FEBS Letters 580 (2006) 1971–1976

# *Yarrowia lipolytica* possesses two plasma membrane alkali metal cation/$H^+$ antiporters with different functions in cell physiology

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Received 12 January 2006; revised 22 February 2006; accepted 22 February 2006

Available online 3 March 2006

Edited by Julian Schroeder

**Abstract The family of Nha antiporters mediating the efflux of alkali metal cations in exchange for protons across the plasma membrane is conserved in all yeast species. *Yarrowia lipolytica* is a dimorphic yeast, phylogenetically very distant from the model yeast *Saccharomyces cerevisiae*. A search in its sequenced genome revealed two genes (designated as *YlNHA1* and *YlNHA2*) with homology to the *S. cerevisiae NHA1* gene, which encodes a plasma membrane alkali metal cation/$H^+$ antiporter. Upon heterologous expression of both *YlNHA* genes in *S. cerevisiae*, we showed that *Y. lipolytica* antiporters differ not only in length and sequence, but also in their affinity for individual substrates. While the *Yl*Nha1 protein mainly increased cell tolerance to potassium, *Yl*Nha2p displayed a remarkable transport capacity for sodium. Thus, *Y. lipolytica* is the first example of a yeast species with two plasma membrane alkali metal cation/$H^+$ antiporters differing in their putative functions in cell physiology; cell detoxification vs. the maintenance of stable intracellular pH, potassium content and cell volume.**


*Keywords:* Alkali metal cation/$H^+$ antiporter; Salt tolerance; Cation efflux; Heterologous expression; *Saccharomyces cerevisiae*; *Yarrowia lipolytica*

## 1. Introduction

The maintenance of intracellular ion homeostasis is crucial to yeast cells [1–4]. The optimal cytoplasmic concentration of potassium, whose presence is necessary for many physiological functions, is much higher than the $K^+$ concentration in many natural environments. On the other hand, sodium (a naturally abundant cation) is toxic to living cells and, when yeasts are in environments with high $Na^+$ levels, they expend a lot of energy maintaining a low sodium concentration in the cytoplasm. In general, yeast cells possess a series of transporters with varying transport mechanisms that help to maintain optimal intracellular concentrations of alkali metal cations by sequestrating surplus cations in organelles or ejecting them from the cell. Two plasma-membrane transport systems are involved in the halotolerance of the most-studied model *Saccharomyces cerevisiae* cells. P-type Ena $Na^+$ ATP-ases mainly export alkali metal cations when the cells are in media with higher pH levels [5], and a $Na^+/H^+$ antiporter (Nha1p) takes part in the alkali-metal-cation efflux at lower external pH levels [5,6]. *Sc*Nha1p is a typical membrane protein of 985 amino acids, composed of a short hydrophilic N-terminus, probably 12 transmembrane domains and a long hydrophilic C-terminus [7]. The substrate specificity of *Sc*Nha1p is broad; it is able to recognize $Na^+$, $Li^+$, $K^+$ and also $Rb^+$ as substrates [5,7].

At the time of writing this paper, plasma membrane alkali metal cation/$H^+$ antiporters from several yeast species have been functionally characterized. These proteins can be divided into two subfamilies, according to their substrate specificity [8]. *Schizosaccharomyces pombe* and *Zygosaccharomyces rouxii* antiporters are only able to recognize sodium or lithium as their substrates [8,9] and thus are probably involved in the detoxification of cells from toxic cations. Members of the second subfamily (antiporters from *S. cerevisiae*, *Pichia sorbitophila*, *Candida albicans* and *Debaryomyces hansenii*) have broad substrate specificity; these proteins do not only export $Na^+$ and $Li^+$, but also $K^+$ and $Rb^+$ [5,7,10–12]. But why should cells possess an efficient system for extruding potassium, when the intracellular level of $K^+$ is usually much higher than the level of $K^+$ in the environment? Furthermore, a stable $K^+$ concentration inside cells is necessary for many physiological functions. A more complex function has been proposed for antiporters with the ability to transport potassium. They may be involved in the regulation of a stable cytoplasmic $K^+$ concentration and cell volume or in the event of a sudden alkalinization of the cytoplasm, they might use the cytoplasmic $K^+$ pool to maintain a stable pH in the cytoplasm, as $H^+$ is the second substrate of these proteins [5,7].

