Contributions of the Response Regulators Ssk1p and Skn7p in the Pseudohyphal Development, Stress Adaptation, and Drug Sensitivity of the Opportunistic Yeast *Candida lusitaniae*\(^{\dagger}^{\dagger}\)

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We recently characterized the histidine kinase receptor genes of *Candida lusitaniae*. For the present study, we have further investigated the role of *SSK1* and *SKN7*, encoding response regulators. The results of functional analysis of mutants indicated that Ssk1p is involved in osmotolerance and pseudohyphal development, whereas Skn7p appears crucial for oxidative stress adaptation.

It is now accepted that two-component signal transduction pathways are essential for stress adaptation, morphogenesis, quorum sensing, and the regulation of virulence in fungi (28). In *Saccharomyces cerevisiae*, the two-component signaling system contains different proteins that work together in a phosphorelay pathway consisting of the sensor histidine kinase receptor (HKR) Sln1p, the histidine-containing phosphotransfer (HPt) protein Ypd1p, and the two response regulators (RR) Ssk1p and Skn7p. Differences in stimulus determine which RR is activated or inactivated. The Ssk1p branch of this pathway regulates a large palette of physiological processes, among which the Hog1-mediated osmoadaptation is the most documented. The Skn7p branch is involved in oxidative stress adaptation, cell wall biosynthesis, and the cell cycle (for a review, see references 9 and 15).

The budding yeast *Candida lusitaniae* (teleomorph, *Claviceps lusitaniae*) is an emerging pathogen which is characterized by its propensity to develop resistance to antifungal agents during treatment (14, 22, 26). Furthermore, this opportunistic yeast can efficiently switch to pseudohyphal growth (13). As a laboratory model, *C. lusitaniae* is an experimentally tractable haploid organism offering formal genetic tools based upon a complete sexual cycle reproducible in vitro (11, 23, 31) and an integrative transformation system for gene disruption (10, 30, 31).

We recently characterized three *C. lusitaniae* genes (*SLN1*, *NIK1*, and *CHK1*) potentially encoding HKRs (6) and showed that Sln1p is crucial (i) for oxidative stress adaptation when *C. lusitaniae* strains grow as budding yeast and (ii) in the early steps of pseudohyphal development, especially in hyperosmotic and oxidative conditions. For the current study, we characterized downstream elements, such as HPt and RR proteins. Blast analysis (2) of the *C. lusitaniae* database (Broad Institute Fungal Genome) revealed a single gene, *YPD1*, encoding a putative HPt protein, and two genes, *SSK1* and *SKN7*, potentially encoding two-component RRs. The characterization of these genes and their corresponding proteins is reported in Table S1 and Fig. S1 in the supplemental material. Null mutants were constructed for both *SSK1* and *SKN7* genes by using an improved integrative transformation system based upon the “URA3-blaster” strategy (6, 25) (see Fig. S2 in the supplemental material). All disruptant and reintegrant strains are listed in Table S2 in the supplemental material. However, we failed to obtain homologous integration of the *YPD1* disruption cassette at the corresponding target locus. The results of Southern blot analysis revealed that Ur*"* transformants derived either from ectopic integrations of the disruption cassette (50%) or from gene replacement at the ura3 locus (50%) (see Fig. S3 in the supplemental material). This result suggested that the *YPD1* mutant could be inviable in *C. lusitaniae*, as reported in several other fungal species (3, 4, 12, 19).

We first analyzed the growth, osmotolerance, drug sensitivities, and oxidative stress responses of *ssk1Δ* and *skn7Δ*, and *ssk1Δ skn7Δ* mutants. The single mutant *ssk1Δ* and the double mutant *ssk1Δ skn7Δ* exhibited increased generation times compared to that of the wild type when cultured in yeast extract-peptone-dextrose (YPD) or yeast nitrogen base medium (see Table S2 in the supplemental material). The growth of both the *ssk1Δ* and the *ssk1Δ skn7Δ* mutants is slightly affected on high-osmolarity medium (1.5 M sorbitol, NaCl or KCl) (Fig. 1), indicating that *C. lusitaniae* Ssk1p could be involved in the growth and in the capacity for adaptation to hyperosmotic conditions of yeast cells. Furthermore, the *ssk1Δ* and the *ssk1Δ*...
skn7Δ mutants exhibited hypersensitivity to UV irradiation (2,880 J·m⁻²) and high temperature (43°C), implying that Ssk1p is also essential for responses to these signals. Moreover, both the ssk1Δ and the ssk1Δ skn7Δ mutants were hypersensitive to methyglyoxal (25 mM), suggesting that Ssk1p is required to protect cells from this metabolic by-product (1, 20). Interestingly, both the skn7Δ and the ssk1Δ skn7Δ mutants exhibited strong sensitivities to H₂O₂, suggesting that Skn7p has a role in the regulation of oxidative stress in C. lusitaniae (Fig. 1).

