SSD1 Is Integral to Host Defense Peptide Resistance in Candida albicans

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Candida albicans is usually a harmless human commensal. Because inflammatory responses are not normally induced by colonization, antimicrobial peptides are likely integral to first-line host defense against invasive candidiasis. Thus, C. albicans must have mechanisms to tolerate or circumvent molecular effectors of innate immunity and thereby colonize human tissues. Prior studies demonstrated that an antimicrobial peptide-resistant strain of C. albicans, 36082S, is hypervirulent in animal models versus its susceptible counterpart (36082R). The current study aimed to identify a genetic basis for antimicrobial peptide resistance in C. albicans. Screening of a C. albicans genomic library identified SSD1 as capable of conferring peptide resistance to a susceptible surrogate, Saccharomyces cerevisiae. Sequencing confirmed that the predicted translation products of 36082S and 36082R SSD1 genes were identical. However, Northern analyses corroborated that SSD1 is expressed at higher levels in 36082S than in 36082R. In isogenic backgrounds, ssd1Δ/ssd1Δ null mutants were significantly more susceptible to antimicrobial peptides than parental strains but had equivalent susceptibilities to nonpeptide stressors. Moreover, SSD1 complementation of ssd1Δ/ssd1Δ mutants restored parental antimicrobial peptide resistance phenotypes, and overexpression of SSD1 conferred enhanced peptide resistance. Consistent with these in vitro findings, ssd1 null mutants were significantly less virulent in a murine model of disseminated candidiasis than were their parental or complemented strains. Collectively, these results indicate that SSD1 is integral to C. albicans resistance to host defense peptides, a phenotype that appears to enhance the virulence of this organism in vivo.

The colonization of human tissues is an essential first step in the pathogenesis of invasive candidiasis. This paradigm is based on multiple lines of evidence as follows: (i) colonization precedes and is an independent risk factor for disseminated candidiasis (16, 21, 28, 37), (ii) colonization burden correlates with the risk of invasive disease (6, 8, 22), and (iii) measures that reduce colonization burden reduce the subsequent risk of fungemia (18, 29). Innate immunity plays a crucial role in controlling candidal colonization and preventing invasive candidiasis. Thus, understanding the relationship among candidal pathogenicity, molecular immunobiology, and host defense is directly relevant to addressing the unacceptably high rates of morbidity and mortality presently associated with candidal infection (1, 12, 23, 27).

In immunocompetent individuals, molecular mechanisms of innate immunity are believed to mediate first-line host defense against invasive candidiasis. High-level cellular inflammatory responses are usually invoked only if these defenses fail to control colonization by Candida albicans. Principal among innate immune defenses are antimicrobial peptides elaborated in skin or upon mucosal surfaces. For example, human β-defensins (e.g., human β-defensin-1 [hBD-1] or hBD-2) are considered integral to innate defense of the integument (3). Likewise, histatins are important molecular effectors of constitutive immunity against Candida in the oral setting (6).

Little is known about the molecular mechanisms that govern C. albicans resistance to antimicrobial peptide-induced growth inhibition or lethality (41). Prior studies have examined the relationship between resistance to thrombin-induced platelet microbicidal protein-1 (tPMP-1) and C. albicans virulence in the rabbit model of infective endocarditis (42). These investigations demonstrated that the peptide-resistant strain C. albicans 36082R proliferated in cardiac vegetations and spleen to densities logarithmically higher than those seen for the orthogenic and peptide-susceptible counterpart, 36082S. Moreover, subsequent studies revealed that fluconazole was significantly less efficacious in reducing tissue burden due to strain 36082R than in reducing that due to strain 36082S (42). Differences in fungal growth rates, levels of adherence to vascular endothelium, levels of clearance from the bloodstream, and susceptibilities to fluconazole were ruled out as potential confounders of the above results. Thus, relative resistance to antimicrobial peptides appears to play a significant role in C. albicans pathogenesis and the efficacy of antifungal therapy. The current studies were undertaken to examine the genetic basis of antimicrobial peptide resistance in this opportunistic human pathogen.

