The High-Osmolarity Glycerol Response Pathway in the Human Fungal Pathogen Candida glabrata Strain ATCC 2001 Lacks a Signaling Branch That Operates in Baker’s Yeast

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The high-osmolarity glycerol (HOG) mitogen-activated protein (MAP) kinase pathway mediates adaptation to high-osmolarity stress in the yeast Saccharomyces cerevisiae. Here we investigate the function of HOG in the human opportunistic fungal pathogen Candida glabrata. C. glabrata sho1Δ(Cgsho1Δ) deletion strains from the sequenced ATCC 2001 strain display severe growth defects under hyperosmotic conditions, a phenotype not observed for yeast sho1Δ mutants. However, deletion of CgSHO1 in other genetic backgrounds fails to cause osmotrigger hypersensitivity, whereas cells lacking the downstream MAP kinase Pbs2 remain osmosensitive. Notably, ATCC 2001 Cgsho1Δ cells also display methylglyoxal hypersensitivity, implying the inactivity of the Sln1 branch in ATCC 2001. Genomic sequencing of CgSSK2 in different C. glabrata backgrounds demonstrates that ATCC 2001 harbors a truncated and mutated Cgssk2-1 allele, the only orthologue of yeast SSK2/SSK22 genes. Thus, the osmophenotype of ATCC 2001 is caused by a point mutation in Cgssk2-1, which debilitates the second HOG pathway branch. Functional complementation experiments unequivocally demonstrate that HOG signaling in yeast and C. glabrata share similar functions in osmostress adaptation. In contrast to yeast, however, Cgsho1Δ mutants display hypersensitivity to weak organic acids such as sorbate and benzoate. Hence, CgSho1 is also implicated in modulating weak acid tolerance, suggesting that HOG signaling in C. glabrata mediates the response to multiple stress conditions.

Yeast cells have developed the ability to counteract changing osmolarity conditions by inducing appropriate physiological responses and adaptive adjustments (4). Osmoadaptation pathways have been studied primarily in Saccharomyces cerevisiae but lately also in pathogenic fungi with clinical relevance, such as Candida albicans (2, 37, 42). The haploid Candida glabrata is the second most prevalent fungal pathogen in humans after C. albicans (15, 35), accounting for about 20% of all systemic diseases caused by Candida spp. C. glabrata populates mucosal surfaces and the guts of healthy individuals, but it also causes life-threatening systemic infections in immunocompromised patients (29).

Candida spp. must be able to adapt to osmotic changes in the microenvironment during host invasion and systemic spreading, as well as to evade host defense strategies (2). However, the underlying mechanisms are not well understood. In yeast, mitogen-activated protein kinase (MAPK) cascades drive important signaling pathways, thus allowing cells to adapt to changing environmental conditions. S. cerevisiae harbors at least five MAPK signaling pathways mediating stress response, including the protein kinase C (PKC) cell integrity pathway (22), the protein kinase A growth control pathway (36), the mating pheromone response pathway (30, 40), and the spore wall assembly pathway (19, 39). Finally, the well-characterized high-osmolarity glycerol (HOG) pathway is essential for yeast survival under-high osmolarity conditions, since it triggers adaptation through intracellular accumulation of glycerol as the adaptive osmolyte (4, 10). Two cell surface membrane sensors, Sho1 and Sln1, constitute two functionally redundant signaling branches that sense and transduce signals through the downstream HOG MAPK pathway (23, 44). Sho1 and Sln1 have been termed osmosensors, but recent studies indicate that Sho1 transduces the stress signal rather than sensing changes in osmolarity (33, 43). Experiments based on systems biology analysis have demonstrated that Sho1 is phosphorylated in a Hog1-dependent manner, indicating a negative-feedback loop acting on the transmembrane protein (11). The key MAPK, Hog1, is activated by phosphorylation through the upstream MAPK kinase (MAPKK) Pbs2. Whereas pbs2Δ and hog1Δ deletion strains are osmosensitive (4), mutations affecting only one of two upstream branches do not cause osmophenotypes, since Sln1 and Sho1 can each independently trigger Hog1 activation (27, 44).

Orthologues of Hog1 are present in other fungi as well as in animals (27). In S. cerevisiae, the HOG pathway responds to a limited range of stress conditions, mainly high osmolality, heat stress (46), intracellular methylglyoxal accumulation (1, 24), citric and acetic acid stresses (21, 25), and oxidative stress (3). Interestingly, Hog1 orthologues play a more general role in regulating a core stress response in C. albicans and Schizosaccharomyces pombe (7, 42). Furthermore, a lack of C. albicans Hog1 (CaHog1) leads to impaired virulence (2). Recent studies demonstrated that CaSho1 plays only a minor role in osmostress adaptation. Nevertheless, CaSho1 is important for...
growth under oxidative stress conditions, and it mediates phosphorylation of the Cek1 MAPK in exponentially growing cells (37).

