Hog1 Mitogen-Activated Protein Kinase Plays Conserved and Distinct Roles in the Osmotolerant Yeast Torulaspora delbrueckii††

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Torulaspora delbrueckii has emerged during evolution as one of the most osmotolerant yeasts. However, the molecular mechanisms underlying this unusual stress resistance are poorly understood. In this study, we have characterized the functional role of the high-osmolarity glycerol (HOG) mitogen-activated protein kinase pathway in mediating the osmotic stress response, among others, in T. delbrueckii. We show that the T. delbrueckii Hog1p homologue TdHog1p is phosphorylated after cell transfer to NaCl- or sorbitol-containing medium. However, TdHog1p plays a minor role in tolerance to conditions of moderate osmotic stress, a trait related mainly with the osmotic balance. In consonance with this, the absence of TdHog1p produced only a weak defect in the timing of the osmostress-induced glycerol and GPD1 mRNA overaccumulation. Tdhog1Δ mutants also failed to display aberrant morphology changes in response to osmotic stress. Furthermore, our data indicate that the T. delbrueckii HOG pathway has evolved to respond to specific environmental conditions and to play a pivotal role in the stress cross-protection mechanism.

† Supplemental material for this article may be found at http://ec.asm.org/.

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The ability of Saccharomyces cerevisiae to withstand increasing osmotic pressures is critical for its survival in natural habitats and is an essential trait in many biotechnological processes. Variation in ambient osmolality is a constant phenomenon occurring in almost all steps from yeast biomass production to downstream applications in which cells have to grow and perform optimal fermentation under hostile conditions (51). However, the success of this industrial microorganism is based on its capacity to transform carbohydrates rapidly into ethanol and CO2 rather than its unusual resistance to environmental stresses. Yeasts other than S. cerevisiae, such as Zygosaccharomyces rouxii, Debaryomyces hansenii, and Torulaspora delbrueckii, display much higher resistance to adverse conditions and in particular to osmotic stress. This characteristic has made these yeasts potential models to explore and understand the mechanisms underlying osmotic tolerance in eukaryotic cells. However, little is known about the molecular basis of this phenotype, and, in most cases, the characterization of signaling pathways, transcription factors, and gene targets in these non-Saccharomyces species remains elusive.

Stress responses in S. cerevisiae have been widely researched. Yeast cells exposed to an osmostressing environment show a particular transcription profile. Thus, over 250 to 400 genes, covering a wide variety of physiological functions, are up-regulated after different conditions of osmotic shock (16, 24, 54). Stimulated expression of these genes appears to depend mainly on well-characterized molecular signaling pathways: the cyclic AMP-activated protein kinase A pathway (61), the high-osmolarity glycerol (HOG) pathway (66), one of the five mitogen-activated protein kinase (MAPK) pathways known in S. cerevisiae (28), and the calcineurin/Crz1p pathway, which is specifically required for adaptation to high-salt conditions (55). The HOG pathway consists of two discrete signaling branches composed of a putative osmosensor coupled to a MAPK cascade, some of which can lead to the phosphorylation and activation of the core MAPK Hog1p, the orthologue to mammalian p38 and fission yeast Sty1p stress-activated protein kinase (22). Osmostress-induced phosphorylation of Hog1p triggers its nuclear accumulation (21, 52) and the later induction of many osmostress-responsive genes (49, 54), among them GPD1 (53), the gene encoding the main enzyme that produces the compatible osmolyte glycerol in S. cerevisiae (2). Although the correlation between Hog1p activation, enhanced glycerol production, and osmotic resistance in S. cerevisiae is well established, it is unclear whether the MAPK pathway plays a similar role in other yeasts, particularly in highly osmotolerant species.

MAPKs homologous to S. cerevisiae Hog1p have been identified in different yeast species such as Schizosaccharomyces pombe (46), Candida albicans (57), Z. rouxii (37), and D. hansenii (9) as well as in multicellular fungi including Aspergillus nidulans (31), Neurospora crassa (70), and the human pathogen Cryptococcus neoformans (8). Although only a few of them have been studied in detail, it appears that Hog1p orthologues share conserved but different roles in response to a variety of environmental cues. Whereas inactivation of MAPK in S. cerevisiae has dramatic effects on growth under hyperosmotic conditions, hog1Δ null mutants from Z. rouxii can grow as well as the parental strain in the presence of 2 M NaCl (37). Similarly, disruption of HOG1 homologues from C. neoformans (8) or A. nidulans (39) has a weak effect or no effects on growth at high osmolyte concentrations. Moreover, in some species, the HOG pathway appears to have evolved to respond to additional extracellular stimuli and carry out different cellular roles in a niche-dependent way. Examples include cell-to-cell signaling and virulence in C. albicans (5, 59) or C. neoformans (8),...
fungicide resistance in *N. crassa* (70), or methylglyoxal tolerance in *S. cerevisiae* (1). However, it is not yet clear whether this niche-specific evolution also applies to highly osmotolerant species.

In this study, we have isolated the *Hog1p* homologue of the yeast *T. delbrueckii*, a facultative fermentative species characterized by its exceptional resistance to osmotic stress (33, 41). The aim of this work was to investigate the functional role of the HOG pathway in the stress response of *T. delbrueckii* and to determine whether this signaling route has evolved to enable this yeast to proliferate in highly osmotically environments. Surprisingly, we found that glycerol accumulation, a key feature of osmotic tolerance, is controlled mainly by regulatory mechanisms other than the HOG pathway. By contrast, the *T. delbrueckii* HOG pathway has undergone a functional specialization, being used as the central module of the stress cross-protection mechanism in this yeast.