*Yarrowia lipolytica* is a dimorphic obligate aerobe, phylogenetically very distant from *S. cerevisiae* or *S. pombe*, two widely studied yeast species. It can typically be found in lipid-rich food, such as dairy products [13]. *Y. lipolytica* secretes proteases, lipases, RNases, phosphatases or esterases under diverse cultivation conditions [14] and thus grows on unusual carbon sources like fatty acids or alkanes. It is used in the production of organic acids, in the food industry and its secretion ability led to the development of an efficient system for the secretion of heterologous proteins [15]. The importance of this unconventional yeast species is documented by the fact that *Y. lipolytica* was included in the Génolevures sequencing program [16], which made the complete genome sequence available [17]. *Y. lipolytica* possesses a larger genome (20 Mbp vs. 10 Mbp) with a lower overall gene density than *S. cerevisiae* [17].

In spite of *Y. lipolytica*'s industrial and scientific potential, very little is known about the mechanisms of salt tolerance or ion transport in this yeast species. Some interesting data

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0014-5793/$32.00 
doi:10.1016/j.febslet.2006.02.064

concerning its sodium-coupled phosphate transport are available [18], but individual plasma membrane transporters have not yet been characterized. Though ion transporters are over-represented in the *Y. lipolytica* genome compared to other yeast species [16], so far only the *YlPMR1* gene has been cloned and the function of its protein product, a P-type secretory pathway $Ca^{2+}$ ATPase, characterized in more detail [19].

In this work, we isolated two *Y. lipolytica* genes homologous to *S. cerevisiae NHA1*. Both *YlNHA* genes were heterologously expressed in the *S. cerevisiae* halosensitive BW31 strain lacking its own systems for the efflux of alkali metal cations (*nha1*Δ *ena1-4*Δ). The *Yl*Nha2 protein exported sodium from BW31 cells very efficiently; by contrast, the *Yl*Nha1 antiporter provided BW31 with a high tolerance to potassium. Based on our results, we propose that the *Y. lipolytica* genome harbours two *NHA* genes encoding for proteins with different putative functions in cell physiology.

## 2. Materials and methods

### 2.1. Yeast strains, media and growth conditions

*Y. lipolytica* W29 (CBS7504) and *S. cerevisiae* X2180-1B wild-type strains were used in this work. For the heterologous expression of *YlNHA* genes, the halosensitive *S. cerevisiae* BW31 strain [20] was used. Cells were grown aerobically in standard YPD media or YNB-$NH_4$ + 2% glucose media at 30 °C. Salts were added to the YNB-$NH_4$ media prior to and auxotrophic supplements after autoclaving. To adjust the pH of the media, tartaric acid was used for pH 3.5. For pH 5.5, the medium was supplemented with 20 mM MES, for pH 7.0 20 mM MOPS was used; the pH was then adjusted to the required value with NaOH.

### 2.2. DNA manipulations

DNA manipulations were performed according to standard protocols [21]. Platinum *Pfx* DNA polymerase (Invitrogen) with intrinsic proofreading activity was used for the PCR amplification of *YlNHA* genes. All constructs were sequenced using an ABI PRISM 3100 DNA sequencer and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

### 2.3. Plasmid constructions

The genomic DNA of *Y. lipolytica* was prepared as previously described [22]. The oligonucleotides used in this work are listed in Table 1. Primers Yl1fw and Yl1rev or Yl2fw and Yl2rev were used for the PCR amplification of the *YlNHA1* or *YlNHA2* genes, respectively. The PCR-amplified *YlNHA* genes were inserted into the YEp352 vector [23] behind the *S. cerevisiae NHA1* promoter using homologous recombination. Using the Yl1rev and Yl2rev primers, sequences corresponding to *Xma*I-STOP were introduced to the 3′ ends of the *YlNHA* genes. The *Xma*I and *Sac*I (upstream of the promoter sequence in YEp352) sites were used to insert *YlNHA* genes into pGRU1 (Daignan-Fornier, Accession No. AJ249649) in frame with the GFP coding sequence at their 3′ ends. As controls, empty YEp352 and pNHA1-985 containing the *ScNHA1* sequence [7] were used.

### 2.4. Fluorescence microscopy

Cells expressing *YlNHA* genes tagged with the GFP sequence were viewed with an Olympus AX70 microscope using a U-MWB cube with a 450–480 nm excitation filter and a 515 nm barrier filter. The micrographs were recorded with a DP70 digital camera using the program DP Controller.

### 2.5. Salt tolerance determination

Drop test experiments were performed on solid YPD or YNB-$NH_4$ media supplemented with NaCl, KCl, LiCl or RbCl as previously described [7].