Because all components of the yeast HOG pathway are present in *C. glabrata*, we constructed strains lacking *C. glabrata* *SHO1* (CgSHO1) and CgPBS2 from strain ATCC 2001, which was sequenced by the Génolevures Consortium (8). Unexpectedly, a Cg*sho1*Δ strain displays severe osmosensitivity, a phenotype not observed for the corresponding *S. cerevisiae* strain *sks2*-1. Genomic sequencing demonstrates restriction to ATCC 2001. Genomic sequencing demonstrates the inactivity of the Sln1 branch in ATCC 2001, since this strain harbors a truncated nonfunctional Cgsk2-1 allele, causing the removal of the Cgsk2 kinase domain. Importantly, analysis of ATCC 2001 and other strain backgrounds revealed that the physiology of the *C. glabrata* HOG pathway is closely related to that of *S. cerevisiae*. Interestingly, it also seems distinct from the yeast HOG pathway, since it performs additional functions, such as involvement in modulating resistance to certain weak organic acids in *C. glabrata*.

**MATERIALS AND METHODS**

Strains, plasmids, and growth conditions. All strains used in this study are listed in Table 1. *S. cerevisiae* strain YCG9A was obtained by crossing W303-1A *sks2*-1 with W303-1B *sho1*. BY4741 *sho1*Δ was obtained by crossing strains BY4741 *sho1*Δ and BY4742 *sks1*Δ. For gene disruption in the ATCC 2001, BG2, and Cg2633 backgrounds, we used the dominant *SAT1* marker cassette amplified from plasmid pSFS2 (34) with primers SAT1-P1 and SAT1-P5 (Table 2). For construction of the CgSSK2 genomic deletion cassette, the nourseothricin marker gene *NAT1* was amplified from plasmid pK663 (41) using primers NAT1-P1 and NAT1-P5. Disruption cassettes were generated by fusion PCRs as described previously (26) and transformed into *C. glabrata* strains via electroporation as described elsewhere (34). For gene disruption using the *HIS3* marker, we amplified the marker gene from plasmid pTW23 (14) using primers HIS3-P2 and HIS3-P5. Sequences of all primers used for PCR amplification of disruption cassettes are listed in Table 2.

For genomic integration of *SSK2*, a 6,970-bp PCR fragment containing the entire *SSK2* open reading frame (ORF) including flanking 5′ and 3′ untranslated regions was amplified from genomic DNA of strain BG2 and transformed into strain ATCC 2001 *sho1*Δ. Transformants were selected on yeast extract-peptone-dextrose (YPD) supplemented with 1.2 M NaCl. Correct genomic replacement regions was amplified from genomic DNA of strain BG2 and transformed into strain ATCC 2001 *sho1*Δ. Transformants were selected on YPD and synthetic medium (SC) for yeast cultures were prepared essentially as described elsewhere (13). Unless otherwise indicated, all strains were grown routinely at 30°C. For the selection of nourseothricin-resistant transformants, 200 μg/ml of nourseothricin (Werner Bioagents, Jena, Germany) was added to YPD agar plates. For reintroducing CgSHO1 into the deletion strain, a 2,330-bp fragment containing the entire *SHO1* ORF, including the 600-bp 5′ promoter and the 700-bp 3′ untranslated region, was PCR amplified from ATCC 2001 genomic DNA ligated into the pEGM-T easy vector (Promega). Primers used for PCR were 5′-EshO1 (5′′-GCAATTTGGGAGGAC CACAGGATC-3′) and 3′-EshO1 (5′′-GAGGAAGAGGTATGGCCG G-3′). The ARS-CEN-TRP cassette was isolated from plasmid pCgACT14 (17) via partial digestion with AatII and ligated into the corresponding restriction site of pGEM-T Easy harboring CgSHO1. For the empty-vector control, the *SHO1* insert was removed from the vector with EcoRI restriction sites, followed by religation. The same EcoRI-digested fragment was used for cloning into EcoRI-digested pRS316 and YEp-CgSHO1, respectively.

**Growth inhibition assays.** To determine susceptibilities to osmостress, methylglyoxal or high temperatures, exponentially growing cultures were adjusted to an optical density at 600 nm (OD600) of 0.1 and diluted 1:10, 1:100, and 1:1,000. Equal volumes of serial dilutions were spotted onto YPD plates containing various concentrations of NaCl, sorbitol, KCl, or methylglyoxal. Plates were incubated at 30°C or 42°C for 24 h to 48 h. For acetate plates, YPD (pH 4.5) (adjusted with HCl) was supplemented with acetate from an 8.7 M acetic acid stock solution adjusted to pH 4.5 with NaOH. Plates containing other weak acids were prepared exactly as previously described (18).

