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 bcr1ΔΔ mutant, like the ssd1ΔΔ mutant, had increased susceptibility to the antimicrobial peptides, protamine, RP-1, and human β defensin-2. Homozygous deletion of BCR1 in the ssd1ΔΔ mutant did not result in a further increase in antimicrobial peptide susceptibility. Exposure of the bcr1ΔΔ and ssd1ΔΔ 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 ssd1ΔΔ mutant, and the forced expression of BCR1 in the ssd1ΔΔ 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 bcr1ΔΔ 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.
TABLE 1 Strains of *C. albicans* used in this study

| Strain | Genotype | Reference |
|--------|----------|-----------|
| CA024  | Wild type (bloodstream isolate) | 9 |
| CA080  | Wild type (bloodstream isolate) | 9 |
| DAY185 | *ura3::imm434/ura3::imm434 his1::hisG:pHis1/1::hisG::ARG4-URA3::arg4::hisG* | This study |
| CW195  | *ura3::imm434/ura3::imm434 his1::hisG:pHis1/1::hisG::ARG4-URA3::arg4::hisG* | This study |
| CW193  | *ura3::imm434/ura3::imm434 his1::hisG::ARG4-BCR1/1::hisG::ARG4-bcr1::URA3* | This study |
| rta2Δ/Δ | *ura3::imm434/ura3::imm434:URA3 his1::hisG/1::hisG::ARG4::hisG::ARG4-bcr1::URA3* | This study |
| APRA-1 (ssd1Δ/Δ-1) | *ura3::imm434/ura3::imm434:URA3 his1::hisG/1::hisG::ARG4-bcr1::URA3* | 6 |
| APRA-1comp (ssd1Δ/Δ-1::SSD1) | *ura3::imm434/ura3::imm434:URA3 his1::hisG/1::hisG::ARG4-bcr1::URA3* | 6 |
| ssd1Δ::bcr1Δ/Δ-1 and ssd1Δ::bcr1Δ/Δ-II | *ura3::His1/1::ARG4::bcr1::URA3::bcr1::NAT1 ura3::imm434/ura3::imm434 arg4::hisG/1::hisG::ARG4-bcr1::URA3* | This study |
| ssd1Δ::BCR1-EO-1 and ssd1Δ::BCR1-EO-II | *ura3::His1/1::ARG4::bcr1::URA3::bcr1::NAT1 arg4::hisG/1::hisG::ARG4-bcr1::URA3* | This study |
| bcr1Δ/Δ::SSD1-EO-1 and bcr1Δ/Δ::SSD1-EO-II | *ura3::His1/1::ARG4::bcr1::URA3::bcr1::NAT1 arg4::hisG/1::hisG::ARG4-bcr1::URA3* | This study |
| CJN1144 | *ura3::imm434/ura3::imm434 arg4::hisG/1::hisG::ARG4-bcr1::URA3* | 19 |
| CJN1153 | *ura3::imm434/ura3::imm434 arg4::hisG/1::hisG::ARG4-bcr1::URA3* | 19 |
| CJN1222 | *ura3::imm434/ura3::imm434 arg4::hisG/1::hisG::ARG4-bcr1::URA3* | 19 |
| CJN1259 | *ura3::imm434/ura3::imm434 arg4::hisG/1::hisG::ARG4-bcr1::URA3* | 19 |
| CJN1276 | *ura3::imm434/ura3::imm434 arg4::hisG/1::hisG::ARG4-bcr1::URA3* | 19 |
| CJN1281 | *ura3::imm434/ura3::imm434 arg4::hisG/1::hisG::ARG4-bcr1::URA3* | 19 |
| CJN1288 | *ura3::imm434/ura3::imm434 arg4::hisG/1::hisG::ARG4-bcr1::URA3* | 19 |
| JF11 | *ura3::imm434/ura3::imm434 arg4::hisG/1::hisG::ARG4-bcr1::URA3* | This study |
| JF25 | *ura3::imm434/ura3::imm434 arg4::hisG/1::hisG::ARG4-bcr1::URA3* | This study |
| bcr1Δ/Δ::MAL31-EO | *ura3::imm434/ura3::imm434 arg4::hisG/1::hisG::ARG4-bcr1::URA3* | This study |
| bcr1Δ/Δ::RTA1-EO | *ura3::imm434/ura3::imm434 arg4::hisG/1::hisG::ARG4-bcr1::URA3* | This study |

