Identification of Genes Upregulated by the Transcription Factor Bcr1 That Are Involved in Impermeability, Impenetrability, and Drug Resistance of Candida albicans a/α Biofilms

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Candida albicans forms two types of biofilm, depending upon the configuration of the mating type locus. Although architecturally similar, a/α biofilms are impermeable, impenetrable, and drug resistant, whereas a/a and α/α biofilms lack these traits. The difference appears to be the result of an alternative matrix. Overexpression in a/a cells of BCR1, a master regulator of the a/α matrix, conferred impermeability, impenetrability, and drug resistance to a/a biofilms. Deletion of BCR1 in a/α cells resulted in the loss of these a/α-specific biofilm traits. Using BCR1 overexpression in a/a cells, we screened 107 genes of interest and identified 8 that were upregulated by Bcr1. When each was overexpressed in a/a biofilms, the three a/α traits were partially conferred, and when each was deleted in a/α cells, the traits were partially lost. Five of the eight genes have been implicated in iron homeostasis, and six encode proteins that are either in the wall or plasma membrane or secreted. All six possess sites for O-linked and N-linked glycosylation that, like glycosylphosphatidylinositol (GPI) anchors, can cross-link to the wall and matrix, suggesting that they may exert a structural role in conferring impermeability, impenetrability, and drug resistance, in addition to their physiological functions. The fact that in a screen of 107 genes, all 8 of the Bcr1-upregulated genes identified play a role in impermeability, impenetrability, and drug resistance suggests that the formation of the a/α matrix is highly complex and involves a larger number of genes than the initial ones identified here.

Like pathogenic bacteria (1–3), the opportunistic yeast pathogen Candida albicans forms biofilms on a number of different substrates, including dentures, catheters, and a variety of host tissues (4, 5). Traditionally, biofilms are formed by microbial pathogens on a surface to help withstand expulsion from the host due to fluid flow, produce a controlled microenvironment, and protect the community of cells from host and therapeutic challenges, such as drug therapy, the invasion of white blood cells, and antibodies (3, 6–11). Biofilms formed in vitro on silicone elastomer in rich media by the majority of C. albicans strains found in nature exhibit all of these traits, which would expect to be a success of opportunistic microbial pathogen. They are highly tethered to the substratum, impermeable to low- and high-molecular-weight molecules, impenetrable by phagocytic white blood cells, and drug resistant (5, 12–16). The last three of these traits, which one would expect to facilitate commensalism and pathogenesis, are henceforth referred to as “pathogenic traits” in this study. However, a minority of C. albicans strains found in nature form a second type of biofilm in vitro, which, although architecturally similar to the majority biofilms, lacks these pathogenic traits. These minority biofilms are highly permeable, penetrable, and drug susceptible. These minority biofilms have been associated with the mating process (16–19; Y.-N. Park, K. J. Daniels, C. Pujol, T. Srikantha, and D. R. Soll, submitted for publication). The fact that the two biofilms are architecturally similar but differ in permeability, penetrability, and drug susceptibility provided us with a screen for identifying genes upregulated by the transcription factor Bcr1 (16, 20–24) that are involved in conferring impermeability, impenetrability, and drug resistance to majority biofilms.

The formation of the two types of biofilm by C. albicans depends upon the configuration of the mating type locus, MTL (16). When yeast cells from strains in the α/α configuration, the majority configuration (>90%) in nature (25–28), are dispersed on silcone elastomer, the material used to form catheters, they form tethered robust biofilms in nutrient-rich media. These biofilms have been shown to be impermeable to low- and high-molecular-weight molecules, impenetrable by human polymorphonuclear leukocytes, and resistant to fluconazole (16). When a/α cells undergo homozygosis to the a/a or α/α configuration, they also form robust biofilms under these conditions when in the white phase of the white-opaque transition (17, 29–34). The white-opaque transition is an essential step for mating competency (35, 36). The biofilms formed by white MTL-homozygous cells in vitro are architecturally similar to biofilms formed by a/α cells. Both contain a basal yeast cell polylayer at the substratum and a thick upper portion of vertically oriented hyphae encapsulated in a polyelectrolyte extracellular matrix. However, in contrast to α/a biofilms, a/a or α/a biofilms are permeable to low- and high-molecular-weight molecules, readily penetrated by human white blood cells, and fluconazole susceptible (16). Formation of the two types of biofilms is regulated by different signal transduction pathways. Formation of an a/a biofilm is regulated by the Ras1/cyclic AMP (cAMP) pathway, whereas formation of a white a/a or α/α biofilm is regulated by the mitogen-activated protein (MAP) kinase pathway (29–34). The Ras1/cAMP pathway targets a transcription fac-

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tor cascade that includes Efg1 → Tec1 → Bcr1, in which both Tec1 and Bcr1 directly regulate genes involved in a/α biofilm formation (16, 24, 37, 38). The MAP kinase pathway, however, has only been shown to date to target Tec1, which regulates genes involved in a/α or α/α biofilm formation (16, 29). Bcr1 has been shown to play no significant role in a/α or α/α biofilm formation (16).

Although MTL-homozygous biofilms formed in vitro do not exhibit those traits deemed important for commensalism and pathogenesis, they have been shown to facilitate chemotropism of conjugation tubes formed by minority (1% to 10%) opaque a/α and opaque α/α cells seeded in them (17). Moreover, using a complementation strategy, we recently found that when MTL-heterozygous and MTL-homozygous biofilms are similarly seeded with minority opaque cells of auxotrophic a/α and α/α strains, complementation frequencies are 10 to over 100 times higher in MTL-homozygous biofilms (Park et al., submitted). These results suggest a hypothesis in which MTL-heterozygous biofilms play a role in commensalism and pathogenesis, whereas MTL-homozygous biofilms play a role in mating. This is consistent with the fact that white MTL-homozygous biofilm formation is regulated by pheromones secreted by a self-induction process (32) or by minority opaque cells of the opposite mating type and that the same MAP kinase pathway that regulates the mating response also regulates MTL-homozygous biofilm formation (18, 19, 30, 31, 33, 34). Thus, white a/α or α/α cell biofilm formation is coordinately regulated by the same extracellular signals, the mating pheromones, that induce the mating response.

To explain the similarity in the general architectures of MTL-heterozygous and MTL-homozygous biofilms but the dissimilarities in permeability, penetrability, and drug susceptibility, we considered the following hypothesis. Since the alternative signal transduction pathways regulating the two types of biofilms both target Tec1, we propose that Tec1 activates the genes responsible for the most fundamental aspects of architecture common to the two types of biofilm. These include formation of the adhesive and cohesive basal polylayer of yeast cells at the substrate, the subsequent formation of germ tubes by these yeast cells, and the elongation of the latter into hyphae. In a/α biofilms, Bcr1, which is activated by Tec1, regulates genes that are responsible for the formation of a matrix that results in the vertical orientation of hyphae, maximum biofilm thickness, and the three pathogenic traits examined here, i.e., impermeability, impenetrability, and drug resistance. Conversely, in white a/α and α/α cells, Tec1 targets an as yet unidentified transcription factor that regulates genes responsible for the formation of a matrix that results in the vertical orientation of hyphae and the traits that facilitate mating of minority opaque cells.

