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# Identification and characterization of putative osmosensors, HwSho1A and HwSho1B, from the extremely halotolerant black yeast *Hortaea werneckii*

Martin Fettich [a], Metka Lenassi [a], Peter Veranič [b], Nina Gunde-Cimerman [c], Ana Plemenitaš [a,*]

[a] Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Vrazov Trg 2, SI-1000 Ljubjana, Slovenia
[b] Institute of Cell Biology, Faculty of Medicine, University of Ljubljana, Vrazov Trg 2, SI-1000 Ljubljana, Slovenia
[c] Biology Department, University of Ljubljana, Biotechnical Faculty, Večna pot 111, SI-1000 Ljubljana, Slovenia

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ABSTRACT

In *Saccharomyces cerevisiae,* the Sho1 protein is one of two potential osmosensors that can activate the kinase cascade of the HOG pathway in response to increased extracellular osmolarity. Two novel *SHO1*-like genes, *HwSHO1A* and *HwSHO1B*, have been cloned from the saltern-inhabiting, extremely halotolerant black yeast *Hortaea werneckii.* The HwSho1 protein isoforms are 93.8% identical in their amino-acid sequences, and have a conserved SH3 domain. When the *HwSHO1* genes were transferred into *S. cerevisae* cells lacking the *SHO1* gene, both of the HwSho1 isoforms fully complemented the function of the native *S. cerevisiae* Sho1 protein. Through microscopic and biochemical validation, we demonstrate that in *S. cerevisiae,* both of the HwSho1 proteins have characteristic subcellular localizations similar to the *S. cerevisiae* Sho1 protein, and they can both activate the HOG pathway under conditions of osmotic stress. To a lower extent, crosstalk to the mating pathway expressing HwSho1 proteins is conserved in the *PBS2* deleted *S. cerevisiae* strain. These data show that the HwSho1 proteins from *H. werneckii* are true functional homologs of the Sho1 protein of *S. cerevisiae.*



## 1. Introduction

Hypersaline environments like salterns and salt lakes are dominated by halophilic prokaryotes (Oren, 2002), with some rare representatives of Eukarya that have also adapted to these extreme conditions (Zalar et al., 2005). Among these Eukarya, the dominant fungal species is the black yeast *Hortaea werneckii,* which can grow in nearly saturated salt solutions (5.2 M NaCl) and completely without salt, with a broad growth optimum under laboratory conditions of 1.0–3.0 M NaCl (Gunde-Cimerman et al., 2000).

To cope with life-threatening environmental changes, microorganisms have developed elaborate and sensitive protection systems through which they can rapidly signal, respond to and correctly adapt to such changes (Tatebayashi et al., 2007). In the budding yeast *Saccharomyces cerevisiae,* exposure to increased osmolarities leads to activation of the high osmolarity glycerol (HOG) signaling pathway, which is to date one of the best understood mitogen-activated protein kinase (MAPK) cascades (Hohmann, 2009).

Among the different MAPK cascades, one of the unique features of the HOG pathway is its composition, as it comprises two functionally redundant, but mechanistically distinct, branches, via SLN1 and SHO1. Each of these branches leads to the activation of the MAPK kinase (MAPKK) Pbs2 and its cognate MAP kinase (MAPK), Hog1. Once activated, this dually phosphorylated MAPK Hog1 rapidly translocates into the nuclear compartment, where it participates in the regulation of several osmo-responsive genes (Brewster et al., 1993; Maeda et al., 1995, 1994). When either of PBS2 or *HOG1* is deleted, crosstalk from a high osmolarity stimulus to the mating response and pseudohyphal growth is observed (Zarrinpar et al., 2004; O'Rourke and Herskowitz, 1998).

Each of these branches of the HOG pathway is regulated by at least one osmosensor, which monitors and generates an intracellular signal in response to extracellular osmolarity variations. There is a wealth of information available on the regulation of the signaling pathways controlled by such osmosensors (Hohmann, 2002, 2009; Tatebayashi et al., 2006), although the molecular mechanism(s) by which these osmosensors detect osmotic changes remains a matter of intensive study. In *S. cerevisiae,* it is believed that for the SLN1 branch, the osmosensor is the transmembrane histidine kinase Sln1, which detects turgor changes and transmits a signal through the Sln1–Ypd1–Ssk1 phosphorelay signaling system to Ssk2/Ssk22, the functionally redundant pair of MAPKK kinases (MAPKKKs) (Reiser et al., 2000). In contrast, neither the

* Corresponding author. Fax: +386 1 5437641.
*E-mail addresses:* martin.fettich@mf.uni-lj.si (M. Fettich), metka.lenassi@mf.uni-lj.si (M. Lenassi), peter.veranic@mf.uni-lj.si (P. Veranič), nina.gunde-cimerman@bf.uni-lj.si (N. Gunde-Cimerman), ana.plemenitas@mf.uni-lj.si (A. Plemenitaš).

