Difference in substrate specificity divides the yeast alkali-metal-cation/H⁺ antiporters into two subfamilies

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Yeast plasma membrane Na⁺/H⁺ antiporters (TC 2.A.36) share a high degree of similarity at the protein level. Expression of four antiporters (*Saccharomyces cerevisiae* Nha1p, *Candida albicans* Cnh1p, *Zygosaccharomyces rouxii* ZrSod2-22p and *Schizosaccharomyces pombe* sod2p) in a *Sacch. cerevisiae* mutant strain lacking both Na⁺-ATPase and Na⁺/H⁺ antiporter genes made it possible to study the transport properties and contribution to cell salt tolerance of all antiporters under the same conditions. The ZrSod2-22p of the osmotolerant yeast *Z. rouxii* has the highest transport capacity for lithium and sodium but, like the *Schiz. pombe* sod2p, it does not recognize K⁺ and Rb⁺ as substrates. The *Sacch. cerevisiae* Nha1p and *C. albicans* Cnh1p have a broad substrate specificity for at least four alkali metal cations (Na⁺, Li⁺, K⁺, Rb⁺), but their contribution to overall cell tolerance to high external concentration of toxic Na⁺ and Li⁺ cations seems to be lower compared to the antiporters of *Schiz. pombe* and especially *Z. rouxii*.

Keywords: Na⁺ efflux, K⁺ efflux, salt tolerance, transport

INTRODUCTION

In natural environments, sodium belongs to the abundant and potassium to the scarce ions. However, high internal concentrations of Na⁺ (or its analogue Li⁺) are generally toxic for cells. On the other hand, K⁺ is required for many physiological functions (regulation of cell volume and intracellular pH, protein synthesis, enzyme activation) and this cation is accumulated in cells at a fairly high concentration (Rodríguez-Navarro, 2000; Rodríguez-Navarro *et al.*, 1994). When cells are exposed to salt stress (e.g. increased external concentration of NaCl), Na⁺ enters the cells (probably as a low-affinity substrate through several cation-transporting systems, mainly those involved in potassium uptake: Gaber *et al.*, 1988; Ko & Gaber, 1991; Ramos *et al.*, 1994), and cells must cope with the increased cytoplasmic concentration of Na⁺. In yeast cells, three mechanisms function cooperatively to prevent the accumulation of Na⁺ in the cytoplasm: restriction of Na⁺ influx, active Na⁺ efflux and compartmentation of Na⁺ in the vacuole (Niu *et al.*, 1995). Two different types of transport systems mediating active sodium efflux from cells exist in yeast plasma membranes: Na⁺-ATPases and Na⁺/H⁺ antiporters. So far, genes encoding specific

plasma membrane Na⁺/H⁺ antiporters have been identified in four different yeast species.

The Na⁺/H⁺ antiporter responsible for sodium and lithium tolerance of the fission yeast *Schizosaccharomyces pombe* is encoded by the *sod2* gene and is believed to be the sole sodium extrusion system in this yeast (Jia *et al.*, 1992). Three genes encoding Na⁺/H⁺ antiporters were identified in two osmotolerant *Zygosaccharomyces rouxii* strains: ZSOD2 and ZSOD22 in ATCC 42981 (Iwaki *et al.*, 1998; Watanabe *et al.*, 1995), and ZrSOD2-22 in CBS 732 (Kinclová *et al.*, 2001a). Although the gene (*ZEN1*) encoding Na⁺-ATPase was also identified in *Z. rouxii*, it was shown that the tolerance of cells to high external concentrations of Na⁺ and Li⁺ depends mainly on the Na⁺/H⁺ antiporter activity (Watanabe *et al.*, 1999). On the other hand, in the model yeast *Saccharomyces cerevisiae*, the most efficient sodium-eliminating system is Na⁺-ATPase (encoded by the *ENA1-4/PMR2A-E* genes: Haro *et al.*, 1991; Wieland *et al.*, 1995), while the Na⁺/H⁺ antiporter (encoded by the *NHA1* gene: Prior *et al.*, 1996) plays a minor role in the salt tolerance of *Sacch. cerevisiae* cells (Bañuelos *et al.*, 1998). Finally, a homologous gene (*CNH1*) encoding the Na⁺/H⁺ antiporter was isolated from two *Candida*

albicans strains, SC5314 (Soong *et al.*, 2000) and MEN (Kinclová *et al.*, 2001b). Deletion of *CNH1* leads to retardation of growth and a highly elongated morphology of cells, but it has little effect on the sensitivity of *C. albicans* cells to high concentrations of sodium and lithium (Soong *et al.*, 2000).