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 10^9 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 was 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).
TABLE 2 Antimicrobial peptides used in this study

| Peptide | Class | Charge | Amino acid sequence | Secondary structure | Tissue source | Proposed mechanism of action | Reference |
|---------|-------|-------|---------------------|---------------------|-------------|-----------------------------|---------|
| LLGDFKSEKKERKFKYQKDFKUNRTS | Defensin | -4 | LLGDFKSEKKERKFKYQKDFKUNRTS | Helix | Epidermis, mucosa | Peptide resistance | 
| GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP | Defensin | -21 | GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP | Hairpin/helix | Epidermis, mucosa | Perturbation of cell membrane and cellwall | 
| PRRRSSSPFRPRSSRRRGRRR | Defensin | -31,32 | PRRRSSSPFRPRSSRRRGRRR | -Defensin | Epidermis, mucosa, cellwall, and energetics | Peptide resistance | 

**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 (Amps), which was more susceptible than the DAY185 reference strain to hBD-2, LL-37, and RP-1 (Fig. 1A). We also selected strain CA080 (Ampr), which was less susceptible than strain DAY185 to all peptides tested. Of note, all of these strains had similar susceptibility to amphoterin 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 *BCRI* are differentially expressed in the Amp^a^ and Amp^f^ strains. To assess the genes whose products mediate antimicrobial peptide resistance in *C. albicans*, the Amp^a^ and Amp^f^ 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^a^ strain, 100 µg/ml for the Amp^f^ 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...
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 Amps and Ampr 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 Amps isolate but not in the Ampr 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 Amps and Ampr strains. Upon exposure to RP-1, BCR1 transcript levels progressively increased in the Amps strains but remained at below basal levels in the Ampr 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 the indicated strains of C. albicans to RP-1 (A and C) and hBD-2 (B and D). 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).

We found that among these genes, only RTA2 and BCR1 were differentially expressed between the Amps and Ampr 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 Amps isolate but not in the Ampr 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 Amps and Ampr strains. Upon exposure to RP-1, BCR1 transcript levels progressively increased in the Amps strains but remained at below basal levels in the Ampr 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 the indicated strains of C. albicans to RP-1 (A and C) and hBD-2 (B and D). Results are the means ± SD from two independent experiments. *, P < 0.05 compared to the wild-type strain (WT).

We found that among these genes, only RTA2 and BCR1 were differentially expressed between the Amps and Ampr 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 Amps isolate but not in the Ampr 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 Amps and Ampr strains. Upon exposure to RP-1, BCR1 transcript levels progressively increased in the Amps strains but remained at below basal levels in the Ampr 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 the indicated strains of C. albicans to RP-1 (A and C) and hBD-2 (B and D). Results are the means ± SD from two independent experiments. *, P < 0.05 compared to the wild-type strain (WT).

We found that among these genes, only RTA2 and BCR1 were differentially expressed between the Amps and Ampr 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 Amps isolate but not in the Ampr 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 Amps and Ampr strains. Upon exposure to RP-1, BCR1 transcript levels progressively increased in the Amps strains but remained at below basal levels in the Ampr 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 the indicated strains of C. albicans to RP-1 (A and C) and hBD-2 (B and D). Results are the means ± SD from two independent experiments. *, P < 0.05 compared to the wild-type strain (WT).

We found that among these genes, only RTA2 and BCR1 were differentially expressed between the Amps and Ampr 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 Amps isolate but not in the Ampr 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 Amps and Ampr strains. Upon exposure to RP-1, BCR1 transcript levels progressively increased in the Amps strains but remained at below basal levels in the Ampr 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.

