Two-component signaling systems are widely used for signal transduction by bacteria, eukaryotic microorganisms, and plants. A two-component phosphorelay system in fungi includes a membrane-bound sensor histidine kinase (HK) protein, which is autophosphorylated on a histidine residue in the histidine kinase domain and then transfers the phosphoryl group to an internal receiver domain. The phosphohistidine is then shuttled through a histidine phosphotransfer (HPT) protein to a terminal response regulator (RR). When stress signals (oxidants, high salt, etc.) are detected by yeast cells, the RR protein is dephosphorylated and is able to activate the downstream mitogen-activated protein kinase (MAPK) pathway to adapt cells to stress (18, 19). The most extensively studied of the two-component MAPK systems in fungi is the hyperosmotic glycerol (HOG) MAPK pathway in Saccharomyces cerevisiae (19, 25). In Candida albicans, Hog1 MAPK regulates glycerol accumulation and adaptation to high osmolarity, as well as oxidative stress, morphogenesis, and cell wall biosynthesis (2, 3, 38). Homologues of the Hog1 pathway, as well as other HKs and the response regulator protein (Ssk1p) of C. albicans, have been extensively studied (21, 27–30, 39, 41).

In C. albicans, there are three HK proteins, Chk1p, Sln1p, and Nik1/Cos1p (1, 12, 17, 32, 43). Sln1p is the homologue of S. cerevisiae Snl1p. While Chk1p is not found in S. cerevisiae, two homologues have been identified in Schizosaccharomyces pombe (9), but a functional pathway has not been assigned. Nik1/Cos1p is a homologue of the Nik1 protein of Neurospora crassa and is also not found in S. cerevisiae. The genes that encode RR proteins include SKK1 and SKN7. Ssk1p of S. cerevisiae activates the Hog1 MAP kinase pathway during osmotic stress, while Skn7p in S. cerevisiae acts as a transcription factor when phosphorylated. While homologues of these proteins are found in C. albicans, it is important to realize that C. albicans homologues have functions that are different from those of their counterparts in S. cerevisiae. We have shown previously that Ssk1p of C. albicans is required for adaptation to oxidative stress and regulates some aspects of cell wall biosynthesis (21), functions not reported for S. cerevisiae SSK1.

We report here that C. albicans possesses an additional response regulator gene, SRR1 (orf19.5843), besides the previously reported response regulators SSK1 and SKN7 (18). The newly identified response regulator SRR1 appears to be unique to the Candida clade (10). Homology searches did not reveal any orthologues of this protein in S. cerevisiae, Candida glabrata, or Candida krusei. However, orthologues of SRR1 are present in pathogenic fungi belonging to the CUG clade of Saccharomycotina (10). In C. albicans, previous studies by us and other groups have shown that both Ssk1 and Skn7 response regulators have important functions in morphogenesis and oxidative stress adaptation (14, 21, 40). In the present study, we demonstrate that a Δsrr1 mutant is defective in hyphal development and sensitive to both osmotic- and oxidative-stress growth conditions. Disruption of SRR1 causes severe virulence attenuation in the mouse model of disseminated candidiasis. These data imply that C. albicans utilizes SRR1 to regulate a number of critical processes to adapt to the host environment, including stress adaptation, hyphal development, and pathogenicity.

The SRR1 gene was identified by searching the Candida genome database (CGD) for proteins containing a receiver domain by performing BLASTP analysis. Because of its apparent functions in stress (oxidative and osmotic) adaptation, this response regulator gene was named SRR1 (stress response regulator 1). The SRR1 gene has an open reading frame of 849 bp, which encodes a 282-amino-acid (aa) protein with an estimated molecular mass of 32 kDa. Srr1p contains a receiver domain at the C terminus (154 to 270 aa) of this protein. The analysis of the promoter region of SRR1 by MatInspector (16) revealed that it contains a consensus sequence (underlined in 5′-TGCAACAGGGGGAGG-3′) of the fungal stress response elements (STRE); it remains to be seen if it is involved in the modulation of expression of SRR1. A putative peroxisomal
targeting signal 1 (PTS1) sequence, NKI, is located at the C terminus of this protein. PTS1 motif prediction was performed by using the PTS1 predictor software (http://psort.hgc.jp/form2 .html). A similar motif has been reported in Skk1p of C. albicans (13), but its functional characterization has not yet been reported.