The *Sacch. cerevisiae* Nha1p mediates the efflux not only of toxic Na^+ and Li^+ , but also of K^+ and Rb^+ (Bañuelos *et al.*, 1998; Kinclová *et al.*, 2001c) and its function in cells seems to be more general than elimination of toxic cations. The Nha1p is perhaps involved in the regulation of intracellular pH (Sychrová *et al.*, 1999) and intracellular potassium concentration (Bañuelos *et al.*, 1998; Kinclová *et al.*, 2001c). Recently, we have shown that *C. albicans* Cnh1p transports, besides toxic sodium and lithium, also potassium and rubidium (Kinclová *et al.*, 2001b). Both *Schiz. pombe* sod2p and *Z. rouxii* ZSod2 antiporters have been already expressed in a *Sacch. cerevisiae* strain, but their transport activity for alkali metal cations has never been directly measured. The Na^+/H^+ antiporter activity was estimated only as improved growth of *Sacch. cerevisiae* transformants (expressing either *Schiz. pombe* sod2p or *Z. rouxii* ZSod2 antiporters) in the presence of LiCl or NaCl (Hahnenberger *et al.*, 1996; Iwaki *et al.*, 1998). In the case of *Schiz. pombe* sod2p, its activity in *Sacch. cerevisiae* cells was observed also as a Na^+ -dependent proton uptake (Hahnenberger *et al.*, 1996). Different vectors and promoters were used to express *Schiz. pombe* and *Z. rouxii* antiporters in a *Sacch. cerevisiae* strain that, moreover, contained a functional *NHA1* copy (Hahnenberger *et al.*, 1996; Iwaki *et al.*, 1998).

In order to compare directly the transport properties of all known yeast Na^+/H^+ antiporters, we expressed them under the same conditions (vector and promoter) in a *Sacch. cerevisiae* mutant lacking sodium-extrusion systems, Na^+ -ATPases and the Na^+/H^+ antiporter. Cells containing different antiporters were tested for their tolerance to Na^+ , Li^+ , K^+ and Rb^+ , and the efflux activity of sod2p and ZrSod2-22p was measured in comparison with that of Nha1p. Our results showed that the expression of *Z. rouxii* and *Schiz. pombe* genes improved considerably the sodium and lithium tolerance of *Sacch. cerevisiae* cells, but neither sod2p nor ZrSod2-22p was involved in the transport of K^+ or Rb^+ . The *Z. rouxii* antiporter seems to be the most efficient sodium- and lithium-extruding system of all yeast antiporters as its activity maintains the lowest Na^+ concentration in cells growing in the presence of NaCl.

METHODS

Strains and media. The alkali-metal-cation sensitive *Sacch. cerevisiae* B31 strain (*ena1Δ::HIS3::ena4Δ nha1Δ::LEU2*) (Bañuelos *et al.*, 1998) was used in this work for expression and phenotypic characterization of yeast Na^+/H^+ antiporters. Yeast cells were grown aerobically at 30 °C in standard YNB media with 2% glucose and appropriate nutritional supplements. The *Schiz. pombe* wild-type strain 972h⁻ was grown in YE medium (0.5% yeast extract, 3% glucose) containing

LiCl (60 mM) to increase the copy number of the *sod2* gene (Jia *et al.*, 1992).

