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SKN7 of *Candida albicans*: Mutant Construction and Phenotype Analysis

Praveen Singh,[1] Neeraj Chauhan,[1] Anup Ghosh,[1] Freddie Dixon,[2] and Richard Calderone[1]*

Department of Microbiology and Immunology, Georgetown University Medical Center,[1] and Department of Biological and Environmental Sciences, The University of the District of Columbia,[2] Washington, D.C.

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The *SKN7* two-component response regulator gene of *Candida albicans* was deleted, and the phenotype of the mutant was established. This mutant exhibited impaired growth on Spider agar and 10% serum agar compared to wild-type and gene-reconstituted strains. The *skn7* mutant was sensitive to H_2O_2 in vitro, but its virulence was only mildly attenuated. A comparison of the Skn7p and Ssk1p response regulators of *C. albicans* is discussed.

Two-component signal transduction pathways are found in prokaryotes and in several eukaryotic microorganisms and higher plants (6, 15, 20, 21, 27, 29, 31, 32, 37, 43). These signal pathways are essential for stress adaptation, quorum sensing, and the regulation of virulence in bacteria and fungi (6, 15, 48). The HOG1 mitogen-activated protein kinase (MAPK) two-component pathway of *Saccharomyces cerevisiae* that adapts cells to osmotic stress consists of a transmembrane, a histidine kinase sensor protein (Sln1p), a phosphohistidine intermediate protein (Ypd1p), and a response regulator protein (Ssk1p). Similar to the case for *S. cerevisiae*, two response regulator proteins (Ssk1p and Skn7p) have been identified in *Candida albicans*, but functional information on Skn7p is not known. The Ssk1p of *C. albicans* is not essential for osmoregulation but is required for oxidative stress and the expression of genes encoding mannosylation functions (*MNN4*), structural mannoproteins (*ALS1*, *FLO1*), and a two-component histidine kinase (*CHK1*) that regulates cell wall assembly (16, 23). The *ssk1* mutant is killed more readily by human neutrophils, is less adherent, and is avirulent (7, 20, 33). In *S. cerevisiae*, Skn7p adapts cells to oxidant and heat stresses but has also been assigned roles in cell cycle control, cell wall regulation, and nitrogen starvation-induced diploid filamentous growth (1, 11–13, 22, 27, 31, 34, 35, 37). In this report, the construction of a *C. albicans skn7* mutant and its phenotype in vitro and in vivo are described.

The sequence of the *C. albicans SKN7* open reading frame (1,680 bp) was obtained from the Candida page (http://alces.med.umn.edu/Candida.html) and from http://www-sequence.stanford.edu/group/candida/. The deduced *C. albicans* protein of 559 amino acids was compared to the Skn7p from *S. cerevisiae* by using the Clustal W program (http://clustalw.genome.ad.jp/). The similarities of the *C. albicans* Skn7p to the *S. cerevisiae*, *Aspergillus nidulans*, and *Schizosaccharomyces pombe* homologues are approximately 31, 4, and 15%, respectively (data not shown). For *C. albicans* and *S. cerevisiae*, the similarity of each protein is generally restricted to the heat shock factor DNA binding domain (amino acids 24 to 229) and the receiver domain (amino acids 424 to 543), with the latter containing the conserved aspartate residue that is putatively phosphorylated during signal transfer. A coiled-coil region (residues 160 to 203), which is thought to be responsible for protein-protein interactions, was also identified.