**MATERIALS AND METHODS**

**Yeast strains and culture conditions.** *T. delbrueckii* strain PYCC5321 (4), *S. cerevisiae* wild-type strain W303-1A (MATa leu2-3112 ura3-1 his3-1 115 ade2-1 can1-100 GAL SU2 maD) (62) and a hog1Δ mutant strain (50) were used throughout this work. The *T. delbrueckii* TdHog1Δ mutant strain was constructed as described below. Escherichia coli strain DH10B was used as a host for plasmid construction. Yeast cells were cultured at 30°C in defined media: yeast-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% glucose) or SD medium (0.17% yeast nitrogen base without amino acids [DIFCO], 0.5% peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% glucose) supplemented with ampicillin (50 mg/liter). Yeasts were transformed by the lithium acetate method (36).

**Western blot.** Yeast cells were grown in Luria Bertani (LB) medium (1% peptone, 0.5% yeast extract, 0.5% NaCl) supplemented with 10 mg/liter of nourseothricin (clonNAT; WERNER Bioagents, Germany). Yeasts were transformed by electroporation using an electroporator according to the manufacturer’s instructions (Eppendorf).

**Northern blot.** The preparation of total RNA and Northern blot hybridization were performed as described previously (58). PCR-amplified fragments of the *S. cerevisiae* *GPD1* and *ACT1* (loading control) genes were prepared and radiolabeled as previously described (1). Spot intensities were quantified with Image Gauge software (Nikon, Japan). Values of spot intensities were corrected with respect to the *ACT1* mRNA level and are represented as the relative mRNA levels. The highest relative *GPD1* mRNA level for each sample analyzed was set at 100.

**RESULTS**

**Cloning and molecular characterization of the *T. delbrueckii* *HOG1* gene.** Functional complementation for growth of an *S. cerevisiae hog1Δ* mutant by a *T. delbrueckii* genomic library (32) on 0.5 M NaCl was used to isolate DNA fragments encoding *Hog1p*. From more than 1,000 transformants, only one plasmid, pMJH28, which hybridized under heterologous conditions with a probe of the *S. cerevisiae* *HOG1* gene (data not shown), was able to restore growth of the osmosensitive strain on selective medium. DNA sequencing of the fragment in pMJH28 revealed the presence of a 1,284-bp uninterrupted open reading frame encoding a putative 427-amino-acid protein, TdHog1p. This peptide showed 94 and 88% overall identity with *Z. rouxii* ZrHog2 and ZrHog1 proteins, respectively, 88% identity with *S. cerevisiae* Hog1p, and 85% identity with *D. hansenii* Dhog1p. High percentages of identity between TdHog1p and other members of the Hog1/Spc/p38 MAPK subfamily, such as *N. crassa* Os-2 (83%), *C. albicans* CaHog1p (78%), *A. nidulans* HogA (76%), *C. neoformans* Hog1p (76%), and mammalian p38 (47%), were also recorded.

**Protein domain analysis.** Protein domain analysis showed that TdHog1p contains a typical catalytic protein kinase domain (positions 23 to 302), which is also present in other Hog1p and p38 MAPK homologues. Within this region, we identified a protein kinase ATP-binding region (positions 29 to 53) and a consensus Ser/Thr protein kinase active site (positions 140 to 152). Moreover, the *T. delbrueckii* Hog1 protein contains a TGY dual phosphory-
loration signature (amino acids 174 to 176) characteristic of MAPKs activated by hyperosmolarity (15), a common docking domain (60), and an alanine-rich region at the C terminus at positions 302 to 316 and 364 to 383, respectively, which is also found in *S. cerevisiae* Hog1p.

To further confirm the identity of the TdHOG1 gene product, we cloned the entire open reading frame into the yeast expression vectors YEplac195 and YCplac33, and the resulting constructs, YEpTdHOG1 and YCPTdHOG1, were transformed into the *S. cerevisiae* W303-1A hog1Δ strain and examined for salt tolerance. As shown in Fig. 1A, expression of TdHOG1 at a high copy number complemented the osmosensitive phenotype of the *S. cerevisiae* hog1Δ mutant strain on 1 M NaCl. However, the replacement of the Hog1p MAPK by its counterpart TdHog1p in a single copy did not improve the intrinsic salt sensitivity of *S. cerevisiae*. Indeed, YCPTdHOG1 and YCPHOG1 transformants, with the latter carrying the gene from *S. cerevisiae*, showed similar growth on SD liquid medium supplemented with 1.5 M NaCl (Fig. 1B).

**TdHog1p is phosphorylated under osmotic stress.** We examined the effect of a shift to NaCl-containing medium on the phosphorylation state of TdHog1p in cells of the wild-type strain. As shown in Fig. 2A, TdHog1p was clearly activated when cells grown in YPD medium (OD₆₀₀ of 1.0) were subjected to mild NaCl stress conditions (0.5 M). The kinetics of phosphorylation were similar to those previously reported for the *S. cerevisiae* counterpart under similar conditions (65). Indeed, the phosphorylation level reached a maximum within 1 to 5 min of exposure to 0.5 M NaCl and decreased markedly after 10 min (Fig. 2A). As expected, TdHog1p was also sensitive to the presence of external sorbitol (Fig. 2B). Thus, both NaCl and sorbitol trigger the activation of the HOG pathway.