Plasmids. Multi-copy plasmids used in this work for expression of genes encoding different yeast Na^+/H^+ antiporters were as follows: pNHA1-985 harbouring the *Sacch. cerevisiae* *NHA1* gene (Kinclová *et al.*, 2001c), pZrSOD2-22 containing the *ZrSOD2-22* gene from *Z. rouxii* CBS732 (Kinclová *et al.*, 2001a) and pCNH1-G23 harbouring the *CNH1* gene from *C. albicans* MEN (Kinclová *et al.*, 2001b). For cloning the *Schiz. pombe* *sod2* gene (which contains an intron: Jia *et al.*, 1992), the total RNA was isolated from *Schiz. pombe* cells (Carlson & Botstein, 1982) and reverse-transcribed with the First Strand cDNA Synthesis Kit (Pharmacia Biotech). The reverse-transcription products were used for PCR amplification (Peltier Thermal Cycler, PTC-200 MJ Research; *Taq* DNA polymerase, Boehringer Mannheim) with the antisense-anchor-specific primer (5'-GAAGAATTTCGCGGCCGAGGAA-3': Bañuelos & Rodríguez-Navarro, 1998) and a sense-*sod2*-gene-specific primer (5'-CGGGATCCATTACTATGGGCTGGAG-3'). This procedure yielded one DNA fragment (1.5 kb) corresponding to cDNA of the *sod2* gene with *Bam*HI and *Eco*RI restriction sites (underlined in the primer sequences) in the upstream and downstream ends of the open reading frame, respectively. The amplified fragment was first inserted into the pCRII-TOPO vector using the TOPO TA Cloning kit (Invitrogen). Subsequently, the 1.5 kb long *Bam*HI-*Eco*RI fragment was cloned into the multi-copy YEp352 vector (Hill *et al.*, 1986) behind the *NHA1* promoter region (Bañuelos *et al.*, 1998), resulting in the plasmid pSpod2.

Genetic and molecular methods; DNA sequencing and sequence analysis. Standard protocols for nucleic acid manipulations, and yeast and *E. coli* transformations, were used (Bloch *et al.*, 1992; Sambrook *et al.*, 1989). DNA sequencing was carried out using the Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham Life Science). Both strands of the *sod2* cDNA fragment were completely sequenced, and Lasergene99 (DNASTAR) was used for DNA and protein sequence analyses.

Salt tolerance determination. The growth capacity of yeast cells in the presence of NaCl, LiCl, KCl and RbCl was tested as described earlier (Kinclová *et al.*, 2001c).

Cation contents and loss. To estimate the intracellular concentration of alkali metal cations, aliquots of cells were withdrawn from the incubation mixture at various time intervals, cells were collected on Synpor membrane filters (0.85 µm pore diameter, Czech Republic), rapidly washed with 20 mM MgCl_2 , acid-extracted and analysed by atomic absorption spectrophotometry (Haro *et al.*, 1991; Rodríguez-Navarro & Ramos, 1984). The alkali metal cation efflux was estimated as described earlier (Kinclová *et al.*, 2001c). Lithium uptake measurements were done according to Camacho *et al.* (1981). Cells were grown in YNB media to $\text{OD}_{600} \approx 0.3$, harvested, washed and resuspended in the incubation buffer [20 mM MES containing 0.1 mM MgCl_2 , 2% glucose and adjusted to pH 5.5 with $\text{Ca}(\text{OH})_2$]. At time zero, LiCl was added to the suspension (final concentration 50 mM) and samples of cells were withdrawn at 5–10 min intervals during 60 min. For estimation of internal cation contents in growing cells, fresh YNB media (40 ml) without or supplemented with the indicated amounts of NaCl were inoculated to $\text{OD}_{600} \approx 0.02$ from cell cultures grown overnight in 20 ml standard YNB. Then growth was assessed by measuring the increase in OD_{600} of the cell suspension for 16 h. At $\text{OD}_{600} \approx 0.2$, 15 ml of the cultures was harvested, washed and

resuspended in 15 ml MES buffer [20 mM MES, 0.1 mM MgCl₂ adjusted to pH 5.5 with Ca(OH)₂], and two aliquots of 5 ml were used for determination of Na⁺ and K⁺ contents. All measurements of cation transport and content were repeated at least three times; the deviation among parallel results was always <5%.