To determine the function(s) of SRR1 in C. albicans, we constructed a \( \Delta srr1 \) mutant by following the method of Noble and Johnson (36) with the SN148 strain background (see Fig. S1 in the supplemental material). To generate strains with matched auxotrophic requirements, the \( URA3 \), \( HIS1 \), and \( LEU2 \) auxotrophic markers were restored by integration of an empty Cip10 (31) and a modified Cip20 (23) plasmid containing the \( LEU2 \) marker Cip20-Leu2 (see Tables S1 and S2 in the supplemental material) at the \( RPS10 \) locus. When these plasmids are integrated at the \( RPS10 \) locus, restoration of wild-type virulence and \( in vitro \) phenotypes to \( ura3 \) strains occurs (7). The \( C. albicans \) strains (see Table S3 in the supplemental material) generated in this way were auxotrophically (\( Ura^+ \) His\(^+\) Leu\(^+\) Arg\(^-\)) identical. The strains thus created were all \( Arg^- \). Arginine auxotrophy has been shown to be neutral for \( C. albicans \) virulence (36). All the experiments reported in the current study were performed by using strains NC1 (\( SRR1/SRR1 \)), NC2 (\( srr1/srr1 \)), and NC3 (\( srr1/srr1/SRR1 \)).

To determine the effect of \( SRR1 \) disruption on growth rates, we compared the generation time of \( SRR1 \) deletion strain NC2 (\( srr1/srr1 \)) with those of wild-type strain NC1 (\( SRR1/SRR1 \)) and gene-reconstituted strain NC3 (\( srr1/srr1/SRR1 \)). When incubated in yeast extract-peptone-dextrose (YPD) at 30°C, the NC2 strain grew significantly more slowly than the wild-type strain NC1, whereas the generation rate of the gene-reconstituted strain NC3 was similar to that of the wild-type strain. The calculated generation time for the null strain NC2 (2.13 h) was significantly higher than the 1.55-h and 1.59-h generation times of the wild type (NC1) and gene-reconstituted strain (NC3), respectively.

The two previously reported response regulators have functions related to the stress resistance, morphogenesis, and virulence of \( C. albicans \) (14, 21, 40). Thus, our initial experiments with \( SRR1 \) were focused to determine its role in stress response. The NC2 (\( srr1/srr1 \)) mutant was sensitive to osmotic stress (1.5 M NaCl), a phenotype not observed with a \( C. albicans \) \( \Delta ssk1 \) or \( \Delta skn7 \) mutant (21, 40), implying that Srr1p is critical to the response of cells to osmotic stress (Fig. 1). The NC2 (\( srr1/srr1 \)) null mutant strain was also sensitive to hydrogen peroxide at a concentration of 8 mM (Fig. 1), suggesting that Srr1p is required for resistance to oxidative stress \( in vitro \) and, in this regard, is somewhat similar to other response regulators in \( C. albicans \), Skn7p and Ssk1p (21, 40). We did not observe any difference between wild-type and null mutant strains in their sensitivities to menadione, potassium superoxide, sodium dodecyl sulfate (SDS), caffeine, Congo red, or calcofluor white (data not shown). Data from MIC assays verify (Table 1) that the null mutant strain NC2 (\( srr1/srr1 \)) is more sensitive to oxidative and osmotic stress than the wild type (NC1) and reintegrant strain (NC3). In \( in vitro \) killing assays also confirmed the hypersusceptibility of the NC2 mutant strain to H\(_2\)O\(_2\) (data not shown). Thus, \( SRR1 \) is required for protection from oxidants, a result similar to that reported for the \( C. albicans \) \( \Delta ssk1 \) null mutant (21). However, unlike the \( \Delta ssk1 \) or \( \Delta skn7 \) response regulator mutant, the \( \Delta srr1 \) mutant is sensitive

![FIG. 1. Growth of NC1 (\( SRR1/SRR1 \)), NC2 (\( srr1/srr1 \)), and gene-reconstituted strain NC3 (\( srr1/srr1/SRR1 \)) at 30°C for 24 h on YPD agar (control plate) (A), YPD agar containing 8 mM H\(_2\)O\(_2\) (B), and YPD agar containing 1.5 M NaCl (C). Five-microliter cell dilutions (5 \times 10\(^5\) to \( 5 \times 10^3 \) cells) were spotted on the YPD agar compounds.

