Cloning and characterization of two K⁺ transporters of *Debaryomyces hansenii*

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Two genes from the halotolerant yeast *Debaryomyces hansenii* were cloned, *DhTRK1* and *DhHAK1*. These genes encode K⁺ transporters with sequence similarities to the TRK and HAK transporters from *Debaryomyces occidentalis* and *Candida albicans*. The DhHAK1p transporter was only expressed in K⁺-starved cells, as shown by Northern blot analysis. Both DhTRK1p and DhHAK1p were expressed in a *trk1Δ trk2Δ* mutant of *Saccharomyces cerevisiae*, unable to grow at low K⁺. This expression resulted in partial recovery of growth and ability to retain K⁺ at low concentrations. In liquid media, 0.5 M NaCl affected growth of these *S. cerevisiae* transformants as it does in *D. hansenii*, resulting in a much less deleterious effect than in wild-type *S. cerevisiae*. Kinetics of Rb⁺ uptake in the transformants suggest that DhTRK1p and DhHAK1p code for moderate-affinity K⁺ transporters exhibiting a sigmoid response against Rb⁺ concentration and presenting a deviation from classic Michaelis–Menten kinetics at low substrate concentrations. Rb⁺ uptake by the DhTRK1p transporter was stimulated by millimolar concentrations of Na⁺ at pH 4.5. The good performance of DhTRK1p in the presence of NaCl may be a key feature in the halotolerance of *D. hansenii*.

Received 16 January 2007

Revised 5 May 2007

Accepted 30 May 2007

INTRODUCTION

Potassium is the most abundant intracellular cation in living cells, playing several important roles in vital physiological processes. For example, K⁺ functions as a counter-ion in the maintenance of electroneutrality and osmotic equilibrium, and may contribute to the adjustment of membrane potential(s) or of protein activities (Walker *et al.*, 1998; Rodriguez-Navarro, 2000). Because these interactions are not mimicked by Na⁺ or by any other alkaline cation, K⁺ has become absolutely necessary for living cells. Furthermore, the majority of eukaryotic cells, with the exception of a few yeasts, algae and halophytes, do not tolerate environments with high Na⁺ concentration and low water potential, and Na⁺ is toxic for most of them (Serrano 1996; Garciadeblas *et al.*, 2003).

Although in most cells the intracellular K⁺ concentrations are quite similar under unstressed conditions, ranging between 100 and 500 mM, the external K⁺ concentrations to which cells are exposed can vary a lot, ranging from millimolar (in sea water and must) to micromolar in K⁺-depleted soils. In environments where K⁺ is scarce, cells need to accumulate K⁺ against a concentration gradient in

order to maintain optimal intracellular concentrations. Several K⁺ transporters from the TRK-HKT and HAK-Kup families, which allow highly efficient K⁺ uptake, have been described in eukaryotic walled cells (Rodriguez-Navarro, 2000). Transporters of the TRK-HKT type seem to be present in all fungi and plants studied so far (Rodriguez-Navarro, 2000), and they probably evolved from K⁺ channels (Durell *et al.*, 1999). This type of transporter is postulated to mediate the symport of two alkaline cations (Haro & Rodriguez-Navarro, 2002). In contrast, transporters of the HAK-Kup type have been identified in all plants but not in all fungi.

In fact, neither in *Saccharomyces cerevisiae* nor in *Schizosaccharomyces pombe* has the existence of HAK transporters been reported. In *Debaryomyces occidentalis*, a HAK-Kup transporter was identified and proposed to function as a K⁺/H⁺ symporter (Rodriguez-Navarro, 2000). Recently, a third type of K⁺ transporter, a K⁺-ATPase, was identified in the filamentous fungus *Ustilago maydis* and in the yeasts *Pichia sorbitophyla* and *Candida albicans* (Benito *et al.*, 2004). Although both TRK and HAK transport K⁺ with high affinity (in the case of TRK when cells are grown in low K⁺), they are competitively inhibited by Na⁺ in all yeasts and plants tested so far (Haro *et al.*, 1999; Haro & Rodriguez-Navarro, 2002). This

The GenBank/EMBL/DBJ accession number for the *DhHAK1* sequence of *Debaryomyces hansenii* is DQ914819.

inhibition is more evident when the Na⁺ : K⁺ ratio is high; this is probably due to a situation of K⁺-starvation even in the presence of K⁺ when the concentration of Na⁺ is high (Gomez *et al.*, 1996).

Debaryomyces hansenii is a yeast that occurs in marine environments and is tolerant to salt and alkaline pH (Norkrans, 1966; Butinar *et al.*, 2005); it can be also isolated from brines, cheese and other salty, low-water-activity environments (Mounier *et al.*, 2005; Cocolin *et al.*, 2006). Over the past 30 years, a few authors have described several features of this yeast related to its extreme halotolerance; these studies have focused on glycerol production and accumulation (Larsson *et al.*, 1990; Lages *et al.*, 1999), cation fluxes (Norkrans & Kylin, 1969; Prista *et al.*, 1997; Gonzalez-Hernandez *et al.*, 2004), and pH (Mortensen *et al.*, 2006) and Na⁺ targets (Aggarwal *et al.*, 2005). Some of these studies have considered *D. hansenii* as a halophilic yeast (Gonzalez-Hernandez *et al.*, 2004) and used this organism as a model for the study of salt tolerance mechanisms in eukaryotic walled cells (Prista *et al.*, 2002, 2005). The recent release of the complete sequence and annotation of the *D. hansenii* genome by the Génolevure consortium (Dujon *et al.*, 2004) revealed that most known pathways involved in salt tolerance in *S. cerevisiae* are also present in *D. hansenii* (Prista *et al.*, 2005) and also brought new perspectives to molecular studies in this yeast.

