a rudimentary biofilm of 20 μm in depth, while the wild-type and *ALS3/ALS3 + pALS3* complemented strains produced biofilms of over 200 μm in depth (Figure 5). CSLM depth images showed that the rudimentary *ALS3/ALS3* mutant biofilm was comprised mainly of yeast cells, with few hyphae, whereas the biofilms of the wild-type and *ALS3/ALS3 + pALS3* complemented strains...
included abundant hyphae (Figure 5). It should be noted that the als3/als3 mutant is not defective in hyphal formation as it forms normal hyphae when assayed under hyphal inducing conditions (Figure 5). Hyphae are also apparent among the cells in the surrounding medium of an als3/als3 mutant biofilm (unpublished data). These findings argue that Als3 has a major role in biofilm formation and suggest that reduced expression of ALS3 in the bcr1/bcr1 mutant may account for its biofilm defect.

If reduced expression of ALS3 is the cause of the bcr1/bcr1 mutant biofilm defect, then increased expression of ALS3 in a bcr1/bcr1 mutant background should promote biofilm formation. To test this prediction, we introduced the TEF1 promoter adjacent to the native ALS3 coding region to create a TEF1-ALS3 allele, permitting Bcr1-independent ALS3 expression. RT-PCR measurement of ALS3 RNA levels confirmed that the TEF1-ALS3 allele permits expression of ALS3 in both BCR1/BCR1 and bcr1/bcr1 backgrounds (Figure 6). In the wild-type reference strain background, TEF1-ALS3 had no obvious effect on biofilm formation (Figure 6, top row). In the bcr1/bcr1 mutant background, TEF1-ALS3 improved biofilm formation substantially (Figure 2; Figure 6, top row; \( p = 0.002 \)). These observations indicate that increased ALS3 expression in the bcr1/bcr1 mutant promotes significant biofilm formation ability.

We used CSLM imaging to examine the structure of biofilms that resulted from increased ALS3 expression. The TEF1-ALS3 allele did not alter biofilm structure in the otherwise wild-type background (Figure 6, CSLM depth and side views); biofilm depth was about 400 \( \mu \)m; little staining occurred in the basal region; and hyphal staining was prominent. The bcr1/bcr1 strain produced a thin rudimentary biofilm comprised largely of yeast form cells, as expected [14]. The bcr1/bcr1 TEF1-ALS3 strain produced a substantial biofilm that included a basal poorly stained region (Figure 6), similar in appearance to those of the wild-type strain (Figure 6) and complemented bcr1/bcr1 mutant [14]. Thus, increased ALS3 expression permits at least partial rescue of the bcr1/bcr1 mutant defect in biofilm formation.

**Bcr1 Function in Biofilm Formation In Vivo**

In order to determine whether Bcr1 may have a role in biofilm formation in vivo, we turned to a rat venous catheter model [18]. Implanted catheters were allowed to stabilize for 24 h and were then inoculated with wild-type, bcr1/bcr1 mutant, or bcr1/bcr1 + pBCR1 complemented strains. Biofilm formation was visualized after 12, 24, and 48 h by scanning...
Figure 2. Biofilm Dry Mass Determinations

Biofilm dry mass determinations were made in quadruplicate after 60 h growth under standard biofilm conditions, as detailed in Materials and Methods. Reference strains DAY185 (shown) and CAI4-URA3 (not shown) gave similar results. Strains are grouped for convenience of comparison. Strain Set A contains CJN896 (tec/tec), CJN1052 (tec1/tec1 + TEF1), CJN1035 (tec1/tec1 + TEF1-BCR1), CJN1023 (tec1/tec1 + pTEC1), CJN702 (bcr1/bcr1), CJN1060 (bcr1/bcr1 + TEF1), CJN1011 (bcr1/bcr1 + TEF1-BCR1), CJN698 (bcr1/bcr1 + pBCR1), respectively. Strain Set B contains FJS2 (hyr1/hyr1), FJS6 (ece1/ece1), FJS5 (cht2/cht2), FJS8 (rbs5/rbs5), CAYC2YF1U (als1/als1), CAH7-1A1E2 (hwp1/hwp1), CAYF178U (als3/als3), CAQTP178U (als3/als3 + pALS3), respectively. Strain Set C contains CJN1153 (bcr1/bcr1 + TEF1-ALS3), CJN1144 (bcr1/bcr1 + TEF1-ALS1), CJN1288 (bcr1/bcr1 + TEF1-ECE1), CJN1222 (bcr1/bcr1 + TEF1-HWP1), CJN1281 (bcr1/bcr1 + TEF1-CHT2), CJN1259 (bcr1/bcr1 + TEF1-HYR1), CJN1276 (bcr1/bcr1 + TEF1-RBT5), respectively.