### TABLE 1. Fungal strains used in this study

| Strain               | Genotype                                             | Reference or source |
|----------------------|------------------------------------------------------|---------------------|
| C. glabrata          | Wild type                                            | ATCC collection*    |
| ATCC 2001 (CBS138)   | Isogenic to ATCC 2001; his3:URA3 ura3Δ trp1Δ         | 45                  |
| ΔHT6                 | Isogenic to ATCC 2001; sho1Δ::SAT1                   | This study          |
| ΔHTU (ATCC 200089)   | Isogenic to ATCC 2001; sho1Δ::SAT1 SSK2              | This study          |
| Cg2633               | Clinical isolate                                     | Helena Bujdakova    |
| BG2                  | Clinical isolate                                     | This study          |
| ATCC 2001 sho1Δ      | Isogenic to ATCC 2001; sho1Δ::SAT1                   | This study          |
| ATCC 2001 pbs2Δ      | Isogenic to ATCC 2001; pbs2Δ::SAT1                   | This study          |
| ATCC 2001 sho1Δ SSK2 C1 | Isogenic to ATCC 2001; sho1Δ::SAT1 SSK2            | This study          |
| ATCC 2001 sho1Δ SSK2 C2 | Isogenic to ATCC 2001; sho1Δ::SAT1 SSK2            | This study          |
| ATCC 2001 sho1Δ SSK2 C3 | Isogenic to ATCC 2001; sho1Δ::SAT1 SSK2            | This study          |
| BG2 sho1Δ            | Isogenic to BG2; sho1Δ::SAT1                        | This study          |
| BG2 pbs2Δ            | Isogenic to BG2; pbs2Δ::SAT1                        | This study          |
| BG2 ssk2Δ            | Isogenic to BG2; ssk2Δ::NAT1                        | This study          |
| Cg2633 sho1Δ         | Isogenic to Cg2633; sho1Δ::SAT1                      | This study          |
| Cg2633 pbs2Δ         | Isogenic to Cg2633; pbs2Δ::SAT1                      | This study          |
| ΔHTU sho1Δ           | Isogenic to ΔHTU; sho1Δ::HIS3                       | This study          |
| ΔHT6 sho1Δ           | Isogenic to ΔHT6; sho1Δ::HIS3                       | This study          |

*S. cerevisiae*

| Strain               | Genotype                                             | Reference or source |
|----------------------|------------------------------------------------------|---------------------|
| W303-1B              | MATα ura3-1 leu2-3;112 his3-11,15 trpl-1 ade2-1 can1-100 | 38                   |
| YCG9A                | Isogenic to W303-1B; trp1::KanMX ssk22Δ::KanMX       | EUROSCARF           |
| BY4741               | MATα ura3-Δ leu2-Δ his3Δ met1-Δ                        | EUROSCARF           |
| BY4741 sho1Δ         | Isogenic to BY4741; sho1Δ::KanMX                      | EUROSCARF           |
| BY4741 pbs2Δ         | Isogenic to BY4741; pbs2Δ::KanMX                       | EUROSCARF           |
| BY4741 ssk1Δ         | Isogenic to BY4741; ssk1Δ::KanMX                       | EUROSCARF           |
| BY4741 sho1Δ ssk1Δ    | Isogenic to BY4741; sho1Δ::KanMX                       | This study          |

*a* Available at www.atcc.org.
Preparation of cell extracts and immunoblotting. To investigate phosphorylation of Hog1 using self-made antibodies, cultures of the Cgsho1Δ strain transformed either with a plasmid expressing CgSHO1 or with the empty-vector control were grown to the exponential-growth phase before addition of 0.5 M NaCl. Cells were harvested from 40 ml samples taken at different time points and were washed with H2O. Cells were then lysed with glass beads in 300 μl of buffer A (50 mM HEPES (pH 8.0), 0.4 M NH4SO4, 2 mM EDTA, 5% (vol/vol) glycerol, 50 mM sodium fluoride, 20 mM tetra-natrium-diphosphate, 1 mM sodium orthovanadate, 10 mM beta-glycerophosphate, protease inhibitor) using the Fast Prep machine (Qbiogene). Extracts were cleared by centrifugation steps at 3,000 × g and 20,000 × g. Aliquots corresponding to 50 μg of total extract per lane were fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% SDS-PAGE) and transferred to nitrocellulose membranes. Immunoblotting was carried out using phosophospecific polyclonal antibodies recognizing activated Hog1 isoforms. For detection of phosphorylated Hog1 isoforms using anti-phospho-p38 MAPK antibodies (Cell Signaling Technologies), trichloroacetic acid extracts were prepared as described elsewhere (9), and cell lysates equivalent to 0.4 OD600 unit were resolved by SDS-PAGE. Anti-ScPkp1 antibodies recognizing CgPkp1 were used to detect the loading control (20).

Preparation of antibodies. Polyclonal anti-phospho-Hog1 antibodies were raised in rabbits against a 16-residue Hog1-specific peptide conjugated to the keyhole limpet hemocyanin carrier protein. The crude Hog1 phosphopeptide NH2-CARIQDPMQMGTYSV-COOH (phosphorylated residues are bold - shaded) was purified by high-performance liquid chromatography through a Phenyl column. Fractions containing peptides with the predicted sequence were obtained by PCR amplification using primers SHO1-SAT1-P1 and SHO1-SAT1-P2 (probe A) as well as SHO1-SAT1-P4 and SHO1-SAT1-P6 (probe B).