RESULTS

Comparison of yeast Na⁺/H⁺ antiporter primary structure

Plasma membrane Na⁺/H⁺ antiporters, as mentioned above, have so far been identified in four yeast species (*Sacch. cerevisiae*, *C. albicans*, *Z. rouxii* and *Schiz. pombe*). Comparison of amino acid sequences deduced from the genes shows similar structural features. Although they differ in total length, all of them contain a short hydrophilic N-terminus (11–12 amino acid residues), a transmembrane hydrophobic part with 12 putative transmembrane segments, and a hydrophilic C-terminus. The amino acid sequences and lengths of the N-termini, transmembrane parts and connecting loops are highly conserved (Table 2). Alignment of these parts shows several highly conserved regions in both the hydrophobic transmembrane domains and the connecting hydrophilic loops. On the other hand, the C-terminal sequences of the antiporters differ in length (Table 1) and show very low similarity in amino acid sequence (Table 2). Detailed analysis revealed only two conserved regions within the C-terminal parts of *Sacch. cerevisiae* Nha1p and *C. albicans* Cnh1p (Kinclová *et al.*, 2001b). These regions are, however, not present in the C-terminus of the *Schiz. pombe* antiporter, which is very short (Table 1), or in the long C-terminus of *Z. rouxii*.

The Nha1p and Cnh1p antiporters transport, in addition to sodium and lithium, also potassium and rubidium. But as was shown for the Nha1p, the recognition, binding and transport of potassium do not depend on the length of the C-terminus (Kinclová *et al.*, 2001c). To find out if also the other two members of the protein family, *Schiz. pombe* sod2p and *Z. rouxii* ZrSod2-22p, can transport potassium and rubidium, and to compare their transport properties with those of Nha1p and Cnh1p, the coding sequences of the four antiporters were cloned behind the *NHA1* promoter and expressed from a multi-copy vector in the alkali-metal-cation-sensitive *Sacch. cerevisiae* strain B31.

Yeast Na⁺/H⁺ antiporters have different substrate specificity

To determine the substrate specificity of *Schiz. pombe* sod2 and *Z. rouxii* ZrSod2-22 antiporters, first the growth of *S. cerevisiae* B31 cells expressing heterologous sod2p and ZrSod2-22p was tested on plates containing increasing amounts of sodium, lithium, potassium and rubidium salts (Fig. 1). Cells transformed with an empty vector (negative control) were very sensitive to all these cations and only the presence of Nha1p (positive control) conferred high potassium and rubidium tolerance (Fig. 1). Cells with the ZrSod2-22 and sod2 antiporters could not grow on plates supplemented with high concentrations of potassium or rubidium. They tolerated the same amounts of external KCl and RbCl as control cells without any antiporter (800 mM and 500 mM, respectively). These results indicated that neither potassium nor rubidium was a substrate for sod2p or ZrSod2-22p.

Table 1. Protein structure, molar mass and pI of yeast Na⁺/H⁺ antiporters

Antiporter	No. of amino acid residues				Mol. mass (kDa)	pI
	Total	N-terminus	TMS* + loops	C-terminus		
<i>Sc</i> Nha1	985	12	419	554	109.4	5.32
<i>Ca</i> Cnh1-G23	800	11	419	370	88.8	5.91
<i>Zr</i> ZrSod2-22	806	11	418	377	90.2	6.14
<i>Sp</i> sod2	468	11	414	43	52.2	6.99

* TMS, transmembrane segments.

Table 2. Sequence identity of yeast Na⁺/H⁺ antiporters

Antiporter	Identity (%) of <u>entire sequence</u> /TMS + loops/C-terminus			
	<i>Sc</i> Nha1	<i>Ca</i> Cnh1-G23	<i>Zr</i> ZrSod2-22	<i>Sp</i> sod2
<i>Sc</i> Nha1	100	53.9/69.3/32.4	57.7/76.7/35.1	41.1/43.5/18.6
<i>Ca</i> Cnh1-G23		100	50.2/65.6/27.0	43.8/44.8/34.9
<i>Zr</i> ZrSod2-22			100	42.1/44.0/18.8

Fig. 1. Growth of *Sacch. cerevisiae* B31 cells harbouring different yeast Na^+/H^+ antiporters in the presence of alkali metal cations. Serial 10-fold dilutions of saturated cultures were spotted onto YNB medium supplemented with NaCl, LiCl, KCl and RbCl as indicated, and growth was followed for 5 days.

