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Bcr1 Functions Downstream of Ssd1 To Mediate Antimicrobial Peptide Resistance in *Candida albicans*

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In order to colonize the host and cause disease, *Candida albicans* must avoid being killed by host defense peptides. Previously, we determined that the regulatory protein Ssd1 governs antimicrobial peptide resistance in *C. albicans*. Here, we sought to identify additional genes whose products govern susceptibility to antimicrobial peptides. We discovered that a *bcr1*Δ/Δ mutant, like the *ssd1*Δ/Δ mutant, had increased susceptibility to the antimicrobial peptides, protamine, RP-1, and human β defensin-2. Homozygous deletion of *BCR1* in the *ssd1*Δ/Δ mutant did not result in a further increase in antimicrobial peptide susceptibility. Exposure of the *bcr1*Δ/Δ and *ssd1*Δ/Δ mutants to RP-1 induced greater loss of mitochondrial membrane potential and increased plasma membrane permeability than with the control strains. Therefore, Bcr1 and Ssd1 govern antimicrobial peptide susceptibility and likely function in the same pathway. Furthermore, *BCR1* mRNA expression was downregulated in the *ssd1*Δ/Δ mutant, and the forced expression of *BCR1* in the *ssd1*Δ/Δ mutant partially restored antimicrobial peptide resistance. These results suggest that Bcr1 functions downstream of Ssd1. Interestingly, overexpression of 11 known Bcr1 target genes in the *bcr1*Δ/Δ mutant failed to restore antimicrobial peptide resistance, suggesting that other Bcr1 target genes are likely responsible for antimicrobial peptide resistance. Collectively, these results demonstrate that Bcr1 functions downstream of Ssd1 to govern antimicrobial peptide resistance by maintaining mitochondrial energetics and reducing membrane permeabilization.

The fungus *Candida albicans* colonizes the skin and mucosal surfaces of healthy individuals, and colonization is necessary for the organism to cause both superficial and invasive disease. In order to successfully colonize the host and cause disease, *C. albicans* must resist killing by antimicrobial peptides produced by epithelial cells, leukocytes and platelets. In humans, these antimicrobial peptides include defensins, histatins, cathelicidins, kinocidins, and lactoferrin and transferrin family peptides (1).

Several mechanisms that enable *C. albicans* to resist the injurious effects of antimicrobial peptides have been identified. These mechanisms include inactivation of antimicrobial peptides via either cleavage by secreted aspartyl proteases (2) or binding by secreted fragments of Msb2 (3). In addition, stress response pathways within the fungus are important for resistance to antimicrobial peptides. For example, an intact Hog1 mitogen-activated protein kinase pathway is required for resistance to multiple antimicrobial peptides (4, 5). Previously, we determined that the regulatory factor Ssd1 plays a key role in antimicrobial peptide resistance in *C. albicans* (6). A strain in which *SSD1* was deleted was hypersusceptible to certain antimicrobial peptides, whereas strains that overexpressed *SSD1* were resistant to these peptides. In addition, in a murine model of disseminated candidiasis, an *ssd1*Δ/Δ null mutant had attenuated virulence, suggesting that Ssd1-mediated antimicrobial peptide resistance may contribute to virulence of *C. albicans*. However, the mechanism(s) through which *SSD1* contributes to antimicrobial peptide resistance in *C. albicans* was unknown.

The goal of the current study was to identify additional genes whose products mediate peptide resistance in *C. albicans*. Using a candidate gene approach, we discovered that the *BCR1* gene product, a transcription factor, functions downstream of *SSD1* to mediate resistance to some antimicrobial peptides.

MATERIALS AND METHODS

Strains and growth conditions. The *C. albicans* mutant strains used in this study are summarized in Table 1. The 93 clinical strains of *C. albicans* that were screened were blood isolates obtained from a multicenter surveillance study of candidemia in South Korea (7) All strains were maintained on YPD agar (1% yeast extract [Difco], 2% peptone [Difco], and 2% glucose plus 2% Bacto agar). *C. albicans* transformants were selected on synthetic complete medium (2% dextrose and 0.67% yeast nitrogen base [YNB] with ammonium sulfate, and auxotrophic supplements). For use in the experiments, the strains were grown in YPD broth in a shaking incubator at 30°C overnight. The resulting yeasts were harvested by centrifugation and enumerated with a hemacytometer as previously described (8).

Strain construction. All *C. albicans* mutant strains constructed for this study were derived from strain BWP17 (9). Deletion of the entire protein-coding regions of both alleles of *RTA2* was accomplished by successive transformation with *ARG4* and *HIS1* deletion cassettes that were generated by PCR using the primers RTA2-5DR and RTA2-3DR (see Table S1 in the supplemental material). The resulting strain was subsequently transformed with a *URA3-IRO1* fragment, which was released from pBSK-URA3 by NotI/PstI digestion, to reintegrate *URA3* at its native

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TABLE 1 Strains of *C. albicans* used in this study