MATERIALS AND METHODS

Fungal strains and culture conditions. The panel of organisms used in this investigation is summarized in Table 1. All wild-type strains were obtained from the American Type Culture Collection (ATCC; Manassas, VA).

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terpart (iPMP-1\(^8\)) of strain 36082\(^a\), designated strain 36082\(^b\), has been characterized previously (42, 43). Prior studies demonstrated that other than in differences in iPMP-1 susceptibility profiles, the 36082\(^a\) and 36082\(^b\) strains are indistinguishable by genotyping and immunotypic profiling, assessment of endothelial cell adhesion, and metabolic, germination, or growth rate characteristics (41-43). Studies have also confirmed that the iPMP-1\(^-\) and iPMP-1\(^+\) phenotypes are stable in vitro following passage in media or serum and upon passage through experimental animal models (42, 43). Moreover, prior investigations in vivo confirmed that these strains are equivalent in their interactions with platelets or cardiac vegetations and do not differ in rates of clearance after hematogenous inoculation in experimental animal models (9, 42). An initial genomic library was developed using strain 36082\(^a\); however, all relevant sequence data were identical to those for the genome sequence strain SC5314, the parent strain of BW27 (10). Thus, all subsequent genetic studies were performed using C. albicans strain SC5314 as the parent strain to create null and complemented SSD1 mutants, as described below.

(ii) S. cerevisiae. Saccharomyces cerevisiae strain ATCC 62956 (LL20), an antimicrobial peptide-susceptible, transformable strain, was used to express the C. albicans genomic library. It was maintained on yeast nitrogen base (YNB; Difco, Detroit MI) medium supplemented with 0.5% ammonium sulfate, 2% \(\alpha\)-glucose, and 100 \(\mu\)g/ml each of \(\alpha\)-lecine and \(\alpha\)-histidine (Sigma-Aldrich, St. Louis MO) and solidified with 1.5% agarose.

All organisms were stored at 4°C on appropriate agar slants; strain 36082\(^a\) was stored at the same temperature on Sabouraud dextrose agar containing 7 mg/ml protamine sulfate (Sigma-Aldrich). Prior to experimentation, organisms were cultured to late logarithmic phase in YNB broth at 30°C for 12 h and prepared and enumerated as previously described (42).

**Construction of the C. albicans 36082\(^a\)** genomic library. Initially, a C. albicans genomic library was constructed using DNA from strain 36082\(^a\) as previously described (9). In brief, C. albicans 36082\(^a\) genomic DNA was isolated and partially digested with Sau3A1 (New England Biolabs). Fragments of the C. albicans genome of 6 to 10 kb in length were ligated into the BamHI site of pE-20H (originally provided by Susan Sandmeyer, University of California, Irvine) (10), which is a \(2\mu\)-based shuttle plasmid, incorporating HIS3 as a selection marker in S. cerevisiae. Plasmid constructs were transformed into Esch. coli JM109 high-efficiency competent cells by use of the pGEM-T system (Easy Vector; Promega, Madison, WI). The resultant library, comprised of \(~15,000\) clones, was amplified in E. coli strain XL10-Gold (Stratagene, Torrey Pines, CA), transformed into S. cerevisiae LL20 by standard methods, and plated on YNB agar with appropriate supplementation.

**Screening for fungal protease susceptibility or resistance.** Protease is a helical cationic polypeptide used to screen for antimicrobial peptide resistance phenotypes (14, 42). Prior studies have demonstrated that resistances of C. albicans strains 36082\(^a\) and 36082\(^b\) to iPMP-1 and other antimicrobial peptides are mirrored by their responses to protease (42, 43). Candida albicans or S. cerevisiae inocula ranging from 10\(^8\) to 10\(^9\) CFU were plated in 10-\(\mu\)l volumes onto Sabouraud agar containing protamine sulfate (concentration range, 0 to 7 mg/ml) along with appropriate supplements for specific fungal strains (e.g., arginine, histidine, uridine). Plates were incubated at 30°C, and growth was recorded every 24 h.

