Insight into the Role of HOG Pathway Components Ssk2p, Pbs2p, and Hog1p in the Opportunistic Yeast Candida lusitaniae

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NOTE

In the present study, we have investigated the role of SSK2, PBS2, and HOG1, encoding modules of the high-osmolality-glycerol mitogen-activated protein kinase pathway in Candida lusitaniae. Functional analysis of mutants indicated that Ssk2p, Pbs2p, and Hog1p are involved in osmotolerance, drug sensitivity, and heavy metal tolerance but not in oxidant stress adaptation.

One important component of the Saccharomyces cerevisiae signaling network is the high-osmolality-glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway, which responds to osmotic, oxidative, and heavy metal stress (for reviews, see references 14 and 30). This transduction module also plays a broad role in regulating morphology, growth, and adaptation to various conditions in a number of other fungal species (1, 3, 7, 8, 10, 12, 13, 15–18, 20, 21, 25, 27, 32, 33, 35, 36). The HOG MAPK signaling pathway consists of MAPK Hog1p, MAPK kinase (MAPKK) Pbs2p, and MAPKK kinase (MAPKKK) Ssk2p, which communicate by a phosphorylation cascade. It is now accepted that the HOG pathway can be regulated by two upstream branches. The first branch is referred to as the two-component system and includes the histidine kinase receptor Shl1p, the histidine-containing phosphotransferase Ypd1p, and the response regulator Ssk1p. Ssk1p modulates the Ssk2p-Pbs2p-Hog1p activity by regulating the Ssk2p MAPKKK. The second branch is initiated by the Sho1p adaptor protein, which makes use of several proteins (Ste20p, Ste50p, and Ste11p) to link the HOG cascade through the Pbs2p MAPK (31).

In two previous studies, we reported the characterization of genes (SLN1, NIK1, CHK1, YPD1, SSK1, and SKN7) encoding members of the two-component system in the opportunistic yeast Candida lusitaniae (5, 29). In the present study, we investigated the contribution of the downstream HOG MAPK pathway elements Ssk2p, Pbs2p, and Hog1p in the stress adaptation, drug sensitivity, mating ability, and yeast-to-pseudohyphae morphological transition of this fungal species.

The sequences of the C. lusitaniae SSK2 (CLUG_02562.1), PBS2 (CLUG_03729.1), and HOG1 (CLUG_01883.1) genes were retrieved from the annotated Candida database of the Broad Institute (http://www.broad.mit.edu/). To determine the role of these genes in C. lusitaniae, null mutants were constructed for each one by using the URA3 blaster strategy (5, 24) (see Fig. S1 in the supplemental material). All disruptant and reintegrant strains are listed in Table 1.

We first investigated the putative involvement of Ssk2p, Pbs2p, and Hog1p in the integration and transduction of various environmental stress signals. For this purpose, we analyzed the growth, osmotolerance, oxidative stress response, drug sensitivity, and metalloid tolerance of the ssk2Δ, pbs2Δ, and hog1Δ mutants. All strains exhibited similar doubling times in liquid yeast extract-peptone-dextrose (YPD) medium (data not shown). The growths of the ssk2Δ, pbs2Δ, and hog1Δ mutants are affected on high-osmolality medium (1.5 M sorbitol, 1 M NaCl or KCl) (Fig. 1). Interestingly, we found that the ssk2Δ mutant is slightly less sensitive to these osmolytes than the pbs2Δ and hog1Δ mutants. However, these results indicate that C. lusitaniae Ssk2p, Pbs2p, and Hog1p could be involved in the capacity of adaptation of yeast cells to hyperosmotic conditions. All the strains tested (wild-type, mutant, and reintegrant strains) displayed similar phenotypes when grown under oxidant conditions (43°C), implying that Ssk2p, Pbs2p, and Hog1p are also essential for responses to these signals. Moreover, the ssk2Δ, pbs2Δ, and hog1Δ mutants were sensitive to methylglyoxal (30 mM), suggesting that HOG pathway components are required to protect cells from this metabolic by-product (2, 19). C. lusitaniae ssk2Δ, pbs2Δ, and hog1Δ cells displayed similarly increased resistance to the cell wall biogenesis inhibitor Congo red relative to wild-type cells. However, we found these mu-
tants slightly sensitive to calcofluor white (a compound that interferes with cell wall formation) and to the purine analogue caffeine. Finally, the ssk2Δ, pbs2Δ, and hog1Δ mutants showed impaired growth on cadmium- or arsenite-containing media, suggesting a role for Ssk2p, Pbs2p, and Hog1p in the regulation of heavy metal detoxification (Fig. 1).