Strain	Genotype	Reference
CA024	Wild type (bloodstream isolate)	
CA080	Wild type (bloodstream isolate)	
DAY185	*ura3*::λ*imm434/ura3*::λ*imm434 his1*::*hisG*::*pHIS1/his1*::*hisG arg4*::*hisG*::*ARG4-URA3/arg4*::*hisG*	9
CW195	*ura3*::λ*imm434/ura3*::λ*imm434 his1*::*hisG*::*pHIS1/his1*::*hisG arg4*::*hisG/arg4*::*hisG bcr1*::*ARG4/bcr1*::*URA3*	This study
CW193	*ura3*::λ*imm434/ura3*::λ*imm434 his1*::*hisG*::*pHIS1-BCR1/his1*::*hisG arg4*::*hisG/arg4*::*hisG bcr1*::*ARG4/bcr1*::*URA3*	This study
rta2 Δ/Δ	*ura3*::λ*imm434/ura3*::λ*imm434*::*URA3 his1*::*hisG/his1*::*hisG arg4*::*hisG/arg4*::*hisG rta2*::*HIS1/rta2*::*ARG4*	This study
APRΔ-1 (*ssd1*Δ/Δ-I)	*ura3*::λ*imm434/ura3*::λ*imm434*::*URA3 his1*::*hisG/his1*::*hisG arg4*::*hisG/arg4*::*hisG ssd1*::*HIS1/ssd1*::*ARG4*	6
APRΔ-1comp (*ssd1*Δ/Δ-I::*SSD1*)	*ura3*::λ*imm434/ura3*::λ*imm434*::*URA3 his1*::*hisG/his1*::*hisG arg4*::*hisG/arg4*::*hisG ssd1*::*HIS1/ssd1*::*ARG4*::*SSD1*	6
*ssd1*Δ/Δ-*bcr1*Δ/Δ-I and *ssd1*Δ/Δ-*bcr1*Δ/Δ-II	*ssd1*Δ::*HIS1/ssd1*Δ::*ARG4 bcr1*Δ::*URA3/bcr1*Δ::*NAT1 ura3*Δ::λ*imm434/ura3*Δ::λ*imm434 arg4*::*hisG/arg4*::*hisG his1*::*hisG/his1*::*hisG*	This study
*ssd1*Δ/Δ+BCR1-OE-I and *ssd1*Δ/Δ+BCR1-OE-II	*ura3*::λ*imm434/ura3*::λ*imm434*::*URA3 his1*::*hisG/his1*::*hisG arg4*::*hisG/arg4*::*hisG ssd1*::*HIS1/ssd1*::*ARG4 BCR1*::*pAgTEF1-NAT1-AgTEF1UTR-TDH3-BCR1/BCR1*	This study
*bcr1*Δ/Δ+SSD1-OE-I and *bcr1*Δ/Δ+SSD1-OE-II	*ura3*::λ*imm434/ura3*::λ*imm434 his1*::*hisG*::*pHIS1/his1*::*hisG arg4*::*hisG/arg4*::*hisG bcr1*::*ARG4/bcr1*::*URA3 SSD1*::*pAgTEF1-NAT1-AgTEF1UTR-TDH3-SSD1/SSD1*	This study
CJN1144	*ura3*::λ*imm434/ura3*::λ*imm434 his1*::*hisG*::*pHIS1/his1*::*hisG arg4*::*hisG/arg4*::*hisG bcr1*::*ARG4/bcr1*::*URA3 TEF1-ALS1*::*NAT1/ALS1*	19
CJN1153	*ura3*::λ*imm434/ura3*::λ*imm434 his1*::*hisG*::*pHIS1/his1*::*hisG arg4*::*hisG/arg4*::*hisG bcr1*::*ARG4/bcr1*::*URA3 TEF1-ALS3*::*NAT1/ALS3*	19
CJN1222	*ura3*::λ*imm434/ura3*::λ*imm434 his1*::*hisG*::*pHIS1/his1*::*hisG arg4*::*hisG/arg4*::*hisG bcr1*::*ARG4/bcr1*::*URA3 TEF1-HWP1*::*NAT1/HWP1*	19
CJN1259	*ura3*::λ*imm434/ura3*::λ*imm434 his1*::*hisG*::*pHIS1/his1*::*hisG arg4*::*hisG/arg4*::*hisG bcr1*::*ARG4/bcr1*::*URA3 TEF1-HYR1*::*NAT1/HYR1*	19
CJN1276	*ura3*::λ*imm434/ura3*::λ*imm434 his1*::*hisG*::*pHIS1/his1*::*hisG arg4*::*hisG/arg4*::*hisG bcr1*::*ARG4/bcr1*::*URA3 TEF1-RBT5*::*NAT1/RBT5*	19
CJN1281	*ura3*::λ*imm434/ura3*::λ*imm434 his1*::*hisG*::*pHIS1/his1*::*hisG arg4*::*hisG/arg4*::*hisG bcr1*::*ARG4/bcr1*::*URA3 TEF1-CHT2*::*NAT1/CHT2*	19
CJN1288	*ura3*::λ*imm434/ura3*::λ*imm434 his1*::*hisG*::*pHIS1/his1*::*hisG arg4*::*hisG/arg4*::*hisG bcr1*::*ARG4/bcr1*::*URA3 TEF1-ECE1*::*NAT1/ECE1*	19
JF11	*ura3*::λ*imm434/ura3*::λ*imm434 his1*::*hisG*::*pHIS1/his1*::*hisG arg4*::*hisG/arg4*::*hisG bcr1*::*ARG4/bcr1*::*URA3 PGA10*::*pAgTEF1-NAT1-AgTEF1UTR-TDH3-PGA10/PGA10*	This study
JF25	*ura3*::λ*imm434/ura3*::λ*imm434 his1*::*hisG*::*pHIS1/his1*::*hisG arg4*::*hisG/arg4*::*hisG bcr1*::*ARG4/bcr1*::*URA3 CSA1*::*pAgTEF1-NAT1-AgTEF1UTR-TDH3-CSA1/CSA1*	This study
*bcr1*Δ/Δ+MAL31-OE	*ura3*::λ*imm434/ura3*::λ*imm434 his1*::*hisG*::*pHIS1/his1*::*hisG arg4*::*hisG/arg4*::*hisG bcr1*::*ARG4/bcr1*::*URA3 MAL31*::*pAgTEF1-NAT1-AgTEF1UTR-TDH3-MAL31/MAL31*	This study
*bcr1*Δ/Δ+RTA1-OE	*ura3*::λ*imm434/ura3*::λ*imm434 his1*::*hisG*::*pHIS1/his1*::*hisG arg4*::*hisG/arg4*::*hisG bcr1*::*ARG4/bcr1*::*URA3 RTA1*::*pAgTEF1-NAT1-AgTEF1UTR-TDH3-RTA1/RTA1*	This study

locus (8). Proper integration of the *URA3-IRO1* fragment was confirmed by PCR using the primers URA3-F and URA3-R (see Table S2 in the supplemental material).

To delete the entire protein-coding region of *BCR1* in the *ssd1*Δ/Δ mutant, deletion cassettes containing *BCR1* flanking regions and the *URA3* or *NAT1* selection marker were amplified by PCR with primers BCR1-5DR and BCR1-3DR (see Table S1 in the supplemental material), using pGEM-URA3 (9) and pJK795 (10) as templates, respectively. These PCR products were then used to successively transform a Ura$^-$ *ssa1*Δ/Δ strain.

To construct gene overexpression strains, a DNA fragment containing the *NAT1* nourseothricin resistance gene and the *TDH3* promoter was integrated upstream and adjacent to the protein-coding region of the gene to be overexpressed (11). The TDH3-*BCR1* overexpression strains were constructed by transforming *C. albicans* with a DNA fragment generated by PCR using plasmid pCJN542 (11) as the template and the primers BCR1OE-5′ and BCR1OE-3′ (see Table S1 in the supplemental material). Following a similar approach, primers SSD1OE-5′ and SSD1OE-3′ (see Table S1 in the supplemental material) were used for overexpression of *SSD1*, primers MAL31OE-5′ and MAL31OE-3′ were used for overexpression of *MAL31*, and primers RTA1OE-5′ and RTA1OE-3′ were used for overexpression of *RTA1*.