DOI: 10.1371/journal.ppat.0020063.g002

electron microscopy of the intraluminal catheter surface (Figure 7). The wild-type and bcr1/bcr1 + pBCR1 complemented strains initiated biofilm formation by 12 h and yielded extensive adherent populations by 24 h (Figure 7A, 7B, 7G, and 7H). Both strains produced mature biofilms by 48 h that included abundant matrix material (Figure 7C and 7I), as previously reported for strain K1 [18]. In contrast, the bcr1/bcr1 mutant yielded few adherent cells at 12 and 24 h (Figure 7D and 7E), and the catheter surface was devoid of biofilm material after 48 h (Figure 7F). Despite the dramatic differences in biofilm formation ability, the three strains grew comparably in a mouse disseminated infection model; median mouse survival time was 13 d after inoculation with the wild-type strain and 10 d after inoculation with either the bcr1/bcr1 mutant or bcr1/bcr1 + pBCR1 complemented strains.

Based on this evidence, Bcr1 is not required for growth in vivo under non–biofilm-forming conditions but is required for biofilm formation in vivo.

Als3 Function in Biofilm Formation In Vivo

Our observations above indicate that Als3 is a key mediator of Bcr1-dependent biofilm formation in vitro. To verify that these findings extend to in vivo biofilm formation, we compared als3/als3 mutant and als3/als3 + pALS3 complemented strains in the rat venous catheter model. Both strains formed extensive biofilms within 24 h (Figure 8A and 8B). Therefore, Als3 is not absolutely required for biofilm formation in vivo.

To determine whether Als3 may contribute to biofilm formation in vivo, we tested the ability of the TEF1-ALS3 expression construct to rescue the bcr1/bcr1 mutant biofilm defect. The bcr1/bcr1 TEF1-ALS3 strain produced an extensive biofilm containing both cells and matrix material (Figure 8C). This biofilm, formed after 24 h, was similar in overall appearance to that formed by the BCR1/BCR1 control strains (Figures 7B and 8B). TEF1-ALS3 expression thus rescues biofilm formation in a bcr1/bcr1 background (compare Figures 7E and 8C). These findings support the model that ALS3 is a critical Bcr1 target gene that functions in biofilm formation in vivo.

Overexpression Assays of Bcr1 Target Gene Function In Vitro

Our in vivo assays suggest that Als3 may be one of several Bcr1 targets that contribute to biofilm formation. The analysis of insertion and deletion mutant strains above pointed toward Als1 and Hwp1 as additional candidate functional targets, although their biofilm defects were mild: biofilm biomass was reduced only slightly (Figure 2), and CSLM visualization revealed no qualitative defects (unpublished data). Thus, we turned to an alternative functional analysis strategy, gene overexpression, which has recently been applied with considerable success on a genome-wide scale in Saccharomyces cerevisiae [20]. Gene overexpression is particularly useful in identifying functions among partially redundant genes, the situation that we postulate to exist here.

To determine if increased expression of Bcr1-activated target genes, other than ALS3, may rescue the biofilm defect of the bcr1/bcr1 mutant, we created genomic fusions of the TEF1 promoter to the CHT2, HYR1, RBT5, ALS1, HWP1, and ECE1 coding regions. The TEF1-ALS1, TEF1-HWP1, and TEF1-ECE1 alleles improved biofilm formation ability considerably (p < 0.004 for all comparisons to bcr1/bcr1), although not to the extent of TEF1-ALS3 (Figure 2, Strain Set C; p < 0.006 for
all comparisons to \( bcr1/bcr1 \) TEF1-ALS3). These same TEF1 promoter fusion alleles did not augment biofilm formation in the \( BCR1/BCR1 \) background (unpublished data). These results indicate that Als1, Hwp1, and Ece1 may act in addition to Als3 to contribute to biofilm formation.

**Discussion**

We have recently taken a genetic approach to elucidate the mechanistic basis of *C. albicans* biofilm formation [14, 15]. A central issue is how in vitro biofilm models are related to biofilm growth in vivo and, thus, to disease. Here we have shown that the transcription factor Bcr1 is required in vivo, as it is in vitro, for biofilm formation. One key target gene under Bcr1 control is ALS3, as demonstrated by the rescue of biofilm formation through increased ALS3 expression in vitro and in vivo. These results argue that Als3-mediated adherence is a key factor in formation of biofilms in vitro and in vivo. However, absence of Als3 causes a biofilm defect only in vitro and not in vivo. One implication from this result is that Bcr1 activates additional biofilm adhesin genes. In support of this model, we find that overexpression of three additional Bcr1 target genes partially restores biofilm formation ability in vitro to a \( bcr1/bcr1 \) mutant. Our findings are summarized in Figure 9. Clearly, the interplay of in vitro and in vivo analyses holds great promise for defining biofilm regulatory mechanisms.

**Relationship of Bcr1 and Hyphal Gene Expression**

Our studies here solidify the concept that Bcr1 relays a signal within the hyphal developmental program because an increase in \( BCR1 \) expression leads to increased expression of the hyphal-specific genes HYR1, HWP1, and ALS3 in a hyphal-defective \( tec1/tec1 \) mutant. However, we find that some Bcr1-dependent genes are expressed substantially in the \( tec1/tec1 \) strain, including RBT5, ECE1, and ALS1, despite the reduced expression of \( BCR1 \). Two simple explanations can account for this apparent paradox. One possibility is that the 4-fold reduced level of Bcr1 in the \( tec1/tec1 \) mutant is sufficient to activate a subset of target genes. These genes may have the highest-affinity Bcr1 binding sites, or their promoter regions may include binding sites for additional transcription factors that interact cooperatively with Bcr1. A second possibility is that some Bcr1 target genes are subject to a compensatory regulatory mechanism in the \( tec1/tec1 \) background. The latter explanation seems particularly plausible for RBT5, which responds to numerous genetic and environmental regulatory