Norkrans & Kylin (1969) reported that higher NaCl concentrations were required to completely inhibit the uptake of potassium in *D. hansenii* than in *S. cerevisiae*. More recently, for *D. hansenii*, our group reported the non-inhibitory effect of Na⁺ on K⁺ uptake, when K⁺ is present at low levels, and the non-toxic effect of Na⁺ under these conditions, even when it is accumulated in relatively high intracellular concentrations (Prista *et al.*, 1997). So far the molecular bases for these features have not been clearly established. As part of a study aimed at

characterizing the major mechanisms involved in the extreme halotolerance of *D. hansenii*, we decided to clone and characterize the two major K⁺ uptake systems previously described in yeasts. The experiments described below analyse important aspects concerning the mechanisms of K⁺ uptake in *D. hansenii* and characterize the effects of Na⁺ on DhHAK1- and DhTRK1-mediated K⁺ transport. We propose that these two K⁺ uptake systems may play an important physiological role as part of the global strategy evolved in *D. hansenii* to achieve K⁺ nutrition while coping with high NaCl concentrations, thereby providing a competitive advantage in many salty environments such as the sea.

METHODS

Strains, plasmids and growth conditions. The yeast strains and plasmids used in this work are listed in Table 1. All *S. cerevisiae* strains used in this work are derivatives of W303-1A (*MATa*). Type strain *D. hansenii* PYCC2968 (CBS 767) was used as the source of genomic DNA. *S. cerevisiae* WΔ3 (W303-1A *trk1::LEU2 trk2::HIS3*) was used as recipient strain in complementation experiments with the plasmids listed in Table 1. *Escherichia coli* DH5α (Hanahan, 1985) was used for routine propagation of the plasmids.

The parental yeast strains were routinely maintained in YPD medium [5 g yeast extract, 10 g peptone, 20 g glucose (dextrose) and 20 g agar per litre], supplemented with 50 mM KCl when necessary. For selective purposes, YNB medium or minimal medium (Rodriguez-Navarro & Ramos, 1984; Prista *et al.*, 1997) with 2 % (w/v) glucose and distinct KCl and NaCl concentrations was used. These media were supplemented, when required, with the adequate requirements for prototrophic growth (Pronk, 2002). *E. coli* DH5α was routinely maintained in Luria-Bertani (LB) medium at 37 °C; ampicillin (100 µg ml⁻¹) and X-Gal (4 µg ml⁻¹) were added (Sambrook *et al.*, 1989) when required.

Recombinant DNA techniques. Plasmid isolation was performed by alkaline extraction as described by Birnboim & Doly (1979), and modified as in Sambrook *et al.* (1989). For plasmid isolation from yeasts, we used the procedure described by Hoffman & Winston

Table 1. List of plasmids and yeast strains used in the present work

Plasmid or strain	Relevant characteristics	Source or reference
Plasmids		
YEp352	Yeast episomal vector, 2 µm, URA3 yeast marker and Amp ^R	Hill <i>et al.</i> (1986)
CPpTRK	YEp352 derivative containing <i>DhTRK1</i> gene	This work
CPpHAK	YEp352 derivative containing <i>DhHAK1</i> gene	This work
Strains		
<i>D. hansenii</i>		
PYCC2968 (CBS767)	Type strain	
<i>S. cerevisiae</i>		
W303-1A	<i>MATa ade2 ura3 trp1 leu2 his3</i>	Wallis <i>et al.</i> (1989)
WΔ3	<i>W303-1A trk1Δ::LEU2 trk2Δ::HIS3</i>	Haro <i>et al.</i> (1999)
CPTRK	WΔ3[CPpTRK]	This work
CPHAK	WΔ3[CPpHAK]	This work
CPZERO	WΔ3[YEp352]	This work

(1987). Agarose gel electrophoresis and restriction site mapping were performed according to standard methods (Sambrook *et al.*, 1989). Yeast genomic DNA from *D. hansenii* for PCR amplification was isolated as described by Cryer *et al.* (1975) after a previous treatment with lyticase (5 mg ml⁻¹).