We found that among these genes, only RTA2 and BCR1 were differentially expressed between the Amps and Ampr 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 Amps isolate but not in the Ampr 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 Amps and Ampr strains. Upon exposure to RP-1, BCR1 transcript levels progressively increased in the Amps strains but remained at below basal levels in the Ampr 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 the indicated strains of C. albicans to RP-1 (A and C) and hBD-2 (B and D). Results are the means ± SD from two independent experiments. *, P < 0.05 compared to the wild-type strain (WT).

We found that among these genes, only RTA2 and BCR1 were differentially expressed between the Amps and Ampr 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 Amps isolate but not in the Ampr 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 Amps and Ampr strains. Upon exposure to RP-1, BCR1 transcript levels progressively increased in the Amps strains but remained at below basal levels in the Ampr 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 the indicated strains of C. albicans to RP-1 (A and C) and hBD-2 (B and D). Results are the means ± SD from two independent experiments. *, P < 0.05 compared to the wild-type strain (WT).

We found that among these genes, only RTA2 and BCR1 were differentially expressed between the Amps and Ampr 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 Amps isolate but not in the Ampr 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 Amps and Ampr strains. Upon exposure to RP-1, BCR1 transcript levels progressively increased in the Amps strains but remained at below basal levels in the Ampr 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.
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 ssd1ΔΔ mutant with that of the bcr1ΔΔ mutant. We found that the ssd1ΔΔ mutant was hypsersusceptible to protamine, the cell membrane stressor SDS, and the cell wall stressor Congo red (Fig. 3). The bcr1ΔΔ 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 ssd1ΔΔ::SSD1 and bcr1ΔΔ::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 ssd1ΔΔ bcr1ΔΔ double mutant had same susceptibility to protamine as the bcr1ΔΔ single mutant (Fig. 3). The bcr1ΔΔ mutant also had increased susceptibility to protamine and SSD1, but it had near-wild-type susceptibility to Congo red. As expected, the susceptibility of the ssd1ΔΔ::SSD1 and bcr1ΔΔ::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.

**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 ssd1ΔΔ/Δ and bcr1ΔΔ/Δ single mutants, the ssd1ΔΔ/Δ PTDH3-BCR1 overexpression strain, and the ssd1ΔΔ/Δ bcr1ΔΔ/Δ double mutant to four different antimicrobial peptides (Table 2; Fig. 5). Both the ssd1ΔΔ/Δ and bcr1ΔΔ/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 ssd1ΔΔ/Δ mutant partially reversed its hypsersusceptibility to these peptides. Interestingly, deletion of BCR1 in the

---

**FIG 3** Susceptibilities of the ssd1ΔΔ/Δ and bcr1ΔΔ/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.
ssd1Δ/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 ssd1Δ/Δ mutant, and not the bcr1Δ/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 ssd1Δ/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' 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' strain was lower than that of the Amp' 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 DiOC6 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' and Amp' strains. Although the baseline propidium iodide membrane permeability of DAY185 was similar to that of the Amp' 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' 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 ssd1Δ/Δ and bcr1Δ/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 ssd1Δ/Δ and bcr1Δ/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 ssd1Δ/Δ or bcr1Δ/Δ mutant under the assay conditions used (data not shown), indicating...
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 \textit{bcr1}/H9004\mu- 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 \textit{in vitro} and \textit{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 \textit{bcr1}/H9004\mu- strains that overexpressed ALS1, ALS3, CHT2, CSA1, ECE1, HYR1, HWPI1, 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.

\section*{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 \textit{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 \textit{ssd1} \Delta \Delta mutant did not result in increased susceptibility to protamine and hBD-2. Thus, Bcr1 and Ssd1 appear to function in the same pathway. Whether SSD1 transcript levels were not reduced in the \textit{bcr1} \Delta \Delta 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

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{Effects of RP-1 on plasma membrane permeability and mitochondrial membrane potential of the \textit{ssd1} \Delta \Delta and \textit{bcr1} \Delta \Delta mutants. The indicated strains of \textit{C. albicans} were exposed to 5 \mu g/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\textsubscript{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.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure8.png}
\caption{Proposed model of the interactions of Ssd1 and Bcr1 in the regulation of \textit{C. albicans} susceptibility to different antimicrobial peptides.}
\end{figure}
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 ssd1Δ/Δ mutant was highly susceptible to Congo red, HNP-1, and LL-37, whereas the bcr1Δ/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 ace2Δ/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 bcr1Δ/Δ mutant had increased susceptibility to several antimicrobial peptides, it seemed paradoxical that BCR1 mRNA levels were upregulated in the Amp′ clinical isolate. We speculate that this upregulation of BCR1 represents a compensatory response and that the Amp′ 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′ 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′ 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 ssd1Δ/Δ and bcr1Δ/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′ 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 programed 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 bcr1Δ/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.