In C. lusitaniae, mutation of NK1 or SK1, encoding histidine kinase receptor or response regulator proteins, respectively, leads to severe dicarboximide and phenylpyrrole resistance (5, 29). We thus tested if the deletion of genes encoding downstream elements could be responsible for a comparable antifungal sensitivity pattern. C. lusitaniae ssk2Δ, pbs2Δ, and hog1Δ cells displayed strong fenpiclonil and iprodione resistance (Fig. 1). Nevertheless, neither hypersensitivity nor resistance toward the clinical antifungals amphotericin B, 5-fluorocytosine, and fluconazole was observed (not shown). Therefore, we conclude that the HOG pathway modulates filamentous-fungus-specific antifungals (dicarboximides and phenylpyrroles) but not clinical antifungal susceptibility in C. lusitaniae.

We then investigated if the deletion of the SSK2, PBS2, or HOG1 genes could have an impact upon the in vitro mating ability of C. lusitaniae. Indeed, morphological changes occur during the sexual reproduction of C. lusitaniae, notably during conjugation (9). Genetic crosses were performed under the same conditions as those described previously, using the reference strain from Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands).

The values are means ± standard deviations based on three individual replicates. ND, not determined.

### Table 1. Candida lusitaniae strains

| Strain | Genotype | Mating type | Parent | Generation (h) |
|--------|----------|-------------|--------|---------------|
| 0936Δ  | Wild type | MATα        |        | 1.23 ± 0.02   |
| CL38Δ  | Clinical isolate | MATα        |        | ND            |
| 0936 ura3Δ::URA3 b | MATα | 6936 | ND |
| PC1 (a) | ura3Δ::URA3 | MATα        | CL38   | ND            |
| ssk2Δ  | ura3Δ::URA3 ssk2Δ::REP-URA3::REP | MATα | 6936 ura3Δ::URA3 | 1.31 ± 0.02 |
| pbs2Δ  | ura3Δ::URA3 pbs2Δ::REP-URA3::REP | MATα | 6936 ura3Δ::URA3 | 1.37 ± 0.03 |
| hog1Δ  | ura3Δ::URA3 hog1Δ::REP-URA3::REP | MATα | 6936 ura3Δ::URA3 | 1.26 ± 0.05 |
| ssk2Δα | ura3Δ::URA3 ssk2Δ::REP-URA3::REP | MATα | PC1 (α) | ND |
| pbs2Δα | ura3Δ::URA3 pbs2Δ::REP-URA3::REP | MATα | PC1 (α) | ND |
| hog1Δα | ura3Δ::URA3 hog1Δ::REP-URA3::REP | MATα | PC1 (α) | ND |
| ssk2ΔRep | ura3Δ::URA3 ssk2Δ::REP pVAX-URA3::SSK2 | MATα | ssk2Δ | ND |
| pbs2ΔRep | ura3Δ::URA3 pbs2Δ::REP pVAX-URA3::PBS2 | MATα | pbs2Δ | ND |
| hog1ΔRep | ura3Δ::URA3 hog1Δ::REP pVAX-URA3::HOG1 | MATα | hog1Δ | ND |

a Reference strain from Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands).
b Described in reference 5.
c The values are means ± standard deviations based on three individual replicates. ND, not determined.

### FIG. 1

Sensitivity of the wild-type strain, representative single mutants ssk2Δ, pbs2Δ, and hog1Δ, and the reintegrand ssk2Δ + SSK2, pbs2Δ + PBS2, and hog1Δ + HOG1 strains to different stresses. All strains were grown overnight in YPD liquid medium (360). The cells were counted, and then dilutions (10³ to 10⁶ cells) were spotted onto a YPD plate supplemented or not supplemented (control) with 1.5 M sorbitol, 1 M NaCl or KCl, 8 mM H2O2, 100 μM menadione, 80 μg ml⁻¹ calcofluor white, 200 μg ml⁻¹ Congo red, 10 mM caffeine, 30 mM methylglyoxal, 4 μg ml⁻¹ fenpiclonil, 12 μg ml⁻¹ iprodione, 50 μM Cd(NO3)2, or 800 mM AsNaO2. 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 1 day 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.
The capacity of mutants to differentiate pseudohyphae was investigated. For this purpose, approximately $10^6$ cells (in drops of 4 µl) of wild-type, ssk2Δ, pbs2Δ, and hog1Δ strains were spotted on various YCB solid media. Figure 2 shows representative pictures of pseudohyphae formation after 48 h of growth on YCB medium supplemented or not supplemented with a discriminatory concentration of each compound used in the drop plate assays described above. The concentrations indicated in Fig. 2 were used because higher and lower concentrations either were found too toxic or had no effect on the pseudohyphal development of the wild-type strain. The lengths of the pseudohyphae emerging from the edges of the colonies are reported in Table S2 in the supplemental material.