Table 3. Dependence of cell salt tolerance on external pH

Maximum sodium, lithium and potassium concentrations in YNB plates at different pH values tolerated by *Sacch. cerevisiae* B31 cells expressing empty vector (control), *Sacch. cerevisiae*, *Z. rouxii* or *Schiz. pombe* Na^+/H^+ antiporters.

Plasmid in B31 (<i>ena1-4Δ nha1Δ</i>)	pH...	KCl (mM)			NaCl (mM)			LiCl (mM)		
		3·5	5·5	7·0	3·5	5·5	7·0	3·5	5·5	7·0
YEp352		800	800	300	300	300	50	25	15	< 5
pNHA1-985		1800	1600	800	1200	800	150	30	20	5
pZrSod2-22		800	800	300	2200	1200	150	200	100	15
pSpSod2		800	800	300	1500	800	100	50	20	5

Dependence of yeast Na^+/H^+ antiporter activity on external pH

The cation-transport activity of Na^+/H^+ antiporters is governed by the electrochemical gradient of protons across the plasma membrane (Niu *et al.*, 1995; Rodríguez-Navarro *et al.*, 1994), and thus it depends on extracellular pH. The dependence of salt tolerance on external pH was tested in drop tests on media buffered to pH 3·5, 5·5 or 7·0, and supplemented with NaCl, LiCl or KCl. Table 3 shows that, in accordance with the nature of the substrate/ H^+ antiport mechanism, the tolerance of B31 cells harbouring different yeast antiporters decreased with increasing external pH. The external pH did not influence the substrate specificity; sod2p and ZrSod2-22p had no effect on cell potassium tolerance at all pH values tested (Table 3). The *Schiz. pombe* sod2 antiporter conferred higher sodium and lithium tolerance to cells at acid pH_{out} 3·5 compared to the presence of Nha1p but at external pH 5·5 and 7·0 cells with sod2p tolerated the same (or even lower) amounts of sodium and lithium than cells containing the Nha1p. On the other hand, the *Z. rouxii* ZrSod2-22 antiporter greatly improved the sodium and lithium tolerance of B31 cells under all conditions tested. At low pH_{out} , B31 cells containing ZrSod2-22p tolerated an eightfold higher concentration of NaCl and LiCl in comparison with control cells and an almost two (six) times higher concentration of NaCl (LiCl) compared to

cells expressing the Nha1p (Table 3). Also at pH_{out} 5·5, the ZrSod2-22p provided cells with the highest tolerance to sodium and lithium. Interestingly, even at pH_{out} 7·0, where the activity of *Sacch. cerevisiae* and *Schiz. pombe* antiporters was negligible, the ZrSod2-22 antiporter significantly improved the lithium tolerance of cells. The influence of the *C. albicans* Cnh1 antiporter on the salt tolerance of B31 cells was studied in a similar experiment earlier (Kinclová *et al.*, 2001b). Under the same conditions as described here, the Cnh1p had a lower activity towards sodium compared to the other three antiporters (compare Table 3 and Kinclová *et al.*, 2001b).

Although the sequence similarity of the transmembrane parts and connecting loops of yeast plasma membrane Na^+/H^+ antiporters is very high and they differ mainly in their C-termini, our results show that the substrate specificity of sod2p and ZrSod2-22p is narrower in comparison with the two other antiporters of the family (Nha1p and Cnh1p). However, the difference in substrate specificity is not correlated with the length of C-termini as we have shown that the long C-terminus of Nha1p is apparently not indispensable for the antiporter's substrate specificity (Kinclová *et al.*, 2001c). Considering only sodium and lithium tolerance, the *Z. rouxii* ZrSod2-22 antiporter was most effective. The activity of sod2p from *Schiz. pombe* appeared to be highly dependent on the external pH value, as the Na^+ and Li^+ tolerance of cells containing this antiporter

Fig. 2. Loss of K^+ (a), Na^+ (b) and Li^+ (c) from *Sacch. cerevisiae* B31 cells at pH_{out} 5.5. Cells transformed with YEp352 as a control ($\blacklozenge$), and with pNHA1-985 ($\blacksquare$), pZrSOD2-22 ($\bullet$) and pSpsod2 ($\blacktriangle$), were preincubated with 100 mM NaCl (or 50 mM LiCl) at pH 7.0 for 60 min, transferred to incubation buffer at pH 5.5, and the internal cation contents were determined. For the loss of K^+ no preloading was necessary.

diminished with increasing pH_{out} more than those of cells harbouring the other antiporters (Table 3).