<table>
<thead>
<tr>
<th>( C. albicans ) strain</th>
<th>( H_2O_2 ) (mM)</th>
<th>NaCl (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC1 (( SRR1/SRR1 ))</td>
<td>10.0</td>
<td>1.8</td>
</tr>
<tr>
<td>NC2 (( srr1/srr1 ))</td>
<td>8.0</td>
<td>1.0</td>
</tr>
<tr>
<td>NC3 (( srr1/srr1/SRR1 ))</td>
<td>10.0</td>
<td>1.8</td>
</tr>
</tbody>
</table>

TABLE 1. Susceptibilities of \( C. albicans \) strains to hydrogen peroxide and sodium chloride.
to osmotic stress, suggesting that \textit{SRR1} is required for mediating resistance to osmotic stress.

Because the two previously reported response regulators Ssk1 and Skn7 have functions in hyphal formation, it was of interest to determine the effect of \textit{SRR1} disruption on morphology by growing \textit{C. albicans} strains on Spider, 10\% serum, and RPMI (pH 7.5) growth media. The NC2 (\textit{srr1/srr1}) null mutant strains displayed suppressed filament formation in hyphae, inducing growth comparable to that of the wild-type and reconstituted strains, indicating that \textit{SRR1} is critical for hyphal development in \textit{C. albicans} under the growth conditions used in our experiments (Fig. 2).

To investigate the role of Srr1p in virulence, wild-type NC1, null mutant NC2, and gene-reconstituted strain NC3 were inoculated intravenously into immunocompetent BALB/c mice.

The survival of mice over a 21-day period of time was determined for each strain (Fig. 3). We found that mice infected with the wild-type strain rapidly succumbed to the infection within 3 days, whereas mice infected with the null mutant strain NC2 (\textit{srr1/srr1}) survived for at least 3 weeks (the duration of experiment). Mice infected with the gene-reconstituted strain NC3 (\textit{srr1/srr1/SRR1}) survived longer than mice infected with the wild type. The colonization (CFU/g) of kidneys was determined for each strain at 24 to 72 h postinfection (Table 2), except for animals infected with the wild-type strain NC1, since all were moribund by 72 h. Fungal burdens, expressed as numbers of CFU/g of kidney (Table 2), were relatively constant for both the wild-type and gene-reconstituted strains over the course of 72 h. However, for the NC2 (\textit{srr1/srr1}) null mutant strain, the highest number of CFU/g kidney was significantly lower ($P < 0.0001$; NC2 versus all strains) than for the wild-type strain, suggesting that the NC2 (\textit{srr1/srr1}) null mutant strain was rapidly cleared from the kidneys. Histopathology of kidney sections supports this observation (Fig. 4). Taken together, the data clearly demonstrate that Srr1p is required for virulence in a murine model of systemic candidiasis.

### FIG. 2. Phenotypes of the NC1 (\textit{SRR1/SRR1}), NC2 (\textit{srr1/srr1}), and NC3 (\textit{srr1/srr1/SRR1}) strains grown on solid media (Spider medium, RPMI medium, and 10\% serum), which induce hyphal development. All the plates were incubated for 5 days at 30\°C, and representative colonies were photographed.

### FIG. 3. Survival of mice infected with \textit{C. albicans} strains in a murine model of hematogenously disseminated candidiasis. Mice were infected with $1 \times 10^6$ CFU of each strain, namely, NC1 (\textit{SRR1/SRR1}), NC2 (\textit{srr1/srr1}), and NC3 (\textit{srr1/srr1/SRR1}). The percent survival of mice infected with each strain was determined 21 days postinfection.

### TABLE 2. Log$_{10}$ CFU/g counts in mice infected with \textit{C. albicans} strains

<table>
<thead>
<tr>
<th>\textit{C. albicans} strain</th>
<th>Log$_{10}$ no. of CFU/g of tissue (mean ± SD) in kidney$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>NC1 (\textit{SRR1/SRR1})$^b$</td>
<td>6.14 ± 0.35</td>
</tr>
<tr>
<td>NC2 (\textit{srr1/srr1})</td>
<td>3.53 ± 0.42</td>
</tr>
<tr>
<td>NC3 (\textit{srr1/srr1/SRR1})</td>
<td>5.99 ± 0.37</td>
</tr>
</tbody>
</table>

$^a$ At 24, 48, and 72 h postinfection, moribund mice were sacrificed, and the number of CFU of each strain was determined by plating homogenates of kidneys on YPD agar. Cultures were incubated at 30\°C, and colonies were counted after 48 h of incubation. ND, not detected.