Total RNA was extracted from exponential-phase yeast cells after 5 h incubation under several conditions. Cells were then collected by membrane filtration as described by Belinchon *et al.* (2004), frozen using liquid nitrogen and kept at -80 °C until RNA extraction was performed. Total RNA was extracted by the hot phenol extraction protocol (Schmitt *et al.*, 1990), modified by Daniela Castro (personal communication) as described below. Frozen cells were resuspended in 470 µl 100 mM sodium acetate (pH 5.0), 5 mM MgCl₂ plus 0.1 vol. SDS 10%, w/v, 5 µl DEPC and 500 µl glass beads, and vortexed for 1 min. After vortexing, cells were subjected to three hot phenol extractions (5 min at 65 °C) with 1 vol. phenol/chloroform/isoamyl alcohol (25:24:1) pH 5.0 and one extraction with 1 vol. chloroform/isoamyl alcohol at room temperature. RNA precipitation was performed as described by Schmitt *et al.* (1990). Total RNA was fractionated through formaldehyde-agarose gels and transferred to N⁺-Hybond membranes (GE Healthcare). Hybridization was performed with digoxigenin (DIG)-labelled probes prepared from internal fragments of *DhTRK1* and *DhHAK1*. Fragments were labelled using the DIG system (Roche) by random priming, according to the manufacturer's instructions. Hybridizations were performed in DIG Easy Hyb (Roche) at 50 °C. Membranes were then washed under high-stringency conditions and exposed to X-ray films for a maximum of 72 h.

Cloning the *DhTRK* and the *DhHAK* genes. Sequence data for *D. hansenii* were obtained from the Génolevures Consortium Website at <http://cbi.labri.u-bordeaux.fr/Genolevures/> by performing TBLASTN search with Trk1p and Hak1p sequences from *D. occidentalis* against the *D. hansenii* genomic sequence. Using the BLASTP 2.2.14 program (NCBI) (Altschul *et al.*, 1997), ORFs showing homology to the Trk1p protein of *S. cerevisiae* and *D. occidentalis* and to the Hak1p proteins of *D. occidentalis* and *C. albicans* were identified. To express both DhTrk1p and DhHak1p from *D. hansenii*, YEp352 vector was used. Based on the nucleotide sequences of these ORFs together with the contiguous upstream and downstream regions, primers were designed in order to amplify a region from approximately 1000 bp upstream of the ATG start codon to 500 bp after the TAA stop codon for each gene. Forward f1TRK (5'-CCGCTCGAGCGGACATACGGCTGTCTGTGGAAAAA-3') and f1HAK (5'-CCGCTCGAGCGGACCAAGACCGCAAGAACA-3') primers, modified to incorporate a restriction site for *XhoI* (underlined), and reverse r1TRK (5'-CGCGGATCCTGTCAAGCTCTTGAATTTTC-3') and r1HAK (5'-CGCGGATCCGCGCAAGAATGGATTGACCTGAAC-3') primers, modified to incorporate a restriction site for *BamHI* (underlined), were used to amplify DNA fragments containing full-length ORFs encoding *DhTRK1* (f1TRK and r1TRK) and *DhHAK1* (f1HAK and r1HAK), using genomic DNA from *D. hansenii* CBS 767 as a template for PCR. PCR amplification was carried out in an Eppendorf thermocycler with DNA polymerase from BIOTOOLS, for 35 cycles, at 68 °C (annealing temperature chosen according to the primer characteristics). The amplified products were digested with *XhoI* and *BamHI*, purified using the 'GFX PCR DNA and Gel Band Purification' kit (GE Healthcare) and cloned into YEp352 vector previously digested with *BamHI* and *Sall* and purified with the same kit. Cloning was performed according to standard protocols described by Sambrook *et al.* (1989). Constructs were named as CPpTRK, for plasmids containing the *DhTRK1* gene, and CPpHAK, for plasmids containing the *DhHAK1* gene (Table 1). The plasmids were cloned into *E. coli* DH5 α , amplified, subjected to extraction and restriction analysis, and finally sequenced using an ABI Prism automated DNA sequencer (Perkin-Elmer). Transformation of *S. cerevisiae* WΔ3 was performed by the lithium acetate method described by Geitz &

Schiestl (1995). Transformants were selected on minimal medium without uracil, leucine and histidine. Fifty-three transformants containing CPpTRK and 159 transformants containing CPpHAK were obtained. One representative clone from each transformant (CPTRK and CPHAK) was used for heterologous expression studies.

Nucleotide sequence and analysis. DNA and protein sequences for comparative analysis were obtained from BLASTP 2.2.14 (Altschul *et al.*, 1997). Multiple protein alignments were performed by using CLUSTAL W version 1.83 (Thompson *et al.*, 1994) and phylogenetic trees were obtained by using TreeView X (Page, 1996).

Growth assays. The capability of yeast strains to grow in the presence of low KCl with or without NaCl was assessed on solid YNB or in minimal liquid medium (Rodríguez-Navarro & Ramos, 1984; Prista *et al.*, 1997), supplemented with KCl and NaCl to the desired final concentrations. For solid media assays, transformants were grown for 24 h in 5 ml YNB liquid medium without amino acids and with the required auxotrophic supplements to a final density of approximately 3×10^7 cells ml⁻¹. Plates were inoculated with 10 µl drops of serial 10-fold dilutions of saturated cultures and incubated at 28 °C. Growth was recorded after 1 and 2 weeks. For liquid media assays, transformants were grown to mid-exponential phase, for 24 h in 5 ml YNB liquid medium with 50 mM KCl without amino acids and with the auxotrophic requirements. After being harvested and washed twice with cold ultrapure water, cells were inoculated in modified arginine phosphate medium (Rodríguez-Navarro & Ramos, 1984) supplemented with KCl and NaCl as indicated, and the pH was adjusted to 5.6. The inoculum was calculated in order to obtain an initial OD₆₄₀ of 0.1 in the arginine phosphate medium. Cells were grown at 28 °C. Growth rates were determined from the absorbance vs time curves measured at 640 nm (Spectronic 20D, Milton Roy).