Efflux of Na^+ , Li^+ and K^+ mediated by yeast alkali-metal-cation/ H^+ antiporters

To verify if the different cell tolerances to alkali metal cations observed in drop tests really resulted from the transport activity of Na^+/H^+ antiporters, the efflux of alkali metal cations from B31 cells harbouring either the heterologous *sod2* or *ZrSod2-22* antiporters or the *Sacch. cerevisiae* *Nha1p* was measured. In the control cells, lacking any antiporter, the internal concentration of all cations tested did not change significantly during the experiment; thus the observed effluxes of cations from cells were mediated exclusively by Na^+/H^+ antiporters (Fig. 2). In agreement with the results obtained in drop tests, no loss of potassium was observed in cells containing either *ZrSod2-22p* or *sod2p*. Only *Nha1p* mediated potassium efflux from cells (Fig. 2a).

As shown in Fig. 2(b, c), the high tolerance to sodium and lithium provided by the *Z. rouxii* antiporter was the result of a high transport capacity of the system. In the case of lithium, the *ZrSod2-22* antiporter was much more active than the *Nha1p* (Fig. 2c). However, the initial rates of sodium efflux were similar for *ZrSod2-22p* and *Nha1p* at the beginning of the experiment but the intracellular concentration of sodium decreased more slowly in cells containing *ZrSod2-22p* at the end of the experiment (Fig. 2b). Surprisingly, at pH 5.5, only a very low efflux of lithium was observed from cells expressing *sod2p* (Fig. 2c) despite the fact that at pH 5.5 cells expressing *sod2p* demonstrated the same level of lithium tolerance as the cells with *Nha1p*.

Lithium uptake

The high lithium efflux activity of *ZrSod2-22* antiporter was also confirmed when lithium uptake was measured. No difference in uptake of Li^+ among cells containing empty vector and cells with *Nha1p* or the *sod2p* was observed (data not shown), but cells harbouring the *ZrSod2-22* antiporter contained considerably lower

Fig. 3. Li^+ uptake in *Sacch. cerevisiae* B31 cells at pH 5.5. Cells transformed with YEp352 as a control ($\blacklozenge$), or with pZrSOD2-22 ($\bullet$), were incubated in buffer at pH 5.5; LiCl was added (final concentration 50 mM) at time zero and its intracellular content was followed for 60 min.

amounts of Li^+ than control cells after only 10 min incubation in the presence of 50 mM LiCl, and this difference increased during the experiment (Fig. 3). The apparently lower rate of lithium uptake was more likely due to the simultaneous *ZrSod2-22* antiporter activity.

Higher activity of Na^+/H^+ antiporters maintains a higher K^+/Na^+ ratio in the cells

It was shown that the level of intracellular K^+/Na^+ ratio must be higher than 1:2 for normal growth of cells in the presence of Na^+ (Camacho *et al.*, 1981) and that the Na^+/H^+ antiporter activity contributes in *Sacch. cerevisiae* to maintaining the appropriate ratio (Bañuelos *et al.*, 1998). To study the influence of external Na^+ concentration on the Na^+/K^+ ratio in B31 cells harbouring yeast Na^+/H^+ antiporters *ZrSod2-22p* and *sod2p* (which do not transport potassium), and *Nha1p* and *Cnh1p* (mediating the efflux of both K^+ and Na^+), cells were grown in liquid media without or with NaCl (100, 300 and 800 mM). The internal concentration of Na^+ and K^+ was estimated when the OD_{600} of cultures reached values around 0.2. In media without NaCl (Fig. 4a), cells grew with similar rates (generation time of