**Identification of C. albicans gene(s) in protamine-resistant clones of S. cerevisiae LL20.** Plasmids from protease-resistant clones of S. cerevisiae LL20 were rescued, amplified in E. coli, and reintroduced into protamine-susceptible (wild-type) S. cerevisiae LL20 as previously detailed (9). Resulting transformants were then confirmed for protamine resistance as described above. Plasmids from randomly selected S. cerevisiae clones with confirmed protamine resistance were rescued and subcloned into pE-20H and transformed into S. cerevisiae LL20, and transformants were then assayed for protamine resistance as described above. In addition, catalytic RNAs from two random protease-resistant clones were digested by ScaI (New England Biolabs), and their restriction maps were analyzed.

**Functional assessment of SSD1 clones.** Clones encoding the protease-resistant phenotype contained a 6.3-kb fragment located within a ScaI site and mapped to 1.9 kb from its end. To assess the function of this 6.3-kb fragment, 4.4- and 1.9-kb fragments were individually subcloned and tested for protamine resistance as described above.

**Comparison of SSD1 genes in C. albicans strains 36082\(^a\) and 36082\(^b\).** SSD1 genes from C. albicans strains 36082\(^a\) and 36082\(^b\) were compared for sequence and expression profiles to assess potential correlations with antimicrobial peptide susceptibility or resistance phenotypes as follows.

1. **PCR amplification and sequencing.** SSD1 was amplified by high-fidelity PCR (Expand high-fidelity system; Roche, Indianapolis, IN) from genomic DNA of strains 36082\(^a\) and 36082\(^b\) by use of primers SSD1-2 primer pairs SSD1-1 and SSD1-CP1 (Table 2) in a 100-\(\mu\)l reaction mixture by following standard cycling protocols. Reaction products were verified by 0.8% agarose gel electrophoresis and independently cloned into pGEM-T vectors for sequencing. Midi-plasmid prepara-
Table 2. Primer sequences used in the current investigation

| Use                  | Name     | Direction* | Sequence       |
|----------------------|----------|------------|----------------|
| Cloning              | SSD1-NP-1| S          | CGGGATCCATGAGCTGTCGCTACAAGATTAC |
|                      | SSD1-CP-1| AS         | GCGTTCGACTGTCGCAACATGCAAAGCCATAC |
| Sequencing           | S1       | S          | CTGACGACATTGGTC |
|                      | S1.5R    | S          | CGTTTCAAGATCATTAGG |
|                      | S1.75    | S          | GGAATCTCGTGGTAGTGGG |
|                      | S2       | S          | GCTCCTCTACTTCCAC |
|                      | S2.5     | S          | GGAATCTCGAATCTGGCT |
|                      | S3       | S          | CGAACATGTTGAGTC |
|                      | S3.5     | S          | CATGGAATTCCTGGGC |
|                      | S4       | S          | CGTGGTCGAGTATGTCG |
|                      | S4.5     | S          | CACACAAAGTGAAATCTG |
|                      | S5       | S          | CTGGAGGATCAAGC |
|                      | S6       | S          | CGATGTCATGTTGAT |
|                      | AS0      | AS         | TCGGCTGGAGTAGTAG |
|                      | AS1      | AS         | GCAACTGTCACATAGT |
|                      | AS1.5    | AS         | GCAATACGTCATTAGT |
|                      | AS2      | AS         | CCAAATCTGGTTCTTC |
|                      | AS2.5    | AS         | CTAACAATGTCAGTAGG |
|                      | AS4      | AS         | CGTGGTAGCTCTTAC |
|                      | AS5.5    | AS         | CAGTGACACATATGTCAT |
|                      | AS6      | AS         | GCCCCATATGCTAAATG |
|                      | AS7      | AS         | GGAATGGCTAAATCCTC |
|                      | AS8      | AS         | GAGCTTGCATCTCTCC |
| Mutant engineering   | HIS      | AS         | AACGTCGTCAGTGGAAAAC |
|                      | ARG      | AS         | TACCGCCTCAATTCACAC |
|                      | SSD1S    | S          | AACTGCGATGGCGATCAAGAAG |
| Northern probes      | ACT1a    | S          | CGCACTGAAGATGGTTC |
|                      | ACT1b    | AS         | CAAACCTTAATAGCCTGGT |
|                      | EFB1a    | S          | GATGAGGATCATACGTC |
|                      | EFB1b    | AS         | CAAACACGACTTGTTAAC |
| SSD1 overexpression  | TDH3a    | S          | TATTAAATCTCAATTGTTGAGTTATCTCATAATTAAAA |
|                      | TDH3b    | AS         | GCAAGGTCTTCCTCTGAGTTCGAGTCG |
|                      | NorThra  | AS         | ATCAAGCTTGGCTTGGTC |
|                      | NorThrb  | AS         | GCTGACACTTCTCAATAG |