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osmosensor nor the signal generator of the SHO1 branch have been clearly defined. The first candidate here is Sho1, which was until recently known to be the most upstream component of the SHO1 branch pathway. In *S. cerevisiae*, the Sho1 protein contains four highly conserved N-terminal transmembrane domains, Ste11-binding region, and a C-terminal cytoplasmic Src homology-3 (SH3) domain that binds to a proline-rich motif of the MAPKK Pbs2 (Maeda et al., 1995; Tatebayashi et al., 2006; Zarrinpar et al., 2004). Sho1 predominantly localizes to the cytoplasmic membrane regions of the cell during polarized growth, such as at the emerging bud and the bud neck. It appears that Sho1 serves as an obligatory adaptor between the MAPKKK Ste11 and its cognate MAPKK Pbs2, and not as an osmosensor, as was original postulated (Tatebayashi et al., 2007). Sho1 homologs have also been studied in the human pathogen *Candida albicans* and in the opportunistic pathogen *Aspergillus fumigatus*, where they have essential roles in cell morphogenesis and they mediate resistance to oxidative stress (Ma et al., 2008; Roman et al., 2005).

The other two potential osmosensor candidates in *S. cerevisiae* are the mucin-like transmembrane proteins Msb2 and Hkr1, which show redundant functions for activation of the HOG pathway. Tatebayashi et al. (2007) showed a complex interplay between Msb2, Hkr1 and Sho1 in osmostress responses, clearly demonstrating that Msb2 and Hkr1 are upstream of the known components of the SHO1 branch of the HOG pathway, and that Msb2 and Hkr1 appear to activate the HOG pathway by more than one mechanism. Recent studies have indicated that Hkr1 has a specific role in the HOG pathway, while Msb2 also participates in the filamentous growth pathway (Vadaie et al., 2008).

Our previous studies on *H. werneckii* have revealed its superior mechanisms for adapting to and surviving in extremely salty environments, which have not been seen in either salt-sensitive or moderately salt-tolerant fungi. The most relevant differences for *H. werneckii* that have been studied to date relate to its plasma-membrane properties (Turk et al., 2007), osmolyte composition (Petrovic et al., 2002), accumulation of ions (Kogej et al., 2005), melanization of the cell wall (Kogej et al., 2006), and differential expression of osmo-responsive genes (Vaupotic and Plemenitas, 2007). The existence of a signaling pathway that is similar to the *S. cerevisiae* HOG pathway was demonstrated by identification of the putative histidine-kinase-like osmosensor HwHhk7 (Lenassi and Plemenitas, 2007), together with two MAPKs: MAPKK HwPbs2 (our unpublished data) and the final MAPK HwHog1 (Lenassi et al., 2007; Turk and Plemenitas, 2002).

The aim of the present study was to further investigate the HOG pathway in this extremely halotolerant organism, with a focus on the identification and characterization of the Sho1 homolog in *H. werneckii*. Here, we report the identification of two HwSho1 isoforms from *H. werneckii* that are involved in the HOG signaling pathway.