Fig. 4. NaCl tolerance of *Sacch. cerevisiae* B31 cells expressing different yeast Na⁺/H⁺ antiporters. (a, b) Growth of cells (OD₆₀₀) in liquid YNB media without (a) or with (b) 800 mM NaCl as indicated was followed for 16 h. At OD₆₀₀ ≈ 0.2 (indicated by the broken line in each plot), 15 ml culture was harvested and the intracellular concentrations of Na⁺ (c) and K⁺ (d) were determined. B31 cells were transformed with YE352 (◆, black bars), pNHA1-985 (■, empty bars), pZrSOD2-22 (●, square-hatched bars), pSpsod2 (▲, diagonally striped bars) and pCNH1-G23 (✕, stippled bars). ND, Not determined, as the cell culture did not reach OD₆₀₀ ≈ 0.2.

about 2 h); only the generation time of cells containing the *C. albicans* Cnh1 antiporter was longer (3 h). In the absence of NaCl, the intracellular concentration of K⁺ was almost the same in all transformants [about 600 nmol (mg dry wt)⁻¹], and a negligible amount of Na⁺ was found (Fig. 4c, d). With increasing Na⁺ concentration in the medium, Na⁺ replaced part of the internal K⁺ but the total intracellular concentration of Na⁺ plus K⁺ was similar to the internal concentration of K⁺ in the absence of NaCl. Low concentration of sodium (100 mM NaCl) had no effect on growth (including the very salt-sensitive control cells without any antiporter). With increasing NaCl in the medium (300 mM NaCl) small differences in growth rate appeared among cells containing different antiporters, and this difference even increased in media with 800 mM NaCl (Fig. 4b), where only the growth of cells containing the *Z. rouxii* antiporter ZrSod2-22 (the most sodium-tolerant cells in drop test experiments) was not very much affected (the generation time of cells harbouring ZrSod2-22p increased by only about 0.75 h in 800 mM NaCl). In all media with NaCl, cells expressing the ZrSod2-22 antiporter had the lowest intracellular concentration of sodium (Fig. 4c, square-hatched bars). The intracellular concentration of cations was not deter-

mined in control cells cultivated in the presence of 800 mM NaCl because the cells did not grow sufficiently (Fig. 4b).

In drop-test experiments, at pH 5.5, where cell growth was followed for several days, cells containing either Nha1p or sod2p tolerated the same concentration of NaCl (800 mM, Table 3). In liquid media (the pH of standard non-buffered YNB media was about 5.5 at the beginning of cultivation, growth observed only for 16 h), we could not see differences in growth rates among cells expressing Nha1p and sod2p growing in 100 or 300 mM NaCl (data not shown); however in 800 mM NaCl media, cells with Nha1p grew better than cells with sod2p (generation time 4.5 h vs 6 h, respectively) (Fig. 4b). In all cases, Nha1p maintained an intracellular concentration of sodium about 50% that of cells with sod2p (Fig. 4c, empty and diagonally striped bars, respectively). These results corresponded to the observation that sodium efflux mediated by sod2p at pH 5.5 was much lower than from cells with the Nha1p (Fig. 2b). B31 cells containing Cnh1p were less tolerant of external sodium than cells with the Nha1 antiporter, both on plates (Kinclová *et al.*, 2001b) and in liquid media with 800 mM NaCl (Fig. 4b). In agreement with

this, a higher internal sodium concentration was found in Cnh1p-expressing cells grown with 800 mM NaCl than in Nha1p-expressing cells (Fig. 4c, stippled and empty bars, respectively).

The high efflux activity of ZrSod2-22p for sodium was reflected also in the highest concentration of potassium maintained in cells (Fig. 4d). The cells with Cnh1p and sod2p had almost the same internal amounts of sodium and potassium, although these two antiporters differ in substrate specificity. This result suggested that Cnh1p probably discriminated between Na⁺ and K⁺ and it transported sodium with higher affinity.

From all these results one can conclude that the functional expression of all four yeast Na⁺/H⁺ antiporters in *Sacch. cerevisiae* influenced the intracellular concentration of sodium in cells (and thus the intracellular K⁺/Na⁺ ratio), which in turn affected Na⁺ tolerance of cells.