**Effect of TdHOG1 on growth under NaCl and sorbitol stress.** In order to confirm the functional role of the *T. delbrueckii* HOG pathway, we constructed a Tdhog1Δ mutant. Detection of the correct disruption of TdHOG1 was done by PCR and Western blotting. From a total of 10 transformants verified, all showed the pattern of PCR-amplified fragments expected for the correct gene disruption of TdHOG1. In addition, they lacked a specific band of hybridization with antiserum against total Hog1p (data not shown). Thus, it seems that a single TdHOG1 wild-type allele is responsible for the MAPK activity of the HOG pathway in the haploid *T. delbrueckii* strain PYCC5321.

Figure 2C shows the results of the phenotypic characterization of Tdhog1Δ mutant cells on YPD plates supplemented with 0.5 M NaCl or 1 M sorbitol, which both give approximately the same water activity, around 0.98 (53). In contrast to the dramatic osmosensitivity of the *S. cerevisiae* hog1Δ mutant, the absence of TdHog1p had scarcely any effect on the growth of *T. delbrueckii* cells under conditions of moderate osmotic stress. Similar results were obtained when the phenotype was tested using 1 M NaCl (Fig. 2C). However, Tdhog1Δ mutant cells were unable to proliferate at 1.5 M NaCl, while the wild-type strain showed noticeable growth (Fig. 2C).

**Osmostress-induced glycerol overaccumulation and GPD1 expression.** In *S. cerevisiae*, hyperosmotic shock triggers the Hog1p-dependent transcriptional induction of *GPD1*, the gene for glycerol production (2) and the main osmoregulator in this yeast (11). Likewise, *T. delbrueckii* overproduces glycerol in response to osmotic stress (33). Consequently, we analyzed the kinetics of glycerol production in YPD cultures of wild-type and Tdhog1Δ mutant strains subjected to osmotic stress. As can be seen in Fig. 3A, the absence of MAPK slightly delayed the accumulation of total glycerol in response to 1 M NaCl. Similar results were obtained at different salt concentrations or in the presence of sorbitol (data not shown). However, after 3 h of incubation with 1 M NaCl, the glycerol content in the culture of the Tdhog1Δ mutant increased to about 80% of the wild-type levels (Fig. 3A). Under identical conditions, the total glycerol content of *S. cerevisiae* hog1Δ cultures treated with NaCl was found to be lower, around 30% of the wild-type levels (Fig. 3A, right panel).

We then used Northern blot analysis to examine the expression profile of *GPD1* in NaCl-stressed *Torulaspora* cells. Since this gene has not been cloned, total RNA samples were hybridized with a probe of its *S. cerevisiae* counterpart under heterologous conditions. Previous work in our laboratory has
were not observed in any case. Instead, cells showed a clear
M NaCl (Fig. 4) or higher concentrations (data not shown) led
the presence and absence of 1 M sorbitol. Only exposure to 1
T. delbrueckii
hog1
S. cerevisiae
cells of
cells (12). Figure 4 shows the budded morphology of wild-type
(shmoo-like cells) that are characteristic of pheromone-treated
conditions, a
hog1
has no effect on their morphology. By contrast, under the same
exposure to 1 M sorbitol (data not shown).

As expected, expression of
GPD1
was induced in wild-type cells exposed to 1 M NaCl, with an approx-
imately 10-fold induction within 60 min (Fig. 3B). The absence
of MAPK did not affect
GPD1
expression in non-
stressed control cells. Similar results were observed for cells
exposed to 1 M sorbitol (data not shown).

Osmostress-induced morphology changes in Tdhog1Δ muta-
nants. Exposure of wild-type S. cerevisiae cells to osmotic stress
has no effect on their morphology. By contrast, under the same
conditions, a hog1Δ mutant displays morphological alterations
(shmoo-like cells) that are characteristic of pheromone-treated
cells (12). Figure 4 shows the budded morphology of wild-type
cells of S. cerevisiae strain W303-1A exposed to 1 M sorbitol or
NaCl, in contrast to the shmoos formed in the S. cerevisiae
hog1Δ mutant. However, wild-type and Tdhog1Δ mutant
strains of T. delbrueckii displayed normal morphologies in both
the presence and absence of 1 M sorbitol. Only exposure to 1
M NaCl (Fig. 4) or higher concentrations (data not shown) led
to a clear morphological response, although shmoo-like cells
were not observed in any case. Instead, cells showed a clear
inability to separate normally. Thus, the response appeared to
depend on the chemical used. However, blocking in cell sepa-
ration was also evident after exposure to 2 M sorbitol (data not
shown).

Cross talk between the T. delbrueckii HOG pathway and
parallel MAPK pathways under hyperosmotic conditions. The
formation of shmoo-like cells in S. cerevisiae Hog1p-deficient
strains occurs by the inappropriate activation of two MAPKs
(17, 30, 48), Kss1p, in the MAPK of the filamentation/invasion
pathway (23), and Fus3p in the pheromone response pathway
(19). Therefore, we investigated the phosphorylation states of
these MAPKs in wild-type and Tdhog1Δ cells exposed to high
osmolarity. Protein extracts were examined with Western anal-
ysis by using a monoclonal anti-phospho-p44/42 MAPK anti-
body, which recognizes the TEY activation sequence found in
mammalian p42 and p44 MAPKs (Erk1/Erk2) and yeast
MAPKs Kss1p and Fus3p. As shown in Fig. 5, a single band of
the expected mobility for either T. delbrueckii Kss1p or Fus3p
MAPK (~41 kDa) appeared in protein extracts from Tdhog1Δ
cells 30 min after osmotic shock. In contrast, there was no
evidence of this effect in control cells (Fig. 5).