Radial diffusion assays. The susceptibilities of the different *C. albicans* strains to the various antimicrobial peptides (Table 2) were determined using a radial diffusion assay (12). Organisms were mixed with 1,4-piperazinediethanesulfonic acid (PIPES) (10 mM, pH 7.5)-buffered agarose at a final concentration of 10^6 CFU/ml and then added to petri dishes. Next, cylindrical wells were cut into the agar, and 10-μg amounts of the antimicrobial peptides human neutrophil defensin 1 (HNP-1), human β-defensin 2 (hBD-2), LL-37, and RP-1 were added to the wells. After 3 h of incubation at 30°C, the plate was overlaid with YNB agar and incubated at 30°C for 24 h, and then the zone of inhibition were measured. Each experiment was performed at least twice.

Susceptibility to protamine sulfate and nonpeptide stressors. The susceptibilities of the various *C. albicans* strains to protamine and nonpeptide stressors were tested using spot dilution assays. Serial 10-fold dilutions of *C. albicans* ranging from 10^5 to 10^1 CFU were plated in 5-μl volumes on YPD agar containing protamine sulfate (Sigma-Aldrich), SDS, or Congo red and incubated at 30°C. The growth was recorded every 24 h.

The susceptibilities of selected clinical isolates of *C. albicans* to amphotericin B, fluconazole, voriconazole, caspofungin, and micafungin were determined by the Clinical and Laboratory Standards Institute M27-A3 method (13).

Real-time PCR. *C. albicans* expression of *BCR1*, *RTA2*, and *SSD1* was determined by real-time PCR. Total RNA was extracted from logarithmic-phase *C. albicans* cells using the hot-phenol method. In some experiments, the *C. albicans* strains (10^7 CFU/ml) were grown in the presence and absence of sublethal concentrations of RP-1 in PIPES (10 mM, pH 7.5) at serial time points prior to RNA extraction. Quantitative real-time PCR was carried out using the SYBR green PCR kit (Applied Biosystems) and an ABI 7000 real-time PCR system (Applied Biosystems) following the manufacturer's protocol. The primers used in these experiments are listed in Table S1 in the supplemental material. The results were analyzed by the $\Delta\Delta C_T$ method, using the transcript level of the *C. albicans ACT1* gene as the endogenous control. The mRNA levels for each gene were determined in at least three biological replicates, and the results were combined.

Flow cytometry. Multicolor flow cytometry was used to assess the effects of the antimicrobial peptides on *C. albicans*. The fluorophores used were as follows: membrane permeabilization, propidium iodide (PI) (Sigma-Aldrich); transmembrane potential, 3,3-dipentyloxacarbocyanine ($DiOC_5$) (Invitrogen); and phosphatidylserine accessibility, annexin V (allophycocyanin conjugate; Invitrogen) (14). In these experiments, 10^6 *C. albicans* cells were incubated with RP-1 (5 μg/ml) in 100 μl PIPES (pH 7.5) for 1 h with shaking at 30°C. The cells were stained for 10 min at room temperature by adding 900 μl stain buffer (PI, 5.0 μg/ml; $DiOC_5$, 0.5 μM; and annexin V, 2.5 μM in 50 mM K^+MEM). Flow cytometry was performed using a FACSCalibur instrument (Becton Dickinson). The fluorescence of at least 5×10^3 cells was analyzed.

Statistical analysis. Differences in *C. albicans* gene expression and susceptibility to antimicrobial peptides were compared by analysis of variance. *P* values of ≤0.05 were considered to be significant.

RESULTS

Antimicrobial peptide resistance in clinical *C. albicans* bloodstream isolates is distinct from antifungal resistance. To identify naturally occurring *C. albicans* strains with altered susceptibility to antimicrobial peptides, a panel of 93 bloodstream isolates was screened for susceptibility to protamine, a helical cationic polypeptide that is frequently used to screen for antimicrobial peptide susceptibility (15, 16). Strains with markedly increased or decreased susceptibility to protamine were subsequently tested for susceptibility to other antimicrobial peptides, including RP-1, hBD-2, HNP-1, and LL-37. From this collection of strains, we selected strain CA024 (Amp^s), which was more susceptible than the DAY185 reference strain to hBD-2, LL-37, and RP-1 (Fig. 1A). We also selected strain CA080 (Amp^r), was which less susceptible than strain DAY185 to all peptides tested. Of note, all of these strains had similar susceptibility to amphotericin B, fluconazole, voriconazole, caspofungin, and micafungin (see Table S2 in the supplemental material), suggesting that susceptibility to antimicrobial peptides is unrelated to susceptibility to conventional antifungal agents.

Expression profiling of candidate genes indicates that *RTA2* and *BCR1* are differentially expressed in the Amp^s and Amp^r strains. To assess the genes whose products mediate antimicrobial peptide resistance in *C. albicans*, the Amp^s and Amp^r strains were exposed for various times to a sublethal concentration of RP-1 at which 90% of the organisms survived after a 1-h exposure (2.5 μg/ml for the Amp^s strain, 100 μg/ml for the Amp^r strain, and 5 μg/ml for DAY185). Next, we used real-time PCR to compare the transcript levels of 9 candidate resistance genes in these strains. The products of the candidate genes were representative of targets or signaling pathway components known or hypothesized to contribute to microbial resistance to host defense peptides. These candidate genes included

TABLE 2 Antimicrobial peptides used in this study

Peptide	Class	Charge	Amino acid sequence	Secondary structure	Tissue source	Proposed mechanism of action	Reference
Protamine	Polyamine	+21	PRRRRSSSRPIRRRRPRRASRRRRRRGGRRRR	Linear/extended	Reproductive tissues	Unknown for fungi	
Rational peptide 1 (RP-1)	Synthetic	+8	ALYKKFKKKLLKSLKRLG	α-Helix	Modeled upon PF-4 α-helices	Perturbation of cell membrane and energetics	Current study
Human β-defensin 2 (hBD-2)	β-Defensin	+6	GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP	β-Hairpin/helix	Epidermis, mucosa	Perturbation of cell membrane, cell wall, and energetics	14
Human neutrophil protein 1 (HNP-1)	α-Defensin	+4	ACYCRIPACIAGERRYGTCIYQGRLWAFCC	β-Hairpin	Neutrophil	Perturbation of cell membrane, ATP efflux and depletion	31, 32
LL-37	Cathelicidin	+6	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	Extended/helix	Epidermis, mucosa, neutrophil	Unknown for fungi	