* S, sense; AS, antisense.
Candida albicans strain BWP17 was disrupted using a previously described PCR method (40). The two alleles of the SSD1 gene were replaced by HIS1 and ARG4, respectively. Primers for amplification of HIS1 and ARG4 disruption cassettes, and those used to confirm the integration of HIS1 and ARG4 markers, were as shown in Table 2. The initial SSD1 null mutant genotype was ura3/H11002 (genotype, ssd1/H9004/ssd1/H9004 ura3/ura3). Because it exhibits a gene locus effect (4), URA3 was then replaced in its original locus through homologous recombination. Thus, a 3.9-kb fragment encompassing the complete URA3 gene was reintroduced, yielding the final URA3 prototrophic null mutant strains. Two independent null mutants were created in this manner for comparison and for assessment of reproducibility.

To complement each null mutant, a plasmid containing a hisG-ura3-hisG cassette (pMB-7) was constructed with the complete SSD1 coding sequence plus upstream and downstream flanking regions (4.5-kb DraI-BglII fragment). The construct was linearized by SacI digestion and integrated into the ssd1/H9004/ssd1/H9004 null mutant to create an initial complemented strain. In each complemented organism, URA3 was then removed by intrachromosomal recombination with 5-fluoroorotic acid selection, and URA3 was reintroduced into its native locus as described above. This process yielded final SSD1 complemented strains with URA3 authentically replaced in the original locus. An independent complemented strain was created for each SSD1 null mutant. Prior to further study, the genotypes of each mutant and complemented strain were verified by Southern analysis. The lack of SSD1 expression in null mutants was confirmed by Northern analyses and quantitative PCR.

Candida albicans strain DAY185 (HIS1 ARG4 URA3 heterozygous) served as an SSD1 prototroph control. Because URA3 in strain DAY185 is not in its original locus, C. albicans strain CAF 2-1 was used as an additional control, as it retains a URA3 allele in its original locus (10, 40). All C. albicans strains exhibited equivalent growth rates at 30°C over 48 h.

**SSD1 overexpression studies.** To generate SSD1 overexpression strains of C. albicans, the strong constitutive promoter TDH3 was integrated immediately upstream of the SSD1 coding sequence in DAY185. To do so, a PCR product containing the NAT1-P_{TDH3} cassette (kindly provided by Aaron Mitchell) and the respective primers indicated in Table 2 was generated. The PCR fragment was introduced into recipient strains by standard methods, and the resulting clones were screened on yeast extract-peptone-dextrose agar containing 400