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**Figure 3. Effect of Increased \( BCR1 \) Expression on Target Gene RNA Levels**

RNA prepared from mid-log phase Spider medium cultures was used to prepare Northern blots or in RT-PCR assays, as indicated. Northern blots were probed for the transcripts indicated along the left side, and PhosphorImager exposures are shown. RT-PCR assays for ALS1, ALS3, and TEF1 were conducted on serial 2-fold dilutions of cDNA preparations and fractionated on agarose gels; only the last two dilutions are shown. TEF1 transcript levels were used as an expression control. Strains included DAY185 (reference strain) (sample 1), CJN1011 (\( bcr1/bcr1 + TEF1-BCR1 \)) (sample 2), CJN1015 (reference strain + TEF1) (sample 3), CJN1035 (\( tec1/tec1 + TEF1-BCR1 \)) (sample 4), CJN1039 (reference strain + TEF1-BCR1) (sample 5), CJN1052 (\( tec1/tec1 + TEF1 \)) (sample 6), and CJN1060 (\( bcr1/bcr1 + TEF1 \)) (sample 7).

DOI: 10.1371/journal.ppat.0020063.g003
signals [21–23]. Identification of the Bcr1 binding site will help to distinguish between these explanations.

One unexpected observation is that \textit{CHT2} expression is both Bcr1 and Tec1 dependent. \textit{CHT2}, which specifies a cell wall chitinase homolog, is expressed at higher levels in yeast cells than hyphal cells under many growth conditions [24, 25]. However, \textit{CHT2} is not exclusively a yeast phase-specific gene. For example, it was found to be coregulated with numerous hyphal-specific genes in a study of pH-regulated gene expression [26], and the \textit{CHT2} transcript has been detected previously in cells induced to form hyphae in Spider medium or serum [21]. Our results indicate that Bcr1 and Tec1 target genes are not restricted to hyphal-specific genes.

Control of Adherence by Bcr1 during Biofilm Formation In Vitro

The proposal that Bcr1 is a positive regulator of biofilm adherence stems from two prior observations. First, Bcr1 is required for biofilm formation, a process that depends upon both cell-cell and cell-substrate adherence. Second, numerous Bcr1-dependent genes encode proteins that contribute to cell wall or cell surface structure. The in vitro studies reported here include three lines of evidence in support of this proposal. First, expression of Bcr1 in a \textit{tec1/tec1} mutant promotes substantial adherence to a silicone substrate. Second, a deletion of one Bcr1-dependent adhesin gene, \textit{ALS3}, causes a biofilm formation defect similar to that of the \textit{bcr1/bcr1} mutant. Third, the \textit{bcr1/bcr1} biofilm formation defect is fully rescued through increased expression of \textit{Als3} and partially rescued through increased expression of two other known adhesins, Als1 and Hwp1. Our results thus indicate that the adhesin expression defect is a major cause of the \textit{bcr1/bcr1} mutant biofilm formation defect.

The \textit{tec1/tec1} mutant has a severe biofilm defect: it grows under our in vitro cultivation conditions as a suspension of yeast cells. Introduction of \textit{TEF1-BCR1} alters that mutant phenotype by promoting growth primarily on the surface of the silicone substrate. The biofilm so formed is unstable in that it disperses into clumps of cells during manipulation, and its biomass is 3-fold less than that of the wild-type and complemented mutant strains. Thus, expression of Bcr1 is not sufficient to promote extensive biofilm formation by yeast cells. However, increased adherence of the \textit{tec1/tec1}
Als3 belongs to a large protein family with consensus hyphal adhesin that serves as a substrate for C. albicans genes disrupted with the Ura-blaster method [34, 35]. However, the ece1ece1 mutant has no apparent phenotypic defect [35]. The idea that Ece1 functions in adhesion is suggested by our observation that its over-expression restores biofilm formation to a ber1ber1 mutant. ECE1, like HWP1, is induced by mating pheromone [33], another possible connection between Ece1 and adherence. Ece1 does not resemble an adhesin: it is comprised of novel 34-residue repeats that surround a possible transmembrane domain [35]. Although its mechanism of action is uncertain, an interesting possibility is that Ece1 promotes surface exposure of adhesins.

Genetic Control of Biofilm Formation In Vivo

Biofilm formation in vivo is considerably more complex than in vitro and involves dynamic interactions with many host proteins, cells, and environmental factors. These differences raise the question of whether the major genetic factors operative in vitro play a commensurate role in vivo. We have addressed this issue for two gene products: Bcr1 and Als3. Although the experimental outcomes were different in detail, they argue that both proteins have significant roles in vivo.