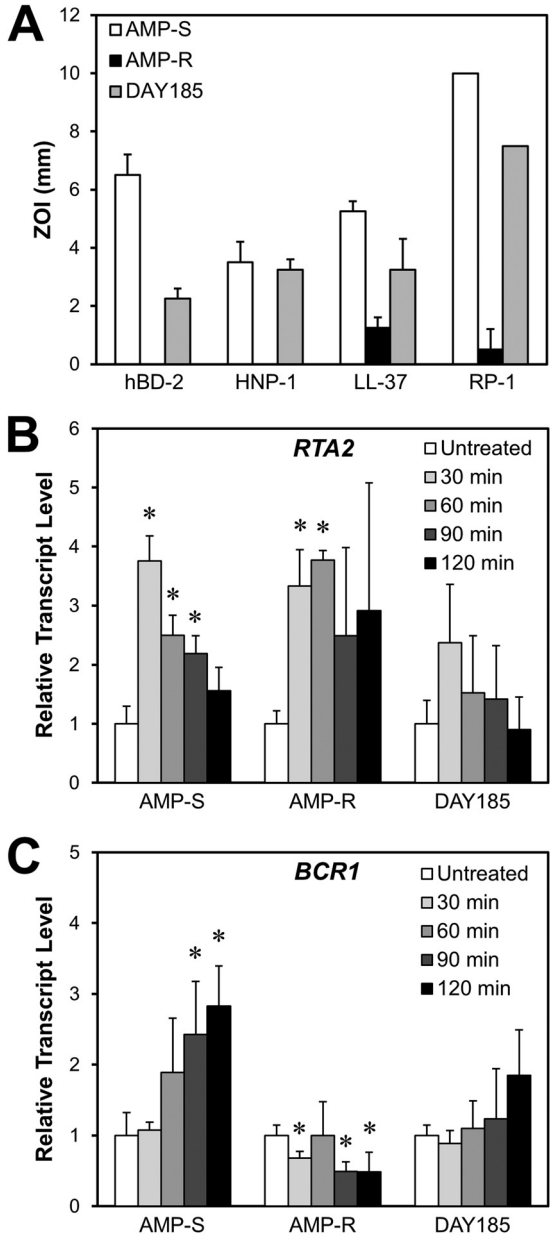

FIG 1 Comparative antimicrobial peptide susceptibilities and time courses of *RTA2* and *BCR1* mRNA levels in two *C. albicans* bloodstream isolates (CA024 [Amp^s] and CA080 [Amp^r]) with differing levels of antimicrobial peptide susceptibility and in the DAY185 reference strain. (A) Susceptibilities of the three *C. albicans* strains to the indicated antimicrobial peptides were determined by a radial diffusion assay at pH 7.5. Antimicrobial susceptibility was measured as the zone of inhibition (ZOI) after incubation at 30°C for 24 h. Results are means ± standard deviations (SD) from three independent experiments. (B and C) *RTA2* (B) and *BCR1* (C) transcript levels in the Amp^s, Amp^r, and DAY185 strains after incubation for the indicated time in the presence of a sublethal concentration of RP-1 (2.5 μg/ml for Amp^s, 100 μg/ml for Amp^r, and 5 μg/ml for DAY185). Transcript levels were measured by real-time PCR using *ACT1* as the endogenous control gene and normalized to organisms incubated for 60 min in medium without RP-1 (untreated). Results are means ± SD for three biological replicates, each measured in duplicate. *, $P < 0.05$ compared to cells grown in the absence of RP-1. HNP-1, human neutrophil peptide 1; hBD-2, human β-defensin 2.

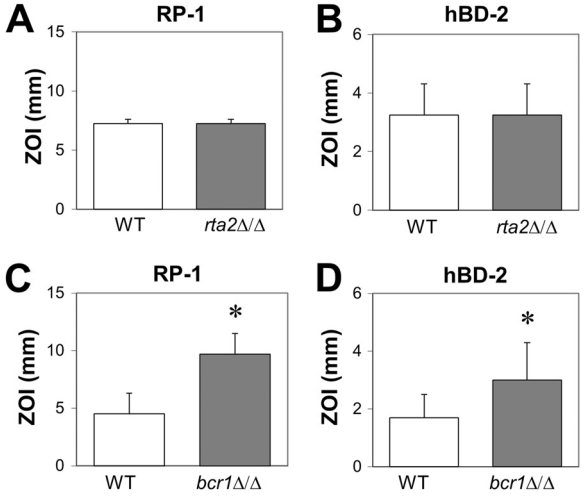

FIG 2 Influence of *RTA2* and *BCR1* on *C. albicans* antimicrobial peptide susceptibility. The susceptibilities of the indicated strains of *C. albicans* to RP-1 (A and C) and hBD-2 (B and D) were determined by a radial diffusion assay after incubation at 30°C for 24 h. Results are the means ± SD from two independent experiments. *, $P < 0.05$ compared to the wild-type strain (WT).

ones involved in cell wall integrity (*GSL1*), cell membrane integrity (*RTA2*), mitochondrial integrity (*MDM10*), transcriptional regulation (*ADA2*, *ACE2*, *BCR1*), stress response (*HOG1*, *PBS2*), and protein trafficking (*VPS51*).

We found that among these genes, only *RTA2* and *BCR1* were differentially expressed between the Amp^s and Amp^r strains in response to the antimicrobial peptide RP-1. *RTA2* mRNA levels increased significantly in both isolates after exposure to RP-1 for 30 min (Fig. 1B). However, upon further exposure to RP-1, *RTA2* transcript levels progressively decreased in the Amp^s isolate but not in the Amp^r isolate. In strain DAY185, there was a trend toward increased *RTA2* transcript levels after 30 min of RP-1 exposure, but this difference did not achieve statistical significance ($P = 0.09$). The pattern of *BCR1* mRNA expression also varied between the Amp^s and Amp^r strains. Upon exposure to RP-1, *BCR1* transcript levels progressively increased in the Amp^s strains but remained at below basal levels in the Amp^r strain (Fig. 1C). Although *BCR1* mRNA levels in strain DAY185 increased slightly after 120 min of exposure to RP-1, this trend was not statistically significant ($P = 0.09$). These findings suggested that *RTA2* and *BCR1* may govern or influence resistance to certain peptides such as RP-1.

Contributions of *RTA2* and *BCR1* to intrinsic antimicrobial peptide resistance. To determine the relationship of *RTA2* and *BCR1* to antimicrobial peptide resistance, we used a radial diffusion assay to assess the susceptibilities of *rta2*Δ/Δ and *bcr1*Δ/Δ mutants to antimicrobial peptides with different structure-activity relationships. The *rta2*Δ/Δ mutant had wild-type susceptibility to RP-1 and hBD-2 (Fig. 2A and B), possibly due to the presence of other members of the *RTA* gene family (*RTA1*, *RTA3*, and *RTA4*). On the other hand, the *bcr1*Δ/Δ mutant was more susceptible to both peptides than the wild-type strain (Fig. 2C and D). Thus, *BCR1* is necessary for *C. albicans* to resist RP-1 and hBD-2, whereas *RTA2* is not.