TABLE 2. Oligonucleotides used in this study

| Name         | Sequence                        |
|--------------|---------------------------------|
| SHO1-SAT1-P1 | 5′-ACACCAGAGGTGATAATTGC-3′     |
| SHO1-SAT1-P3 | 5′-CCGAGGCGCCCTAGAGCCGCCTG-3′ |
| SHO1-SAT1-P4 | 5′-CTACAGTCATGTCCGTGAGCATCTT-3′ |
| SHO1-SAT1-P6 | 5′-CAACTTCCATCAGTGGAAATCT-3′  |
| PBS2-SAT1-P3 | 5′-CTACAGTCATGTCCGTGAGCATCTT-3′ |
| PBS2-SAT1-P4 | 5′-CTACAGTCATGTCCGTGAGCATCTT-3′ |
| PBS2-SAT1-P6 | 5′-CAACTTCCATCAGTGGAAATCT-3′  |
| SAT1-P2      | 5′-CCGCTGATGCGGCCCTGGTCG-3′   |
| SAT1-P5      | 5′-GACGAGGAGGCGCGGCCTGACG-3′  |
| SHO1-HIS3-P1 | Identical to SHO1-SAT1-P1   |
| SHO1-HIS3-P3 | Identical to SHO1-SAT1-P3   |
| SSK2-P1      | 5′-CAACTTAACTACAGGACAG-3′     |
| SSK2-P3      | 5′-GTCTAGATGCTGGCCCTCTCT-3′  |
| SSK2-P4      | 5′-CTACAGTCATGTCCGTGAGCATCTT-3′ |
| NAT1-P2      | 5′-CCGCTGATGCGGCCCTGGTCG-3′   |
| NAT1-P5      | 5′-GACGAGGAGGCGCGGCCTGACG-3′  |

a Overlapping sequences required for the PCR fusion are italicized and boldfaced.
marker SAT1, since no auxotrophic marker is available in these clinical strains. SHO1 and PBS2 were disrupted using the three-way PCR method in BG2, Cg2633, and ATCC 2001. Correct deletion of the SHO1 locus was tested by Southern blot analysis (Fig. 1B). The Cgsho1Δ deletion strains obtained were then tested for growth under high-osmolarity conditions (Fig. 1A). Only the ATCC 2001 Cgsho1Δ strain showed severely impaired growth on high salt concentrations, whereas a lack of SHO1 in BG2 and Cg2633 did not result in increased osmosensitivity (Fig. 1A). As expected, however, deletion of PBS2 led to osmostress hypersensitivity in all three strains (Fig. 1A). Thus, deletion of the MAPKK Pbs2 causes a severe phenotype on high NaCl concentrations that is independent of the genetic background. The observed salt stress sensitivity of a C. glabrata Cgsho1Δ strain is hence restricted to ATCC 2001, supporting the idea of an inactive Sln1 branch in strain ATCC 2001. Disruption of SHO1 in this strain could therefore lead to complete inactivation of the HOG pathway.

Deletion of CgSHO1 in ATCC 2001 causes general osmostress sensitivity and thermosensitivity. To investigate the role of Sho1 and the HOG pathway in the ATCC 2001 background, we constructed further SHO1 deletion strains. We chose strains ΔHT6 and ΔHTU (16, 45), since auxotrophic markers are available in these strains. Genomic disruption cassettes were generated by three-way PCR using the CgHS3 gene as a selectable marker and were used for transformation. Several independent transformants were obtained, and homologous recombination leading to gene disruption was confirmed by PCR and Southern blot analysis (data not shown).

Transformants of ΔHT6 and ΔHTU derivatives in which SHO1 was correctly replaced were then tested for growth under high-osmolarity conditions. Strain ΔHTU Cgsho1Δ showed impaired growth on plates containing 0.7 M NaCl and completely failed to grow on 1 M NaCl, whereas the wild-type strain tolerated concentrations as high as 1.2 M NaCl (Fig. 2). Deletion of CgSHO1 also led to reduced growth on plates supplemented with other types of osmolytes, namely, sorbitol and KCl. Interestingly, we observed an increased temperature sensitivity of ΔHTU Cgsho1Δ at 42°C, although at 37°C no altered sensitivity was observed (data not shown). Reintroducing SHO1 on a plasmid could restore growth at high osmolarity and elevated temperatures (Fig. 2). Similar phenotypes were also observed for ΔHT6 Cgsho1Δ strains (data not shown). In S. cerevisiae, the HOG pathway is also involved in the response to increasing concentrations of methylglyoxal (1, 24), a toxic intermediate of carbon metabolism. Similarly, the ΔHTU Cgsho1Δ strain displayed slightly increased sensitivity to 40 mM and 50 mM methylglyoxal relative to that of wild-type strains (Fig. 2).