If this hypothesis was correct, then deleting BCR1 in a/α cells should compromise the matrix, as shown in an in vivo vaginal model (39), but not the formation of the basic biofilm, and should eliminate the characteristics of impermeability, impenetrability, and drug resistance. On the other hand, overexpressing BCR1 in a white a/α biofilm may partially or completely confer the pathogenic characteristics exhibited by a/α biofilms. These were indeed the results we obtained here. They in turn provided us with a strategy to screen for genes that regulate the pathogenic traits of an a/α biofilm. In the screen we performed here, BCR1 was overexpressed in white a/α biofilms, and the effect this had on the expression of 107 genes of interest was tested by reverse transcription-PCR (RT-PCR). Eight of the 107 genes were upregulated by overexpression of Bcr1. These genes were then individually overexpressed in an a/α background and deleted in an a/α background. The results revealed that all eight of the Bcr1-upregulated genes identified in the screen contribute, to various degrees, to the pathogenic traits specific to a/α biofilms of C. albicans. Five of the proteins encoded by the eight genes (CSA1, CSA2, PGA7, PGA10, and RBT5) are either secreted or located at the cell surface and play roles in iron homeostasis (40–42), one (AQY1) is a plasma membrane protein that functions as a water channel (43, 44), one (CHK1) functions in a signaling pathway (45), and one (WHI1) is expressed in white but not opaque cells, with no known function (46, 47). We discuss whether the roles of the molecules on the cell surface involve their previously elucidated functions, such as in iron homeostasis, or whether they may interplay directly with the matrix architecture through linkages with β-glucan and other proteins in the matrix. Whatever roles these first eight proteins play, it seems clear from our results that the formation of an extracellular a/α matrix exhibiting pathogenic traits is highly complex and that the genes involved may have diverse functions. Clearly, many more Bcr1-regulated genes that play a role in the pathogenic traits of a/α matrix formation will emerge in future screens and provide further insights into how an a/α matrix is generated and functions.

MATERIALS AND METHODS

Strains and media. The names, genotypes, and origins of the wild-type strains and mutants used in this study are listed in Table S1 in the supplemental material. Strains were maintained at 25°C on agar plates containing modified Lee’s medium (48, 49) prior to use.

Biofilm development. Biofilms were generated as previously described (17), with small modifications. Briefly, cells were grown to stationary phase at 25°C in modified Lee’s medium (48), conditions conducive to both a/α and a/α biofilm formation. Biofilms were developed on 10-mm silicone elastomer disks (0.04 in. thick; Bentec Medical). Each disk was placed in the well of a 12-well cluster plate (Costar; Corning Inc.), and the well was filled with a 2.5-ml suspension containing a total of 2 × 107 stationary-phase cells (grown in modified Lee’s liquid medium at 25°C for 48 h) in RPMI medium (Gibco). The cells were allowed to adhere to the disks for 90 min. Each disk was removed from its well, rinsed very gently with DPBS (Dulbecco’s phosphate-buffered saline without CaCl2 or MgCl2, pH 7.2; Gibco) to remove nonadherent cells, and placed in a well with fresh RPMI medium. The disks were incubated at 29°C on a rocker (Immunetics) with a 6°/s deflection for 16 h or 48 h. To induce genes under the control of the tetracycline-inducible promoter in biofilms, RPMI medium was supplemented with 50 μg/ml doxycycline (+ doxy) at 0 h. An additional dose of 25 μg/ml doxycycline was added after 24 h.

Biofilm thickness and architecture. Forty-eight-hour biofilms were rinsed in PBS, fixed in 10% formalin solution, rinsed again, and stained with calcofluor white M2R (Fluorescent Brightener 28; Sigma) (50). Biofilm thickness and architecture were analyzed using a Radiance 2100 two-photon laser scanning confocal microscope (2-photon LSCM) (Bio-Rad) to visualize calcofluor white-stained cells, hyphae, and extracellular matrix (ECM) (Mai-Tai IR laser with excitation at 780 nm and emission at 460 nm; Spectra Physics). Fluorescence images were acquired as a z series at 2-μm intervals through a 125-μm thickness. Images were gathered using LaserSharp software (Bio-Rad). Side views of stacked images of the z series were used for thickness measurements. Matrix density was analyzed by determining the pixel intensity. LSCM parameters were set to optimize the pixel intensity in the control matrix, and the same parameters were used to compare pixel intensities of mutant biofilms.

Biofilm permeability. To determine dye penetration, 48-h biofilms were overlaid with a solution containing Film Tracer Sipro Ruby dye (Invitrogen) and incubated for 45 min prior to live confocal imaging as
previously described (16), with minor modifications. Sypro Ruby fluorescence images were acquired by LSCM (argon laser with excitation at 457 nm and emission at 610 nm) as a z series at 2-μm intervals through a 125-μm thickness. After z-series acquisition, a z image through the image stack, perpendicular to the substrate, was generated to assess the limits of dye permeation.

**Biofilm penetrability.** To assess human polymorphonuclear leukocyte (PMN) penetrability, PMNs were purified from venous blood as previously described (51). The purified cells were suspended in Hanks’ balanced salt solution (Gibco-BRL) at a final concentration of 1.5 × 10⁶ cells/ml, labeled with 1 μM Vybrant CM-Dil ( Molecular Probes, Invitrogen) for 5 min, and washed with RPMI medium. Twenty-microliter aliquots of PMNs were overlaid on 48-h biofilms and incubated for 3 h at 37°C in 5% CO₂. Dil fluorescence images were acquired as described above (HeNe laser with excitation at 543 nm and emission at 570 nm). Perpendicular z-image slices were obtained by LSCM to assess the extent of PMN penetrability.