***ssd1*Δ/Δ and *bcr1*Δ/Δ mutants have increased susceptibility to both antimicrobial peptide and membrane stressors.** Previ-

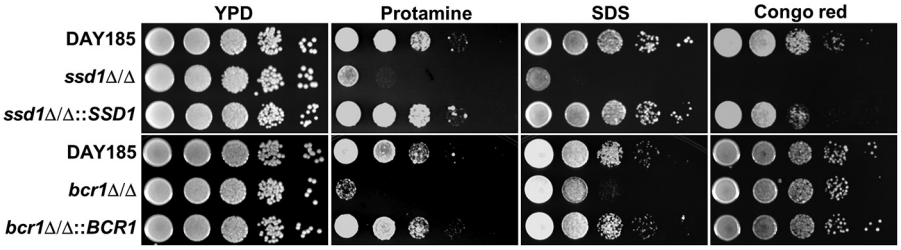

FIG 3 Susceptibilities of the *ssd1*Δ/Δ and *bcr1*Δ/Δ mutants to protamine and nonpeptide stressors. Images of serial 10-fold dilutions of the indicated strains that were plated onto YPD agar containing 2 mg/ml protamine sulfate, 0.1% SDS, or 300 μg/ml Congo red and incubated at 30°C for 2 days are shown.

ously, we found that *SSD1* was required for *C. albicans* to resist multiple antimicrobial peptides (6). Therefore, we compared the susceptibility to protamine and nonpeptide stressors of an *ssd1*Δ/Δ mutant with that of the *bcr1*Δ/Δ mutant. We found that the *ssd1*Δ/Δ mutant was hypersusceptibile to protamine, the cell membrane stressor SDS, and the cell wall stressor Congo red (Fig. 3). The *bcr1*Δ/Δ mutant also had increased susceptibility to protamine and SDS, but it had near-wild-type susceptibility to Congo red. As expected, the susceptibility of the *ssd1*Δ/Δ::*SSD1* and *bcr1*Δ/Δ::*BCR1* complemented strains to all stressors was similar to that of the wild-type strain. Collectively, these data indicate that both *SSD1* and *BCR1* are required for wild-type resistance to both protamine and SDS.

***BCR1* functions downstream of *SSD1*.** Next, we investigated the genetic relationship between *SSD1* and *BCR1* in governing antimicrobial peptide resistance in *C. albicans*.

To determine if *SSD1* and *BCR1* function in either a common pathway or parallel pathways, we constructed and analyzed a mutant that lacked both *SSD1* and *BCR1*. The *ssd1*Δ/Δ *bcr1*Δ/Δ double mutant had same susceptibility to protamine as the *bcr1*Δ/Δ single mutant (Fig. 4A), indicating that *SSD1* and *BCR1* likely function in the same pathway.

To determine whether *BCR1* was upstream or downstream of *SSD1*, we used real-time PCR to measure *BCR1* mRNA expression in the *ssd1*Δ/Δ mutant and *SSD1* mRNA expression in the *bcr1*Δ/Δ mutant. We found that *BCR1* transcript levels were reduced in the *ssd1*Δ/Δ mutant compared to the wild-type strain, whereas *SSD1* mRNA levels were unchanged in the *bcr1*Δ/Δ mutant. These findings suggest that *BCR1* is downstream of *SSD1* (Fig. 4B).

To verify that *BCR1* acts downstream of *SSD1*, we overexpressed *BCR1* in the *ssd1*Δ/Δ mutant and overexpressed *SSD1* in the *bcr1*Δ/Δ mutant by placing a copy of each of these genes under the control of the strong *TDH3* promoter. The forced expression of *BCR1* in the *ssd1*ΔΔ mutant partially restored resistance to protamine (Fig. 4C). On the other hand, overexpression of *SSD1* in the *bcr1*Δ/Δ mutant had no effect on resistance to protamine (Fig. 4D). Taken together, these findings suggest that *BCR1* governs antimicrobial peptide resistance at least in part by functioning downstream of *SSD1*.

***SSD1* and *BCR1* have differing effects on susceptibility to different antimicrobial peptides.** Because different antimicrobial peptides have different structures and modes of action (17), it is likely that resistance to different antimicrobial peptides is governed by distinct signaling pathways. To investigate this possibility, we compared the susceptibilities of the *ssd1*Δ/Δ and *bcr1*Δ/Δ single mutants, the *ssd1*Δ/Δ P_{TDH3}-*BCR1* overexpression strain, and the *ssd1*Δ/Δ *bcr1*Δ/Δ double mutant to four different antimicrobial peptides (Table 2; Fig. 5). Both the *ssd1*Δ/Δ and *bcr1*Δ/Δ single mutants had increased susceptibility to the α-helix peptide RP-1 and to the β-hairpin peptide hBD-2. Also, overexpression of *BCR1* in the *ssd1*Δ/Δ mutant partially reversed its hypersusceptibility to these peptides. Interestingly, deletion of *BCR1* in the

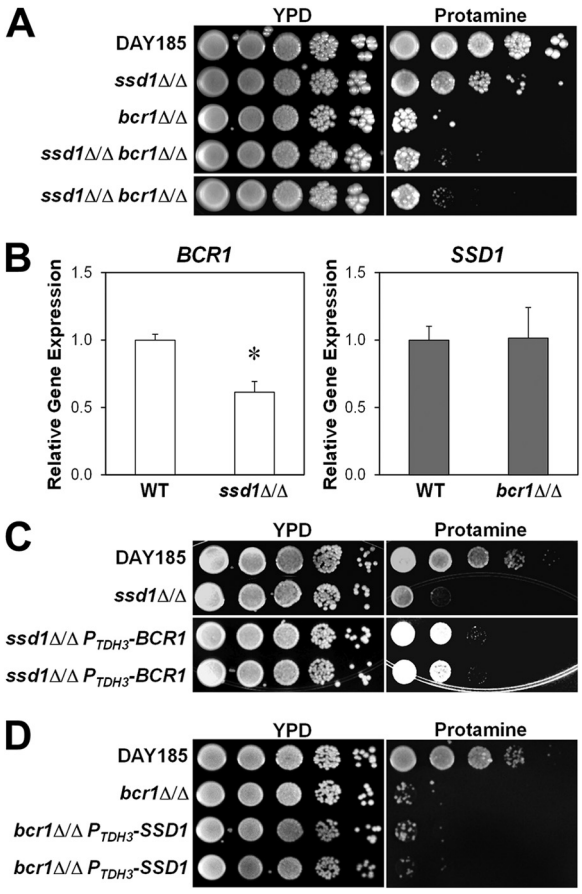

FIG 4 Epistasis analysis of *BCR1* and *SSD1*. (A) Susceptibilities of independent *ssd1*Δ/Δ *bcr1*Δ/Δ double deletion mutants to protamine (1.8 mg/ml). (B) Effects of deletion of *SSD1* and *BCR1* on *BCR1* and *SSD1* mRNA expression. Total RNA was isolated from the indicated strains grown in YPD at 30°C to early log phase, after which expression of *BCR1* and *SSD1* was determined by real-time PCR and normalized *ACT1*. Results are means ± SD for three biological replicates, each measured in duplicate. *, $P < 0.05$ compared to the wild type (DAY185). (C and D) Effects of overexpression of *BCR1* in the *ssd1*Δ/Δ mutant (C) and overexpression of *SSD1* in the *bcr1*Δ/Δ mutant (D) on susceptibility to protamine (2 mg/ml).