*FIG. 1. Comparative susceptibilities of C. albicans strains containing native or altered SSD1 genotypes. (A) Equivalence in growth rates of a range of inocula (10^6 to 10 CFU) on media lacking protamine after 48 h of incubation. (B) S. cerevisiae was transformed with pE-20H plasmid constructs containing genomic DNA from Candida albicans 36082R. Clones of interest were isolated after 120 h of culture at 30°C on YNB with protamine (5 mg/ml). Plasmid-specific protamine resistance was verified by rescue of plasmids from resistant clones, reintroduction into susceptible S. cerevisiae, and culture on YNB-protamine agar. Quadrants: 1, S. cerevisiae containing the empty plasmid pE-20H; 2 to 4, S. cerevisiae transformed with pE-20H containing a 6.3-kb region of genomic DNA from C. albicans strain 36082R (peptide resistant). Inocula of each strain were equivalent. (C) Differential susceptibilities of strains on media containing protamine (5 mg/ml) after 48 h of incubation.*
μg/ml nourseothricin (25). Next, resulting nourseothricin-resistant clones were verified by colony PCR using cognate primers (Table 1). Overexpression strains were evaluated for protamine resistance as described above.

**Murine model of hematogenously disseminated candidiasis.** To assess the influence of SSD1-mediated antimicrobial peptide resistance in *C. albicans* virulence in vivo, the null and complemented SSD1 strains were compared with their wild-type parent in a murine model of disseminated candidiasis (31). All mouse experiments were carried out according to the NIH guidelines for the ethical treatment of animals. Organisms were confirmed to retain original genotypic and antimicrobial peptide resistance phenotypes following passage in serum (data not shown), as demonstrated for strain 36082 following in vivo infection (42, 43). Groups of 10 male BALB/c mice were randomly selected to receive either the wild-type parent (DAY185), the null mutant (APRΔ-2: ssd1Δ/ ssd1Δ-II), or the complemented (APRΔ-2comp: ssd1Δ/ ssd1Δ-II:SSD1) strain of *C. albicans*. To induce hematogenously disseminated candidiasis, mice were inoculated via the tail vein with 5 × 10³ blastospores of *C. albicans* in 0.3 ml of phosphate-buffered saline (31). Mice were monitored for survival three times daily for 14 days. Animals still surviving at day 14 were censored at that time point. These studies were repeated twice and the results were combined for analysis.

**Statistical analyses.** In vitro experiments were performed a minimum of two independent times on different days. Two-way analysis of variance was used to compare differences in data; the Bonferroni correction for multiple comparisons was used where appropriate. Data exhibiting discontinuous distributions (e.g., MICs) were analyzed by nonparametric Wilcoxon rank sum statistics. Differences in survival among mouse groups infected with comparative strains in which both alleles of *SSD1* resulted in reduced susceptibility to his-5, which exerted relatively low but equivalent activities divergent from those of protamine (Table 3). The null mutant of *SSD1* (e.g., APRΔ-2) in the SC5314 background with *C. albicans* DNA were screened for capability of growth on protamine agar and assessed for candidal gene(s) (Fig. 1B). Eight such clones were chosen randomly and their plasmids analyzed as described above. Each of these clones contained an equivalent 6.3-kb insert, identified by sequencing to be *C. albicans* SSD1. SacI digestion of plasmids containing *C. albicans* SSD1 (e.g., pMYK-2) yielded identical restriction maps comprised of two fragments (1.9 and 4.4 kb). Neither fragment alone conferred protamine resistance when subcloned into *S. cerevisiae* LL20 recipient strain (data not shown).

**Identification of *C. albicans* SSD1-mediated protamine resistance in *S. cerevisiae*.** Clones of *S. cerevisiae* transformed with *C. albicans* DNA were screened for capability of growth on protamine agar and assessed for candidal gene(s) (Fig. 1B). Eight such clones were chosen randomly and their plasmids analyzed as described above. Each of these clones contained an equivalent 6.3-kb insert, identified by sequencing to be *C. albicans* SSD1. SacI digestion of plasmids containing *C. albicans* SSD1 (e.g., pMYK-2) yielded identical restriction maps comprised of two fragments (1.9 and 4.4 kb). Neither fragment alone conferred protamine resistance when subcloned into *S. cerevisiae*.