**Fluconazole susceptibility.** To assess the effect of fluconazole (Sigma) on biofilm cell viability, 1 ml of RPMI medium was removed from each well containing a 48-h biofilm culture (17) and replaced with 1 ml of fresh RPMI medium. Fluconazole was added to a final concentration of 25 μg/ml. Biofilms were incubated for a further 24 h at 29°C with rocking. Biofilms were rinsed with DPBS and placed in 1.5-ml Eppendorf tubes containing 1 ml of DPBS supplemented with 20 mM EDTA for 5 min. Biofilms were then vortexed to remove biofilm cells from their silicone elastomer support. Cells were pelleted and resuspended in TBS (Tris-buffered saline; 20 mM Tris, 120 mM NaCl, pH 7.5) twice. After the final rinse, the cell pellet was resuspended in TBS containing 5 mM Sytox Green, a dead cell double-stranded DNA (dsDNA) stain (Invitrogen), for 5 min, with intermittent mixing. The Sytox Green was removed by sequential rinse, the cell pellet was resuspended in TBS containing 5 nM Sytox Green, Hoechst 33342 (Mai-Tai IR laser with excitation at 788 nm and emission at 488 nm), and finally rinsed with RPMI medium. Twenty-microliter aliquots of PMNs were overlaid on 48-h biofilms and incubated for 3 h at room temperature. Samples were stored at 4°C. For analysis, all cell nuclei were acquired and the number of nuclei per image determined using ImageJ software (52). Three fields were counted for each of three duplicate biofilm formation (20, 39, 58–61). We subsequently extended these observations (16), showing that although deletion of BCR1 in a/α cells has a major effect on biofilm formation, deletion in a/α cells does not. In this study, we continued this analysis and found, in particular, deletion caused reduction in the expression of a number of genes in biofilms that were known to be expressed at the surfaces of hyphae, several of which are involved in adhesion (21, 22, 37, 57), thus arguing that this transcription factor plays a fundamental role in biofilm formation.
TABLE 1 Architectural and pathogenic traits of biofilms formed by BCR1 deletion mutants of a/a and a/a strains and by BCR1 overexpression mutants of an a/a strain

| Strain          | Doxycycline treatment | Biofilm architecture | Commensal/pathogenic traits |
|-----------------|-----------------------|----------------------|-----------------------------|
|                 |                       | Thickness (μm)       | Sypro Ruby impermeability   |
|                 |                       | Basal yeast          | Human PMN impermeability    |
|                 |                       | Formation            | Fluconazole resistance (%)  |
|                 |                       | of hyphae            |                             |
|                 |                       | Vertical orientation |                             |
| SC5314 (a/a)    | −                     | 86 ± 14              | +++++                       | 3 ± 2                        |
| a/a-Δbcrl/Δbcrl | −                     | 56 ± 8               | +++++                       | 41 ± 8                       |
| P37005 (a/a)    | −                     | 72 ± 3               | −                           | 35 ± 7                       |
| a/a-Δbcrl/Δbcrl | −                     | 64 ± 2               | +++++                       | 33 ± 2                       |
| a/a-Δbcrl/Δbcrl | −                     | 68 ± 4               | −                           | 40 ± 11                      |
| a/a-Δbcrl/Δbcrl | +                     | 36 ± 6               | +++++                       | 17 ± 5                       |

* Treatment with 50 μg per ml of doxycycline at 0 min and then with 25 μg per ml at 24 h. +, treated; −, untreated.

* Biofilm parameters were assessed with reference to the parental strain SC5314, which attained a maximum of +++++ for every parameter. Graded decreases in a parameter were qualitatively assessed from +++++ to +. A minus sign represents the absence of a characteristic. ECM, density of the extracellular polymolecular matrix. For fluconazole resistance, the proportion of cells that stained with Sytox Green, a dead cell dsDNA stain, was determined.

* The architecture of biofilms was assessed by examining calcofluor white-treated preparations by LSCM.

* See examples of images for impermeability and impenetrability and data for fluconazole resistance in Fig. 2.

Calciofluor white-stained biofilms viewed from the side and an analysis of individual scans at various depths revealed that the hyphae of a/a Δbcrl/Δbcrl biofilms, rather than orienting vertically, as was the case for parental a/a biofilms, had collapsed and fanned out horizontally, as is apparent in comparisons of LSCM scans of the upper regions of parental biofilms (Fig. 1A) and mutant biofilms (Fig. 1B). These results suggest that the supporting matrix in the upper portion of the a/a Δbcrl/Δbcrl biofilms was severely compromised. Decreased staining with calciofluor white also indicated a diminished extracellular matrix (Table 1).

Biofilms formed by cells of the natural a/a strain P37005 (17) exhibited the same basic architecture as that of wild-type a/a biofilms (16). They were similarly composed of a basal yeast cell poly-layer and an upper region of vertically oriented hyphae embedded in a supporting matrix (Table 1). On average, however, they were approximately 20% thinner than a/a biofilms (P = 0.0001) (Table 1), as previously reported (32). We previously showed that in contrast to a/a cells, when BCR1 was deleted in an a/a strain, the thickness of the biofilm decreased by only 10% (16), and the repeat experiment performed here revealed a decrease of 11% (P = 0.001). Using LSCM to scan calciofluor white-stained biofilms, we found that the basic architecture of a/a Δbcrl/Δbcrl biofilms was indistinguishable from that of biofilms formed by the parent strain P37005 (a/a). Biofilms of the a/a Δbcrl/Δbcrl strain contained a basal poly-layer of yeast cells and an upper region containing vertically oriented hyphae embedded in a dense matrix (Table 1). Most importantly for the present study, these results indicated that in an a/a background, but not an a/a background, Bcr1 played a major role in the formation of the extracellular matrix. Our results, however, did not exclude a minor role for Bcr1 in a/a biofilms, given the small but reproducible decrease in a/a Δbcrl/Δbcrl 1 biofilm thickness (Table 1).

In addition to affecting the architecture of the upper portion of an a/a biofilm, deleting BCR1 affected the three a/a biofilm traits examined: impermeability to the low-molecular-weight stain Sypro Ruby, impenetrability by human PMNs, and fluconazole resistance. While wild-type a/a biofilms were relatively impermeable to Sypro Ruby (Fig. 2A; Table 1), impenetrable by PMNs (Fig. 2E; Table 1), and highly resistant to fluconazole (Fig. 2I; Table 1), a/a Δbcrl/Δbcrl biofilms were far more permeable to Sypro Ruby (Fig. 2C; Table 1), readily penetrated by PMNs (Fig. 2G; Table 1), and far more susceptible to fluconazole (Fig. 2I; Table 1). In contrast, deletion of BCR1 in a/a cells did not affect these characteristics in a/a biofilms (Table 1), obviously because a/a biofilms were already highly permeable, penetrable, and fluconazole susceptible (16) (see Table S1 in the supplemental material for a genetic description of the a/a Δbcrl/Δbcrl mutant).

FIG 1 Deletion of BCR1 results in the collapse of hyphae in the upper portion of an a/a biofilm. (A) LSCM scans through the upper portion of a calciofluor white-stained a/a SC5314 (parental strain) biofilm. The stained (white) dots are cross sections of vertically oriented hyphae. (B) LSCM scans through the upper portion of a BCR1 deletion mutant (a/a) Δbcrl/Δbcrl biofilm. The stained wavy threads are collapsed hyphae. Bar, 100 μm.
These results demonstrate that in a/a biofilms, Bcr1, although not essential for the formation of the basic yeast cell polylayer or the basic formation of hyphae, is essential for the formation of a matrix that not only mediates the vertical orientation of the hyphae, and therefore contributes to maximum thickness, but also is essential for impermeability, impenetrability, and fluconazole resistance.