K⁺ content and K⁺ and Rb⁺ uptake assays. K⁺ content assays were performed essentially as described by Ramos *et al.* (1990). Cells were grown in minimal medium without uracil, leucine and histidine, with 500 µM KCl, to an OD₆₄₀ of 0.3. The culture was split into two portions. One portion was immediately collected on Millipore filters (0.45 µm), rapidly washed with 20 mM MgCl₂ and treated with HCl for a minimum of 24 h (Rodríguez-Navarro & Ramos, 1984). The other portion was harvested by centrifugation, washed twice with cold ultrapure water, resuspended in 10 mM MES (pH 5.6, adjusted with Ca(OH)₂, 2%, w/v, glucose) to the original OD₆₄₀ and incubated at 28 °C in an orbital shaker for K⁺ starvation. After 5 h incubation, cells were collected and treated by the methods described above for K⁺ non-starved cells.

The intracellular K⁺ content was analysed by atomic emission spectrophotometry (Rodríguez-Navarro & Ramos, 1984).

For Rb⁺ uptake, influx assays in K⁺-starved cells were performed as described previously (Ramos *et al.*, 1994). Cells grown in minimal medium (pH 4.5) with 10 mM KCl to mid-exponential phase were harvested, washed twice with cold ultrapure water and potassium-starved in the same medium without KCl. After a 5 h starvation period, cells were harvested by centrifugation, washed twice with cold ultrapure water, and resuspended at OD₆₄₀ 0.5–0.6 in Tris/citrate buffer (20 mM citric acid adjusted to pH 4.5 with Tris/HCl, 2%, w/v, glucose) with or without NaCl. At time zero, RbCl was added at the required concentration. Samples were taken periodically and treated as previously described. In short-term experiments, uptake was linear with time and initial uptake rates were obtained from the slope of this line.

Data analysis. All the experiments were repeated at least twice. The agreement among repetitions was high and typically the standard deviations were lower than 10% of the mean. In order to study the complex kinetics of Rb⁺ influx, we used the equation from

Garcia-deblas *et al.* (2003):

$$v = \frac{V_{\max 1} [\text{Rb}^+]}{K_m + [\text{Rb}^+]} + \frac{V_{\max 2} [\text{Rb}^+]^2}{[\text{Rb}^+]^2 + K_1 [\text{Rb}^+] + K_2}$$

in which the first term corresponds to the rate of ectopic transport of *trk1trk2* mutants (Madrid *et al.*, 1998) and the second term to the rate through a transporter with two binding sites for Rb⁺ (Haro & Rodriguez-Navarro, 2002). In this case, the kinetic parameters presented in Table 3 were obtained fitting the experimental data points to the equation by non-linear regression analysis using GraphPad Prism version 4.00 for Mac OS X (Graphpad Software, www.graphpad.com). Several fittings were performed for the whole range and partial ranges of concentrations. Tentative parameters were always obtained by graphic approaches (Eddie–Hofstee or double-reciprocal plots).

RESULTS

Cloning and sequence analysis of the *DhTRK1* gene

We compared some of the known TRK protein sequences against all the translated sequences from the *D. hansenii* genome sequence database released by Génolevure, by TBLASTN search. We found an ORF sequence (DEHA0A10648 g) located in chromosome A (anti-sense strand) and previously annotated as a gene for a putative TRK-type K⁺ uptake transporter protein of 1050 aa with high homology to DoTRK1p from *D. occidentalis* (Dujon *et al.*, 2004). The phylogenetic analysis of DhTRK1p and other fungal transporters of the same family revealed that DhTRK1p is most closely related to the DoTRK1p transporter from *D. occidentalis* and the CaTRK1p transporter from *C. albicans*, and less related to *Schiz. pombe* and *S. cerevisiae* TRK transporters (Fig. 1a).

Looking further into the protein sequence of DhTRK1p, all the basic characteristics of TRK-HKT transporters (Gaber *et al.*, 1988; Durell *et al.*, 1999) were found, with the sequence of four M1PM2 segments consisting of an N-terminal outer-

Table 3. Kinetic parameters of DhTRK1- and DhHAK1-mediated Rb⁺ influx

Assays were performed by measuring concentration-dependent Rb⁺ uptake (in a range between 1 μM and 100 mM) by cells K⁺-starved for 5 h. The initial Rb⁺ uptake in two types of cells expressing DhTRK1p and DhHAK1p were fitted to the equation given in Methods. The fits are shown in Fig. 4.