Neither hypersensitivity nor resistance of mutants toward the clinical antifungals amphoterin B, 5-fluorotin, and fluconazole (FLC) was observed. The cell-mediated immunities (CMIs) of all null mutants were similar to those observed for the wild-type strain (amphotericin B CMI, ≤1 μg·ml⁻¹; 5-fluorotin CMI, ≤4 μg·ml⁻¹; and FLC CMI, ≤8 μg·ml⁻¹) (data not shown). Because in the filamentous fungi Neurospora crassa and Cochliobolus heterostrophus, mutations of HKR or RR genes are responsible for severe dicarboximide and phenylpyrrole resistance (16, 17, 24), we tested the effects of these antifungal drugs on our null-mutant strains. Both the ssk1Δ and the ssk1Δ skn7Δ mutants were resistant to fenpiclonil and iprodione (Fig. 1). These data demonstrate that in C. lusitaniae cells, as with the NIK1 gene disruption (6), the ssk1 deletion confers resistance to these compounds.

We next assessed the effects of the deletion of SSK1 and SKN7 on the morphological changes occurring during the sexual reproduction of C. lusitaniae, notably during conjugation (11). All mutants were still able to reproduce sexually in vitro when mated unilaterally or bilaterally (crosses are listed in the mating test section of the supplemental material) with the appropriate opposite mating type. Our data imply that, contrary to the human pathogen Cryptococcus neoformans, in which the Tco1p HKR governs sexual reproduction via the Ssk1p branch (3), the RR proteins of C. lusitaniae do not play an obvious role in the mating process.

We finally investigated the capacity of the mutants to differentiate pseudohyphae as described in reference 6. Figure 2 shows representative pictures of pseudohyphal formation after 48 h of growth on yeast carbon base (YCB) medium supplemented or not with the most discriminatory concentration of sorbitol (1 M), NaCl (0.5 M), KCl (0.5 M), or H₂O₂ (1 mM). Those concentrations were used because higher and lower

FIG. 1. Sensitivity of the wild-type strain, representative single mutants ssk1Δ and skn7Δ, double mutant ssk1Δ skn7Δ, and reintegrant strains ssk1Δ+SKSI and skn7Δ+SKN7 to different stresses. All strains were grown overnight in YPD liquid medium, the cells were counted, and then dilutions (10⁶ to 10² cells) were spotted onto YPD plate supplemented or not (control) with 1.5 M sorbitol, NaCl or KCl, 6 mM of H₂O₂, 25 mM methyglyoxal, 2 μg ml⁻¹ fluiclovin, or 25 μg ml⁻¹ iprodione. To test UV sensitivity, cells on YPD plates were exposed to UV for 12 s (2,880 J·m⁻²). Each plate was further incubated for 2 days at 35°C and photographed. For high-temperature sensitivity, spotted cells were incubated for 1 day at 43°C and photographed. This experiment was done in triplicate, and pictures show representative plates for each condition. WT, wild type.

FIG. 2. Pseudohyphal-growth ability of mutants. Amounts of 10⁶ cells (contained in drops of 5 μl) of the wild-type strain, representative single mutants ssk1Δ and skn7Δ, double mutant ssk1Δ skn7Δ, and reintegrant strains ssk1Δ+SKSI and skn7Δ+SKN7 were spotted onto YCB solid medium supplemented or not with discriminatory concentrations of sorbitol (1 M), NaCl (0.5 M), KCl (0.5 M), and H₂O₂ (1 mM). Observations were made 48 h after spotting. This experiment was done in triplicate, and pictures show representative structures observed for each sample. The lengths of pseudohyphae emerging from the edge of the colonies are reported in Table S4 in the supplemental material. WT, wild type. Bar, 300 μm.
concentrations were either too toxic or had no effect on the pseudohyphal development of the wild-type strain. The lengths of pseudohyphae emerging from the edge of the colonies are reported in Table S4 in the supplemental material. Homogeneously distributed pseudohyphae were obtained for all strains on unsupplemented YCB medium, but both the ssk1Δ and the ssk1Δ skn7Δ mutants displayed a reproducible reduction of pseudohyphal length. This reduction in pseudohyphal development could result either from a delay in the morphogenetic transition induced by the mutation or from global growth defects. The addition of 1 M sorbitol produced a homogeneous transition induced by the mutation or from global growth defects. However, despite these similarities, the results of the current study (in addition to those of our previous study (6)) show that in spite of a close conservation of the C. albicans MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase.