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Research Foundation of Korea (NRF-2010-013-E00023) and the National Institutes of Health (RO1AI054928, R01DE017088, and R01AI39001). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

1. Yount NY, Yeaman MR. 2012. Emerging themes and therapeutic prospects for anti-infective peptides. Annu. Rev. Pharmacol. Toxicol. 52:337–360.
2. Meißner TF, Hube B, Schild L, Shirliff ME, Scheper MA, Winkler R, Ton A, Jbara-Rizk MA. 2009. A novel immune evasion strategy of Candida albicans: proteolytic cleavage of a salivary antimicrobial peptide. PLoS One 4:e4509. doi:10.1371/journal.pone.0004509.
3. Szafranski-Schneider E, Swidergall M, Cottier F, Tieler D, Roman E, Pla J, Ernst JF. 2012. Msb2 shedding protects Candida albicans against antimicrobial peptides. PLoS Pathog. 8:e1002501. doi:10.1371/journal.ppat.1002501.
4. Vykova S, Jang WS, Li W, Nayyar N, Edgerton M. 2007. Histatin 5 initiates osmotic stress response in Candida albicans via activation of the Hog1 mitogen-activated protein kinase pathway. Eukaryot. Cell 6:1876–1888.
5. Argimon S, Fanning S, Blankenship JR, Mitchell AP. 2011. Interaction between the Candida albicans high-osmolarity glycerol (HOG) pathway and the response to human beta-defensins 2 and 3. Eukaryot. Cell 10:272–275.
6. Gank KD, Yeaman MR, Kojima S, Yount NY, Park H, Edwards JE, Jr, Filler SG, Fu Y. 2008. SSD1 is integral to host defense peptide resistance in Candida albicans. Eukaryot. Cell 7:1318–1327.
7. Jung SI, Shin JH, Song JH, Peck KR, Lee K, Kim MN, Chang HH, Moon CS. 2010. Multicenter surveillance of species distribution and antifungal susceptibilities of Candida bloodstream isolates in South Korea. Med. Mycol. 48:669–674.
8. Park H, Myers CL, Sheppard DC, Phan QT, Sanchez AA, Edwards JE, Jr, Filler SG. 2005. Role of the fungal Ras-protein kinase A pathway in governing epithelial cell interactions during oropharyngeal candidiasis. Cell. Microbiol. 7:499–510.
9. Wilson RB, Davis D, Mitchell AP. 1999. Rapid hypothesis testing with Candida albicans through gene disruption with short homology regions. J. Bacteriol. 181:1868–1874.

10. Shen J, Guo W, Kohler JR. 2005. CaNAT1, a heterologous dominant selectable marker for transformation of Candida species and other pathogenic Candida species. Infect. Immun. 73:1239–1242.

11. Nobile CJ, Solis N, Myers CL, Fay AJ, Deneault JS, Nantel A, Mitchell AP, Filler SG. 2008. Candida albicans transcription factor Rim101 mediates pathogenic interactions through cell wall functions. Cell. Microbiol. 10:2180–2196.

12. Yount NY, Yeaman MR. 2004. Multidimensional signatures in antimicrobial peptides. Proc. Natl. Acad. Sci. U. S. A. 101:7363–7368.

13. Clinical and Laboratory Standards Institute. 2008. Reference method for broth dilution antifungal susceptibility testing of yeasts, 3rd ed. Clinical and Laboratory Standards Institute, Wayne, PA.