Strain	$V_{\max 1}$ (nmol min ⁻¹ mg ⁻¹)	K_m (mM)	$V_{\max 2}$ (nmol min ⁻¹ mg ⁻¹)	K_1 (mM)	K_2 (mM)
CPTRK	30	10.97	6.7	1.38	0.023
CPHAK	23	13.21	2.4	0.53	0.012

membrane helix (M1), a P-loop region (P) and a C-terminal inner transmembrane helix (M2). The glycine residues conserved in the four P fragments, in M2b, and immediately after Pd were also present (Durell & Guy, 1999). We also observed a smaller region located between the third and fourth transmembrane regions, the absence of a highly hydrophobic region located at Q¹⁰⁴⁰ and a reduced number of conserved potential N-linked glycosylation sites (data not shown), which is consistent with the high similarity between DhTRK1p and ScTRK2p (Ko & Gaber, 1991).

Cloning and sequence analysis of the *DhHAK1* gene

Using the same approach that was applied for the TRK transporter, we found two almost consecutive ORFs in chromosome E (anti-sense strand) for the HAK-type transporter; these ORFs were separated by 39 bp and were annotated as pseudogenes in the Génolevure database. The complete region containing the two pseudogenes was amplified and sequenced, confirming the existence of the stop codon at the position +1287 of the putative ORF, the same position as described in the Génolevure database (Dujon *et al.*, 2004). The translation of the amplified sequence revealed two in-frame and consecutive sequences, which corresponded to a hypothetical protein of 817 aa, divided by a stop codon located at the position of amino acid 430, and showing a strong homology to DoHAK1p and several other HAK-KUP transporters. Phylogenetic analysis of DhHAK1p (Fig. 1b) showed that the protein clustered within the group of yeast HAK transporters. As expected, the protein had both the 12 transmembrane regions and the conserved regions usually found in HAK transporters (Rodriguez-Navarro, 2000). This was not the case for the conserved amino acid sequence in the first transmembrane domain GXXXGDXGTSPLY, described by Senn *et al.* (2001) as characteristic of some high-affinity HAK transporters from plants like HvHAK1p.

Expression of *DhTRK1* and *DhHAK1* transcripts

We performed Northern blot analysis for *DhTRK1* and *DhHAK1* transcripts in *D. hansenii* using total RNA

Table 2. Doubling time of transformants of *S. cerevisiae*

Transformants with the empty plasmid YEp352 (CPZERO) and with plasmids harbouring *DhTRK1* (CPTRK) and *DhHAK1* (CPHAK) were grown in arginine phosphate medium (0.5 mM KCl, pH 5.6) with and without 0.5 M NaCl. Results for *S. cerevisiae* W303 and *D. hansenii* are also shown.

Strain	Doubling time (h)	
	0 M NaCl	0.5 M NaCl
CPZERO	5.9	—
CPTRK	5.3	9.3
CPHAK	6.2	9.5
W303	2.4	15.3
<i>D. hansenii</i>	3.7	5.6

—, No growth observed after 2 weeks.

Fig. 1. (a) Phylogenetic tree of TRK-HKT transporters. GenBank accession numbers are: XP456781 (DhTRK1), CAB91046 (DoTRK1), AFF72203 (CaTRK1), CAA89494 (ScTRK1), CAA82128 (ScTRK2), gi1717749 (SpTRK1), CAB93300 (SpTRK2), CAA08813 (Nctrk-1) and NP567354 (AtHKT1). (b) Phylogenetic tree of HAK transporters. GenBank accession numbers are: DQ914819 (DhHAK1), AAB17122 (DoHAK1), XP718752 (CaHAK1), AAC39315 (HvHAK1), AAC36491 (HvHAK2), AAK53758 (McHAK1), AAF36490 (AtHAK5) and CAA08814 (Nchak-1). In both panels the protein alignment and tree were obtained using CLUSTAL W and TreeView, and the scale bar corresponds to a distance of 10 changes per 100 amino acid positions.

prepared from *D. hansenii* cells incubated for 5 h in minimal medium, under potassium starvation and non-starvation conditions, and in the absence or presence of 0.5 M NaCl. Under K^+ starvation conditions, the expression of *DhHAK1* was only observed in the absence of NaCl (results not shown). Under the same conditions, we did not observe expression of DhTRK1p. This negative result has been previously reported for Nctrk-1p, ScTRK1p and ScTRK2p (Haro *et al.*, 1999), and DoTRK1p (Banuelos *et al.*, 2000).

Functional characterization of DhTRK1p and DhHAK1p

To verify the K^+ transport capability of the proteins encoded by *DhTRK1* and *DhHAK1* we amplified the chromosomal regions corresponding to *DhTRK1* and *DhHAK1* together with their own putative promoter and termination regions. The amplified fragments were cloned and used to transform a *trk1Δtrk2Δ* *S. cerevisiae* mutant, WΔ3, lacking the endogenous TRK transporters and incapable of growing under low K^+ conditions. Characterization of all transformants harbouring *DhTRK1* or *DhHAK1* was performed. Two of each category were selected for phenotypic characterization.

DhTRK1p and DhHAK1p improve growth under low K^+ conditions. In comparative growth tests performed in solid minimal medium with different K^+ concentrations (pH 4.5, 5.6 and 7.5), all the transformants bearing *DhTRK1* or *DhHAK1* behaved in a similar way. Fig. 2

shows the results in minimal medium supplemented with 10 μ M and 50 mM KCl. Strain CPZERO, harbouring YEp352, was used as a control, showing that the plasmid had no effect on the phenotype of the *trk1Δtrk2Δ* strain. The presence of either CPpTRK or CPpHAK led to partial recovery of the ability to grow under low K^+ concentrations, which is a characteristic of *S. cerevisiae* W303 carrying its native K^+ transporters.