## 2. Materials and methods

### 2.1. Strains and growth conditions

The cultures used in this study comprised: the black yeast *H. werneckii* (MZKI B736), *Aureobasidium pullulans* (EXF 150), *Cladosporium sphaerospermum* (EXF 385), *Cladosporium cladosporoides* (EXF 381) and *Eurotium amstelodami* (MZKI A561). These were obtained from the culture collection of the Slovenian National Institute of Chemistry (MZKI), from the Department of Biology, Biotechnical Faculty, University of Ljubljana (EXF), and from the Centraalbureau voor Schimmelcultures Ultrecht, The Netherlands. The cells were grown at 28 °C in a rotary shaker (180 rpm) in a defined yeast nitrogen-base (YNB) medium: 0.17% (w/v) yeast nitrogen-base, 0.08% (w/v) complete supplement mixture (both Qbiogene and Illkirch, France), 0.5% (w/v) ammonium sulfate, 2.0% (w/v) glucose, in deionized water, pH 7.0. The cells were harvested in mid-exponential growth phase by centrifugation at 4000*g* for 10 min, and then frozen in liquid nitrogen. The reference *S. cerevisiae* deletion strain YER118c (BY4741; MATa; his3D1; leu2D0; met15D0; ura3D0; YER118c::kanMX4) was acquired from the European *S. cerevisiae* archive for functional analysis (EUROSCARF). The AZ116 (*ade2Δ1*; *can1Δ100*; *his3Δ11*; *leu2Δ3*; *trp1Δ1*; *ura3Δ1*; Δ*sho1*; Δ*ssk2*; Δ*ssk22*) and the AZ117 (*ade2Δ1*; *can1Δ100*; *his3Δ11*; *leu2Δ3*; *trp1Δ1*; *ura3Δ1*; Δ*sho1*; Δ*ssk2*; Δ*ssk22*; Δ*pbs2*) deletion strains were kindly provided by A. Zarrinpar and W. Lim from the University of California (San Francisco).

### 2.2. Amplification of SHO1-like gene fragments from the genomes of halotolerant fungi

Highly purified fungal genomic DNA was isolated from mid-exponential phase cells grown in YNB medium without NaCl, according to a phenol/chloroform/isoamyl alcohol method that was modified for DNA isolation from filamentous fungi, as previously described (Rozman and Komel, 1994). Partial sequences of the *SHO1* orthologs from the halotolerant fungi were amplified by PCR using the degenerate primer pair dSho1.1 and dSho1.2 (Table 1), which corresponded to the conserved amino-acid regions of the known fungal Sho1 orthologs. About 50 ng genomic DNA was used as a template in 20 μl PCR reactions with Taq DNA polymerase (Fermentas). Touch-down PCR was performed using 10 touch-down cycles with an annealing temperature decreasing from 60 °C to 55 °C. The expected 500 bp PCR products were cloned into the pGEM-T Easy Vector (Promega) and sequenced (Sequiserve, Vaterstetten, Germany), and then confirmed using the BLAST program.

### 2.3. Cloning and sequencing of the HwSHO1 gene(s)

To clone the *HwSHO1* gene, first, a partial sequence of the *SHO1* homolog was amplified using PCR, with the primers dHwSho1.1 and dHwSho1.2 (Table 1) and the *H. werneckii* genomic DNA as a template. A 528 bp PCR product was labeled with [$^{32}$P]-dCTP using the Prime-it random primer labeling kit (Stratagene), according to the manufacturer instructions. The probe was then used for screening *H. werneckii* gDNA and cDNA libraries, constructed in the ZAP Express vector, as described previously (Turk and Plemenitas,

**Table 1**
Primers used in this study.

| Oligonucleotide | Sequence (5′–3′)[a] | Strand[b] |
|---|---|---|
| dSho1.1 | GTGTGGATYTTTTAYTTYGG | + |
| dSho1.2 | IGAGGAAGATIARRTARTT | – |
| dHwSho1.1 | GTGTGGATYTTTTAYTTYGG | + |
| dHwSho1.2 | TCAIAGCAAAATMARRTARTT | – |
| PrSho1.1 | GTCGATGGTACCTTCTCTTGTTTATCTGGGGGCTA | + |
| PrSho1.2A | GTTGTTGTAGTCCATATTTTGAAAACGTGA | – |
| PrSho1.2B | GTTGTTGTAATCCATATTTTGAAAACGTGA | – |
| HwSho1.1A | TCACGTTTTCAAAATATGGACTACAACAAC | + |
| HwSho1.1B | TCACGTTTTCAAAATATGGATTACAACAAC | + |
| HwSho1.2 | GCCGACGGAATTCAAGCAAGATCAAATAATT**CG**A | – |
| PrSho1.3 | TAGTGAATTCCATATTTTGAAAACGTGATTTTTGG | – |
| ScSho1.1 | GTCGATAGGATCCATGTCAATATCATCAAAGATAAG | + |
| ScSho1.2 | GCCGAGCAAGCTTACGATGCATTTCTTCTGGAC | – |
| HwSho1.3 | GTCGATAGGATCCGATGGACTACAACAAC | + |
| HwSho1.4 | GCCGAGCAAGCTTAAGCAAGATCAAATAATTCGA | – |

[a] Restriction sites are single underlined; Overlapping extensions are double underlined.
[b] (+) Coding strand of the multiplied gene; (–) noncoding strand of the multiplied genes.