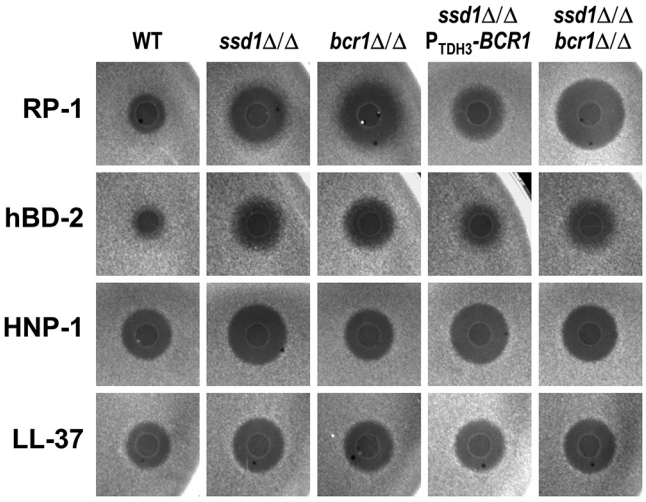

FIG 5 Effects of *SSD1* and *BCR1* deletion or overexpression on susceptibility of *C. albicans* to diverse antimicrobial peptides. The susceptibilities of the indicated strains of *C. albicans* to HNP-1, hBD-2, LL-37, and RP-1 were measured using a radial diffusion assay. The zones of growth inhibition were imaged after incubation at 30°C for 24 h.

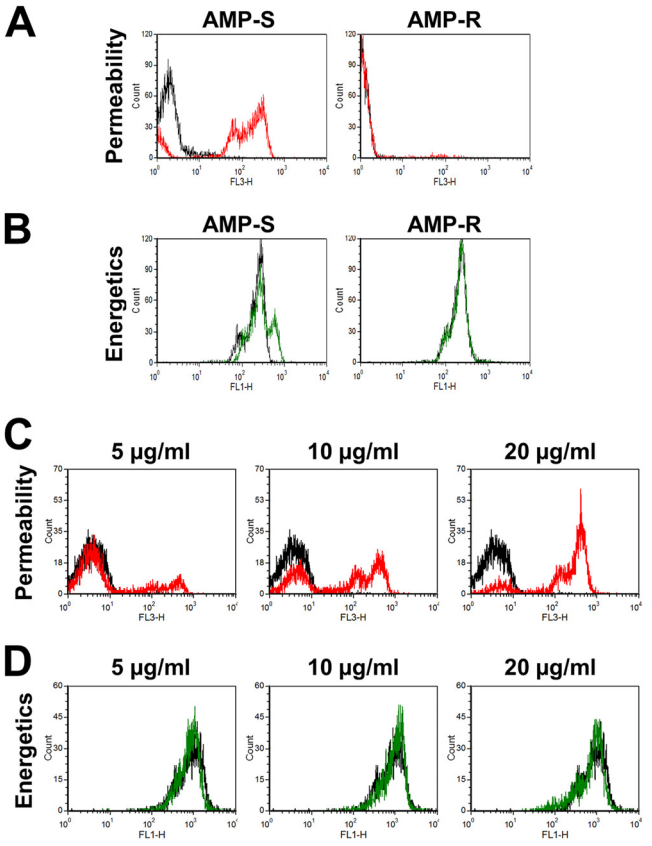

FIG 6 Effects of RP-1 on *C. albicans* plasma membrane permeability and mitochondrial membrane potential. The indicated strains of *C. albicans* were exposed to RP-1 at pH 7.5 for 1 h and then analyzed by flow cytometry. (A and C) Histograms of propidium iodide fluorescence, a measure of membrane permeabilization, of the Amp^s and Amp^r strains exposed to 5 μg/ml RP-1 (A) and of strain DAY185 exposed to 5 to 20 μg/ml RP-1 (C). The fluorescence of untreated control cells is indicated by the black lines, and the fluorescence of cells exposed to RP-1 is indicated by the red lines. (B and D) Histogram of $DiOC_5$ fluorescence, a measure of mitochondrial membrane potential, of the Amp^s and Amp^r strains exposed to 5 μg/ml RP-1 (B) and of strain DAY185 exposed to 5 to 20 μg/ml RP-1 (D). The fluorescence of untreated control cells is indicated by the black lines, and the fluorescence of cells exposed to RP-1 is indicated by the green lines.

*ssd1*Δ/Δ mutant resulted in even greater susceptibility to RP-1 but did not further increase susceptibility to hBD-2. These results suggest that both *SSD1* and *BCR1* mediate *C. albicans* resistance to RP-1 and hBD-2, and they are consistent with the hypothesis that *BCR1* functions downstream of *SSD1*.

In contrast, *SSD1* was necessary for resistance to the β-hairpin peptide HNP-1 and the linear peptide LL-37, whereas *BCR1* was not. Only the *ssd1*Δ/Δ mutant, and not the *bcr1*Δ/Δ mutant, had increased susceptibility to these peptides (Fig. 5). In addition, neither overexpression of *BCR1* nor deletion of *BCR1* influenced the susceptibility of the *ssd1*Δ/Δ mutant to these peptides. Collectively, these results indicate that while *SSD1* governs resistance to multiple antimicrobial peptides, *BCR1* mediates resistance to only a subset of them.