Interestingly, inspection of the Western blot revealed the
presence of an additional ~61-kDa strong immunoreactive
band in both wild-type and Tdhog1Δ mutant cells (Fig. 5). This
band might correspond to the phosphorylated form of Slt2p,
the MAPK in the protein kinase C (PKC) pathway (28), which
also contains the same TEF activation loop as Fus3p and
Ssk1p and is therefore recognized by the antibody. Notably, we
also observed that Slt2p exhibited sustained phosphorylation
upon exposure to osmotic stress (Fig. 5). Indeed, only the 30-
to 60-min samples showed a significant reduction of the phos-
pho-Slt2p signal. In addition, a loss of Slt2p phosphorylation
was not observed in the Td
hog1
/H9004
strain (Fig. 5). This is in
sharp contrast to the situation reported previously for
S. cerevisiae,
where Slt2p is rapidly dephosphorylated (within 1 min),
in a Hog1p-independent way, upon osmotic stress (68). Hence,
it seems that a functional
T. delbrueckii
HOG pathway is es-
sential for the proper deactivation of the PKC signal in re-
sponse to hyperosmolarity.

The functional role of
T. delbrueckii
Hog1p in response to several types of stress. Functions other than osmostress pro-
tection have been identified for HOG homologue pathways in
some yeast species. Based on this, we analyzed the need for
TdHog1p in the response of
T. delbrueckii
to diverse stimuli
(Fig. 6A). Clearly, the MAPK was essential for tolerance to
methylglyoxal and citric acid. However, it appeared to be dis-
ispensable for growth at a high temperature, 34°C (Fig. 6A). We
also noted that the
T. delbrueckii
MAPK was not essential for the response to H2O2. To further confirm this point, we ana-
lized the kinetics of TdHog1p phosphorylation in
T. delbrueckii
wild-type cells following oxidative stress. As shown in
Fig. 6B, the level of phospho-TdHog1p did not vary after
exposure of yeast cells to 4 mM H2O2. Similar results were

FIG. 4. Effect of TdHog1p on the osmostress-induced morphological
changes of
T. delbrueckii
. Wild-type (wt) strains of
S. cerevisiae
(W303-1A) and
T. delbrueckii
(PYCC5321) and the corresponding
hog1
/H9004
mutants
hog1
/H9004
and Td
hog1
/H9004
were pregrown in YPD medium, collected, and then trans-
ferred to the same medium containing 1 M NaCl. At the indicated
times, aliquots of the cell cultures were withdrawn and analyzed for
glycerol content as described in Materials and Methods. Data rep-
resent the mean values of at least three independent experiments. Errors
were calculated by using the formula (1.96 × SD)/n, where n is the
number of measurements (SD, standard deviation).dw, dry weight.

FIG. 5. The
T. delbrueckii
HOG pathway modulates signaling through
parallel MAPK pathways in response to osmotic stress. Cells of wild-type
T. delbrueckii
strain PYCC5321 (wt) and the isogenic
Tdhog1
mutant
(Tdhog1
) were cultured in YPD medium to mid-log phase at 30°C and
transferred to the same medium containing 1 M NaCl. At the indicated
times, cells were collected and processed, and protein extracts were ana-
lyzed by Western blotting with anti-phospho-p44/42 MAPK antibody, which
allows the visualization of the phosphorylated (P) form of Slt2p, Kss1p, and
Fus3p MAPKs. Molecular weight (Mw) markers are shown (in thousands).
Putative
T. delbrueckii
proteins Kss1 and Fus3 are expected to comigrate
under the conditions applied. A representative experiment is shown.

FIG. 3. Glycerol production and
GPD1
induction in wild-type and
Td
hog1
mutant cells of
T. delbrueckii
. (A) Total glycerol levels after
hyperosmotic shock in cultures of
T. delbrueckii
(left graph)
PYCC5321 (wild type [wt]) (black bars) and Td
hog1
mutant (gray bars)
strains and
S. cerevisiae
(right graph) W303-1A wild-type (black bars) and
hog1
/H9004
mutant (gray bars) strains. Cells were grown in YPD medium to mid-exponential phase (OD600 of 1.5), collected, and trans-
ferred to the same medium containing 1 M NaCl. At the indicated
times, aliquots of the cell cultures were withdrawn and analyzed for
glycerol content as described in Materials and Methods. Data repre-
sent the mean values of at least three independent experiments. Errors
were calculated by using the formula (1.96 × SD)/n, where n is the
number of measurements (SD, standard deviation). dw, dry weight.

(B) Cells of
T. delbrueckii
wild-type (■) and Td
hog1
mutant (□)
strains grown in YPD medium were transferred to medium containing
1 M NaCl. Samples were taken at the indicated times and analyzed by
Northern blotting as described in Materials and Methods. Filters were
probed for
GPD1
mRNA. The graph in panel B represents the quan-
tification of the mRNA levels of
GPD1
relative to those of
ACT1.
observed for cells shifted to 34°C (Fig. 6B). Hence, the T. delbrueckii HOG pathway appears to be necessary under specific environmental conditions.

**Stress cross-protection in T. delbrueckii.** S. cerevisiae cells exposed to nonlethal doses of one type of stress develop tolerance to higher doses of the same stress as well as to unrelated stresses; these phenomena are described as induced tolerance and cross-protection, respectively (20, 43). It is commonly accepted that yeasts other than *Saccharomyces* display induced tolerance. However, the existence of cross-protection mechanisms is not always evident and has frequently been the subject of controversy (18). Therefore, we were interested in investigating this phenomenon in the osmotolerant yeast *T. delbrueckii*.