We used the urablaster technique (26) to construct a *skn7*Δ null mutant (N2) (Table 1 and Fig. 1) according to standard methods of transformation and Southern hybridization (17, 20, 42, 45). In addition, a strain that was reconstituted with one allele of *SKN7* and *URA3* (strain R15) was also constructed to ensure that the phenotypes of the strains, described below, were not the result of manipulation of strains during transformation (Fig. 1B). In Fig. 1A, the cassette that was used to disrupt the *SKN7* is shown. We replaced a 1.289-kb fragment of *SKN7* that included the coding regions for the heat shock factor DNA binding coiled-coil domain and the receiver domain (Fig. 1A) with the *hisG-URA3-hisG* disruption cassette containing the 5′ and 3′ flanking sequences of *SKN7*. Following the first transformation, Ura^+ clones were selected on yeast nitrogen base minimal medium. A total of 10 transformants were isolated. DNA from strain N1 was digested with KpnI and HindIII and analyzed by Southern blot hybridization (Fig. 1C). We observed a 1.365-kb fragment that corresponded to the parental allele and a 5.57-kb fragment consistent with the replacement in one allele of *SKN7* with the disruption cassette. Ura^- segregants were isolated from 5-fluoroorotic acid (5-FOA) plates and examined by Southern blotting. The 5.57-kb fragment seen in strain N1 was absent in the 5-FOA segregant (N1.5), and a new 2.86-kb hybridizing fragment was present (Fig. 1C), consistent with the loss of the *URA3* and one copy of the *hisG* fragment from the entire disruption cassette. A second transformation was performed with strain N1.5 ($Ura3^-$), and the remaining allele was disrupted. Southern hybridization of a representative $Ura3^+$ transformant with

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Georgetown University Medical Center, SE 312 Med Dent Building, 3900 Reservoir Rd. NW, Washington, DC 20007. Phone: (202) 687-1137. Fax: (202) 687-1800. E-mail: calderor@georgetown.edu.

TABLE 1. *C. albicans* strains used in this study

Strain	Genotype	Source
CAF2-1	Δ*ura3*::*imm434*/*URA3*	26
CAI4	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	26
N1	Δ*ura3*::*imm434*/Δ*ura3*::*imm434* Δ*skn7*::*hisG-URA3-hisG*/*SKN7*	This work
N1.5	Δ*ura3*::*imm434*/Δ*ura3*::*imm434* Δ*skn7*::*hisG*/*SKN7*	This work
N2	Δ*ura3*::*imm434*/Δ*ura3*::*imm434* Δ*skn7*::*hisG*/Δ*skn7*::*hisG-URA3-hisG*	This work
R15	Δ*ura3*::*imm434*/Δ*ura3*::*imm434* Δ*skn7*::*hisG*/*SKN7*::*hisG-URA3-hisG*	This work

both alleles of *SKN7* deleted (N2) is shown in Fig. 1C. In strain N2, two hybridizing fragments were seen, and these fragments correspond to the second disrupted allele (5.57 kb) and the 2.86-kb fragment as described for the other disrupted allele lacking *URA3* and one *hisG*. Then, a strain reconstituted for one allele was constructed after transformation of the *skn7* Ura3$^-$ mutant (N2.5) and selection on 5-FOA as described above. The *SKN7-URA3-hisG* cassette used for that transformation is shown in Fig. 1B. Several Ura3$^+$ transformants (strain R15) were selected on yeast nitrogen base minimal medium as described above, and the integration of the transforming DNA in the Δ*skn7*::*hisG* locus was confirmed by PCR analysis of five transformants (Fig. 1D). A 3.05-kb fragment corresponding to *SKN7* and *URA3* was observed, indicating the restoration of one *SKN7* allele. The reconstituted strain (R15) was also confirmed by Southern hybridization (data not shown). Transcripts in all strains except the *skn7* mutant were measured by reverse transcription (RT)-PCR using a standard protocol (33) with the primer set CAACAACAGTCACTT GGAC and CCGACATACCATTCTGC (data not shown).