Both *SSD1* and *BCR1* are required for resistance to membrane permeabilization and maintenance of mitochondrial membrane potential upon exposure to RP-1. Next, using flow cytometric assays of plasma membrane permeability and mitochondrial membrane potential, we investigated the effects of RP-1 on the different *C. albicans* strains. Treatment of the Amp^s strain with RP-1 at 5 μg/ml caused a substantial increase in propidium iodide fluorescence, indicating an increase in membrane permeability (Fig. 6A). Interestingly, the baseline propidium iodide fluorescence of the Amp^r strain was lower than that of the Amp^s strain, and it did not increase after exposure to RP-1. In addition, at the concentration of RP-1 that was used, there was no change $DiOC_5$ fluorescence in either strain, indicating that there was no detectable change in mitochondrial membrane energetics (Fig. 6B). As predicted by the susceptibility data, the response of DAY185 to RP-1 was intermediate to those of the Amp^s and Amp^r strains. Although the baseline propidium iodide membrane permeability of DAY185 was similar to that of the Amp^s strain, exposure of DAY185 to 5 μg RP-1 per ml resulted in only a modest increase in permeability (Fig. 6C). However, exposure of DAY185 to increasing concentrations of RP-1 resulted in a progressive increase in permeability but had only a modest effect on mitochondrial energetics (Fig. 6D). These data indicate that under the conditions tested, the main effect of RP-1 on susceptible strains of *C. albicans* is to increase membrane permeability and that any significant effect of RP-1 on mitochondrial energetics must occur after 1 h. The data also suggest that the Amp^r strain has an altered plasma membrane, which results in decreased permeability even in the absence of RP-1.

Next, we investigated the mechanisms by which the *ssd1*Δ/Δ and *bcr1*Δ/Δ mutants became hypersusceptible to RP-1. Treatment of both of these mutants with RP-1 caused greater membrane permeabilization than in the DAY185 control strain (Fig. 7A). Furthermore, RP-1 exposure resulted in a reduction in mitochondrial membrane potential in the *ssd1*Δ/Δ and *bcr1*Δ/Δ mutants (Fig. 7B). Importantly, complementation of the *ssd1*Δ/Δ and *bcr1*Δ/Δ mutants largely restored the wild-type phenotype in these assays. Of note, RP-1 did not lead to increased surface exposure of phosphatidylserine in either the *ssd1*Δ/Δ or *bcr1*Δ/Δ mutant under the assay conditions used (data not shown), indicating

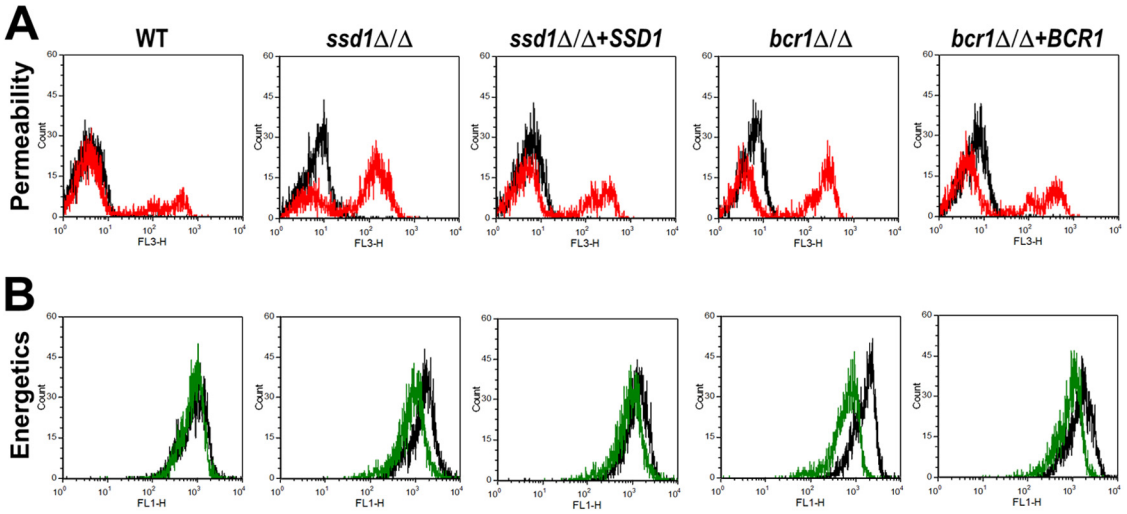

FIG 7 Effects of RP-1 on plasma membrane permeability and mitochondrial membrane potential of the *ssd1*Δ/Δ and *bcr1*Δ/Δ mutants. The indicated strains of *C. albicans* were exposed to 5 μg/ml RP-1 at pH 7.5 for 1 h and then analyzed by flow cytometry. (A) Histogram of propidium iodide fluorescence, a measure of membrane permeabilization. The fluorescence of untreated control cells is indicated by the black lines, and the fluorescence of cells exposed to RP-1 is indicated by the red lines. (B) Histogram of $DiOC_5$ fluorescence, a measure of mitochondrial membrane potential. The fluorescence of untreated control cells is indicated by the black lines, and the fluorescence of cells exposed to RP-1 is indicated by the green lines.

that these mutants did not have greater susceptibility to RP-1-induced programmed cell death within the 1-h time period tested. Therefore, these findings suggest that *SSD1* and *BCR1* mediate early resistance to RP-1 by maintaining homeostatic membrane integrity and mitochondrial energetics.

The antimicrobial peptide susceptibility of the *bcr1*Δ/Δ mutant cannot be rescued by previously known *BCR1* target genes. Prior studies have shown that Bcr1 governs the expression of genes that specify cell surface proteins involved in adherence and biofilm formation, both *in vitro* and *in vivo* (18–20). We used an overexpression-rescue approach in an attempt to identify Bcr1 target genes that govern antimicrobial peptide resistance. The susceptibility to protamine was determined for *bcr1*Δ/Δ strains that overexpressed *ALS1*, *ALS3*, *CHT2*, *CSA1*, *ECE1*, *HYR1*, *HWP1*, *PGA10*, *RBT5*, *MAL31*, and *RTA1*. However, none of these strains had restoration of protamine resistance, indicating that other Bcr1 target genes mediate antimicrobial peptide resistance.

DISCUSSION

The current data support the model that Bcr1 mediates resistance to some antimicrobial peptides by functioning downstream of Ssd1. In support of this model, we found that homozygous deletion of either *SSD1* or *BCR1* rendered *C. albicans* hypersusceptible to similar stressors, including protamine, RP-1, hBD-2, and SDS. In addition, deletion of either gene resulted in a similar response to RP-1, namely, loss of mitochondrial membrane potential and increased membrane permeabilization. Finally, deletion of *BCR1* in the *ssd1*Δ/Δ mutant did not result in increased susceptibility to protamine and hBD-2. Thus, Bcr1 and Ssd1 appear to function in the same pathway. However, Ssd1 appears to govern resistance to a broader spectrum of antimicrobial peptides, and Bcr1 contributes to resistance to a subset of these peptides (Fig. 8).