**Overexpressing Bcr1 in a/a biofilms.** Because it appeared from the deletion mutant data that Bcr1 regulates genes that confer these pathogenic traits in a/a biofilms, we considered the possibility that overexpressing BCR1 in a white a/a biofilm might confer these pathogenic traits. We therefore transformed cells of the natural a/a strain P37005 with a construct that contained BCR1 under the regulation of the tetracycline-inducible promoter TETp in one of the two copies of the ADH1 gene (53), generating the a/a-TETp-BCR1 strain (see Table S1 in the supplemental material for a genetic description). We then compared untreated and doxycycline-treated a/a-TETp-BCR1 biofilms for general architecture, impermeability, impenetrability, and fluconazole resistance. An analysis of transcript levels by RT-PCR revealed that doxycycline added at 0 h and again at 24 h upregulated BCR1 at both 16 and 48 h of a/a biofilm development (Fig. 3). a/a biofilms treated with doxycycline were reproducibly thinner than parental biofilms (P < 0.001) but were indistinguishable architecturally (Table 1). However, overexpression did result in a partial increase in impermeability to Sypro Ruby (Fig. 2D; Table 1), an increase in impenetrability by human PMNs (Fig. 2H; Table 1), and an increase in fluconazole resistance (P = 0.0001) (Fig. 2I; Table 1). To measure biofilm resistance to fluconazole, 48-h biofilms were treated with the drug for 24 h and then disrupted, and the proportion of cells that stained with Sytox Green, a dead cell dsDNA dye, was measured. Overexpression of BCR1 in a white a/a biofilm therefore brought the level of each pathogenic trait closer to that of wild-type a/a biofilms. These results are consistent with the hypothesis that Bcr1 regulates the expression of genes that encode matrix proteins conferring impermeability, impenetrability, and fluconazole resistance to a/a biofilms.

**Identifying genes upregulated by Bcr1.** The a/a-TETp-BCR1 overexpression mutant provided us with a screen for identifying genes upregulated by Bcr1 that facilitate the pathogenic traits of a/a biofilms. In the screen, the expression of each of 107 genes (Fig. 3) in biofilms formed by the a/a-TETp-BCR1 strain in the absence and presence of the inducer doxycycline was compared by RT-PCR. A number of these test genes were selected because they encode proteins associated with the plasma membrane or cell surface or were considered potential candidates because of their putative functions or association with a particular phenotype (see Table S5). The transcription levels were assessed at 16 h of biofilm development, when the basal yeast cell polylayer had formed, and after 48 h, when the more extensive upper region of vertically oriented hyphae and surrounding matrix had formed. The 107 genes tested and the RT-PCR primers employed for each are provided in Table S2 in the supplemental material.

Overexpression of BCR1 by the addition of doxycycline to developing a/a-TETp-BCR1 biofilms downregulated or upregulated 34 of the 107 genes (Fig. 3; Table 2). Twenty-four of these genes were downregulated at 16 or 48 h, eight were upregulated at 16 or 48 h, and two (HAP3 and KRE62) were downregulated at 16 h and then upregulated to their original levels at 48 h (Fig. 3; Table 2). Seven genes (CSA1, CSA2, PGA7, PGA10, RB5T, WH11, and CHKI) were upregulated by doxycycline at 16 and 48 h (Fig. 3; Table 2). One (AQY1) was upregulated only at 16 h but increased at 48 h to the same level in the absence or presence of doxycycline (Fig. 3; Table 2).

Here we focused on the eight genes upregulated by Bcr1 in a/a biofilms at 16 h or at 48 h. These included the genes for Csa1, Csa2, Pga7, Pga10, and Rbt5, which contain the “common

**FIG 2** Bcr1 plays a fundamental role in conferring upon a/a biofilms the traits of impermeability, impenetrability, and fluconazole resistance, deemed important in commensalism and pathogenicity. (A to D) Sypro Ruby impermeability (staining). (E to H) Impenetrability of Dil-stained human polymorphonuclear leukocytes. (I) Proportions of biofilm cells that underwent nuclear staining with Sytox Green dye after fluconazole treatment. All tested biofilms were developed on silicone elastomer in RPMI medium for 48 h before analysis. See Table S1 in the supplemental material for genetic descriptions and origins of stains. − doxy, no doxycycline treatment; + doxy, doxycycline treatment.
in fungal extracellular membrane" motif (CFEM) and have been shown to be involved in iron homeostasis (40–42); *AQY1*, which encodes a transmembrane water channel (43, 44); *CHK1*, which encodes an intracellular histidine kinase that functions in a two-component signaling pathway (45); and *WH11*, which encodes an intracellular white-phase-specific protein of unknown function (46, 47). Although we were able to analyze *CHK1* in a deletion mutant in an *a/a/H9251* background (see below), we could not study it in an overexpression mutant in an *a/a* background, because repeated attempts to generate the overexpression construct for transformation were unsuccessful, presumably because of the large size of the gene.

**Effects of overexpressing Bcr1-upregulated genes in a/a biofilms.** We first tested whether overexpressing any of the selected genes in an a/a strain partially or completely conferred one or more of the pathogenic traits of *a/a/H9251* biofilms as did overexpression of Bcr1. Overexpression strains were generated in the parent strain P37005 (*a/a*) (26), in which each of seven genes, *CSA1*, *CSA2*, *AQY1*, *PGA7*, *PGA10*, *RBT5*, and *WH11*, was individually placed under the regulation of the tetracycline promoter (53). The overexpression constructs were inserted into one of the alleles of the *ADH1* gene, which has been shown to serve as a neutral site for overexpression studies (31, 53, 62). The seven overexpression strains generated included the *a*/a-*TETp-CSA1*; *a*/a-*TETp-CSA2*; *a*/a-*TETp-AQY1*; *a*/a-*TETp-PGA7*; *a*/a-*TETp-PGA10*; *a*/a-*TETp-RBT5*; and *a*/a-*TETp-WH11* strains (see Table S1 in the supplemental material). LSCM analysis of calcofluor white-stained preparations revealed that in the absence of doxycycline, all seven strains formed biofilms similar to that of the parent strain P37005 (*a/a*). After 48 h, they had all formed a basal polylayer of unbudded, tightly cohering yeast cells that bound tightly to the substratum and an upper region of vertically ori-
Genes upregulated by overexpression of Bcr1

| Gene          | Regulation 16 h | Regulation 48 h |
|---------------|----------------|----------------|
| ECE1          | –              | –              |
| GCA1/2        | –              | –              |
| GSL1          | ↓              | ↓              |
| KRE6          | –              | –              |
| orf19.3338    | –              | –              |
| PGA31         | ↓              | ↓              |
| BRG1          | –              | –              |
| HSK1          | –              | –              |
| PHR1          | –              | –              |
| VPS20         | –              | –              |
| HWP1          | –              | –              |
| KRE1          | –              | –              |
| PHR2          | –              | –              |
| PMT4          | –              | –              |
| TOK1          | –              | –              |
| UGP1          | ↓              | ↓              |
| EAP1          | –              | –              |
| KRE5          | –              | –              |
| KTR4          | ↓              | ↓              |
| orf19.716     | ↓              | ↓              |
| orf19.2460    | ↓              | ↓              |
| PGA13         | ↓              | ↓              |
| PMT6          | –              | –              |
| TPO4          | –              | –              |