As shown in Fig. 7A, cells of the *T. delbrueckii* wild-type strain were unable to grow at 37°C on YPD plates lacking (control) or containing 0.5 M sorbitol. However, this phenotype was suppressed when cells were challenged with 1 M (Fig. 7A) or 1.5 M sorbitol (data not shown). Nevertheless, we reasoned that this phenomenon does not necessarily imply previous adaptation of the cells by osmotic stress. Reduction of water activity by sorbitol or salts is indeed an effective way to reduce thermal death, as demonstrated for osmotolerant yeasts like *D. hansenii* (3). Consequently, we looked into how TdHog1p is involved in this phenomenon. As can be seen in Fig. 7A, the thermal protection afforded by 1 M sorbitol was retained in the Td*hog1Δ* strain. Nevertheless, growth of mutant cells was clearly impaired at 37°C compared to the wild type, whereas at 30°C, the differences were less pronounced. Hence, adaptation to thermal stress appears to be dependent upon TdHog1p. Presumably, growth at high temperatures is also improved by water activity reduction mechanisms, being more pronounced at high osmolyte concentrations.

To investigate the role of TdHog1p in the above-described findings in greater depth, we first tested the levels of phospho-TdHog1p in cells exposed to increased temperatures plus high osmolarity (Fig. 7B). Exposure of yeast cells at a temperature of 37°C induced a modest increase in the level of phospho-TdHog1p, around 1.5- to 2-fold over basal levels, compared with that observed after exposure to 1 M sorbitol, about ninefold (Fig. 2B). Moreover, overphosphorylation of TdHog1p took place late and was only evident within 15 to 30 min after the thermal transfer, a temporary pattern often linked to an indirect mechanism of activation (34). In contrast, cells subjected to highly osmotic conditions at 37°C showed the same kinetics of TdHog1p phosphorylation that are characteristic for hyperosmolarity (Fig. 2A and 7B).

Next, we investigated the thermal protection conferred by pretreatment of *T. delbrueckii* cells with sorbitol. Cells grown in YPD medium were exposed to 1 M sorbitol at 30°C for 3 h and then transferred to fresh prewarmed (37°C) YPD medium lacking sorbitol (Fig. 7C). As can be seen in Fig. 7C, pretreatment of wild-type cells with osmotic stress afforded higher growth ability at 37°C. However, this effect was lost in Td*hog1Δ*
cells. Hence, cross-protection exists in *Torulaspora* and is mediated by the HOG pathway.

**DISCUSSION**

Phylogenetically, *T. delbrueckii* and *S. cerevisiae* are closely related, as previously demonstrated by sequence comparisons (10, 32). Despite this genealogical relationship, *T. delbrueckii* is able to survive and proliferate at high osmolyte concentrations, a property not shared by *S. cerevisiae* (33). As a result, this nonconventional yeast is frequently found in different habitats compared to those of *S. cerevisiae*, including cane molasses, syrups, honey, and foods with a high sugar content (41). Hence, they would be expected to differ in the regulatory mechanisms and molecular targets that enable adaptability to high-osmolarity environments.

These differences were first evident in our analysis of the functional response of the *T. delbrueckii* HOG pathway to osmotic stress. Although the MAPK TdHog1p was rapidly phosphorylated after transfer to NaCl- or sorbitol-containing medium, this was not essential for the growth of *T. delbrueckii* cells under moderate stress conditions, 0.5 M NaCl or 1 M sorbitol. Only when severe stress conditions were tested, for example, 1.5 M NaCl, was the need for a functional HOG pathway clearly evident. This result fully agrees with previous observations of *Z. rouxii hog1Δ* mutant cells, which show NaCl sensitivity only at concentrations above 2 M (37). Therefore, we predicted that this fact could reflect a distinct role of the HOG pathway in the osmoadaptation mechanisms employed by *T. delbrueckii* and *S. cerevisiae*. Consistent with this, neither osmostress-induced glycerol accumulation nor the expression of *GPD1* was strongly affected in Td*hog1Δ* mutant cells exposed to moderate hyperosmolarity. This is in sharp contrast with the situation in *S. cerevisiae*, where Hog1p is the main regulator of these responses (2, 53). Nevertheless, we noted that glycerol production and *GPD1* mRNA levels were upregulated upon osmotic shock. Therefore, a sudden change in the water activity of the environment is perceived by *T. delbrueckii* as a stressful condition. However, *T. delbrueckii*, like *S. cerevisiae*, must be frequently exposed to significant osmolarity fluctuations in natural habitats. Therefore, adaptation to these

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**FIG. 7.** The HOG pathway mediates stress cross-protection in *T. delbrueckii*. (A) YPD-grown cells of *T. delbrueckii* PYCC5321 wild-type (wt) and Td*hog1Δ* mutant strains were spotted onto YPD plates (control) and YPD plates containing 0.5 or 1 M sorbitol. Plates were incubated at 30 or 37°C for 2 to 4 days. (B) Cells of the *T. delbrueckii* wild-type strain were grown in YPD medium at 30°C and then subjected to heat stress alone (37°C) or in combination with high osmolarity, 1 M sorbitol (37°C + 1 M sorbitol). At the indicated times, cells were harvested and processed, and the crude protein extracts were assayed for TdHog1p phosphorylation (P-TdHog1) levels as described in the legend of Fig. 2A. (C) Exponentially growing cells of the wild-type (squares) and Tdhog1Δ mutant (circles) strains in YPD medium were either left unstressed (open symbols) or exposed to 1 M sorbitol (closed symbols) at 30°C. After 3 h, cells were washed and shifted to prewarmed (37°C) YPD medium lacking sorbitol, and the OD600 was measured at regular intervals. In all cases, representative experiments are shown.
changes requires a strict regulatory system that allows adaptability, thus avoiding the continuous stimulation of this response. Glycerol production is one of the processes requiring most energy by far (64). Hence, both yeasts appear to follow a similar strategy in response to osmotic stress. However, they have diverged in the regulatory mechanisms that control glycerol accumulation, at least under moderate osmotic stress conditions.