Ssd1 is an RNA-binding protein and a component of the regulation of Ace2 and morphogenesis (RAM) pathway (21). In *C. albicans*, this pathway governs multiple processes, including filamentation and cell wall integrity (22, 23). Consistent with our results, others have found that deletion of *SSD1* in *C. albicans* results in increased susceptibility to Congo red (23). In *Saccharomyces cerevisiae*, Bck1 phosphorylates Ssd1, thereby governing its activity (24). Ssd1 is almost certainly a substrate of Bck1 in *C. albicans* as well (22). Although the transcription factor Bcr1 was initially found to regulate adherence and biofilm formation, a recent study found that Bcr1 and Ace2 share multiple common target genes, suggesting that Bcr1 may function in the RAM pathway (25). Most importantly, Bck1 was discovered to phosphorylate Bcr1 and regulate its transcriptional activity (26). Thus, Bcr1 and Ssd1 are targets of the same kinase, and this commonality is consistent with the model that Bcr1 and Ssd1 act in the same response pathway to govern susceptibility to certain antimicrobial peptides and SDS.

We also found that *BCR1* mRNA expression was reduced in the *ssd1*Δ/Δ mutant and that overexpression of *BCR1* in the *ssd1*Δ/Δ mutant partially restored resistance to protamine, RP-1, and hBD-2. Conversely, *SSD1* transcript levels were not reduced in the *bcr1*Δ/Δ mutant, and overexpression of *SSD1* in this strain failed to restore antimicrobial peptide resistance. Collectively, these results indicate that Bcr1 functions downstream of Ssd1. Whether

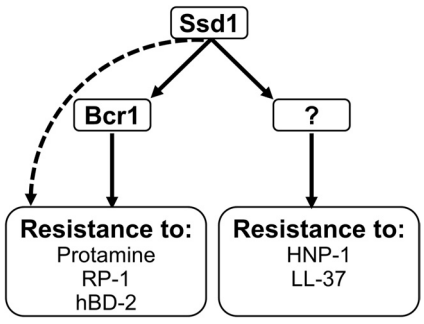

FIG 8 Proposed model of the interactions of Ssd1 and Bcr1 in the regulation of *C. albicans* susceptibility to different antimicrobial peptides.

Ssd1 governs *BCR1* mRNA expression directly or indirectly is not yet known. However, in *S. cerevisiae*, Ssd1 binds to specific mRNAs, governing their localization within the cell and inhibiting their translation (24). If Ssd1 functions similarly in *C. albicans*, we would predict that it influences *BCR1* mRNA levels by an indirect mechanism.

Although Bcr1 and Ssd1 function in the same pathway, our data indicate that Ssd1 governs resistance to a wider range of stressors than Bcr1. For example, the *ssd1*Δ/Δ mutant was highly susceptible to Congo red, HNP-1, and LL-37, whereas the *bcr1*Δ/Δ mutant was not. These results indicate that Ssd1 governs resistance to these stressors independently of Bcr1 and suggest that there must be incomplete overlap among Bcr1 and Ssd1 target genes.

Interestingly, although Ace2 and Bcr1 are both members of the RAM pathway and both govern biofilm formation, we found that an *ace2*Δ/Δ mutant had wild-type susceptibility to protamine and RP-1 (S. Jung and S. Filler, unpublished data). Therefore, either Ace2 target genes are not involved in resistance to the study antimicrobial peptides under the conditions tested or the compensatory changes in the cell wall induced by deletion of *ACE2* mask any increase in susceptibility to these peptides under these experimental conditions.

Even though the *bcr1*Δ/Δ mutant had increased susceptibility to several antimicrobial peptides, it seemed paradoxical that *BCR1* mRNA levels were upregulated in the Amp^s clinical isolate. We speculate that this upregulation of *BCR1* represents a compensatory response and that the Amp^s strain is hypersusceptible to antimicrobial peptides by another mechanism.

Although the mechanisms by which antimicrobial peptides kill bacteria have been studied extensively, less is known about how they antagonize fungi. Under the specific time and conditions tested, we found that the major effect of RP-1 on the Amp^s and DAY185 strains was to cause an increase in membrane permeability. At the concentration tested, RP-1 did not increase membrane permeability in the Amp^r strain, which was highly resistant to the growth-inhibitory effects of RP-1. The finding that the capacity of RP-1 to induce membrane permeabilization directly correlated with its capacity to inhibit growth supports the model that induction of membrane permeabilization is a key component of the antifungal activity of this peptide.

RP-1 had different effects on mitochondrial energetics in different strains. Under the conditions tested, RP-1 had minimal effects on the mitochondrial energetics of any of the wild-type strains. However, it markedly reduced the mitochondrial energetics of both the *ssd1*Δ/Δ and *bcr1*Δ/Δ mutants. These results suggest that Ssd1 and Bcr1 are required for *C. albicans* to sustain mitochondrial membrane potential when exposed to RP-1. Furthermore, it is possible that the upregulation of *BCR1* that occurred when the Amp^s strain was exposed to RP-1 prevented this strain from losing mitochondrial membrane potential, even though it was still killed. In prior studies, we have shown that certain antimicrobial peptides can induce programmed cell death-like effects (e.g., phosphatidylserine accessibility) in *C. albicans*, particularly after 2 hours or more of exposure (14). However, in the current study, which focused on the early (1-h) response profile, no such effects were observed. Future studies will investigate the roles of Bcr1 and Ssd1 in early versus late mechanisms of resistance to antimicrobial peptides.

It is notable that the signaling pathways that govern biofilm formation in the bacterium *Pseudomonas aeruginosa* also regulate susceptibility to antimicrobial peptides. For example, a *P. aeruginosa* mutant that lacks the transcriptional regulator PsrA is defective in biofilm formation and has increased susceptibility to the bovine neutrophil antimicrobial peptide indolicidin (27). Moreover, PhoQ, which is a member of a two-component regulatory system, governs both biofilm formation and antimicrobial peptide resistance in *P. aeruginosa* and *Salmonella enterica* serovar Typhimurium (28–30). Based on the link between biofilm formation and antimicrobial peptide resistance in organisms from two different phyla, it seems probable that both processes depend on factors that influence the cell wall and cell surface.

We attempted to identify Bcr1 target genes that mediate antimicrobial peptide resistance using an overexpression-rescue approach that was focused on genes involved in biofilm formation and cell wall structure. However, none of the overexpressed genes reversed the antimicrobial peptide susceptibility of the *bcr1*Δ/Δ mutant. Thus, it is likely that other Bcr1 target genes are responsible for resistance to antimicrobial peptides. Alternatively, Bcr1-mediated resistance may require the simultaneous action of multiple downstream genes. Future work to identify the Bcr1 target genes that mediate resistance to antimicrobial peptides holds promise to provide new insights into the mechanisms by which *C. albicans* resists this key host defense mechanism. In turn, identification of such resistance genes and proteins may reveal novel antifungal targets for improved prevention or therapy of fungal infections.

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