**In vitro protamine susceptibility of SSD1 null and complemented strains of *C. albicans*.** To verify that *SSD1* governs protamine susceptibility in *C. albicans*, we constructed mutant strains in which both alleles of *SSD1* were disrupted. Null mutation of *SSD1* (e.g., APRΔ-2) in the SC5314 background correlated with significantly high susceptibility to protamine compared to that of the parental strain (DAY185) or those of *SSD1*-complemented strains (e.g., the APRΔ-2comp strain) (Fig. 1C and 2).

**Influence of *SSD1* on resistance to diverse antimicrobial peptides.** Next, we investigated whether *SSD1* also mediates resistance to antimicrobial peptides of sources and structures divergent from those of protamine (Table 3). The null mutant was more susceptible to all antimicrobial peptides tested, except his-5, which exerted relatively low but equivalent activities against all *C. albicans* strains. Conversely, the expression of *SSD1* in *S. cerevisiae* resulted in reduced susceptibility to his-5,
TABLE 3. Comparative influence of C. albicans SSD1 on C. albicans and S. cerevisiae susceptibilities to diverse antimicrobial peptides

| Peptide (conc [mg/ml]) | S. cerevisiae | C. albicans |
|------------------------|---------------|-------------|
| LL20(pE-20H) APR-Δ2 (suda/Δssd1-I) | LL20(pMYK-2) | DAY185 |
| Pro (0.2) | 4.0 | 3.8 | 4.6* | 2.6 |
| Pro (0.4) | 5.0* | 4.3 | 5.5* | 3.3 |
| Pro (0.5) | 6.3* | 5.8 | 6.0* | 4.0 |
| Pro (2) | 8.9* | 8.0 | 10.3* | 5.3 |
| Pro (7) | 12.3 | 9.8 | 12.2* | 8.2 |
| his-5 | 2.2 | 1.2 | 1.6 | 1.8 |
| hNP-1d | 3.8 | 3.9 | 5.6* | 4.0 |
| hJΔ2-Δ2 | 4.6* | 3.4 | 6.0* | 3.3 |
| RP-1f | 6.3* | 4.8 | 6.0* | 3.0 |
| RP-11g | 3.3 | 2.8 | 4.5* | 1.9 |
| RP-13h | 2.2 | 1.7 | 2.7* | 1.0 |

a. Results represent mean values with standard deviations of ±1 mm derived from a minimum of three independent experiments performed on different days, with equivalent results (standard error, ±5%). Asterisks indicate significant differences (P < 0.05) between respective organism strains. Note that a larger zone represents a relative increase in susceptibility; smaller zones represent relative decreases in susceptibility. Results shown for APR-Δ (suda/Δssd1-II) were indistinguishable from those for APR-Δ (suda/Δssd1-Δ1) (see Table 1).

b. hJNP-1 is a 30-amino-acid neutrophil antimicrobial peptide that conforms predominantly to a β-sheet structure; the calculated net charge at pH 7 is +3 (32).

c. hJΔ2 is a 41-amino-acid peptide of β-sheet and turn structure present in several human tissues, such as skin and mucosal secretions; the calculated net charge at pH 7 is +6 (46, 48).

d. RP-1 is an 18-amino-acid α-helical peptidomimetic designed in part based on microbial helices of human platelet factor-4 family kinocidins (44, 49, 50); the calculated net charge at pH 7 is +8.

e. RP-13 is an 18-amino-acid α-helical peptidomimetic designed in part based on microbial helices of human platelet factor-4 family kinocidins (44, 49, 50); the calculated net charge at pH 7 is +6.

f. JS. The authenticity of differential SSD1 expression was verified by independent normalization to ACT1 (Fig. 4), EFB1, or total RNA (data not shown). Importantly, the elevated level of SSD1 transcription was relatively constitutive and not significantly altered by growth in the presence versus the absence of sublethal levels of protease.