The differential specialization of Hog1p functions in \textit{S. cerevisiae} and \textit{T. delbrueckii} was further evidenced by analyzing the osmocortex-induced morphological changes in the Tdhog1Δ strain. High osmolality induces an aberrant morphology in \textit{S. cerevisiae} hog1Δ mutants, resembling that of cells exposed to pheromones, shmoos, or pear-shaped cells (13). As we demonstrated, osmotic stress induced the overphosphorylation of Fus3p and Kss1p, the MAPKs in the pheromone response and filamentation/invasion pathways, respectively, in the Tdhog1p-deficient strain. Thus, the MAPK Tdhog1p prevents osmolarity-induced cross talk between the HOG and parallel MAPK pathways in \textit{T. delbrueckii}. However, shmoos were not formed by Tdhog1Δ mutant cells exposed to a high level of sorbitol or NaCl. Therefore, \textit{S. cerevisiae} and \textit{T. delbrueckii} appear to control a different set of genes by the Fus3p-Kss1p MAPKs. It is also possible that \textit{T. delbrueckii} has evolved additional mechanisms to ensure the specificity of the osmocortex signal. Nevertheless, further work is required to identify upstream elements of the \textit{T. delbrueckii} HOG pathway and clarify the activation mechanisms operating in this yeast.

Regarding the morphological effects of osmotic stress, we also noted that Tdhog1Δ mutant cells remain attached after budding at high osmolyte concentrations, a phenotype that was missing in the wild-type strain. This apparent cell division defect led to large branched aggregates, similar to those observed for chitinase-negative \textit{S. cerevisiae} strains (40). This phenotype has also been reported for hog1Δ mutant cells of \textit{C. albicans} subjected to 1 M NaCl, suggesting a link between cell wall metabolism and the activity of CaHog1p in this pathogenic yeast (5). Such a relationship has also been suggested for \textit{S. cerevisiae}, since components of the HOG pathway appear to be involved in cell wall maintenance (6).

A connection between cell wall metabolism and the \textit{T. delbrueckii} HOG pathway was evidenced by the repressing effects that this pathway exerts on the activity of the PKC pathway following osmotic stress. The PKC signaling pathway is induced during budding and mating (69) as well as in response to environmental conditions such as high temperature, hypoosmotic stress, and cell wall-damaging conditions (38, 44). As we have shown, overphosphorylation of Slt2p, the MAPK of the PKC pathway, was maintained in Tdhog1Δ mutant cells after a shift to conditions of high osmolality. This lack of repression might thus account for the cellular aggregation displayed by the Tdhog1Δ mutant strain upon osmotic stress. On the other hand, the level of phospho-Slt2p was notably high in cells grown at 30°C. This observation indicates that \textit{T. delbrueckii} perceives stressful temperature fluctuations that do not stimulate a stress response in \textit{S. cerevisiae}. Moreover, the kinetics of Slt2p dephosphorylation after osmotic stress were unusually slow in \textit{T. delbrueckii} compared to that exhibited by \textit{S. cerevisiae} (68). Thus, this result suggests that there has been a divergence in the mechanisms that down-regulate the PKC pathway in \textit{T. delbrueckii} and \textit{S. cerevisiae}.

Another aspect of relevance addressed in our work is the functional role of Tdhog1p under stress conditions other than high osmolality. Interestingly, Tdhog1p has no apparent function at supraoptimal temperatures or at high levels of H$_2$O$_2$. The lack of Tdhog1p activity at high temperatures can account for the previous observation that \textit{S. cerevisiae} Hog1p is not essential for growth at 37°C (67). Recently, Smith et al. (59) also showed that \textit{C. albicans} Hog1p is not activated by temperature upshifts. However, the finding that Tdhog1p is not essential for the oxidative stress response was quite unexpected. Previous reports demonstrated that the HOG pathway provides protection against this stressful condition in \textit{S. cerevisiae} (29) as well as in other yeasts and fungi species such as \textit{C. albicans} (7), \textit{Schizosaccharomyces pombe} (14), and \textit{C. neoformans} (8). Although we have no obvious explanation for this result, it could reflect a wider ability of this yeast to cope with high levels of reactive oxygen species. Consistent with this, we found that \textit{T. delbrueckii} was able to grow at high relative levels of H$_2$O$_2$ (4 mM) compared with other yeasts and in particular with \textit{S. cerevisiae}. Thus, the \textit{T. delbrueckii} HOG pathway appears to have evolved not to respond to oxidative stress.