### 2.6. Cation loss experiments and cation content measurements

For $K^+$ loss experiments, cells in the early exponential phase of growth ($OD_{600}$ approx. 0.2) were harvested, washed with deionized water and resuspended in a pH 4.5 incubation buffer (10 mM Tris, 0.1 mM $MgCl_2$, 2% glucose, adjusted to pH 4.5 with citric acid and $Ca(OH)_2$), supplemented with 20 mM $RbCl_2$ to prevent $K^+$ reuptake. Aliquots of cells were withdrawn over a period of 120 min. To measure $Na^+$ loss, cells from the early exponential phase of growth were preloaded with sodium cations in YNB-$NH_4$ media (pH 7.0, adjusted with $NH_4OH$) containing 100 or 600 mM NaCl for 1 h. After this preincubation, cells were harvested, washed and resuspended in the pH 4.5 incubation buffer, supplemented with 20 mM KCl to prevent $Na^+$ reuptake. Aliquots of cells were withdrawn over a period of 40 min. For $Na^+$ content measurements, cells from the exponential phase of growth were NaCl preloaded as described above, washed and resuspended in 10 mM Tris, 0.1 mM $MgCl_2$ at pH 7.0 (adjusted with citric acid and $Ca(OH)_2$). Three aliquots of cells were withdrawn immediately. The cation content in the withdrawn aliquots of cell suspensions was determined using atomic absorption spectrophotometry [7]. Experiments were repeated at least three times and representative results are shown; the standard deviations of the results were less than 10% of the corresponding mean.

## 3. Results and discussion

### 3.1. Y. lipolytica *salt tolerance*

To compare the salt tolerance of the *Y. lipolytica* and *S. cerevisiae* wild-type strains, drop test experiments were performed on YPD plates with increasing concentrations of NaCl (0–2.5 M) or KCl (0–2.5 M). *Yarrowia* cells were able to grow in the presence of 2 M NaCl, while *Saccharomyces* tolerated only 1.75 M sodium in the medium. The tolerance to potassium was also higher with *Y. lipolytica*; it tolerated 2.5 M KCl whereas the maximal potassium concentration that allowed *S. cerevisiae* to grow was 2 M. This higher salt tolerance suggested that *Y. lipolytica* could possess very efficient efflux systems for maintaining the appropriate intracellular concentration of alkali metal cations.

### 3.2. Sequence analysis of Yl*Nha proteins*

Two *Y. lipolytica* genes with homology to *S. cerevisiae NHA1* were found in the Génolevures database (genes YALI0C00759g and YALI0E02156g). We have designated the genes *YlNHA1* and *YlNHA2*, respectively. Table 2 shows the length of the proteins and their sections (according to a model proposed for *Sc*Nha1p [7]) encoded by the *YlNHA* genes and their *S. cerevisiae* and *S. pombe* homologues. *Yl*Nha2p is significantly shorter than *Yl*Nha1p; the difference in length of the proteins is especially remarkable in the length of their C-termini. Similarly to *Yl*Nha2p, the *S. pombe* sod2 antiporter has a very short C-terminus, and in contrast to *Sc*Nha1p, *Sp*sod2p exports only toxic $Na^+$ or $Li^+$ cations [8]. *Yl*Nha2p is the only known yeast alkali metal cation/$H^+$ antiporter whose N-terminus is only 9 amino acids long; *Yl*Nha1p and the antiporters from other yeast species (whose sequences can be found in published databases) possess N-termini of 11 to 12 amino acids.

Multiple sequence alignment of *Sc*Nha1p, *Sp*sod2p and *Y. lipolytica* Nha antiporters is shown in Supplementary material 1. Table 3 shows that the identity of *Y. lipolytica* putative antiporters with each other is low (33.9%). This finding is not significantly affected by the difference in the length of the C-terminal sections of the proteins, as the identity of the protein sections corresponding to the transmembrane domains and

Table 1
Oligonucleotides used in this work

| Primer | Sequence (5′–3′) |
|---|---|
| Yl1fw[a] | *TTTTTTGTACATTATAAAAAAAAATCCTGAACTTAGCTAGATATT*ATGGGTTGGGACCAGCTGGGTATC |
| Yl1rev[a,b,c] | *GTAAAACGACGGCCAGTGCCAAGCTTGCATGC*TAA**TCCCGGG**GACCTCCTCTCACCGTATCGCCCCAC |
| Yl2fw[a] | *TTTTTTGTACATTATAAAAAAAAATCCTGAACTTAGCTAGATATT*ATGCCAGTTCTCAACATCTCCAAC |
| Yl2rev[a,b,c] | *CGTTGTAAAACGACGGCCAGTGCCAAGCTTGCATGC*TAA**TCCCGGG**GACCAACTTGATCTCCTCTTCGGTCAG |

[a]Sequences homologous to *ScNHA1* promoter or YEp352 are in italics.
[b]*Xma*I site is in bold.
[c]STOP codon is underlined.