Lack of CgSHO1 in the ATCC 2001 genetic background inactivates the HOG pathway. In S. cerevisiae, HOG pathway activation leads to dual phosphorylation of Hog1 to ultimately activate the MAPK cascade. We therefore investigated the phosphorylation status of Hog1 in C. glabrata in response to osmostress. Cultures of the Cgsho1Δ strain transformed either with a plasmid expressing CgSHO1 or with the empty vector were grown to the exponential-growth phase before addition of 0.5 M NaCl. Samples were taken at several time points and extracts separated by SDS-PAGE. Activated Hog1 isoforms were detected using phosphospecific polyclonal antibodies rec-
ognizing phosphorylated Hog1 (P-Hog1). Whereas in the strain harboring wild-type CgSHO1 a transient phosphorylation signal in response to osmostress was detectable, this signal was absent in the Cgsho1/H9004 strain (Fig. 3A). This finding supports a functional block of the signaling cascade upstream of CgHog1. Even increasing the NaCl concentration to 1 M failed to trigger phosphorylation and activation of CgHog1 in the absence of Sho1 (data not shown).

In C. albicans, deletion of components of the HOG pathway influences cell morphology and filamentation (37). Likewise, in S. cerevisiae, cells lacking HOG1 exhibit a cell morphology phenotype (5). Therefore, we microscopically inspected Cgsho1Δ cells with respect to their morphology under osmostress. We took pictures of exponentially growing cells 2 h after incubation with or without 0.5 M NaCl (Fig. 3B). Cells containing wild-type SHO1 looked normal under both conditions, whereas growth of the Cgsho1Δ strain caused abnormal cell morphology under high-osmolarity conditions (Fig. 3B). About 50 to 80% of the cells were elongated or swollen. We then immobilized cells on a microscope slide and inspected the growth patterns of the same cells under stress and nonstress conditions for as long as 4 h, covering several cell divisions. In response to osmotic stress, the Cgsho1Δ strain seemed to display severe budding defects (Fig. 3C). Therefore, Sho1 must play a major role in mediating the osmostress response and Hog1 phosphorylation in C. glabrata strains H9004 HT6 and H9004 HTU, demonstrating an inactive Sln1 branch in the ATCC 2001 background.

C. glabrata Sho1 can functionally complement the lack of S. cerevisiae Sho1. Next, we investigated whether the function of Sho1 within the signaling cascade of the HOG pathway is conserved between C. glabrata and S. cerevisiae. To check the functionality of the CgSHO1 gene in S. cerevisiae, we cloned the CgSHO1 ORF, including the 600-bp promoter and 700-bp untranslated regions, into the CEN-based yeast vector pRS316 and the multicopy vector YEp352. Plasmids pRS-CgSHO1 and YEp-CgSHO1 were used for transformation of the S. cerevisiae recipient strain YCG9A (sho1/H9004 ssk2/H9004 ssk22/H9004). Positive transformants were then analyzed for growth on plates containing 1 M or 1.2 M NaCl, concentrations that severely impaired the growth of the control strain YCG9A (Fig. 4A). Introducing CgSHO1 either on a multicopy plasmid or on a CEN-based plasmid fully restored growth of the S. cerevisiae
sho1Δ ssk2Δ ssk22Δ strain YCG9A (Fig. 4A). These data suggest that we have indeed identified CgSho1 as the orthologue of Sho1 from S. cerevisiae.

CgSSK2 carries a point mutation in ATCC 2001 but not in other C. glabrata strains. We initially found that only ATCC 2001 sho1Δ is hypersensitive to osmolarity. In other backgrounds, the same deletion did not influence resistance to high concentrations of NaCl. Hence, we assumed that the Sln1 branch in ATCC 2001 either is not functional or is absent, perhaps due to inactivity of one of the proteins of this branch. The annotated genome sequence of ATCC 2001 published by the Genolevures Consortium contains two ORFs highly similar to the 5′ and 3′ halves of the S. cerevisiae SSK2 gene. These are separated by an unsequenced gap, implying the possibility of a pseudogene. To fill this sequence gap completely, we sequenced this region by standard primer walking, covering a sequence of 1,680 bp. The deduced amino acid sequence is identical for all three strains, ATCC 2001, BG2, and Cg2633. This newly sequenced region combines both previously identified ORFs into a single ORF encoding a 1,667-residue protein. However, the genome sequence of the ATCC 2001 CgSSK2 locus carries a translational stop codon within the predicted protein kinase domain, which is otherwise highly conserved between S. cerevisiae and C. glabrata (Fig. 4B). Sequencing of the equivalent genomic region showed that BG2 and Cg2633 did encode the conserved Cys1668 at the identical position. Furthermore, we sequenced the relevant genomic region in the SSK2 genes of seven C. glabrata clinical isolates (kindly provided by Helena Budjakova). However, none of the clinical isolates carried this stop mutation present in the ATCC 2001 strain (data not shown). Moreover, we sequenced the entire SSK2 gene in the BG2 wild-type strain, potentially encoding a 1,755-residue CgSsk2 protein containing the entire protein kinase domain (Fig. 4B).