FIG. 3. Models for the action of Ssk1p and Skn7p in C. lusitaniae and comparison with simplified models for S. cerevisiae (9) and C. albicans (8, 18, 27). A possible mechanism that incorporates our experimental results from this study and our previous study (6) is proposed. The RR protein Skn7p acts in oxidative stress adaptation, possibly through Sln1p and Ypd1p elements. Dotted arrows indicate mechanisms or interactions not yet fully elucidated. MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase.

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REFERENCES

1. Aguilera, J., S. Rodriguez-Vargas, and J. A. Prieto. 2005. The HOG MAP kinase pathway is required for the induction of methylglyoxal-responsive genes and determines methylglyoxal resistance in Saccharomyces cerevisiae. Mol. Microbiol. 56:225–239.

2. Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402.

3. Bahia, Y. S., K. Kojima, G. M. Cox, and J. Heitman. 2006. A unique fungal two-component system regulates stress responses, drug sensitivity, sexual development, and virulence of Cryptococcus neoformans. Mol. Biol. Cell 17:3122–3135.
4. Banno, S., R. Noguchi, K. Yamashita, F. Fukumori, M. Kimura, I. Yamaguchi, and M. Fujiyama. 2007. Roles of putative His-to-Asp signaling modules HPT-1 and RRG-2, on viability and sensitivity to osmotic and oxidative stresses in Neurospora crassa. Curr. Genet. 51:197–208.

5. Calera, J. A., X. J. Zhao, and R. Calderone. 2000. Defective hyphal development and avirulence caused by a deletion of the SSK1 response regulator gene in Candida albicans. Infect. Immun. 68:518–525.

6. Chapeland-Leclerc, F., P. Paccallet, G. Ruprich-Robert, D. Reboutier, C. Chastin, and N. Papon. 2007. Differential involvement of histidine kinase receptors in pseudohyphal development, stress adaptation, and drug sensitivity of the opportunistic yeast Candida lusitaniae. Eukaryot. Cell 6:1782–1794.

7. Chauhan, N., M. Krupa, and R. Calderone. 2007. The Sko1p response regulator and Chk1p histidine kinase mutants of Candida albicans are hypersensitive to fluconazole and voriconazole. Antimicrob. Agents Chemother. 51:3747–3751.

8. Chauhan, N., J. P. Latge, and R. Calderone. 2006. Signalling and oxidant adaptation in Candida albicans and Aspergillus fumigatus. Nat. Rev. Microbiol. 4:435–444.

9. Chen, R. E., and J. Thorner. 2007. Function and regulation in MAPK signaling pathways: lessons learned from the yeast Saccharomyces cerevisiae. Biochem. Biophys. Acta 1773:1311–1340.

10. Francois, F., F. Chapeland-Leclerc, J. Villard, and T. Noel. 2004. Development of an integrative transformation system for the opportunistic pathogenic yeast Candida lusitaniae using URA3 as a selection marker. Yeast 21:95–106.

11. Francois, F., T. Noel, R. Pepin, A. Bruffert, C. Chastin, A. Favel, and J. Villard. 2001. Alternative identification test relying upon sexual reproductive abilities of Candida lusitaniae strains isolated from hospitalized patients. J. Clin. Microbiol. 39:3900–3914.

12. Furukawa, K., Y. Hoshi, T. Maeda, T. Nakajima, and K. Abe. 2005. Aspergillus nidulans HOG pathway is activated only by two-component signalling pathway in response to osmotic stress. Mol. Microbiol. 56:1246–1261.

13. Goumar, A., A. Bruffert, C. Martin, J. Villard, and T. Noel. 2004. Selection and genetic analysis of pseudohyphal defective mutants with attenuated virulence in Candida lusitaniae. J. Med. Mycol. 14:3–11.