Genes downregulated by overexpression of Bcr1

| Gene          | Regulation 16 h | Regulation 48 h |
|---------------|----------------|----------------|
| CSA1          | ↑              | ↑              |
| CSA2          | ↑              | ↑              |
| AQY1          | ↑              | –              |
| PGA7          | ↑              | ↑              |
| PGA10         | ↑              | ↑              |
| RBT5          | ↑              | ↑              |
| WH11          | ↑              | ↑              |
| CHK1          | ↑              | ↑              |

Genes upregulated and then upregulated by Bcr1

| Gene          | Regulation 16 h | Regulation 48 h |
|---------------|----------------|----------------|
| HAP3          | ↓              | ↑              |
| KRE6          | –              | –              |

Notes:
- a See Fig. 2 for RT-PCR results for 107 screened genes. ↓, decrease; ↑, increase; –, no change.
- b Overexpression in the a/a-TETp-MCR1 strain was induced by doxycycline. See the legend to Fig. 2 for details.

In the presence of doxycycline, the biofilms of the six analyzable overexpression strains contained a basal yeast cell polylayer and an upper layer of vertically oriented hyphae embedded in extracellular matrix (Table 3). There were, however, architectural peculiarities. The biofilms of doxycycline-treated a/a-TETp-CSA1 and a/a-TETp-CSA2 cells contained pockets of dense extracellular matrix and tangled hyphae within the upper region, the biofilms of doxycycline-treated a/a-TETp-AQY1 cells contained an unusually dense extracellular matrix, and the biofilms of doxycycline-treated a/a-TETp-PGA7 and a/a-TETp-PGA10 cells contained tangled hyphae at the upper (distal) border (Table 3). Calcofluor white staining revealed that none of the biofilms of the overexpression strains were as uniform when treated with doxycycline as when left untreated. Doxycycline had no effect on the uniformity of the biofilms formed by the parent strain P37005 (data not shown).

We next tested whether overexpression of the six analyzable strains increased impermeability, human PMN penetration, or fluconazole resistance ([16]). Sypro Ruby remained at the top of biofilms formed by the a/a strain SC5314 (Fig. 4A) but penetrated the entire depth of biofilms formed by the a/a parent strain P37005 (Fig. 4B; Table 3), as previously reported ([16]). The dye penetrated through the entire or close to the entire depth of biofilms formed by the six overexpression strains in the absence of doxycycline (Table 3; Fig. 4C to G). However, the dye penetrated through only approximately half to three-fourths the depth of the doxycycline-treated biofilms of the six mutants (Table 3; Fig. 4C to G). Permeability was not uniform in most of these biofilms (Fig. 4C to G), consistent with the decrease in matrix uniformity.

Biofilms formed by the a/a strain SC5314 were highly impermeable by human PMNs (Fig. 4H; Table 3), whereas biofilms formed by the a/a parent strain P37005 were highly penetrable (Fig. 4I; Table 3) ([16]). PMNs penetrated the biofilms formed by all overexpression strains not treated with doxycycline but were partially or completely inhibited from penetrating the biofilms of the doxycycline-treated biofilms of the six analyzable overexpression strains (Fig. 4J to N; Table 3).

Finally, biofilms formed by the a/a strain SC5314 were highly resistant to fluconazole, whereas biofilms formed by the a/a parent strain P37005 were susceptible (Fig. 4O; Table 3) ([16]). In the absence of doxycycline, the proportions of cells that stained with Sytox Green, a dead cell dsDNA stain, after fluconazole treatment of the parental a/a strain P37005 and the six a/a overexpression derivatives were 4- to 6-fold greater than those of biofilms of the a/a strain SC5314 (Fig. 4O; Table 3). The addition of doxycycline decreased the number of cells stained with Sytox Green by 32 to 49% compared to the numbers in the absence of doxycycline for five of the six overexpression mutants (for CSA1, CSA2, AQY1, PGA7, and PGA10) (P values ranged from 0.0036 to 0.0001) (Fig. 4O; Table 3). Overexpressing WH11 had a smaller effect on fluconazole resistance that was not significant (P = 0.5) but was reproducible (Fig. 4O; Table 3). Together, the results on impermeability, impenetrability, and fluconazole resistance indicate that for each of the six testable Bcr1-upregulated genes, overexpression in an a/a biofilm conferred, to various degrees, the pathogenic traits exhibited by a/a biofilms.

Deleting Bcr1-regulated genes in an a/α strain. The overexpression results in an a/a background suggested that none of the six testable genes were involved in forming the basal yeast cell polylayer or in the basic formation of hyphae, but they were all...
involved, to various degrees, in the uniformity of the matrix and in conferring, to various degrees, the pathogenic traits of an a/α biofilm. However, overexpression in these cases was performed in an a/a genetic background, which we assume results in the activation and deactivation of genes specific for generating a specialized matrix that facilitates mating (17, Park et al., submitted). The most direct test for the involvement of the eight identified Bcr1-upregulated genes in an/a/a biofilm formation, therefore, was to assess the phenotypes of biofilms formed by a/α strains in which each of the eight genes were individually deleted (see Table S1 in the supplemental material for genetic descriptions). LSCM of calcofluor white-stained preparations revealed that all eight deletion mutants formed a basic biofilm composed of a basal polylayer of uninned yeast cells from which hyphae emerged (Table 4). Biofilms of all eight mutants, like those of the parent strain SC5314, contained hyphae embedded in an extracellular matrix in the upper biofilm region. For calcofluor white-stained preparations of the deletion mutants of CSA1, CSA2, AQY1, PGA7, and PGA10, however, small abnormalities in the basic architecture were observed, most notably decreases in the vertical orientation of hyphae and decreases in the density or a loss in the uniformity of the matrix, especially in the most distal regions of the upper hyphae-matrix region (Table 4). In many cases, there were mats or tangles of hyphae in the top layer of the upper biofilm region, suggesting a lack of matrix support. There were also highly stained pockets of matrix in the upper layer for several of the deletion mutants. Although this made the assessment of thickness less accurate, the averages for the eight mutants varied between 52 and 84 μm, with a mean (± SD) of 72 ± 10 μm, which, on average, was 13% below that of the parent strain SC5314 (Table 4).