Because methylglyoxal sensitivity and acetic acid hypersensitivity in S. cerevisiae are caused mainly by an inactive Sln1 branch (24, 25), we performed additional spot tests using these compounds. These experiments revealed that wild-type ATCC 2001 was also significantly more sensitive to methylglyoxal and acetate than other strains investigated (Fig. 5A). On methylglyoxal, ATCC 2001 showed growth defects similar to those of a BG2 Cgpbs2Δ deletion strain (Fig. 5A); disruption of CgSHO1 further increased sensitivities, as described above. The Cgsho1Δ and Cgpbs2Δ deletions in ATCC 2001 did not further increase acetate sensitivity. For complementation analysis, we introduced a functional SSK2 allele into the genome of the ATCC 2001 sho1Δ strain. Since all attempts to clone CgSSK2 into a C. glabrata vector failed, we integrated the CgSSK2 allele encoded in the BG2 genome into the corresponding genomic locus of ATCC 2001 sho1Δ. Cells carrying the replaced SSK2 gene were selected on plates containing 1.2 M NaCl and verified by genomic sequencing. Growth inhibition analysis with three independent transformants (C1 to C3) revealed that introduction of the gene encoding full-length CgSSK2 into ATCC 2001 sho1Δ restored growth at high osmolarity and elevated temperatures. Moreover, all clones grew on higher acetate and methylglyoxal concentrations than the corresponding wild-type strain (Fig. 5B).

Furthermore, we deleted parts of the CgSSK2 ORF (bp 2063 to 5005) in wild-type BG2. As expected, the mutant strain obtained, BG2 Cgssk2Δ, displayed higher sensitivity on acetate and, to a lesser extent, on methylglyoxal than wild-type BG2 but growth similar to that of wild-type ATCC 2001 (Fig. 5C). Furthermore, we performed immunoblot analysis to determine the phosphorylation status of CgHog1 in strains lacking SSK2 or cells expressing restored CgSsk2. In strain BG2 with
FIG. 5. The ATCC 2001 genome carries a nonfunctional truncated ssk2-1 allele. (A) Cultures of the C. glabrata strains ATCC 2001 and BG2 as well as their isogenic Cgbs2Δ and Cgsho1Δ deletion strains were diluted to an OD$_{600}$ of 0.1, 0.01, or 0.001 and spotted onto YPD and YPD agar plates containing the indicated amounts of methylglyoxal (MG) or onto YPD (pH 4.5) and YPD (pH 4.5) agar plates containing the indicated amounts of acetate. Plates were incubated at 30°C for 2 days. (B) Cultures of the C. glabrata strain ATCC 2001 and the isogenic Cgbs2Δ and Cgsho1Δ deletion strains, as well as three independent clones of ATCC 2001 sho1Δ SSK2 (C1 to C3), were grown to the exponential-growth phase, diluted to an OD$_{600}$ of 0.1, and spotted along with serial 1:10 dilutions onto plates containing the indicated amounts of NaCl, MG, or acetate. Plates were incubated at 30°C for 2 days or at 42°C for 1 day. (C) Cultures of ATCC 2001, BG2, and BG2 Cgssk2Δ were diluted to an OD$_{600}$ of 0.1, 0.01, or 0.001; serial dilutions were spotted onto YPD and YPD agar plates containing 50 mM MG or onto YPD (pH 4.5) and YPD (pH 4.5) agar plates containing 80 mM acetate. Plates were incubated at 30°C for 2 days. (D) Cultures of BG2 and ATCC 2001 and the indicated isogenic deletion strains, as well as three clones of ATCC 2001 sho1Δ SSK2 (C1 to C3), were grown to the early-exponential-growth phase before 0.5 M NaCl was added. Samples were taken at the indicated time points and crude trichloroacetic acid extracts prepared. Aliquots corresponding to 0.4 OD$_{600}$ equivalent per lane were fractionated through a 10% SDS-PAGE gel. Immunoblotting was carried out using polyclonal anti-phospho-p38 MAPK or anti-Pgk1 antibodies. Extracts of ATCC 2001 sho1Δ SSK2 C2 and C3 were detected on different immunoblots, as indicated by the separation of these gels.
CgSSK2 deleted, the Hog1 phosphorylation signal decreased more rapidly after osmostress than that in the wild type. Strikingly, reintegration of the functional CgSSK2 allele in the ATCC 2001 background with CgSHO1 deleted led to a phosphorylation signal in the unstressed situation, indicating constitutive Hog1 phosphorylation. This hyperphosphorylation phenotype was observed for all three independent clones tested (Fig. 5D). Taken together, our results show that the truncated allele of Cg

**FIG. 6.** HOG pathways operating in *S. cerevisiae* and *C. glabrata*. Activation of the MAPKK Pbs2 can occur through at least two distinct upstream osmosensing mechanisms. One branch links the osmosensing protein Sln1 via Ypd1, Ssk1, and the MAPKKKS Ssk2 and Ssk22 to Pbs2. In the other branch, Sho1 functions to link an as yet unidentified osmosensor to the downstream components Cdc42, Ste20, Ste50, and the MAPKKK Ste11. Activated Pbs2 phosphorylates the MAPK Hog1, which in turn activates a variety of transcription factors. As indicated, Ssk22 does not exist in *C. glabrata*, and ATCC 2001 contains the ssk2-1 allele, encoding a truncated and nonfunctional Ssk2 version.