In the present work, we have also observed stress cross-protection in \textit{T. delbrueckii}, suggesting that a general stress response (20) exists in this yeast. In \textit{S. cerevisiae}, the general stress response is mediated by a common pathway, the cyclic AMP-protein kinase A pathway (61), which controls the activity and nuclear localization of the transcriptional factors MSN2/MSN4 (45). In addition, \textit{S. cerevisiae} uses strategies of coinduction of defense mechanisms. Instead of a common pathway, different stresses control a common set of genes via different signaling pathways and transcription factors (16, 24). Far from the classical concept of a general stress response, we observed that growth of \textit{T. delbrueckii} at 37°C, a temperature that inhibits the proliferation of this yeast, was dependent on the activity of Tdhog1p coupled with the thermal protection provided by sorbitol. Furthermore, we find that pretreatment with sorbitol provides a growth advantage at 37°C for wild-type cells but not for Tdhog1Δ mutant cells. Therefore, stress cross-protection exists in \textit{T. delbrueckii}, but this is mediated by Tdhog1p. Similarly, Smith et al. (59) previously reported Hog1p-dependent stress cross-protection in \textit{C. albicans}, a result that fits well with the lack of a functional role of homologues of \textit{S. cerevisiae} MSN2/MSN4 in this yeast (47).

Overall, the results presented in this study emphasize the divergence of a classical stress signaling pathway between different yeasts. Our current knowledge indicates that the HOG pathway has evolved in different yeasts in a niche-specific way (8, 59). We have also demonstrated that changes have taken place in the Hog1p–GPD1 relationship during the evolutionary divergence of \textit{S. cerevisiae} and \textit{T. delbrueckii}. While it is easy to imagine the different mechanisms through which gene expression regulation evolves (25), it is much more difficult to understand how these events determine the adaptability of yeasts to changing environments.

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REFERENCES

1. Aguiller, 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 redox resistance in Saccharomyces cerevisiae. Mol. Microbiol. 56:228–239.

2. Alberts, B., J. D. Dahan, J. M. Thevelein, and B. A. Prior. 1994. GDPi, which encodes glyceral-3-phosphate dehydrogenase, is essential for growth under osmotic stress in Saccharomyces cerevisiae, and its expression is regulated by the high-osmolarity glycerol response pathway. Mol. Cell. Biol. 14:4135–4144.

3. Almagro, A., C. Prista, S. Castro, C. Quintas, A. Madeira-Lopes, J. Ramos, and R. Madeira. 2002. Cytoskeletal effects of cytoskeletal mutants. Deharyomoneshanseni and Saccharomyces cerevisiae under stress conditions. Int. J. Food Microbiol. 56:191–197.

4. Almeida, M. J., and C. Pais. 1996. Leavening ability and freeze tolerance of yeasts isolated from arid inland areas. Appl. Environ. Microbiol. 62:4401–4404.

5. Alonso-Monge, R., E. Real, I. Wojda, J. P. Bebelman, W. H. Mager, and M. Alonso-Monge. 2003. Yeast genome expression in response to environmental changes. Mol. Biol. Cell 14:1460–1467.

6. Bahn, Y. S., K. Kojima, G. M. Cox, and J. Heitman. 2000. Pyp1 and Pyp2 PTPases have obvious roles in the stress responses of the fungal pathogen Candida albicans. Yeast 17:1431–1435.

7. Bahn, Y. S., K. Kojima, G. M. Cox, and J. Heitman. 2000. Pyp1 and Pyp2 PTPases have obvious roles in the stress responses of the fungal pathogen Candida albicans. Yeast 17:1431–1435.

8. Bahn, Y. S., K. Kojima, G. M. Cox, and J. Heitman. 2000. Pyp1 and Pyp2 PTPases have obvious roles in the stress responses of the fungal pathogen Candida albicans. Yeast 17:1431–1435.

9. Bahn, Y. S., K. Kojima, G. M. Cox, and J. Heitman. 2000. Pyp1 and Pyp2 PTPases have obvious roles in the stress responses of the fungal pathogen Candida albicans. Yeast 17:1431–1435.

10. Bahn, Y. S., K. Kojima, G. M. Cox, and J. Heitman. 2000. Pyp1 and Pyp2 PTPases have obvious roles in the stress responses of the fungal pathogen Candida albicans. Yeast 17:1431–1435.

11. Bahn, Y. S., K. Kojima, G. M. Cox, and J. Heitman. 2000. Pyp1 and Pyp2 PTPases have obvious roles in the stress responses of the fungal pathogen Candida albicans. Yeast 17:1431–1435.

12. Bahn, Y. S., K. Kojima, G. M. Cox, and J. Heitman. 2000. Pyp1 and Pyp2 PTPases have obvious roles in the stress responses of the fungal pathogen Candida albicans. Yeast 17:1431–1435.

13. Bahn, Y. S., K. Kojima, G. M. Cox, and J. Heitman. 2000. Pyp1 and Pyp2 PTPases have obvious roles in the stress responses of the fungal pathogen Candida albicans. Yeast 17:1431–1435.

14. Bahn, Y. S., K. Kojima, G. M. Cox, and J. Heitman. 2000. Pyp1 and Pyp2 PTPases have obvious roles in the stress responses of the fungal pathogen Candida albicans. Yeast 17:1431–1435.

15. Bahn, Y. S., K. Kojima, G. M. Cox, and J. Heitman. 2000. Pyp1 and Pyp2 PTPases have obvious roles in the stress responses of the fungal pathogen Candida albicans. Yeast 17:1431–1435.

16. Bahn, Y. S., K. Kojima, G. M. Cox, and J. Heitman. 2000. Pyp1 and Pyp2 PTPases have obvious roles in the stress responses of the fungal pathogen Candida albicans. Yeast 17:1431–1435.

17. Bahn, Y. S., K. Kojima, G. M. Cox, and J. Heitman. 2000. Pyp1 and Pyp2 PTPases have obvious roles in the stress responses of the fungal pathogen Candida albicans. Yeast 17:1431–1435.

18. Bahn, Y. S., K. Kojima, G. M. Cox, and J. Heitman. 2000. Pyp1 and Pyp2 PTPases have obvious roles in the stress responses of the fungal pathogen Candida albicans. Yeast 17:1431–1435.