DISCUSSION

Yeast Na⁺/H⁺ antiporters belong to a broad family of secondary active-transport systems that possess 12 transmembrane hydrophobic segments with their N- and C-termini most probably facing the cytosol (Saier *et al.*, 1999). The protein primary structures of yeast Na⁺/H⁺ antiporters show sequence similarity with sodium-specific antiporters from both prokaryotes and higher eukaryotes. The fact that such a transport system is conserved in evolution from bacteria to humans confirms the general need for cells to eliminate toxic sodium efficiently. Besides their detoxicating function, some of the Na⁺/H⁺ antiporters are believed to be involved in the regulation of intracellular pH (Jia *et al.*, 1992). The family of yeast Na⁺/H⁺ antiporters, so far identified in four different yeast species, shares a high level of primary structure identity but its members have different transport properties. Tests of cell salt tolerance showed that the sod2p and ZrSod2-22p substrate specificity is, in contrast to Nha1p and Cnh1p, restricted to Na⁺ and Li⁺. Expression of either of these two antiporters in *Sacch. cerevisiae* had no effect on the potassium and rubidium tolerance of cells, and measurements of K⁺ efflux confirmed that neither sod2p nor ZrSod2-22p mediated K⁺ transport. On the other hand, sod2p and ZrSod2-22p improved the tolerance of B31 cells to sodium and lithium. *Schiz. pombe* is a halosensitive yeast, and sodium export from wild-type cells is very slow and highly affected by pH_{out} (Jia *et al.*, 1992), which corresponds to our results with *Schiz. pombe* sod2p expressed in *Sacch. cerevisiae*, as follows. (1) Compared to the other antiporters, the function of sod2p in the tolerance of cells to sodium and lithium was most dependent on external pH (Table 3). (2) The cation-loss measurements showed only a slow efflux of sodium from cells with sod2p (Fig. 2b). (3) The low sodium export caused a higher accumulation of Na⁺ in cells harbouring sod2p and consequently a slow growth rate in the liquid media with NaCl (Fig. 4). Drop tests and efflux experiments indicated that lithium is a poorer

substrate than sodium for sod2p, especially at higher pH_{out}. sod2p is the shortest member of the Na⁺/H⁺ antiporter family; its C-terminus has only 43 amino acid residues (Table 1). The absence of the long C-terminus in sod2p may be the reason for the low transport capacity for lithium and sodium, as we have shown previously that truncation of the *Sacch. cerevisiae* Nha1p C-terminus to the size corresponding to *Schiz. pombe* sod2p (41 amino acid residues after the last transmembrane domain) resulted in a significant decrease of the protein's transport activity for sodium and lithium (Kinclová *et al.*, 2001c).

The Na⁺/H⁺ antiporter ZrSod2-22, isolated from the osmotolerant *Z. rouxii*, seems to be the most effective sodium and lithium antiporter system of the yeast family. It improved considerably the tolerance of *Sacch. cerevisiae* B31 cells to sodium and especially to lithium at all pH values tested. Sodium and lithium efflux measurements and NaCl growth experiments confirmed its high export capacity. We have already shown that the ZrSod2-22 antiporter could substitute very effectively for the low Na⁺-ATPase activity as its overexpression improved the salt tolerance of some *Sacch. cerevisiae* wild-type strains (Kinclová *et al.*, 2001a). Thus the ZrSod2-22 antiporter could be a good candidate for heterologous expression to improve the salt tolerance of plants.

To conclude, in spite of their high sequence homology, the family of yeast plasma membrane Na⁺/H⁺ antiporters can be divided, as regards substrate specificity and probably cell function, into two distinct subfamilies: (1) the subfamily with substrate specificity only for Na⁺ and Li⁺ (*Schiz. pombe*, *Z. rouxii* antiporters) and with a primary detoxication function in cells, and (2) the subfamily (*Sacch. cerevisiae*, *C. albicans* antiporters) mediating transport of all alkali metal cations that, besides elimination of toxic cations, probably have a role in other cell functions (regulation of intracellular K⁺ concentration, pH and cell volume). The distribution of yeast antiporters into subfamilies does not reflect the level of protein identity, as the closest by sequence comparison, *Sacch. cerevisiae* Nha1p and *Z. rouxii* ZrSod2-22p (Table 2), belong by their substrate specificity to different subfamilies.

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