Mutants of the *C. glabrata* HOG pathway display sensitivity to weak organic acids. Recent studies in yeast revealed that Hog1 plays a role in resistance to acetic acid and that the response to acetic acid stress is mediated through the Sln1 branch of the HOG pathway (25). Our results indicate similar roles of the HOG pathway in *C. glabrata* and baker’s yeast. A CgPBS2Δ mutant in the BG2 background showed defects in growth on plates containing 100 mM acetate relative to the wild type, whereas the Cgsho1Δ mutant in the same background did not show altered sensitivities (Fig. 5A). However, as already observed for methylglyoxal, ATCC 2001 is clearly more sensitive to acetic acid than BG2. The Cgsho1Δ and CgPBS2Δ deletions did not further increase sensitivities in ATCC 2001. Since we observed acetate hypersensitivity in HOG pathway mutants, we tested the growth of the same set of *C. glabrata* strains on plates containing the weak organic acids sorbate, propionate, and benzoate. Surprisingly, we also observed increased sensitivities of mutant strains under these conditions, a phenotype that was not observed in *S. cerevisiae*. Disruption of CgPBS2 in BG2 led to slightly impaired growth on 5 mM benzoate and 2.5 mM sorbate, whereas deletion of CgSHO1 did not cause hypersensitivity in a strain background with a functional Sln1 branch (Fig. 7A). Similar sorbate and benzoate hypersensitivities were observed for CgPBS2Δ in ATCC 2001. Notably, a Cgsho1Δ mutant was even more sensitive to sorbate, benzoate, and propionate in this genetic background. In contrast, neither blockage of one or both upstream branches nor a lack of the downstream MAPKK Pbs2 caused any detectable weak-acid sensitivities in *S. cerevisiae* (Fig. 7B).

**DISCUSSION**

Adaptation to changes in the microenvironment during disease progression is an essential mechanism for the survival of fungal pathogens. MAPK cascades are important components in such cellular adaptation programs. This work aimed to define the role of orthologues of the *S. cerevisiae* HOG MAPK pathway in the pathogenic yeast *C. glabrata*. The analysis of physiological roles for the HOG pathway in the well-studied human fungal pathogen *C. albicans* revealed a strong phenotype, quite distinct from its osmosensing function in *S. cerevisiae*. However, blockage of the signaling of both upstream branches caused a more severe phenotype than disruption of the downstream MAPKK Pbs2, perhaps implying yet another signal transfer bypassing Pbs2 or involvement in another parallel and yet undisclosed pathway in stress sensing.

**C. glabrata** is a close relative of *S. cerevisiae*, considering genome evolution (8). Therefore, most adaptive functions of the HOG pathway are most likely also conserved. However, we show here that the genome of *C. glabrata* ATCC 2001 carries a putative orthologue of yeast SSK2 in a mutated form. Moreover, the SSK2 homologue SSK22 is absent from the *C. glabrata* genome. This fact hinted at possibly distinct functions of the MAPK pathways in these fungi, since one of the two major upstream signaling branches is debilitated. Here we show that (i) the overall functions of HOG signaling in *C. glabrata* are closely related to its functional counterpart in baker’s yeast; (ii) CgSho1 can complement the corresponding *S. cerevisiae* mutant; and (iii) the Sln1 branch is inactive in the ATCC 2001 strain background due to a point mutation in the CgSSK2 gene but active in all other *C. glabrata* strain backgrounds tested.

Our analysis of HOG pathway mutants revealed a spectrum of phenotypes very similar to the respective mutants in baker’s yeast. In *S. cerevisiae*, two HOG branches drive Hog1 activation; one branch uses Sln1, Ypd1, Ssk1, and the MAPKKKS Ssk2 and Ssk22, which then activate downstream Pbs2 (4, 32). The second branch senses through Sho1 and transduces via
Cdc42 and Ste20, Ste50, and Ste11 to converge with the Sln1 branch in the activation of Pbs2 and the key kinase Hog1 further downstream (31). It is known that \textit{S. cerevisiae} Sln1 carries an intrinsic histidine kinase activity and is able to control Skl1 activity using a phosphorelay system involving Ypd1. Skl1 then interacts with the MAPKKKs Ssk2 and Ssk22, which in turn activate Pbs2. Even though the two upstream branches of the HOG pathway seem highly specialized in detecting slightly different concentrations of salt (28), only mutations that block both branches cause severe osmosensitivities.

Our deletion analysis demonstrates that loss of \textit{C. glabrata} \textit{SHO1} alone can cause osmostress hypersensitivity as a function of the genetic background. Experiments with different \textit{C. glabrata} strains demonstrated that osmosensitivity of Cg\textit{sho1} mutants is restricted to ATCC 2001-derived “wild-type” strains. Our results are in line with the conclusion that the Sln1 branch is inactivated in the ATCC 2001 background. This was not observed for other clinical isolates and is therefore not a general peculiarity of \textit{C. glabrata}. Although we did not detect an enhanced sensitivity of this strain to osmotic stress, methyglyoxal and acetate were markedly less well tolerated.