Table 2
Structure of yeast alkali metal cation/$H^+$ antiporters

| Yeast | Antiporter | Number of amino acid residues | | | |
|---|---|---|---|---|---|
| | | Total | N-terminus | TMS + loops | C-terminus |
| *S. cerevisiae* | *Sc*Nha1p | 985 | 12 | 419 | 554 |
| *S. pombe* | *Sp*sod2p | 468 | 11 | 414 | 43 |
| *Y. lipolytica* | *Yl*Nha1p | 853 | 11 | 418 | 424 |
| *Y. lipolytica* | *Yl*Nha2p | 515 | 9 | 411 | 96 |

Table 3
Sequence identity of yeast alkali metal cation/$H^+$ antiporters

| Antiporter | Percent identity of **entire protein sequences**/*transmembrane domains + loops* | | | |
|---|---|---|---|---|
| | *Sc*Nha1p | *Sp*sod2p | *Yl*Nha1p | *Yl*Nha2p |
| *Yl*Nha1p | **37.2**/*59.3* | **38.7**/*40.3* | 100 | **33.9**/*39.2* |
| *Yl*Nha2p | **34.3**/*39.7* | **30.3**/*32.4* | **33.9**/*39.2* | 100 |

connecting loops is only 39.2%. Neither *Yl*Nha1p nor *Yl*Nha2p are much related to *Sp*sod2p. The percent identity of *Yl*Nha2p and *Sc*Nha1p is also low, but the core membrane section of *Yl*Nha1p is almost 60% identical to the transmembrane section of the *S. cerevisiae* protein. Nevertheless, *Yl*Nha1p is still less related to *Sc*Nha1p than the antiporters from *Z. rouxii*, *C. albicans* or *D. hansenii* [8,12].

### 3.3. Heterologous expression of YlNHA genes in S. cerevisiae BW31 cells

To gain some knowledge about the substrate specificity, transport properties and putative functions of the two different *Y. lipolytica* antiporters, *YlNHA* genes were inserted into the YEp352 vector behind the promoter of the *ScNHA1* gene and heterologously expressed in halosensitive *S. cerevisiae* BW31 cells. Their production in BW31 cells also enables the comparison of *Yl*Nha antiporters with other yeast $Na^+(K^+)/H^+$ antiporters that have already been characterized under the same experimental conditions [8,11,12].

### 3.4. Influence of YlNha antiporters on the salt tolerance of BW31 cells

The salt tolerance of BW31 cells expressing *YlNHA* genes was compared to cells containing the empty vector (negative control) or pNHA1-985 (positive control, cells producing *Sc*Nha1p). The presence of a functional plasma membrane antiporter can be detected by an increase in the salt tolerance of BW31 cells as the protein activity eliminates surplus cations from the cytosol. Fig. 1 shows that neither the overexpression of *Yl*Nha1p nor of *Yl*Nha2p was toxic to *S. cerevisiae*. Cells producing *Yl*Nha proteins grew the same as cells producing *Sc*Nha1p or containing the empty vector on the plate without

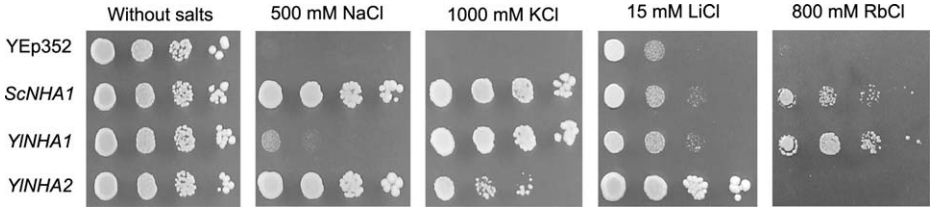


Fig. 1. Growth of BW31 cells producing *Yl*Nha proteins in the presence of different alkali metal cations. Tenfold serial dilutions of cell suspensions were spotted on YNB-$NH_4$ plates supplemented as indicated and growth was followed for 5 days. Cells containing the empty vector (YEp352) were used as negative, and cells expressing *ScNHA1* as positive control, respectively.