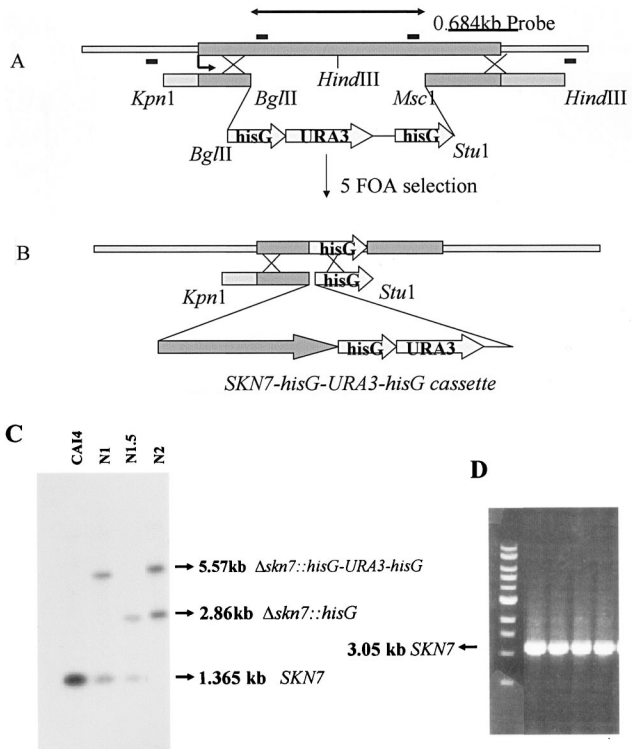

FIG. 1. (A) The cassette used to disrupt the *SKN7* allele. (B) The *SKN7-URA3-hisG* cassette used to reintroduce a wild-type allele into strain N2 (*skn7*/*skn7*) lacking the *URA3*. The primers used to amplify the flanking sequences are indicated (▬). A 1.29-kb section of the gene, including the receiver domain, was deleted. Also, the 0.68-kb 3′ end of the gene was used as a probe for the Southern hybridizations. (C) Southern blot analysis of strains obtained during the disruption of *SKN7*. Genomic DNA was digested with KpnI and HindIII and probed with the 0.68-kb Msc1-HindIII PCR product (panel A). The size of each hybridizing band is indicated. The Ura3$^+$ heterozygote (N1), the Ura3$^-$ heterozygote (N1.5), and the Ura3$^+$ null mutant (N2) are shown (see text for details). (D) PCR of four clones of the gene-reconstituted strain (R15) indicates a single PCR product of 3.05 kb. Strain R15 was confirmed by Southern hybridizations.

The effect of the *SKN7* disruption on morphogenesis was evaluated on 10% serum, M-199 (pH 7.5), and Spider agar media (Fig. 2) (17). The *skn7* mutant produced smooth colonies on Spider agar (Fig. 2, bottom) and M-199 (pH 7.5) agar (Fig. 2, middle) and exhibited reduced growth on 10% serum agar (Fig. 2, top) compared to CAF2. All strains grew similarly on synthetic low-ammonium dextrose agar. Thus, the *SKN7* gene product is required for morphogenesis under some of the conditions used in our experiments (data not shown).

We used t-butyl hydroperoxide, menadione, KO_2, and H_2O_2 to measure the sensitivities of all strains to several types of oxidants (Fig. 3). These experiments were predicated on the oxidant adaptation function of *S. cerevisiae* Skn7p (37). For the oxidant assays, drop plates of several concentrations of yeast cells of each strain (10^5 to 10 cells per 5 μl) were spotted on agar containing either 1 mM t-butyl hydroperoxide or 3 mM H_2O_2. Growth of the null mutant (N2) after 48 h at 30°C in the presence of either H_2O_2 or t-butyl hydroperoxide was significantly lower than that of CAF2, while both the heterozygote (N1) and the gene-reconstituted (R15) strains were similar in their growth but slightly more sensitive than CAF2 (Fig. 3). Sensitivities to menadione, KO_2, 1.5 M NaCl, and the antifungals amphotericin B, miconazole, and nikkomycin Z were similar for all strains (data not shown).

In *S. cerevisiae*, Skn7p regulates mannosylation (27, 34). Therefore, to determine whether the *C. albicans* Skn7p has a similar function, we performed an analysis of total cell wall hexose content as well as the hexose content of alkali-soluble, alkali-insoluble, and Zymolyase-soluble and -insoluble cell wall fractions by using standard methodologies (10, 24, 30). However, we did not observe any significant differences in the hexose contents of alkali-soluble, alkali-insoluble, Zymolyase-soluble, and Zymolyase-insoluble fractions of purified cell walls from strains CAF2, N1, N2, and R15. Likewise, chitin contents were similar for all strains (data not shown).