There were also defects in the three pathogenic traits. The a/α deletion mutants of CSA1, CSA2, and AQY1 completely lost Sypro Ruby impermeability (Fig. 5C, D, and E, respectively; Table 4), while the remaining five a/α deletion mutants, for PGA7, PGA10, RBT5, CHK1, and WH11, underwent partial decreases (Fig. 4F, G, and H; Table 4). The biofilms of all eight of the a/α deletion mutants were completely or almost completely penetrated by PMNs (Fig. 5K to P; Table 4), in contrast to the impenetrability of biofilms of the a/a parent strain SC5314 (Fig. 5A; Table 4). Finally, the biofilms of all eight deletion mutants lost drug resistance. The percentage of cells that stained with Sytox Green after fluconazole treatment for the a/a strain P37005 was 11-fold higher than that of biofilms of the a/a strain SC5314 after treatment, and those of the eight deletion mutants varied between 7- and 13-fold higher than that of parental SC5314 (Fig. 4I; Table 4). These results together demonstrate that individually deleting each of the eight Bcr1-induced genes had various but reproducible effects on a/α biofilm architecture related to the extracellular matrix but, more importantly, showed major defects in the three pathogenic traits.

**Rescuing deletion mutants by complementation.** To demonstrate that the defects observed in a/α deletion mutants were due to the loss of each targeted gene, the deletion mutants for the CSA1, CSA2, AQY1, and PGA10 genes were transformed with a construct in which the coding region of each deleted gene was placed under the regulation of the tetracycline (doxycycline)-regulated promoter TETp (53). The derived complementation mutants were the a/α-Δcsa1/TETp-CSA1, a/α-Δcsa2/TETp-CSA2, a/α-Δaqy1/TETp-AQY1, and a/α-Δpga10/TETp-PGA10 strains (see Table S1 in the supplemental material for genetic descriptions). In the absence of doxycycline, the biofilms of all four of the strains formed a basal yeast cell polylayer with hyphae in the upper layer, encased in an extracellular matrix, that stained uniformly with calcofluor white (Table 4). But like the case with the parental deletion strains, these bio-

### Table 3: Architectural and pathogenic traits of biofilms formed by P37005 (a/a) derivatives in which genes found to be upregulated by overexpression of BCR1 in a/a cells were in turn individually overexpressed in a/a cells

| Strain          | Doxycycline treatment | Biofilm architecture<sup>b</sup> | Commensal/pathogenic traits<sup>b</sup> |
|-----------------|------------------------|-----------------------------------|-----------------------------------------|
|                 | Thickness (μm)          | Basal yeast polylayer             | Formation of hyphae                      | Vertical orientation of hyphae | ECM | Sypro Ruby impermeability | Human PMN impenetrability | Fluconazole resistance (%) |
| SC5314          | −                      | 86 ± 14                           | ++++                                    | ++++                          | ++++ | +++++                     | 3 ± 2                       |
| P37005 (a/a)    | −                      | 72 ± 3                            | ++++                                    | ++++                          | +    | −                         | 35 ± 7                      |
| a/a-TETp-CSA1   | −                      | 76 ± 13                           | ++++                                    | ++++                          | +    | −                         | 37 ± 3                      |
|                 |                        | +                                  | 84 ± 4                                  | ++++                          | +++  | −                         | 20 ± 2                      |
| a/a-TETp-CSA2   | −                      | 75 ± 15                           | ++++                                    | ++++                          | +    | −                         | 25 ± 3                      |
|                 |                        | +                                  | 78 ± 3                                  | ++++                          | +++  | −                         | 17 ± 2                      |
| a/a-TETp-AQY1   | −                      | 72 ± 7                            | ++++                                    | ++++                          | +    | −                         | 31 ± 5                      |
|                 |                        | +                                  | 75 ± 3                                  | ++++                          | +++  | −                         | 16 ± 6                      |
| a/a-TETp-PGA7   | −                      | 64 ± 11                           | ++++                                    | ++++                          | +    | −                         | 34 ± 3                      |
|                 |                        | +                                  | 80 ± 3                                  | ++++                          | +++  | −                         | 20 ± 5                      |
| a/a-TETp-PGA10  | −                      | 64 ± 6                            | ++++                                    | ++++                          | +    | −                         | 37 ± 7                      |
|                 |                        | +                                  | 84 ± 18                                  | ++++                          | +++  | −                         | 20 ± 4                      |
| a/a-TETp-RBT5   | −                      | 76 ± 6                            | ++++                                    | ++++                          | +    | −                         | 33 ± 5                      |
|                 |                        | +                                  | 75 ± 2                                  | ++++                          | +++  | −                         | 29 ± 3                      |

<sup>a</sup> Overexpression derivatives for CHK1 could not be generated, presumably because of the large size of the gene.

<sup>b</sup> See the footnotes to Table 1 for details on headings.

<sup>c</sup> Because the biofilm formed by the a/a-TETp-RBT5 strain in the presence of doxycycline was composed of pseudohyphae and did not bind to the silicone elastomer, we could not analyze it sufficiently for architecture or the commensal/pathogenesis characteristics of a/a biofilms.

<sup>d</sup> The upper regions of doxycycline-treated biofilms of the a/a-TETp-CSA1 and a/a-TETp-CSA2 strains contained pockets of dense extracellular matrix and tangled hyphae.

<sup>e</sup> The upper regions of doxycycline-untransformed biofilms of the a/a-TETp-AQY1 strain contained an unusually dense matrix.

<sup>f</sup> The surfaces of doxycycline-treated biofilms of the a/a-TETp-PGA7 and a/a-TETp-PGA10 strains contained tangled hyphae.
films were thinner than those of the original a/α parent strain SC5314 and exhibited defects in the uniformity of the upper layer, including decreases in the vertical orientation of hyphae and defects in matrix distribution, that were obvious in calcofluor white-stained preparations (Table 4). For each of the four strains, addition of doxycycline to the biofilm caused increases in thickness to values closer to that of the original parent strain SC5314, vertical orientation of hyphae, and uniformity of the matrix (Table 4). The addition of doxycycline to all four complemented strains caused almost a complete rescue of impermeability to Sypro Ruby (Fig. 6A to D; Table 4), a complete rescue of human PMN penetrability (Fig. 6E to H; Table 4), and a partial rescue of fluconazole resistance (Fig. 6I; Table 4). The decreases in susceptibility of the CSA1-, CSA2-, AQY1-, and PGA10-complemented strains were 2.5-, 2.7-, 1.8-, and 2.6-fold, respectively, compared to the parental deletion mutants (Table 4).