The significance of Bcr1 is clearest: it is required in vivo for biofilm formation but not for growth. The fact that the mutant leaves catheter surfaces essentially clear of material suggests that there is a defect in early events of biofilm formation in vivo, much as observed in vitro. The defects under the two circumstances, however, are slightly different: a thin layer of ber1ber1 mutant cells is associated with the substrate transiently in vivo but stably in vitro. It is possible that the few substrate-bound cells that appear early in vivo may be destroyed later by host defenses. An alternative possibility is that larger cell masses are dislodged efficiently by blood flow if their adherence is compromised by the ber1 defect. In either case, it is clear that Bcr1 governs a mechanism that contributes to biofilm formation in vivo.

The potency of TEF1-ALS3 as a suppressor of the ber1ber1 defect argues that Als3 also has a critical role in biofilm formation in vivo. How can that observation be reconciled...
with the fact that an als3Δals3 null mutant has no biofilm defect in vivo? One simple model is that additional adhesins can partially compensate for the absence of Als3 in vivo but not in vitro. Our overexpression studies implicate Als1 and Hwp1 as candidate compensatory adhesins, in keeping with this model. The distinction between the in vivo and in vitro situations may reflect a higher level of expression of the compensatory adhesins in vivo than in vitro. A second possibility is that host constituents, for example serum components, may contribute to adherence. Thus, the same low level of surface adhesin activity may support biofilm formation in vivo but not in vitro.

The restoration of biofilm formation through ALS3 overexpression in the bcr1Δbcr1 mutant both in vitro and in vivo indicates that Bcr1 governs one main function relevant for biofilm formation: adherence. Although transcription factor mutants are useful for definition of functionally related genes, the mechanistic basis for their phenotypic defects can be complex because of the extent of their gene expression defects. Moreover, functional overlap among targets can obscure loss-of-function target gene mutant phenotypes. Our results here illustrate the utility of gene overexpression for identification of critical target genes that govern a complex process.

Figure 6. Overexpression of ALS3 in the bcr1Δbcr1 Mutant Restores Substantial Biofilm Formation In Vitro

Biofilms were grown under standard conditions and stained with concanavalin A conjugate for CSLM visualization. The top panels show the visual appearance. The next set of panels show depth views, in which blue color represents cells closest to the silicone and red color represents cells farthest from the silicone. The next set of panels show side views. For the depth views of reference strain DAY185 (BCR1/BCR1), blue = 0 μm and red = 600 μm; CJN1149 (BCR1/BCR1 + TEF1-ALS3), blue = 0 μm and red = 500 μm; CJN702 (bcr1/bcr1), blue = 0 μm and red = 80 μm; CJN1153 (bcr1/bcr1 + TEF1-ALS3), blue = 0 μm and red = 180 μm. For the side views, the scale bars represent 50 μm for DAY185 (BCR1/BCR1), CJN1149 (BCR1/BCR1 + TEF1-ALS3), and CJN1153 (bcr1/bcr1 + TEF1-ALS3); and 20 μm for CJN702 (bcr1/bcr1). The next set of panels show RT-PCR analysis of ALS3 expression of the indicated strains with successive 2-fold dilutions of cDNA from left to right. The bottom panels show RT-PCR of control TEF1 transcript levels.

DOI: 10.1371/journal.ppat.0020063.g006
Materials and Methods

**Media.** *C. albicans* strains were grown at 30 °C in either YPD (2% Bacto Peptone, 2% dextrose, 1% yeast extract) for Ura+ strains or in YPD+uri (2% Bacto Peptone, 2% dextrose, 1% yeast extract, and 80 μg/ml uridine) for Ura- strains. *C. albicans* transformants were selected for on synthetic medium (2% dextrose, 6.7% YNB with ammonium sulfate, and auxotrophic supplements) or on YPD+clonNAT (2% Bacto Peptone, 2% dextrose, 1% yeast extract, and 400 μg/ml clonNAT [WERNER BioAgents, Jena, Germany]) for Nat+ strains. For biodil growth, strains were grown at 37 °C in Spider medium [36]. Assays for hyphal induction of the *tec1*/*tec1* mutant (+ vector) (CJN1052), the *tec1*/*tec1* mutant overexpressing BCR1 (CJN1035), the reference strain (+ vector) (CJN1015), the reference strain (DAY185), the *als3*/*als3* mutant (CAYF178U), and the *als3*/*als3* +pALS3 complemented strain (CAQTP178U) were also done at 37 °C in Spider medium.

**Plasmid and *C. albicans* strain construction.** All strains used in this study are listed in Table 1. All strains are derived from BWP17 (*ura3Δ::kmm434/ura3Δ::kmm434 arg4::hisG/arg4::hisG his1::hisG/ his1::hisG*) [37] except for the following CA14 derivatives [34]: CA14-URA3 [38], CAYC2YF1U, the *als1/als1* mutant strain [39], and CAH7-1A1E2 [28], the *hap1/leu1* mutant strain. Construction of the *bcr1*/*bcr1* insertion mutant strain, CJN459; the *tec1*/*tec1* insertion mutant strain, CJN308; the *bcr1*/*bcr1* deletion mutant strain, CJN702, and its complemented strain, CJN698, was described previously [14].