Homogeneously distributed pseudohyphae of equivalent lengths were obtained for all four strains on unsupplemented YCB medium (Fig. 2). More precisely, morphogenesis per se was not altered, since the numbers of lateral cells branching along the pseudohyphae do not significantly differ between the mutants and the wild-type strain. Addition of 0.8 M sorbitol produced a homogeneous reduction of pseudohyphae growth of the wild-type strain and partially inhibited pseudohyphal development of ssk2Δ. Similar results were observed in the presence of 0.4 M NaCl or KCl, in which ssk2Δ formed some sporadic bunch-like pseudohyphae (Fig. 2). The most important result of these experiments was that both mutants exhibited even stronger sensitivities to osmotic stress since the pseudohyphal growths of the pbs2Δ and hog1Δ strains were completely abolished on this high-osmolarity medium. These observations imply that Ssk2p, Pbs2p, and Hog1p likely play a role in osmotolerance during the pseudohyphal development of C. lusitaniae. A significant effect of methylglyoxal was also observed since the growths of the ssk2Δ, pbs2Δ, and hog1Δ mutants were restricted to bunch-like pseudohyphae, compared to the homogenous pseudohyphal formation of the wild type at the same concentration. As expected, although the switch of the wild-type strain was altered on YCB medium containing a discriminatory concentration of fenpicilonil or iprodione, the pseudohyphal developments of the ssk2Δ, pbs2Δ, and hog1Δ mutants were similar to those observed on drug-free YCB medium. The dicarboximide and phenylpyrrole resistance mediated by inactivation of HOG pathway components also occurred during the morphogenetic transition in C. lusitaniae. Finally, the effects of an oxidant (H2O2 or menadione), cell wall assembly inhibitors (Congo red and calcifluor white), caffeine, and metalloids on yeast cells, as detailed above, were also investigated on pseudohyphal formation. Strikingly, the mutants showed no significant reduction of pseudohyphal development in the presence of discriminatory concentrations of these compounds relative to the growth of the wild type (Fig. 2).

To summarize, the results obtained in this work strengthen the putative architecture of the C. lusitaniae two-component signaling pathway recently proposed (see reference 29; an update is provided in Fig. 3). Indeed, Ssk2p, Pbs2p, and Hog1p are clearly implicated in osmoreadaptation, as reported for a large pallet of yeast and filamentous-fungus models (3, 6, 12, 16, 26). In addition, the HOG MAPK components appear to be essential for UV and heat shock tolerance, as recently described for Cryptococcus neoformans (3). However, as suggested in a previous work, the Ssk1p-Ssk2p-Pbs2p-Hog1p branch seems not to be required for oxidant adaptation in C. lusitaniae. In this way, the
Sln1p-Ypd1p-Snk7p pathway likely takes charge of this function in *C. lusitaniae* (5, 29). Our data also provide evidence that the HOG pathway is involved in response to the heavy metals cadmium and arsenite, as recently reported for *C. albicans* and *S. cerevisiae* (6, 34). The present study (in addition to previous studies [5, 29]) supports the hypothesis that a Nik1p-Ypd1p-Ssk1p-Ssk2p-Pbs2p-Hog1p-mediated transduction pathway probably regulates dicarboximide (dicarb.) and phenylpyrrole (phenylp.) sensitivity. Genes encoding Sho1p, Cdc42p, Ste20p, Ste50p, and Ste11p (dotted boxes) are located in the *C. lusitaniae* genome (unpublished data), but it is likely that the Sho1 branch does not regulate the HOG MAPK cascade (see Fig. S2 in the supplemental material). It is possible that the HOG pathway could integrate signal from a novel osmotic-stress signaling branch, as recently proposed for *C. albicans* (6, 28). Dotted arrows indicate mechanisms or interactions not yet fully elucidated.

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