14. Hawkins, J. L., and L. M. Baddour. 2003. Candida lusitaniae infections in the era of fluconazole availability. Clin. Infect. Dis. 36:e14–e18.

15. Hohmann, S., M. Kranta, and B. Nordlander. 2007. Yeast osmoregulation. Methods Enzymol. 428:29–45.

16. Izumitsu, K., A. Yoshimi, and C. Tanaka. 2007. Two-component response regulators Sko1p and Skn7p additively regulate high-osmolarity adaptation and fungicide sensitivity in Cochliobolus heterostrophus. Eukaryot. Cell 6:171–181.

17. Jones, C. A., S. E. Greer-Phillips, and K. A. Borkovich. 2007. The response regulator RRG-1 functions upstream of a mitogen-activated protein kinase pathway impacting asexual development, female fertility, osmotic stress, and fungicide resistance in Neurospora crassa. Mol. Biol. Cell 18:2123–2136.

18. Krupa, M., and R. Calderone. 2006. Two-component signal transduction in human fungal pathogens. FEBS Yeast Res. 6:149–159.

19. Maeda, T., S. M. Wurger-Murphy, and H. Saito. 1994. A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature 369:242–245.

20. Maeta, K., S. Izawa, and Y. Inoue. 2005. Methylglyoxal, a metabolite derived from glycolysis, functions as a signal initiator of the high osmolarity glyceralmitogen-activated protein kinase cascade and calmodulin/Crz1-mediated pathway in Saccharomyces cerevisiae. J. Biol. Chem. 280:253–260.

21. Menon, V., D. Li, N. Chauhan, R. Rajnarayanan, A. Dubrovskova, A. H. West, and R. Calderone. 2006. Functional studies of the Ssk1p response regulator protein of Candida albicans as determined by phenotypic analysis of receiver domain point mutants. Mol. Microbiol. 62:997–1013.

22. Minari, A., R. Hachem, and I. Raad. 2001. Candida lusitaniae: a cause of breakthrough fungemia in cancer patients. Clin. Infect. Dis. 32:186–190.

23. Noel, T., A. Favel, A. Michel-Nguyen, A. Goumar, K. Fallague, C. Chastin, F. Leclerc, and J. Villard. 2005. Differentiation between atypical isolates of Candida lusitaniae and Candida pulcherrima by determination of mating type. J. Clin. Microbiol. 43:1430–1432.

24. Ochiai, N., M. Fujimura, T. Nishiyama, A. Ichishi, R. Usami, K. Horikoshi, and I. Yamaguchi. 2001. Characterization of mutations in the two-component histidine kinase gene that confer fludioxonil resistance and osmotic sensitivity in the os-1 mutants of Neurospora crassa. Pest Manag. Sci. 57:437–442.

25. Papon, N., T. Noel, M. Florent, S. Gibot-Leclerc, D. Jean, C. Chastin, J. Villard, and F. Chapeland-Leclerc. 2007. Molecular mechanism of flucytosine resistance in Candida lusitaniae: contribution of the FCY2, FCY1, and FUR1 genes to 5-fluorouracil and flucytosine cross-resistance. Antimicrob. Agents Chemother. 51:369–371.

26. Pfälzer, M. A., S. A. Messer, and R. J. Hollis. 1994. Strain delineation and antifungal susceptibilities of epidemiologically related and unrelated isolates of Candida lusitaniae. Diagn. Microbiol. Infect. Dis. 20:127–133.

27. Roman, E., D. M. Arana, C. Nombela, R. Alonso-Monge, and J. Pla. 2007. MAP kinase pathways as regulators of fungal virulence. Trends Microbiol. 15:181–190.

28. Santos, J. L., and K. Shiozaki. 2001. Fungal histidine kinases. Sci. STKE 2001:RE1.

29. Singh, P., N. Chauhan, A. Ghosh, F. Dixon, and R. Calderone. 2004. SKN7 of Candida albicans: mutant construction and phenotype analysis. Infect. Immun. 72:2390–2394.

30. Young, L. Y., C. M. Hull, and J. Heitman. 2003. Disruption of ergosterol biosynthesis confers resistance to amphotericin B in Candida lusitaniae. Antimicrob. Agents Chemother. 47:2717–2724.

31. Young, L. Y., M. C. Lorenzo, and J. Heitman. 2000. A STE12 homolog is required for mating but dispensable for filamentation in Candida lusitaniae. Genetics 155:17–29.