For construction of the insertion mutant strains for Bcr1 target genes, we took advantage of a Tn7-UAU1 plasmid insertion mutant library containing our genes of interest, made by The Institute for Genome Research (TIGR). Each TIGR plasmid containing the *orf-Tn7-
Role of Bcr1 in Biofilm Formation

Biofilm formation in C. albicans is a complex process involving the coordinated expression of adhesins, which are essential for the initial attachment to surfaces. Bcr1 is a transcriptional activator that plays a critical role in biofilm formation, as it is required for the expression of several adhesins, including Als3, Als1, and Hwp1.

The study described the role of Bcr1 in biofilm formation using a combination of gene mutation and overexpression analyses. The Bcr1 target genes were identified using a library of deletion mutants and overexpression plasmids. The authors observed that the overexpression of Als3, Als1, and Hwp1 was restored in the absence of Bcr1 when these genes were overexpressed, indicating that Bcr1 is necessary for their expression.

Furthermore, the authors found that overexpression of Ece1, a novel protein, was also able to restore biofilm formation in the absence of Bcr1, suggesting that Bcr1-dependent adherence is critical for biofilm formation.

The role of Bcr1 in biofilm formation was also assessed in vivo by catheter infection experiments. The results showed that Bcr1-dependent adherence is critical for biofilm formation in vivo, and the overexpression of Als3, Als1, and Hwp1 was also restored in vivo when these genes were overexpressed.

In summary, the study highlights the importance of Bcr1 in biofilm formation by C. albicans, emphasizing the need for further research to understand the molecular mechanisms underlying this process.

References:
[40] except that C. albicans cells were heat shocked at 44°C for 20 min, which increased efficiency of the standard 42°C for 1 h. The Arg+ heterozygous transformants were then used to obtain Arg+ Ura+ homозygous insertion mutant strains FJS2 (hyr1/ hyr1), FJS3 (cht2/ cht2), FJS6 (oe1/ oe1), FJS8 (bht5/bht5), and FJS10 (cem31/ cem31) using methods described previously [40]. These homozygous insertion mutants were then screened by colony PCR to ensure absence of the wild-type allele. We used strain DAY286 (Arg+ Ura+ His+) [40] as a reference strain for these mutants.

For construction of the TEF1-BCR1 overexpression plasmid pCJN491, PCR was done using primers OE723-ATG (5′-ATGTGAGG GACATCACAAGTACTTCA-3′) and OE723-900S (5′-GACATCACAAGTACTTCA-3′) to generate a 2,723-bp fragment beginning from the ATG of the BCR1 ORF (orf19.8342) to 500 bp downstream of the stop codon. This fragment was inserted into the pGEMT-Easy vector (Promega, Madison, Wisconsin, United States) and then digested with EcoRI and SpeI (releasing a 1,650-bp fragment containing the larger portion of the BCR1 ORF including the start codon and 1,650 bp downstream of the start codon), and cloned into an EcoRI- and SpeI-linearized vector pTEF1 [15], to yield plasmid pCJN491 in the correct orientation. pTEF1 [15] is a vector that harbors the constitutively active A. gossypii TEF1 promoter that is derived from pDDB78, a A. gossypii TEF1 vector [41]. A unique SbfI site lying within the 1,650-bp portion of BCR1 was used to direct integration of the plasmid to the natural BCR1 locus via SbfI digestion. The TEF1-BCR1 overexpression C. albicans strains CJN1011, CJN1035, and CJN1055 were constructed by transforming CJN459 (a His−/ His+) reference strain, respectively, with SbfI-linearized pCJN491 to generate His+ strains overexpressing BCR1. The TEF1 vector alone C. albicans strains CJN1006, CJN1052, and CJN1015 were constructed by transforming CJN459, CJN308, and DAY286, respectively, with NruI-linearized pTEF1 to generate His+ strains with the vector alone.

The NAT1-TEF1 overexpression plasmid pCJN498 was generated as follows. PCR was done using primers AgNat1F (5′-ATGTTTCCCAATTGAAAAAAGAGGAC-3′) and AgNat1R (5′-GAATCGACAG-3′) to generate a 800-bp product containing the sequences in pJK799 surrounding the NAT1 ORF and followed by the A. gossypii TEF1 terminator. The use of A. gossypii sequences instead of C. albicans sequences in pJK799 surrounding the NAT1 ORF prevents misintegration of the construct [42]. This fragment was inserted into the pGEMT-Easy vector (Promega) in the correct orientation to create plasmid pCJN498. PCR was done using primers TEF1-SpeIF (5′-AAAGGCACATGCCCATCCGAGAAGAC-3′) and TEF1-Nde1R (5′-GATGAAAAAGGAGAAGACGAGAAGAC-3′) to generate an 800-bp product containing the C. albicans TEF1 promoter with
Table 1. *C. albicans* Strains Used in This Study