We also studied the effect of NaCl on growth of the transformants carrying *D. hansenii* genes. Although in solid

Fig. 2. DhTRK1- and DhHAK1- mediated growth of WΔ3 transformants at low K^+ . The WΔ3 yeast mutant, defective in the endogenous TRK K^+ -transport systems, was transformed with the empty plasmid YEp352 (CPZERO) or with the plasmids CPpTRK (CPTRK) and CPpHAK (CPHAK). Drops of serial diluted suspensions were used to inoculate arginine phosphate medium (pH 5.6) supplemented with (a) 10 μ M and (b) 50 mM KCl.

medium the effect of NaCl was not clear (results not shown), an effect became evident when the strains were grown in liquid medium (Table 2). In minimal medium with 500 μ M KCl and 0.5 M NaCl, the behaviour of CPTRK and CPHAK was similar to that of *D. hansenii*. In *D. hansenii* and in the transformants the specific growth rate decreased by 30 %, while in *S. cerevisiae* W303, bearing the endogenous K⁺ transporters, 0.5 M NaCl had a strong inhibitory effect on growth.

DhTRK1p and DhHAK1p improve K⁺ accumulation. The good performance of the transformants was confirmed by their intracellular K⁺ contents. In media with 500 μ M K⁺, the presence of the plasmids containing *D. hansenii* genes raised the K⁺ content compared with the host strain, with CPpTRK being more effective. In this case, the K⁺ content was 445 nmol (mg dry weight)⁻¹, similar to those described for wild-type *S. cerevisiae* (Bañuelos *et al.*, 2002). Incubation in K⁺-free medium for 5 h resulted in loss of K⁺ for all the strains, but K⁺ content was still higher for both strains containing *D. hansenii* transporters [235 nmol (mg dry weight)⁻¹ and 250 nmol (mg dry weight)⁻¹ for CPTRK and CPHAK, respectively] than for CPZERO [155 nmol (mg dry weight)⁻¹]. Interestingly, strain CPHAK lost relatively less K⁺ than CPTRK, supporting the view that K⁺ depletion increases DhHAK1p expression, as previously described for DoHAK1p and other HAK transporters from fungi and plants (Rodriguez-Navarro, 2000; Rodriguez-Navarro & Rubio, 2006) and in accordance with the data obtained by Northern blot analysis described above.

DhTRK1 and DhHAK1 encode two K⁺ transporters. To investigate if *DhTRK1* and *DhHAK1* code for K⁺ uptake transporters, we used Rb⁺ as a K⁺ analogue to perform uptake assays in CPTRK and CPHAK K⁺-starved cells. pH 4.5 was chosen for detailed kinetic studies, since it was previously shown that, at this pH, Na⁺ stimulated Rb⁺ uptake and K⁺ accumulation in *D. hansenii* (Prista *et al.*, 1998).

When we analysed the initial Rb⁺ uptake rates for CPTRK and CPHAK, the kinetics analysis of Rb⁺ uptake indicated the existence of two transport components, one corresponding to the ectopic low-affinity uptake presenting estimated kinetic parameters similar to those described for the *trk1Δtrk2Δ S. cerevisiae* mutant (Table 3) (Ramos *et al.*, 1994), and the other exhibiting a sigmoid response against Rb⁺ concentration and presenting a deviation from Michaelis–Menten kinetics which yielded a convex Eadie–Hofstee plot at low Rb⁺ concentrations (Fig. 3).

The kinetic behaviour of DhTRK1p and of DhHAK1p can be described by the equation proposed by Garciadeblas *et al.* (2003), composed of two terms, a component for the low-affinity system and a quadratic rate equation similar to the one described for ScTRK1 (Haro & Rodriguez-Navarro, 2002) (see Methods). At very low Rb⁺ concentrations (below 50 μ M) it was possible to detect the effect of the

Fig. 3. Complex kinetics of DhTRK1- and DhHAK1-mediated Rb⁺ influx. Eadie–Hofstee plot of the initial rates of Rb⁺ uptake in (a) CPTRK and (b) CPHAK strains expressing *D. hansenii* K⁺-transporters. Fitting the experimental data points (dots) to the equation in Methods produced the kinetic parameters summarized in Table 3. The continuous line is the actual fit by the equation.

constant K_2 (usually much lower than K_1). The customary K_m is really the K_1 constant corresponding to the kinetic constant for the second binding site as was proposed by Haro & Rodriguez-Navarro (2002). The kinetic parameters estimated after fitting all the experimental data to the equation are summarized in Table 3. The values of K_1 of 1.38 and 0.53 mM Rb⁺ for DhTRK1p and DhHAK1p, respectively, suggest that these genes encode moderate-affinity Rb⁺ transporters. As reported for other members of the HAK-Kup family (Bañuelos *et al.*, 1995; Haro *et al.*, 1999), the affinity of DhHAK1p was higher than that of DhTRK1p.