19. Bahn, Y. S., K. Kojima, G. M. Cox, and J. Heitman. 2000. Pyp1 and Pyp2 PTPases have obvious roles in the stress responses of the fungal pathogen Candida albicans. Yeast 17:1431–1435.

20. Bahn, Y. S., K. Kojima, G. M. Cox, and J. Heitman. 2000. Pyp1 and Pyp2 PTPases have obvious roles in the stress responses of the fungal pathogen Candida albicans. Yeast 17:1431–1435.

21. Bahn, Y. S., K. Kojima, G. M. Cox, and J. Heitman. 2000. Pyp1 and Pyp2 PTPases have obvious roles in the stress responses of the fungal pathogen Candida albicans. Yeast 17:1431–1435.
HOG MAPK pathway components as revealed by whole-genome expression analysis. Mol. Biol. Cell 15:532–542.
50. Panadero, J., C. Pallotti, S. Rodriguez-Vargas, F. Randez-Gil, and J. A. Prieto. 2006. A downshift in temperature activates the HOG pathway, which determines freeze tolerance in Saccharomyces cerevisiae. J. Biol. Chem. 281: 4638–4645.
51. Randez-Gil, F., J. Aguilera, A. Codón, A. M. Rincón, F. Estruch, and J. A. Prieto. 2003. Baker’s yeast: challenges and future prospects, p. 57–97. In J. H. de Winde (ed.), Functional genetics of industrial yeasts. Springer-Verlag, Heidelberg, Germany.
52. Reiser, V., H. Ruis, and G. Ammerer. 1999. Kinase activity-dependent nuclear export opposes stress-induced nuclear accumulation and retention of Hog1 mitogen-activated protein kinase in the budding yeast Saccharomyces cerevisiae. Mol. Biol. Cell 10:1147–1161.
53. Rep, M., J. Albertyn, J. M. Thevelein, and S. Hohmann. 1999. Different signalling pathways contribute to the control of GPD1 gene expression by osmotic stress in Saccharomyces cerevisiae. Microbiology 145: 715–727.
54. Rep, M., M. Krantz, J. M. Thevelein, and S. Hohmann. 2000. The transcriptional response of Saccharomyces cerevisiae to osmotic shock Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. J. Biol. Chem. 275:8290–8300.
55. Rusnak, F., and P. Mertz. 2000. Calcineurin: form and function. Physiol. Rev. 80:1483–1521.
56. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467.
57. San Jose, C., R. A. Monge, R. Perez-Diaz, J. Pla, and C. Nombela. 1996. The mitogen-activated protein kinase homolog HOG1 gene controls glycerol accumulation in the pathogenic fungus Candida albicans. J. Bacteriol. 178: 5850–5852.
58. Sherman, F., G. R. Fink, and J. B. Hicks. 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
59. Smith, D. A., S. Nicholls, B. A. Morgan, A. J. Brown, and J. Quinn. 2004. A conserved stress-activated protein kinase regulates a core stress response in the human pathogen Candida albicans. Mol. Biol. Cell 15:4179–4190.
60. Tanoue, T., M. Adachi, T. Moriguchi, and E. Nishida. 2000. A conserved docking motif in MAP kinases common to substrates, activators and regulators. Nat. Cell Biol. 2:110–116.
61. Thevelein, J. M., and J. H. de Winde. 1999. Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast Saccharomyces cerevisiae. Mol. Microbiol. 33:904–918.
62. Thomas, B. J., and R. Rothstein. 1989. Elevated recombination rates in transcriptionally active DNA. Cell 56:619–630.
63. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific penalties and weight matrix choice. Nucleic Acids Res. 22:4673–4680.
64. van Dijken, J. P., and W. A. Scheffers. 1986. Redox balances in the metabolism of sugars by yeasts. FEMS Microbiol. Rev. 32:199–224.
65. van Wuytswinkel, O., V. Reiser, M. Siderius, M. C. Kelders, G. Ammerer, H. Ruis, and W. H. Mager. 2000. Response of Saccharomyces cerevisiae to severe osmotic stress: evidence for a novel activation mechanism of the HOG MAP kinase pathway. Mol. Microbiol. 37:382–397.
66. Westfall, P. J., D. R. Ballon, and J. Thorner. 2004. When the stress of your environment makes you go HOG wild. Science 306:1511–1512.
67. Winkler, A., C. Arkind, C. P. Mattison, A. Burkholder, K. Knoshe, and I. Ota. 2002. Heat stress activates the yeast high-osmolarity glycerol mitogen-activated protein kinase pathway, and protein tyrosine phosphatases are essential under heat stress. Eukaryot. Cell 1:163–173.
68. Wojda, I., R. Alonso-Monge, J. P. Bebelman, W. H. Mager, and M. Siderius. 2003. Response to high osmotic conditions and elevated temperature in Saccharomyces cerevisiae is controlled by intracellular glycerol and involves coordinate activity of MAP kinase pathways. Microbiology 149:1193–1204.
69. Zarzov, P., C. Mazzoni, and C. Mann. 1996. The Slt2(MPK1) MAP kinase is activated during periods of polarized cell growth in yeast. EMBO J. 15:83–91.
70. Zhang, Y., R. Lamm, C. Pillonel, S. Lam, and J. R. Xu. 2002. Osmoregulation and fungicidal resistance: the Neurospora crassa os-2 gene encodes a HOG1 mitogen-activated protein kinase homologue. Appl. Environ. Microbiol. 68:532–538.