| Strain       | Genotype                                                                 | Reference |
|--------------|---------------------------------------------------------------------------|-----------|
| BWP17        | ura3::LMM43 arg4::hisG his1::hisG                                       | [37]      |
| CAH7-1A1E2   | ura3::LMM43 arg4::hisG his1::hisG                                       | [28]      |
| CAJ4         | ura3::LMM43 arg4::hisG                                                  | [34]      |
| CAJ4-URA3    | ura3::LMM43 arg4::hisG                                                  | [38]      |
| CAYF178U     | ura3::LMM43::URA3-1R01 arg4::hisG                                       | This study|
| CAQTP178U    | ura3::LMM43::URA3-1R01 arg4::hisG                                       | This study|
| CAYC2Y1F1U   | ura3::LMM43::URA3-1R01 arg4::hisG                                       | [39]      |
| CJN308       | ura3::LMM43 arg4::hisG his1::hisG tec1::Tn7-UAA1                         | [14]      |
| CJN459       | ura3::LMM43 arg4::hisG his1::hisG bcr1::Tn7-UAA1                         | [14]      |
| CJN659       | ura3::LMM43 arg4::hisG his1::hisG bcr1::Tn7-UAA1                         | [14]      |
| CJN702       | ura3::LMM43 arg4::hisG his1::hisG bcr1::Tn7-UAA1                         | [14]      |
| CJN896       | ura3::LMM43 arg4::hisG his1::hisG bcr1::Tn7-UAA1                         | [14]      |
| CJN1011      | ura3::LMM43 arg4::hisG his1::hisG bcr1::Tn7-UAA1                         | This study|
| CJN1015      | ura3::LMM43 arg4::hisG his1::hisG bcr1::Tn7-UAA1                         | This study|
| CJN1023      | ura3::LMM43 arg4::hisG his1::hisG bcr1::Tn7-UAA1                         | [14]      |
| CJN1035      | ura3::LMM43 arg4::hisG his1::hisG bcr1::Tn7-UAA1                         | This study|
| CJN1039      | ura3::LMM43 arg4::hisG his1::hisG bcr1::Tn7-UAA1                         | This study|
| CJN1052      | ura3::LMM43 arg4::hisG his1::hisG bcr1::Tn7-UAA1                         | This study|
| CJN1060      | ura3::LMM43 arg4::hisG his1::hisG bcr1::Tn7-UAA1                         | This study|
| CJN1144      | ura3::LMM43 arg4::hisG his1::hisG bcr1::Tn7-UAA1                         | This study|
| CJN1149      | ura3::LMM43 arg4::hisG his1::hisG bcr1::Tn7-UAA1                         | This study|
| CJN1153      | ura3::LMM43 arg4::hisG his1::hisG bcr1::Tn7-UAA1                         | This study|
| CJN1222      | ura3::LMM43 arg4::hisG his1::hisG bcr1::Tn7-UAA1                         | This study|
| CJN1259      | ura3::LMM43 arg4::hisG his1::hisG bcr1::Tn7-UAA1                         | This study|
| CJN1276      | ura3::LMM43 arg4::hisG his1::hisG bcr1::Tn7-UAA1                         | This study|
| CJN1281      | ura3::LMM43 arg4::hisG his1::hisG bcr1::Tn7-UAA1                         | This study|
| CJN1288      | ura3::LMM43 arg4::hisG his1::hisG bcr1::Tn7-UAA1                         | This study|
| DAY185       | ura3::LMM43 arg4::hisG his1::hisG                                        | [43]      |
| DAY286       | ura3::LMM43 arg4::hisG his1::hisG                                        | [40]      |
| FS52         | ura3::LMM43 arg4::hisG his1::hisG                                        | This study|
| FS55         | ura3::LMM43 arg4::hisG his1::hisG                                        | This study|
| FS26         | ura3::LMM43 arg4::hisG his1::hisG                                        | This study|
| FS58         | ura3::LMM43 arg4::hisG his1::hisG                                        | This study|
| FS10         | ura3::LMM43 arg4::hisG his1::hisG                                        | This study|

DOI: 10.1371/journal.ppat.0020063.t001

Critical *C. albicans* Biofilm Adhesins
transformants were checked by colony PCR. We used strain DAY185

TGCTGGTTGGAAT GCTTATTTG-3

plates for 2 d at 30°C for expression. The cells were then plated onto YPD

ACTAGAAGAAATGATAGGTGTGGAATTGTGAGCGGATA-3

media containing 5-FOA and uridine. A 3.9-kb fragment of these mutants were selected by plating on synthetic