**DISCUSSION**
The major role played by Bcr1 in a/α biofilm formation was first identified by Nobile and Mitchell (24). They found in their model, using Spider medium, that Bcr1 was necessary for forming a normal biofilm on silicone elastomer squares, and they and others further showed that Bcr1 regulates a number of surface molecules, several of which are adhesins associated with hypha formation (20–22, 37, 57, 59, 63–65). In this study, using a model containing a rich medium, in which a/a cells form an adhesive and cohesive basal yeast cell polylayer and an upper layer of vertically oriented hyphae embedded in a dense matrix, we showed that deletion of BCR1 has no effect on the formation of the adhesive and cohesive basal yeast cell polylayer at the substratum and no effect on the basic capacity to form hyphae. It does, however, lead to selective changes in the architecture of the upper region of the biofilm as well as to the loss of biofilm traits deemed important for commensalism and pathogenesis. In the BCR1 deletion mutant of a/a cells, the hyphae in the upper region of the biofilm collapse, presumably because the matrix is reduced. The reduction of matrix in Δbcr1/Δbcr1 biofilms formed in an *in vivo* vaginal model had previously been demonstrated by Noverr and coworkers (39). When BCR1 is deleted in a/a cells, again no changes occur in the formation of the

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**FIG 4** Effects of overexpressing Bcr1-upregulated genes in white a/a biofilms upon impermeability, penetrability, and fluconazole resistance. (A to G) Sypro Ruby impermeability. (H to N) Impenetrability of Dil-stained human polymorphonuclear leukocytes. (O) Proportions of biofilm cells that underwent nuclear staining with Sytox Green, a dsDNA stain, after fluconazole treatment. All tested biofilms were developed for 48 h. See Table S1 in the supplemental material for genetic descriptions and histories of the strains.
Therefore, all eight of the genes identified in this screen play roles, 
tantly, caused major decreases in the three pathogenic traits tested.

Because hyphae of the yeast cell polylayer or the capacity to extend hyphae from the basal 
layer, but in this MTL-homozygous background, there are also no changes in the general matrix-dependent architecture of the upper 
region of the biofilm, as visualized by calcofluor white staining (i.e., the vertical orientation of hyphae and a uniformly dense 
matrix), and no changes in the already absent pathogenic traits. Together, these results suggest that Bcr1 plays a major role in 
regulating genes involved in a/α matrix formation. It should be noted, however, that 
because the biofilms of the BCR1 deletion mutant a/a strains are reproducibly thinner (by 10%), as shown here and previously 
(16), we cannot rule out a minor role for Bcr1 in a/a biofilm formation. Because hyphae of the BCR1 deletion mutant a/a 
biomodels remain vertical and the matrix is dense and uniform, we conclude that a transcription factor other than Bcr1 plays a major 
role in regulating genes involved in the formation of the a/a-specific matrix, which facilitates mating (17; Park et al., submitted).

**Bcr1-regulated genes.** Based on the observation that overexpressing BCR1 in an a/a biofilm partially confers the a/α biofilm 
traits of impermeability, impenetrability, and drug resistance, we screened 107 genes in a/a biofilms overexpressing BCR1 for genes 
upregulated by BCR1 overexpression by using RT-PCR. Although we identified 24 genes that were downregulated and 2 genes that 
were downregulated and then upregulated to their original levels, we focused upon 8 genes that were only upregulated at 16 h or at 
16 and 48 h of biofilm development. Six of them, which lent themselves to overexpression studies, partially or fully conferred one or 
more of the three pathogenic traits of a/a biofilms when overexpressed in a/a biofilms. Furthermore, individually deleting each of the eight genes in a/α cells affected, to different degrees, the architecture of the upper portion of a/α biofilms and, more importantly, caused major decreases in the three pathogenic traits tested. Therefore, all of the genes identified in this screen play roles, 

| Strain   | Doxycycline treatment | Thickness (μm) | Basal yeast polylayer | Formation of hyphae | Vertical orientation of hyphae | ECM | Sypro Ruby impermeability | PMN impenetrability | Fluconazole resistance (%) |
|----------|-----------------------|---------------|-----------------------|---------------------|-----------------------------|-----|------------------------|----------------------|--------------------------|
| SC5314 (a/α) | -                     | 83 ± 10       | ++ + +                | +++ +               | +++ +                     | +++ | + + +                  | + + +                 | 3 ± 2                    |
| P37005 (a/a) | -                     | 73 ± 4        | ++ + +                | +++ +               | +++ +                     | +   | -                      | -                    | 35 ± 7                   |
| a/α−Δcas1/Δcas1 | -                     | 80 ± 4        | ++ + +                | +++ +               | +++ +                     | ++ b| -                      | -                    | 38 ± 12                  |
| a/α−Δcas2/Δcas2 | -                     | 70 ± 5        | ++ + +                | +++ +               | +++ +                     | ++ b| -                      | -                    | 32 ± 11                  |
| a/α−Δngy/Δngy | -                     | 63 ± 4        | ++ + +                | +++ +               | +++ +                     | ++ b| -                      | -                    | 23 ± 13                  |
| a/α−Δpga7/Δpga7 | -                     | 84 ± 5        | ++ + +                | +++ +               | +++ +                     | ++ b| -                      | -                    | 20 ± 9                   |
| a/α−Δpga10/Δpga10 | -                    | 52 ± 8        | ++ + +                | +++ +               | +++ +                     | ++ b| -                      | -                    | 36 ± 13                  |
| a/α−Δbrf5/Δbrf5 | -                     | 78 ± 9        | ++ + +                | +++ +               | +++ +                     | +   | -                      | -                    | 26 ± 7                   |
| a/α−Δwhk1/Δwhk1 | -                     | 71 ± 5        | ++ + +                | +++ +               | +++ +                     | +   | -                      | -                    | 20 ± 6                   |
| a/α−Δwhl11/Δwhl11 | -                    | 78 ± 2        | ++ + +                | +++ +               | +++ +                     | +   | -                      | -                    | 22 ± 8                   |
| a/α−Δcas1/Δcas1−TETp CSA1 | -                 | 67 ± 3        | ++ + +                | +++ +               | +++ +                     | +   | -                      | -                    | 37 ± 2                   |
| a/α−Δcas2/Δcas2−TETp−CSA2 | +                 | 78 ± 3        | ++ + +                | +++ +               | +++ +                     | +   | -                      | -                    | 15 ± 3                   |
| a/α−Δngy/Δngy−TETpQY1 | +                 | 84 ± 6        | ++ + +                | +++ +               | +++ +                     | +   | -                      | -                    | 30 ± 10                  |
| a/α−Δpga10/Δpga10−TETp PGA10 | +                | 54 ± 8        | ++ + +                | +++ +               | +++ +                     | +   | -                      | -                    | 11 ± 5                   |
| a/α−Δpga10/Δpga10−TETp PGA10 | +                | 82 ± 8        | ++ + +                | +++ +               | +++ +                     | +   | -                      | -                    | 14 ± 5                   |