We measured the virulence of strains CAF2, N1, N2, and R15 in the hematogenously disseminated murine model of candidiasis by standard procedures (18, 20). All strains have a single copy of *URA3*. The percentage of survival of mice in-

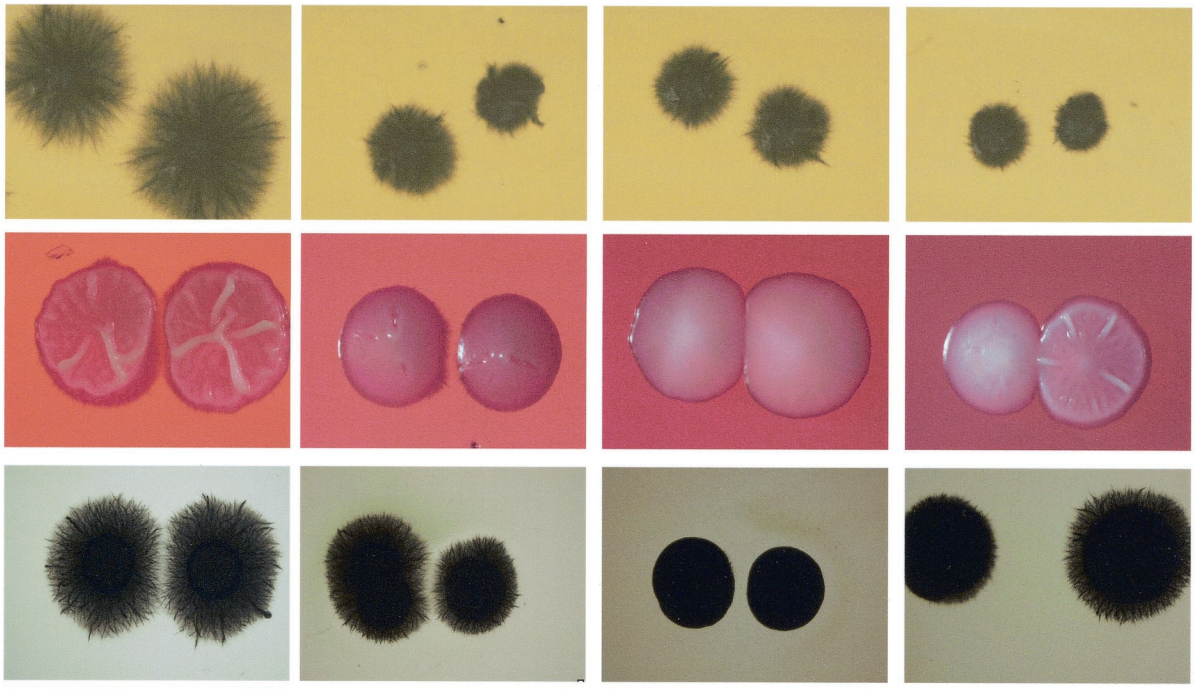

FIG. 2. Phenotypes of the parent (CAF2; row 1), and heterozygote (N1; row 2), null (N2; row 3), and reconstituted (R15; row 4) strains of *C. albicans*. All strains are shown on 10% serum agar (top), M-199 (pH 7.5) (middle), or Spider agar (bottom). All cultures were incubated for 3 to 5 days at 37°C on each medium.

fected with CAF2 (wild type) was reduced significantly, and by 3 days, all animals were euthanized. The virulence of strains N1, N2, and R15 was only slightly attenuated, since by 6 to 8 days, all animals showed signs of morbidity (data not shown). Additionally, CFU levels from the kidneys for all strains at 24 and 48 h postinfection were similar, and filamentous growth in the kidneys of all animals was observed at 48 h.

Two-component signaling is critical to stress adaptation of fungi such as *S. cerevisiae* and *C. albicans* (15, 27, 32, 37, 43). In *C. albicans*, two-component proteins and the downstream MAPK Hog1p regulate adaptation to stresses, including oxidant and osmostress, adherence, cell wall synthesis, morphogenesis, and virulence (3, 7, 20, 23, 33, 38, 49). In addition to the Sln1p histidine kinase of the HOG MAPK pathway mentioned above, *C. albicans* has two other histidine kinases (Chk1p and Nik1p), neither of which is found in *S. cerevisiae* (2, 4, 17–19, 38, 43, 44, 46, 49). Chk1p is a cytoplasmic HK protein that plays a major role in the cell wall biosynthesis and virulence of *C. albicans* (18, 30), while Nik1p is a homologue of the *Neurospora crassa* histidine kinase and the *FOS1* of *Aspergillus fumigatus* (41).