TTGCTTTCTATTTGATAACCCGCCTCAAATCAAGATTGGGAGG

C. albicans NAT1 ORF, followed by the A. gossypii TEF1 terminator, followed by the A. gossypii TEF1 promoter in the correct orientation. The TEF1-ALS3 overexpression C. albicans strains CJN1149 and CJN1153 were constructed by transforming DAY185 (a His− reference strain) [43] and CJN702 (a His−/ura1/1 deletion mutant), respectively, using PCR products from template plasmid pCJN96 and primers ALS3-F-OE-Ag-NAT-Ag-TEF1p (5′-AGCGAACTAATCCGAAACACACAT-3′) and 3′-Confir-2 (5′-AGCGAAATCAATCCGAAACACACAT-3′). These primers amplify the entire A. gossypii TEF1 promoter, the C. albicans NAT1 ORF, the A. gossypii TEF1 terminator, and the C. albicans TEF1 promoter with 100 bp of hanging homology to 3′ for the forward primer and 100 bp of hanging homology from exactly the start codon of the ALS3 ORF. The homology in these primers allows for homologous recombination of the entire cassette directly upstream of the promoter so that ALS3 could be overexpressed with the A. gossypii TEF1 promoter instead of its natural promoter. The transformation into C. albicans strains was done as described above except an additional 5-h recovery step in YPD at 30°C after the cells were heat shocked at 44°C for 20 min in order to allow for NAT1 expression. The cells were then plated onto YPD + 400 μg/ml clonNat plates (0°C to select for transformants) and transformants were checked by colony PCR. We used strain DAY185 (Arg− Ura− His+) [43] as a reference strain for these strains. The als3Δ/Δ3a mutant, CAYF178U, was constructed from strain BW17. The two alleles of ALS3 were serially disrupted using the markers HIS1 and ARG4. The disruption cassettes were amplified with the following primers: ALS3-5′R (5′-CCTCCTATACACACATACAAATGTTGCTCACAACATTTGGAATTGAGAACAAAACAAA CAGTTTCCCCCAGTACGAGATGT-3′) and ALS3-3′R (5′-GGTTGGTCACAGTACGAGATGTACAGTCTTTCACGC ACTGAAAGTAATAGTTGTTGAAATTGTTGAGGATG-3′). The disruption of ALS3 was verified by PCR using the following primers: 3′Confirm-1 (5′-ATGACACCATGTCAAGATCCAG-3′) and 3′Confirm-2 (5′-GGTTGGTTGTTCAATGACATGG-3′). To complement the als3Δ/Δ3a mutant with a wild-type copy of ALS3, a full-length version of ALS3 was digested from pGEMT with PvuII and SpheI [29], and then subcloned into pDS10 at the SpheI site [44]. The construct was linearized with BspI and digested with into ALS3 locus of the als3Δ/Δ3a Ura− strain, selecting Ura+. Excision of the URA3-Δp2600 marker was then sequenced on the plate. The URA3-Δp2600 knock-out region was confirmed by PCR using primers 5′-TGAAGCAGCTTT TAGTGCCC-3′ and 5′-AGAAGTGGAAAGCAGCTTGAGG-3′. URA3 and the adjacent IRO1 locus was restored in the als3Δ/Δ3a strain [39]; als3Δ/Δ3a, and als3Δ/Δ3a ALS3 strains as follows. Ura+ derivatives of these mutants were selected by plating on synthetic media containing 5-FOA and uridine. A 3.9-kb URA3-IRO1 fragment was released from pBSK-URA3 by NotI digestion and used to transform the Ura- strains [44]. The restoration of URA3 to its native loci was confirmed by PCR using the primers 5′-TGGTTGGTTGAAATGGTATTGG-3′ and 5′-TGGAAATATTTGCT TAGTGCCC-3′.

In vitro biofilm growth conditions. For in vitro biofilm growth assays, strains were grown in YPD overnight at 30°C, diluted to an OD600 = 0.5 in 2 ml of Spider medium (with auxotrophic supplements) and added to a sterile 12-well plate with a prepared silicone square (1.5 × 1.5 cm cut from Cardiovascular Instrument silicone sheets [Wakefield, Massachusetts, United States]). The silicone square was previously treated with bovine serum (B-9435; Sigma, St. Louis, Missouri, United States) overnight and washed with PBS to use as the substrate for the biofilm assay. The plates were incubated at 37°C for 3 h at 90 rpm agitation for initial adhesion of cells. To remove unadhered cells, the squares were washed with 2 ml of PBS, and the squares were moved to a fresh 12-well plate containing 2 ml of fresh Spider medium. This plate was incubated at 37°C for 24 h to allow for additional 60 h at 130-rpm agitation to allow for biofilm formation.

Microscopic visualization of in vitro biofilms. For the in vitro experiments, biofilms were observed visually and by CSLM. For in vitro CSLM imaging, biofilms were stained with 25 μg/ml concanavalin A Alexa Fluor 540 conjugate (C-11253; Molecular Probes, Eugene, Oregon, United States) for 1 h in the dark at 37°C with 150 rpm agitation. CSLM was performed with an upright Zeiss AxioSkop2 FS MOT LSM 510 multiphoton microscope using a Zeiss Achroplan ×400/0.8W objective. In order to visualize conacavalin A conjugates, a 393-nm wavelength was used. All in vitro CSLM images were assembled into side and depth views using the Zeiss LSM Image Browser (version 3.2.0.115) software. For all side views, the silicone is located at the top of the image. Depth views are artificially colored images indicating cell density using a color gradient, where blue represents cells closest to and red represents cells farthest from the silicone substrate.

RNA isolation and expression analysis. Overnight cultures were inoculated in 5 ml of YPD at 30°C. The next day, 100 ml of Spider medium was inoculated with the YPD overnight culture to obtain an OD600 = 0.05, and was grown at 37°C for 12 h (OD600 = ~8). Cells were immediately harvested by vacuum filtration. RNA extraction and Northern analysis were performed as previously described [40]. For RT-PCR analysis for detection of ALS1 and ALS3, 10 μg of total RNA was DNase treated at 37°C for 1 h, ethanol precipitated, and resuspended in 100 μl of DEPC water. cDNA was synthesized and RT-PCR was done as previously described for ALS1 and ALS3 [45] with reverse transcriptase and without reverse transcriptase (as a control).