**TABLE 4 Architectural and pathogenic traits of biofilms formed by deletion mutants of Bcr1-regulated genes in an a/α strain and by four of the deletion mutants complemented with the relevant intact gene**

- See the footnotes to Table 1 for details on headings.
- The biofilms showed a lack of uniformity of the matrix when analyzed after calcofluor white staining as described in the text.

directly or indirectly, in conferring impermeability, impenetrability, and drug resistance to a/α biofilms. Five of the eight identified Bcr1-upregulated genes had previously been shown to be involved in iron homeostasis (CSA1, CSA2, PGA7, PGA10, and RBT5) (40–42). This may be no accident given the importance of competition for iron between bacterial biofilms and the host (66). In the case of Candida albicans, the proteins encoded by all five of the iron homeostasis genes had a common domain, the CFEM motif (common in fungal extracellular membrane), containing eight cysteine residues (41). Four of the eight proteins (Cas1, Pag7, Pga10, and Rbt5) contained a glycosylphosphatidylinositol (GPI) anchor that could result in covalent linkages of GPI glycan to β-glucan in the cell wall and matrix (67–70). Cas1 localizes in the hyphal wall (67, 71) and was previously shown to be upregulated by Bcr1 (20). Consistent with our results, Perez et al. (72, 73) showed a decrease in the thickness of biofilms formed by an a/α CSA1 deletion mutant. Although Csa2 does not contain a GPI anchor, it has been shown to be an extracellular protein (74). It has not previously been shown to be upregulated by Bcr1 or to play a role in biofilm formation. PGA7 has been shown to be upregulated in hyphae (75) and regulated by Bcr1 (20, 76). It also has not previously been shown to be involved in a/α biofilm formation. PGA10 has been shown to be upregulated in hyphae (73) and regulated by Bcr1 (76). Deleting PGA10 in both a/α and a/a cells was previously shown to affect both a/α and a/a biofilm formation (72, 73, 77). Finally, RBT5 has been shown to be upregulated in hyphae (67) and regulated by Bcr1 (20, 24, 76). Deletion of RBT5 was previously shown to cause a decrease in the thickness of a/α biofilms (72, 73). Therefore, the products of four of the five iron homeostasis genes that are involved in conferring a/α biofilms with pathogenic traits (Cas1, Pag7, Pga10, and Rbt5) contain GPI anchors that can cross-link these proteins to β-glucan (78). Hence, these proteins may be
involved directly in linking hyphal walls to the extracellular matrix, given that the wall and matrix both contain \(\beta\)-glucans (69, 79, 80). Although Csa2 does not contain a GPI anchor, it is extracellular (74) and thus could also interact directly with the matrix. The covalent linkage of GPI anchors to \(\beta\)-glucans is not the only mechanism by which these cell wall and plasma membrane proteins can be cross-linked to the cell wall and matrix. Glycoproteins can also be cross-linked to the cell wall and matrix through their O-linked or N-linked oligosaccharides (81). In *Neurospora crassa*, it has been shown that \(\alpha\)-1,6-mannan residues of N-linked oligosaccharides are involved in the covalent association of glycoproteins with the cell wall, possibly through a cross-linkage with glucans (82). Similar cross-linking could participate in *C. albicans* cell wall and matrix architecture, since orthologs of Dfg5 and Dcw1, the \(\alpha\)-1,6-mannanases required for this process, are present in *C. albicans* and have been shown to have the same specificity (81). We analyzed the five CFEM proteins for the presence of putative glycosylation sites by using two glycosylation prediction servers (see Table S6 in the supplemental material). The 5 proteins had predicted O-linked glycosylation, and both Csa1 and Csa2 contained putative N-linked glycosylation sites. These glycosylation sites could play a cross-linking role between CFEM proteins and glucans, most notably between the hyphal cell wall and the extracellular matrix of the upper region of a biofilm. Such an interaction may explain why deleting Bcr1 results in the collapse of hyphae in the upper region of an a/α biofilm. Therefore, although all five of these proteins have been shown to play roles in iron homeostasis, they may also play a direct role in the architecture of the matrix, which may be responsible for the vertical orientation of hyphae, biofilm thickness, impermeability, impenetrability, and drug resistance.

**Aqy1**, an aquaporin water channel (44), is located in the plasma membrane (43) and has been shown to play a role in resistance to freeze-thawing (83). It has not previously been shown to play a role in biofilm development. It contains one putative O-linked oligosaccharide with the potential for cross-linking with the cell wall (see Table S5 in the supplemental material). Chk1 is a cytoplasmic histidine kinase involved in a signaling pathway that regulates cell wall biosynthesis (45), presumably through mannan and \(\beta\)-glucan synthesis, which could indirectly affect matrix composition and architecture. While Kruppa et al. (84) found a slight biofilm defect in a CHK1 deletion mutant with a metabolic assay using a tetrazolium dye, Perumal et al. (85) found no difference in thickness between wild-type and deletion mutant biofilms. While Wh11 is also a cytoplasmic protein expressed specifically in the white phase of the switching process in MTL-homozygous *C. albicans* (46, 47), nothing is known of its basic function, and nothing had been known regarding a role for it in biofilm development. Its possible role in a/α matrix formation remains obscure.

**Conclusions.** The results presented here on the role of Bcr1 in

![FIG 5](image-url)
a/α biofilm formation support the hypothesis that while Tec1 regulates the formation of an adherent and coherent basal yeast cell polylayer and the extension distally of hyphae from the surface of the polylayer in a/α biofilms, Bcr1 regulates matrix formation. The Bcr1-regulated a/α matrix in turn appears to confer the a/α biofilm-specific traits of impermeability, impenetrability by human PMNs, and drug resistance. Although Tec1 also regulates the formation of the yeast cell polylayer and the basic formation of hyphae in a/α biofilms, Bcr1 does not appear to regulate the formation of the permeable, penetrable, and drug-susceptible a/α matrix. The hypothesized transcription factor that regulates a/α matrix formation remains unidentified. In this study, by screening 107 genes of interest, we identified eight Bcr1-upregulated genes, all of which play a role in conferring the three traits studied, i.e., permeability, PMN penetrability, and fluconazole resistance, all three of which are presumed to be due to the specialized a/α matrix. Interestingly, three of the eight genes identified encode proteins that either are in the wall or plasma membrane or are secreted. All six have glycosylation sites, and four have GPI anchors, all of which can potentially link them to the wall and matrix. A seventh protein, which is intracellular, is involved in the synthesis of matrix polysaccharides. Our results demonstrating that all eight of the Bcr1-upregulated genes proved to be involved in the integrity of the a/α matrix and the impermeability, impenetrability, and drug resistance traits underscore the importance of BCR1 (23). Also, given that all eight were so readily identified in a screen of 107 genes, we suggest that many more genes with similar roles will be identified, attesting to the complexity of a/α matrix formation.

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