Influence of SSD1 on C. albicans virulence in vivo. Results from in vivo studies comparing virulence levels of SSD1 null, complemented, and wild-type C. albicans strains are summarized in Fig. 5. Mice infected the wild-type strain (DAY185) had a median survival of 7 days. In contrast, mice infected with the null mutant (suda/Δssd1 Δ II) exhibited significantly prolonged survival (median survival, 9 days) compared with their wild-type strain-infected counterparts (P < 0.05). Importantly, complementation of the null mutant with SSD1 restored virulence to that of the wild-type strain.

DISCUSSION

Previous studies have demonstrated that reduced in vitro susceptibility to antimicrobial peptides correlates with increased virulence of C. albicans in a rabbit model of infective endocarditis and hematogenous dissemination (42, 43). More recently, this concept has been extended to show that antimicrobial peptide resistance negatively influences antifungal efficacy in this model (42). In the above-described studies, the
antimicrobial peptide-resistant *C. albicans* strain 36082R achieved significantly greater densities in cardiac vegetations and splenic tissue and was less susceptible to fluconazole therapy than its peptide-susceptible counterpart, 36082S. The current studies were undertaken to explore the genetic basis for antimicrobial peptide resistance in *C. albicans*.

The current data support the hypothesis that increased SSD1 expression in *C. albicans*, or heterologous expression in *S. cerevisiae*, confers increased resistance to antimicrobial peptides. This conclusion derives from the following complementary lines of evidence: (i) genome screening identified SSD1 from *C. albicans* as being uniquely capable of permitting *S. cerevisiae* growth in the presence of protamine; (ii) *S. cerevisiae* clones transformed to overexpress *C. albicans* SSD1 had increased resistance to antimicrobial peptides; (iii) although equivalent in sequence in the peptide-resistant strain 36082R and in its susceptible counterpart, 36082S, SSD1 is constitutively overexpressed in 36082R compared with what is seen for 36082S; (iv) null mutation of SSD1 rendered *C. albicans* hypersusceptible to antimicrobial peptides; (v) complementation of this gene in *C. albicans* restored the parental resistance phenotype; and (vi) constitutive SSD1 overexpression in the DAY185 background conferred enhanced protamine resistance. Collectively, these results support the concept that resistance to host defense peptides is mediated by constitutively high or rapidly induced SSD1 expression early in the face of antimicrobial peptide exposure.

In the present study, the hypothesis that in vitro resistance to antimicrobial peptides would translate into increased virulence during invasive candidiasis was examined by using the murine model. In these studies, null mutation of SSD1 significantly impaired *C. albicans* virulence compared with what was seen

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**FIG. 3.** Impact of SSD1 overexpression on protamine resistance in *C. albicans*. Serial dilutions of parental (DAY185), APRΔ-2 (ssd1Δ/ssd1Δ-II), and DAY185ovex (*P_{TDH3}-SSD1/SSD1*) strains were plated onto Sabouraud agar containing protamine ranging in concentration from 0 to 7 mg/ml. This figure illustrates the impact of SSD1 overexpression compared with parental and null mutant strains in the presence of 5 mg/ml protamine at 48 h postinoculation. Similar results were observed for protamine concentrations of 3 mg/ml or greater.

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**TABLE 4.** Influence of SSD1 on *C. albicans* susceptibility to nonpeptide antifungal disruptants*<sup>a</sup>

| Genetic background | Specific strain (genotype) | MIC of: | MFC of: |
|--------------------|---------------------------|---------|---------|
|                    |                           | *H₂O₂* (mM) | SDS (% [wt/vol]) | AMB (μg/ml) | *H₂O₂* (mM) | SDS (% [wt/vol]) | AMB (μg/ml) |
| 36082              | 36082<sup>S</sup>         | 2.5     | 0.013   | 2.5      | 6.25       | 0.013   | 5          |
| 36082              | 36082<sup>R</sup>         | 1.25    | 0.013   | 1.25     | 3.13       | 0.013   | 2.5        |
| DAY185             | APRΔ-2 (ssd1Δ/ssd1Δ-II)   | 3.13    | 0.013   | 1.25     | 3.13       | 0.013   | 1.25       |
|                    | APRΔ-2comp (ssd1Δ/ssd1Δ-II::SSD1) | 3.13 | 0.013 | 2.5 | 3.13 | 0.013 | 2.5 |