$^b$ All mice infected with NC1 succumbed to infection by 48 h.
There are functional differences between the three response regulator proteins of \textit{C. albicans}, Srr1p, Ssk1p, and Skn7p, that may have an impact on the contribution of each protein to disease. The characterization of the NC2 (\textit{srr1/srr1}) mutant strain clearly demonstrates a role for SRR1 in resistance to osmotic and oxidative stress. The avirulence of the \textit{\Delta srr1} null mutant is similar to that of the \textit{\Delta ssk1} mutant, while the virulence of the \textit{\Delta skn7} mutant was only slightly attenuated (40).

Previous reports have suggested that \textit{C. albicans} mutants with growth defects tend to have reduced virulence in a mouse model of systemic candidiasis (37). Because the NC2 (\textit{srr1/srr1}) null mutant has a longer generation time than the wild type, there is a concern that its avirulence might be attributed to its longer generation time. In this context, it has been reported that mutants with long generation times still cause disease and death in mice at slightly higher inoculum densities than those used here (20), indicating that the generation time is less critical than the inoculum density to the development of candidiasis. A recent study has also revealed that growth rate is not essential for \textit{C. albicans} pathogenicity, as previously thought (35). In that study, it was reported that mutants with low growth rates showed normal infectivity in a mouse model of disseminated candidiasis (35). There are other examples in the literature about \textit{C. albicans} mutants exhibiting normal virulence despite an \textit{in vitro} growth defect phenotype. For example, the \textit{C. albicans} protein kinase \textit{ckA2} null mutant strain has a growth defect but still exhibits normal virulence, like the wild-type strain, in the mouse model of hematogenously disseminated candidiasis (22). Taken together, data from these studies suggest that \textit{C. albicans} virulence is a complex interplay of multiple (host as well as pathogen) factors. \textit{In vitro} assays cannot replicate complex \textit{in vivo} systems, and growth rate assays performed \textit{in vitro} cannot be the exclusive determinants of \textit{C. albicans} virulence.

The virulence of \textit{C. albicans} is multifactorial, including the requirement for adhesins, yeast- to hyphal-phase transition (morphogenesis), and expression of extracellular invasive enzymes, such as secreted aspartyl proteases and phospholipases (11). The ability to overcome stress has also been reported to be important for \textit{C. albicans} virulence. Several studies have also shown that stress adaptation is essential for \textit{C. albicans} virulence (8). \textit{C. albicans} strains with mutations in genes that encode the transcription factor \textit{Cap1p} (5), superoxide dismutase (26), catalase (33, 42), the stress-activated MAPK Hog1p (3), the upstream two-component histidine kinase Chk1p (15), and the response regulator Ssk1p (14) are reported to be avirulent in a murine model of disseminated candidiasis. Our findings, with SRR1, also reveal a positive relationship between stress adaptation and virulence. Data presented in this paper strongly support a role for SRR1 in morphogenesis, stress adaptation, and virulence of \textit{C. albicans}. Two-component proteins have also been reported to be essential for stress adaptation and virulence in other pathogenic fungi, including \textit{Aspergillus fumigatus} (24), \textit{Cryptococcus neoformans} (4, 6), and \textit{Blastomyces dermatitidis} (34), suggesting that their exploitation could lead to the development of drugs that may have broad spectra.

This work is supported in part by an American Heart Association National Scientist Development Grant (0635108N) to N.C.

We thank Suzanne Noble, UCSF, for the generous gift of pSN40 and pSN52 plasmid vectors and the wild-type \textit{C. albicans} SN148 strain for this work. We also thank Al Brown, University of Aberdeen, and Michael Lorenz, University of Texas Health Science Center at Houston, for plasmid Clp20. We gratefully acknowledge Guillaume Delmas, PHRI Center at UMDNJ, for performing animal experiments.

REFERENCES


FIG. 4. Periodic acid-Schiff (PAS)-stained kidney sections from mice infected with \textit{C. albicans} strains NC1 (SRR1/SRR1), NC2 (\textit{srr1/srr1}), and NC3 (\textit{\Delta srr1/srr1}). Arrows indicate the locations of yeast cells.