We demonstrated that, in *C. albicans*, Skn7p is required for adaptation to some types of oxidant stress in vitro and in this regard is somewhat similar to another response regulator in *C.*

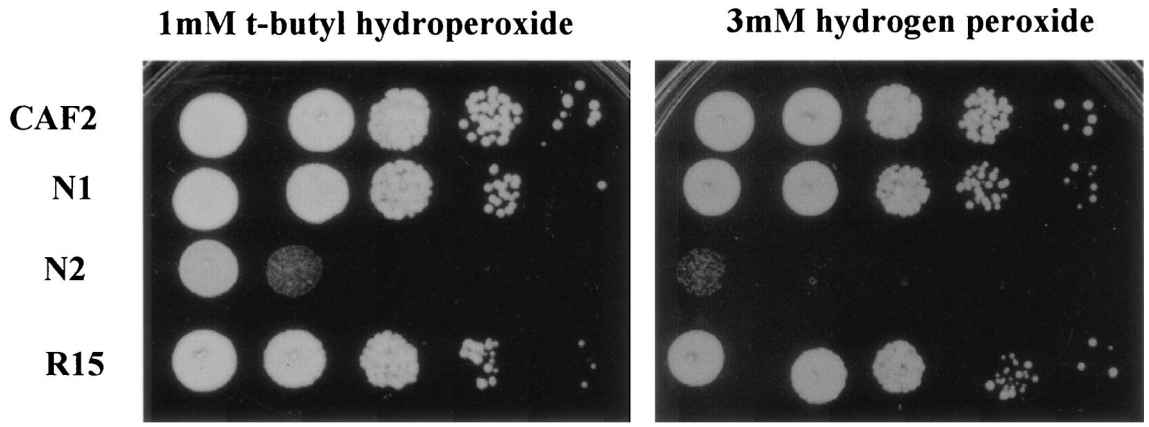

FIG. 3. Growth of CAF2, N1, N2, and R15 at 30°C for 48 h on YPD agar containing 1 mM t-butyl hydroperoxide or 3 mM hydrogen peroxide. Plates were spotted with yeast cells of each strain at concentrations of 10^5 (left) to 10 (right) cells in a total volume of 5 μl.

albicans, Ssk1p (23). However, there are differences in function between the two response regulator proteins of *C. albicans* that interestingly impact on the contribution of each to disease. For example, the *ssk1* mutant is sensitive to other oxidants, including menadione, while the *skn7* mutant is not (23). Further, preliminary studies have shown that the *ssk1* mutant is killed by neutrophils to a significantly greater extent than the *skn7* mutant (D. Chen, J. Richert, and R. Caldarone, submitted for publication). In addition, the *ssk1* mutant is avirulent (20), and in this study, we demonstrated only a slight attenuation of virulence in the *skn7* mutant compared to that in CAF2. The two response regulator proteins also appear to transmit their signals to different downstream proteins. Ssk1p is required for the phosphorylation of Hog1p under oxidant stress (23), while Skn7p is not required for that adaptation (data not shown). In *S. cerevisiae*, oxidative stress response is also regulated by a second protein, Yap1p, that appears to have an even broader range of functions than Skn7p (27, 37). The *C. albicans* homologue of Yap1p has been studied in some detail and is required for the morphogenesis and virulence of *C. albicans* (5). Further, the *CHK1* histidine kinase of *C. albicans* has antioxidant functions (unpublished data). Thus, it appears that redundancy in the adaptation of *C. albicans*, a commensal and an important pathogen of humans, to oxidative stress is critical to its survival as a commensal, to its survival within neutrophils and to the removal of its own oxidant waste (4, 8, 9, 25, 28, 36, 47). The latter issue has been extensively studied for *S. cerevisiae* (40). Adaptation along with the expression of several other factors (secreted enzymes, host recognition proteins, and morphogenesis) probably contributes to the virulence of this organism (14, 32, 39).

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