Sho1, the transmembrane protein of one branch of the HOG pathway, was previously thought to be a sensor of osmostress. However, recent studies also reveal an important role of Sho1 downstream of Ste11 by binding to both the activated Ste11–Ste50 complex and Pbs2, perhaps tethering a “signaling complex” at the cell surface (43). Bringing together Ste11 and Pbs2 is required for activation of Pbs2 and therefore of Hog1. The \textit{C. glabrata} orthologue of yeast \textit{SHO1} can functionally complement \textit{S. cerevisiae sho1} mutants. Studies of \textit{C. albicans} report that Ca\textit{SHO1} plays only a negligible role in osmostress resistance, whereas Casho1 mutants are sensitive to oxidative stress (37). In our work, we failed to detect hypersensitivity of the \textit{C. glabrata} Cg\textit{sho1} mutant on H\textsubscript{2}O\textsubscript{2} or menadione (data not shown). Furthermore, CgHog1 phosphorylation was undetectable in response to oxidative stress (data not shown), indicating a negligible role of the \textit{C. glabrata} HOG pathway in the oxidative-stress response.

It is unclear when during evolution the Cg\textit{SSK2} mutation was acquired by the ATCC 2001 strain. Since inactivity of the Sln1 branch might decrease fitness in the host environment, and because other isolates do not carry the Cg\textit{ssk2-1} allele, it appears likely that the Cg\textit{ssk2-1} mutation was acquired during or after the isolation of the strain from its human source. Interestingly, ATCC 2001 strains carrying restored SSK2 showed increased levels of phosphorylated Hog1. Hence, acquisition of the mutated \textit{ssk2-1} allele might be a natural suppressor mutation of constitutively active Hog1 in order to bypass associated toxicities, a mechanism similar to dominant-negative activities observed for a constitutive Pbs2 kinase in baker’s yeast (12). Nevertheless, this work provides highly relevant information for the fungal pathogen community, since one should keep in mind that all mutations or deletions gen-

![FIG. 7. C. glabrata HOG pathway mutants display sensitivity to weak acids. (A) Cultures of the C. glabrata strains ATCC 2001 and BG2, as well as their isogenic Cg\textit{pbs2Δ} and Cg\textit{sho1Δ} deletion strains, were diluted to an OD\textsubscript{600} of 0.1, 0.01, or 0.001 and spotted onto YPD (pH 4.5) and YPD (pH 4.5) agar plates containing the indicated amounts of sorbate, propionate, or benzoate. Plates were incubated at 30°C for 2 days. WT, wild type. (B) Cultures of WT \textit{S. cerevisiae} W303-1A and its isogenic \textit{sho1Δ}, \textit{ssk1Δ}, \textit{pbs2Δ}, and \textit{ssk1Δ sho1Δ} deletion strains were grown to the exponential-growth phase, and serial dilutions were spotted onto YPD (pH 4.5) and YPD (pH 4.5) agar plates containing the indicated amounts of sorbate, propionate, or benzoate or onto YPD containing 1.2 M NaCl.](image-url)
erated in the ATCC 2001-derived genetic backgrounds are in fact double mutants also carrying a Cgsks2-1 allele. Thus, any stress-related phenotypes and molecular cross talk between different signaling pathways may be caused by synthetic genetic interactions due to the presence of the mutated Cgsks2-1 allele and the lack of the second gene. This is particularly relevant and should be considered for genome-wide knockout approaches on C. glabrata or studies addressing the molecular cross talk between several MAPK pathways in stress response and adaptation. Determining possible differences in the virulence of HOG pathway mutants would indeed be interesting, since this may relate to the in vivo host situation. However, available mouse models for Candida glabrata are suboptimal and difficult to establish. Therefore, we are in the process of establishing a novel Drosophila melanogaster insect model with mutations in the toll pathway to be employed for Candida glabrata virulence studies (D. Ferrandon et al., unpublished data).

Response to weak organic acids, apart from acetic acid, fails to trigger Hog1 signaling in yeast. By contrast, we show that C. glabrata double mutants in the Ssk2 and Sho1 branch are highly sensitive against weak acids of medium chain length such as sorbic acid. No similar phenotype is observed for yeast. The observation that Cgpb2 mutants are less sensitive than the Cgsks2 Cgsol double mutant perhaps points to a bypass to compensate for the lack of CgPbs2, although indirect genetic effects cannot be ruled out. However, the hypersensitivity of the Cgsks2 Cgsol double mutant implies a compensatory mechanism that is operative in S. cerevisiae but missing or nonfunctional in C. glabrata. In conclusion, our data demonstrate that several characteristics of HOG signaling are conserved between baker’s yeast and the human pathogen C. glabrata, although fundamental differences exist with regard to the activating cues. Exploring these differences will also shed further light on networks and cross talk between MAPK pathways in yeast.

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