In vivo biofilm model. A rat central venous catheter infection model [18] was selected for in vivo biofilm studies. The catheter diameter was chosen in an attempt to permit blood flow across the extraluminal catheter substrate. To mimic material used in patients, polyethylene tubing (inner diameter ~0.76 mm, outer diameter 1.52 mm) was chosen. Specific-pathogen-free Sprague-Dawley rats weighing 400 g were used (Harlan Sprague-Dawley, Indianapolis, Indiana, United States). A heparinized (100 U/ml) catheter was surgically inserted into the external jugular vein and advanced to a site above the right atrium (2 cm length). The catheter was secured to the vein and the proximal end tunneled subcutaneously to the mediastinal space and externalized through the skin. The catheters were left in place for 24 h prior to infection to allow a conditioning period for deposition of host protein on the catheter surface. Infection was achieved by intraluminal instillation of 500 μl of C. albicans cells (106 cells/ml). After a dwelling period of 4 h, the catheter volume was withdrawn and the catheter was flushed with heparinized 0.15 M NaCl.

Catheters from two animals were removed at three time points (12, 24, and 48 h) after C. albicans infection to determine biofilm development on the internal surface of the intravascular devices. The distal 2 cm of the catheter was cut from the entire catheter length. Biofilms were then evaluated using both scanning electron microscopy. Scanning electron microscopy was used for architectural investigation of the biofilm process. Catheter segments were washed with 0.1 M phosphate buffer (pH 7.2) and placed in fixative (1% glutaraldehyde and 4% formaldehyde). The samples were immersed in 20% ethanol for 1 min and then 30% ethanol for 30 min. The samples were then dehydrated in a series of 10-min ethanol washes (30%, 50%, 70%, 85%, 95%, and 100%). Final desiccation was accomplished by critical point drying [Tousimis, Rockville, Maryland, United States]. Specimens were mounted on aluminum stubs and sputter-coated with gold. Samples were imaged in a scanning electron microscope (Hitachi S-5700 or JOEL JSM-6100) in the high-vacuum mode at 10 kV. The images were processed for display using Adobe Photoshop 7.0.1.

Disseminated murine candidiasis models. Groups of ten 20-g male Balb/C mice were inoculated via the lateral tail vein with 5 × 106 blastospores with each strain of C. albicans. The mice were monitored three times daily for survival.

Biofilm dry mass measurements. For dry mass measurements, each silicone square was weighed prior to inoculation with the strain of interest. Biofilms were grown for 60 h on the silicone square (as described above). The silicone square and any adherent biofilms were then removed from the wells, dried overnight in a fume hood, and weighed the following day. Total biofilm of each biofilm was calculated by subtracting the weight of the silicone prior to biofilm growth from the weight of the silicone after biofilm growth. The average total biomass for each strain was calculated from four independent samples after subtracting the weight of a blank silicone square with no cells added. Statistical significance (p-values) was calculated with the Student’s two-tailed t-test function in Microsoft Excel.
Supporting Information

Figure S1. Mouse Survival Data
Disseminated murine candidiasis assays. Groups of ten 20-g male Balb/C mice were inoculated via the lateral tail vein with 5 × 10^6 blastospores with each strain of C. albicans. The mice were monitored three times daily for survival.

Found at DOI: 10.1371/journal.ppat.0020063.sg001 (44 KB PDF).

Accession Numbers
Information for the following C. albicans genes can be found at the Candida Genome Database (CGD). Web site (http://www.candidagenome.org): BCR1 (orf19.723), TEC1 (orf19.5908), WHP1 (orf19.1321), ALS3 (orf19.1816), ALS1 (orf19.5741), HVR1 (orf19.4975), CHT2 (orf19.3895), ECE1 (orf19.3537), RBT5 (orf19.5636), and ECM331 (orf19.4255).

Acknowledgments
We are grateful for the online availability of the Candida genome database (CGD) and the CandidaDB Web Server. We thank all members of the Mitchell laboratory for insightful discussions and comments on this manuscript. We also acknowledge our manuscript reviewers, who suggested that we broaden our gene overexpression analysis. We are grateful to Allison Fay for her help in constructing plasmid pCJN498. Paula Sundstrom for generously providing us with her hop1Δhup1 mutant strain, Julia Kohler for sharing her plasmid pEF1G, and Bill Nierman, and his colleagues at The J. Craig Venter Institute for Genome Research (TIGR) for constructing a plasmid insert mutant library from which several C. albicans insertion mutants were made. We are indebted to Theresa Swayne, Sudhindra Swamy, and Sharmile Ramcharan for CSLM advice.

Author contributions.
CJN, DRA, JEN, SGF, and APM conceived and designed the experiments. CJN, DRA, JEN, JEE, SGF, and APM analyzed the data. FJS, FY, QTP, JEF, and SGF contributed reagents/materials/analysis tools. CJN, DRA, JEE, SGF, and APM wrote the paper.

Funding.
This study was supported by National Institutes of Health (NIH) grants R01 AI057804 and R01 AI067703 (APM), NIH K08 AI01767 (DRA), NIH T32 HL07899 (JEN), RO1 AI19990 (JEE), and R01 AI05928 (SGF).

Competing interests.
The authors have declared that no competing interests exist.

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