14. Yount NY, Kuperwasser D, Spisni A, Dutz SM, Ramjan ZH, Sharma S, Waring AJ, Yeaman MR. 2009. Selective reciprocity in antimicrobial activity versus cytotoxicity of hBD-2 and crotamine. Proc. Natl. Acad. Sci. U. S. A. 106:14972–14977.

15. Yeaman MR, Soldan SS, Ghannoum MA, Edwards JE, Jr, Filler SG, Fanning S, Xu W, Solis N, Woolford CA, Filler SG, Mitchell AP. 2004. Multidimensional signatures in antimicrobial peptides. Proc. Natl. Acad. Sci. U. S. A. 101:7363–7368.

16. Wilson RB, Davis D, Mitchell AP. 1999. Rapid hypothesis testing with Candida albicans through gene disruption with short homology regions. J. Bacteriol. 181:1868–1874.

17. Yeaman MR, Soldan SS, Ghannoum MA, Edwards JE, Jr, Filler SG, Fanning S, Xu W, Solis N, Woolford CA, Filler SG, Mitchell AP. 2004. Multidimensional signatures in antimicrobial peptides. Proc. Natl. Acad. Sci. U. S. A. 101:7363–7368.

18. Xiong Y, Cheon SA, Lee KE, Lee SY, Lee BK, Oh DB, Kang HA, Kim JY. 2008. Role of the RAM network in cell polarity and hyphal morphogenesis in Candida albicans. Mol. Biol. Cell 19:5456–5477.

19. Jansen JM, Wanless AG, Seidel CW, Weiss EL. 2009. Cbk1 regulation of the RNA-binding protein Ssd1 integrates cell fate with translational control. Curr. Biol. 19:2114–2120.

20. Finkel JS, Xu W, Huang D, Hill EM, Desai JV, Woolford CA, Nett JE, Taff H, Norice CT, Andes DR, Lanni F, Mitchell AP. 2012. Portrait of Candida albicans adherence regulators. PLoS Pathog. 8:e1002525. doi:10.1371/journal.ppat.1002525.

21. Gutierrez-Escribano P, Zeidler U, Suarez MB, Bachellier-Bassi S, Clemente-Blanco A, Bonhomme J, Vazquez de Aldana CR, d’Enfert C, Correa-Bordes J. 2012. The NDR/LATS kinase Cbk1 controls the activity of the transcriptional regulator Bcr1 during biofilm formation in Candida albicans. PLoS Pathog. 8:e1002683. doi:10.1371/journal.ppat.1002683.

22. Gooderham WJ, Bains M, McPhee JB, Wiegand I, Hancock RE. 2008. Induction by cationic antimicrobial peptides and involvement in intrinsic polymyxin and antimicrobial peptide resistance, biofilm formation, and swarming motility of PsrA in Pseudomonas aeruginosa. J. Bacteriol. 190:5624–5634.

23. Gooderham WJ, Gellatly SL, Sanschagrin F, McPhee JB, Bains M, Cosseau C, Levesque RC, Hancock RE. 2009. The sensor kinase PhoQ mediates virulence in Pseudomonas aeruginosa. Microbiology 155:699–711.

24. Prouty AM, Gann JS. 2003. Comparative analysis of Salmonella enterica serovar Typhimurium biofilm formation on gallstones and on glass. Infect. Immun. 71:1574–1578.

25. Shprung T, Peleg A, Rosenfeld Y, Trieu-Cuot P, Shai Y. 2012. Effect of PhoP-PhoQ activation by broad repertoire of antimicrobial peptides on bacterial resistance. J. Biol. Chem. 287:4544–4551.

26. Vylkova S, Li XS, Berner JC, Edgerton M. 2006. Distinct antifungal mechanisms: beta-defensins require Candida albicans Saa1 protein, while Trk1p mediates activity of cysteine-free cationic peptides. Antimicrob. Agents Chemother. 50:324–331.

27. Edgerton M, Koslukova SE, Araujo MW, Patel RG, Dong J, Bruenn JA. 2000. Salivary histatin 5 and human neutrophil defensin 1 kill Candida albicans via shared pathways. Antimicrob. Agents Chemother. 44:3310–3316.