<sup>a</sup> MICs and MFCs were determined by standard assays as detailed in Materials and Methods. Prototypic agents were *H₂O₂* (oxidative injury), SDS (phospholipid disruptant), and AMB (polyene ergosterol antagonist). Data represent means of three independent experiments, which yielded highly reproducible results. Strains are as defined in Table 1.
for wild-type or complemented strains competent with respect to SSD1. Thus, the present data substantiate the concept that relative susceptibility or resistance to antimicrobial peptides significantly influences C. albicans virulence. These results support prior correlates between increased candidal virulence and resistance to host defense peptides in the rabbit model of disseminated infection (41–43).

The current findings suggest that SSD1 governs C. albicans resistance to host defense peptides in multiple anatomic contexts. For example, SSD1 affords resistance to α-defensins (e.g., hNP-1) or β-defensins (e.g., hβD-2) originating from mucosa, phagocytes, or other sites. Likewise, SSD1 expression reduced susceptibility to synthetic peptides RP-1, RP-11, and RP-13, which are modeled upon α-helical or β-hairpin determinants of platelet microbicidal proteins found in the bloodstream (44, 50). It should be noted that mice express α-defensins (e.g., cryptdins) (26) and β-defensins (32) as well as helical cathelicidin-related antimicrobial peptide in skin, mammary glands, and other tissues and in saliva (24). Despite structural distinctions, many host defense peptides have a net cationic charge at physiologic pH (45, 49), contain structural archetypes such as the γ-core motif (47), and have similar microbial targets, including the cell membrane and cellular energetics (45, 48). The fact that SSD1 appears to confer protection against host defense peptides differing in structure but sharing the mode of action suggests commonalities in peptide-induced injury against which SSD1 protects C. albicans. In contrast, an absence of candidal SSD1 in either S. cerevisiae or C. albicans did not render organisms hypersusceptible to his-5, which is found in human saliva. These findings imply that his-5 has targets different from those of other antimicrobial peptides examined in this study. Finally, we observed that peptide-susceptible strains predominated in cases of twofold-increased MICs or MFCs to nonpeptide stressors (e.g., H2O2 or AMB). This inverse relationship, where SSD1 confers resistance to some but not all peptides and does not confer resistance to nonpeptide stressors, substantiates our hypothesis that SSD1 mediates relatively specific resistance to relevant host defense effector molecules.

Paradoxically, deletion of SSD1 in S. cerevisiae has been

**FIG. 4.** Comparative kinetics of SSD1 expression in C. albicans strains 36082S (S) and 36082R (R) in the presence or absence of protamine (0.4 mg/ml) by Northern analysis. In the bottom panel, relative levels of expression of SSD1 in strains 36082S and 36082R were derived from densitometric analysis normalized to the housekeeping gene ACT1. Key: white bars, 36082S; black bars, 36082R. Note the consistently higher expression of SSD1 in strain 36082R in the presence or absence of protamine. Normalization to EF1 and to total 28S rRNA produced equivalent patterns of results (data not shown).
FIG. 5. Influence of SSD1 on C. albicans virulence in the murine model of hematogenously disseminated candidiasis. Male BALB/c mice were injected via the tail vein with 5 × 10^5 blastospores of C. albicans in 0.3 ml of phosphate-buffered saline. Each strain was injected into 9 or 10 mice per group per experiment, and the mice were monitored for survival three times daily for 14 days. Animals still surviving at day 14 were censored at that time point. The experiment was repeated twice and the results were combined. Strain key: O, DAY185 (wild type); □, APRΔΔ (ssd1Δ/ssd1Δ-H1 null mutant); ▲, APR32comp (ssd1Δ/ssd1Δ-H1::SSD1 complemented). The asterisk indicates a significant difference from the parent or complemented strain (P < 0.05).