Effect of Na⁺ on Rb⁺ transport by DhTRK1p and DhHAK1p

Several studies on K⁺ (Rb⁺) influx have shown that Na⁺ is taken up by *S. cerevisiae* and produces a competitive inhibition effect on K⁺ (Rb⁺) uptake (Ramos & Rodriguez-Navarro, 1986), probably leading to a situation of K⁺ deprivation deleterious to the cells. Previous studies on Rb⁺ uptake in *D. hansenii* have shown that Na⁺ concentrations up to 50 mM do not inhibit Rb⁺ uptake at pH 4.5 (Prista *et al.*, 1997). The differences in K⁺ transport performance between *D. hansenii* and *S. cerevisiae*, together with the weaker growth inhibition observed for the CPTRK and CPHAK strains (see above), suggested that, if DhTRK1 and DhHAK1 mediate the main pathway of K⁺ (Rb⁺) uptake in *D. hansenii*, as seems to be the case, they could show different behaviour in response to Na⁺, compared to *S. cerevisiae*. To address this possibility we tested the effect of NaCl on Rb⁺ influx in CPTRK and CPHAK. Fig. 4 shows the initial Rb⁺ uptake

rates for CPTRK and CPHAK K^+ -starved cells in the absence and in the presence of 1, 5 and 10 mM NaCl. In CPHAK, increasing NaCl concentrations led to an increasingly higher inhibitory effect, with 10 mM NaCl resulting in a threefold decrease in Rb^+ uptake rate. The results obtained indicate that the K_i of Na^+ for DhHAK is in the millimolar range, much higher than for Rb^+ (results not shown). In contrast, in the transformant bearing the DhTRK1p transporter no inhibition was observed up to 10 mM NaCl, at pH 4.5. At this pH, in CPTRK, we were even able to observe significant stimulation (40 %) at 10 mM NaCl relative to uptake in the absence of NaCl.

DISCUSSION

In the present work we identified and cloned the *DhTRK1* and *DhHAK1* genes, which displayed strong similarities with other genes for K^+ transporters belonging to the TRK and HAK families. In the case of *DhHAK1*, the detection of a stop codon in a conserved region of the putative *DhHAK1* gene was inconsistent with the experimental results obtained for the CPHAK transformant. The first hypothesis to explain these anomalous results was the existence of an intron in that region. Although this could not be completely discarded since we did not sequence the cDNA or mRNA, this is a highly improbable situation since firstly no sequences corresponding to putative introns present in the genomic cloned sequence were predicted, and secondly there is a high level of conservation between the region where the stop codon was found and the same region of the DoHAK1 sequence obtained from a cDNA. Another possibility was that *D. hansenii* could have a suppression mechanism, as has been described for several

viral and cellular systems including *S. cerevisiae* (Kim *et al.*, 1990; Bonetti *et al.*, 1995) and *C. albicans* (Santos *et al.*, 1993). This mechanism could lead to a non-standard translation process of termination codon readthrough in which the stop codon UGA, often described as a particularly leaky codon (Surguchov, 1988), would be avoided. The fact that CPHAK cells showed a clearly different phenotype with respect to growth, K^+ content and Rb^+ uptake, as compared with CPZERO, supported the idea that a functional DhHAK1p was being expressed in CPHAK clones, consistent with the hypothesis of an existing non-standard translation process of termination codon readthrough. If the stop codon was effective, this would imply that a truncated protein lacking 47 % of the C-terminal amino acid sequence (the last four transmembrane regions and the hydrophilic C terminus) was functional. This is very improbable since in the case of HAK-Kup transporters it has been shown that the hydrophilic C terminus has an essential role in HAK-Kup transporter functionality (Schleyer & Bakker, 1993).

Several studies have shown that high-affinity K^+ uptake systems are induced upon K^+ starvation and internal K^+ depletion in yeast and plant cells (Haro *et al.*, 1999; Martinez-Cordero *et al.*, 2004). A difficult point to explain in our results is the observation that DhHAK was not detectably expressed in K^+ -starved cells when NaCl was present. It is conceivable that, rather than a low extracellular K^+ concentration, intracellular K^+ depletion is required for HAK expression. The presence of NaCl would activate DhTRKp. In this case starvation would be less efficient in promoting internal K^+ depletion, and for this reason the expression of DhHAK would not be detectable.

In growth assays performed at 0.5 mM KCl, a concentration 100 times lower than that reported for optimal growth of the *trk1Δtrk2Δ* strain (Gaber *et al.*, 1988), the presence of 0.5 M NaCl affected growth parameters of CPTRK and CPHAK in a similar way to that observed for *D. hansenii* growth (Table 2), and in a way much less dramatic than for wild-type *S. cerevisiae*. In *D. hansenii*, even though a lower capacity of the K^+ transport systems was observed, it was proposed that a higher Na^+ intracellular content could help cells to overcome the lack of K^+ when it is scarce (Gonzalez-Hernandez *et al.*, 2004). This cannot be the case for the *S. cerevisiae* transformant strains, since Na^+ should still be toxic when accumulated inside these cells.

In the transformants bearing *D. hansenii* K^+ transporters, the growth phenotype of wild-type *S. cerevisiae* was not completely recovered (Fig. 2). This result can be explained by the lower K^+ (Rb^+) affinity in the transformant strains (Table 3) as compared to *S. cerevisiae* (Ramos *et al.*, 1994). This may lead to a decrease in growth rate due to a lower intracellular K^+ content, a consequence of the reduced ability to capture K^+ from a medium where the availability of this cation is limited. The lower K^+ (Rb^+) affinity exhibited by DhTRK1p and DhHAK1p compared with the affinities described for the same type of transporters in

Fig. 4. Effect of NaCl on DhTRK1- and DhHAK1-mediated Rb^+ uptake. Rb^+ influx at 0.2 mM was measured in (a) CPTRK and (b) CPHAK strains, in the absence (○) and presence of 1 mM (●), 5 mM (△) and 10 mM (▲) NaCl. Assays were performed in Tris/citrate buffer pH 4.5, as described in Methods.

other yeasts (Rodriguez-Navarro, 2000) is in accordance with the lower affinity for Rb⁺ observed in *D. hansenii* (Prista *et al.*, 1997).

Both DhTRK1p and DhHAK1p showed similar Michaelis-Menten-deviated kinetic behaviours. For DhTRK1p, this type of deviation is in accordance with previously described kinetics for other known TRK-HKT transporters (Garcia-deblas *et al.*, 2003; Haro & Rodriguez-Navarro, 2003) and can be attributed to the existence of two Rb⁺ (K⁺) binding sites with different affinities, the first of them being occupied by the residual K⁺ (up to 1.5 µM) resulting from cell content loss in K⁺-free buffer, as suggested by Haro & Rodriguez-Navarro (2002). In the case of DhHAK1p, this kind of deviation from Michaelis-Menten kinetics has never been described for HAK transporters. Nevertheless, it agrees with the idea that some HAK transporters may be, in some conditions, K⁺/H⁺ symporters as was described for plant HAK transporters (Santa-Maria *et al.*, 1997; Senn *et al.*, 2001). At pH 4.5, the H⁺ concentration is sufficiently high to interfere with the binding of Rb⁺ to the first site especially when Rb⁺ is low, as was described for K⁺ transporters in the case of K⁺ and Rb⁺ (Borst-Pauwels, 1981; Haro & Rodriguez-Navarro, 2002). The fact that Rb⁺-uptake assays to estimate kinetic parameters are usually performed at pH 6.0, a proton concentration significantly lower than we used (1 µM H⁺ vs 32 µM H⁺ in our case) may explain the differences in the results. This inhibition is relieved upon increasing the Rb⁺ concentration.

The initial Rb⁺ influx rates obtained at pH 4.5 with increasing NaCl concentrations (Fig. 4) show that NaCl has different effects on each transporter. The effect of NaCl on the DhTRK1p-mediated Rb⁺ uptake at pH 4.5 was significantly different from that usually observed for transporters of this type such as ScTRK1p and trk-1 from *Neurospora crassa*. For ScTRK1p, 2 mM NaCl inhibited Rb⁺ influx by 35% at the same Rb⁺ concentration (Haro & Rodriguez-Navarro, 2002) and for Nctrk-1 we estimated that 10 mM NaCl inhibited Rb⁺ influx by 30% (Haro *et al.*, 1999). Although these results were obtained for a different pH, the known independence of pH of ScTRK1 K⁺ influx (Rodriguez-Navarro & Ramos, 1984), together with consistent results presented by Haro & Rodriguez-Navarro (2002), led us to conclude that the effect of NaCl on K⁺ transport is quite peculiar in *D. hansenii*.

Taken together, these results suggest that DhTRK1p and DhHAK1p are the main K⁺ transporters in *D. hansenii*. They may have evolved to become rather low-affinity transporters, since potassium is relatively abundant in the marine habitats of this yeast. Potassium is an essential cation in all living systems. Since it is involved in several crucial cellular processes such as protein synthesis and the control of membrane potential and intracellular pH, cells need to maintain minimum intracellular levels of this cation in order to survive. Since NaCl is a competitive inhibitor of K⁺ uptake, under high NaCl concentrations

most yeasts have problems in maintaining intracellular K⁺ content, a deleterious situation to the cell. Functional and robust K⁺ transport systems with the remarkable characteristic of not being, or at least being less, inhibited by NaCl may be critical features to guarantee survival and growth of *D. hansenii* in salty environments.

ACKNOWLEDGEMENTS

This work was supported by Fundação para a Ciência e a Tecnologia (Project POCTI/ 2000/BIO/32749), by grant SFRH/BPD/19913/2004 to J.C.G.-H., by grant BFU2005-06388-C04-03 from Ministerio de Educación y Ciencia (Spain) to J.R. and by Acção Integrada Luso-Espanhola E-81/04 and HP2003-0050 from Ministerio de Ciencia y Tecnología (Spain). We thank A. Rodriguez-Navarro, from the Universidad Politécnica de Madrid, for kindly supplying strains and plasmids, and for helpful discussions, D. Castro and A. Peña, from the Universidad Nacional Autónoma de México, for helpful suggestions and I. Henriques from Virginia Tech for reviewing the manuscript.

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Edited by: D. J. Jamieson