Table 4
Maximum salt concentrations allowing the growth of BW31 expressing *NHA* genes in YNB-$NH_4$ media with different pH levels

| Antiporter in BW31 | $pH_{out}$ | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 3.5 NaCl (mM) | 5.5 | 7.0 | 3.5 KCl (mM) | 5.5 | 7.0 | 3.5 LiCl (mM) | 5.5 | 7.0 |
| None (YEp352) | 300 | 200 | 50 | 600 | 800 | 300 | 15 | 10 | <5 |
| *Sc*Nha1p | 1000 | 500 | 100 | 1800 | 1800 | 1200 | 20 | 15 | 5 |
| *Yl*Nha1p | 400 | 300 | 50 | 1800 | 1800 | 1000 | 20 | 15 | <5 |
| *Yl*Nha2p | 1500 | 1500 | 250 | 1000 | 1000 | 300 | 50 | 50 | 10 |

added salts; i.e., in non-stressed conditions. As far as changes in cell salt tolerance are concerned, *Yl*Nha1p production mainly increased BW31 tolerance to potassium and rubidium, and only to a small extent to sodium or lithium. By contrast, *Yl*Nha2p provided BW31 cells with a high tolerance to sodium and lithium, while their tolerance to potassium was only weakly influenced (Fig. 1).

Table 4 shows how the pH level of the media influenced the ability of *Yl*Nha antiporters to increase BW31 salt tolerance. At an external pH of 7.0, cells grew in the presence of the lowest levels of cations in the media. This finding of pH dependence is consistent with antiporter energization by an electrochemical gradient of $H^+$ across the plasma membrane [24]. *Yl*Nha1p increased BW31 tolerance to potassium very effectively, similarly to *Sc*Nha1p (in both cases, the cells tolerated 1800 mM KCl in media at pH 3.5 and 5.5). We also observed a small positive effect of *Yl*Nha1p on BW31 tolerance to $Na^+$ and $Li^+$ at pH 3.5 and 5.5. *Yl*Nha2p was the most effective antiporter in providing BW31 with a high tolerance to sodium and lithium over a broad pH range, including pH 7.0. This result suggests that *Yl*Nha2p functionality was less seriously affected by high external pH than that of *Sc*Nha1p. Its positive effect on the potassium tolerance of BW31 could be observed at pH 3.5 and 5.5; nevertheless, *Yl*Nha2p provided BW31 cells with potassium tolerance much less effectively than *Yl*Nha1p or *Sc*Nha1p.

### 3.5. *Localization of* Yl*Nha antiporters in* S. cerevisiae *cells*

To verify that *YlNHA* genes encode plasma membrane proteins, and not the antiporters localized in the membranes of cell organelles, the 3′ ends of *YlNHA* genes were tagged with the GFP coding sequence. The localization of *Yl*Nha-GFP proteins was visualized using fluorescence microscopy. GFP tagging did not influence the activity of proteins, as the salt tolerance of strains producing tagged and non-tagged *Yl*Nha versions was the same (not shown). Fig. 2 shows that both *Yl*Nha proteins tagged with GFP were localized close to the cell surface and not in the membranes of the intracellular organelles of BW31 cells growing in YNB-$NH_4$ media without added salts. These results confirm *Yl*Nha proteins as plasma membrane antiporters.

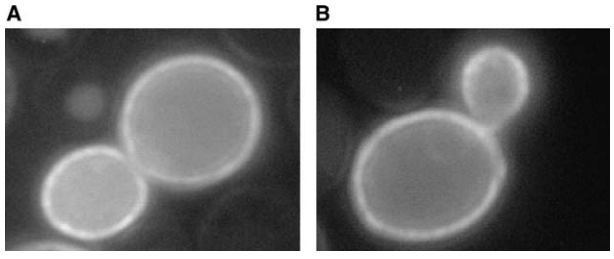


Fig. 2. Localization of GFP-tagged *Yl*Nha proteins in exponentially growing BW31 cells. Cells were grown overnight in YNB-$NH_4$ media without added salts and viewed with a fluorescence microscope. Cells producing *Yl*Nha1p (A) and *Yl*Nha2p (B).

### 3.6. $Na^+$ *efflux mediated by* Yarrowia *antiporters*

To verify that the increase in BW31 salt tolerance was really caused by active cation efflux, we measured the loss of alkali metal cations from BW31 cells producing *Yl*Nha proteins. For $Na^+$ efflux measurements, the intracellular sodium level must be increased by the incubation of cells in the presence of NaCl. Firstly, cells were sodium preloaded by incubation in media containing 100 mM NaCl at pH 7.0 for 60 min. This preloading was successfully used for measurements of the $Na^+$ efflux caused by several yeast $Na^+/H^+$ antiporters [7,8,11,12], and a small $Na^+$ efflux could also be measured with the *Yl*Nha1 protein after such a preloading (Fig. 3A). The observed small $Na^+$ loss from cells expressing *YlNHA1* is in agreement with a weak increase in BW31 tolerance to $Na^+$ mediated by *Yl*Nha1p in drop test experiments (Fig. 1). Nevertheless, the growth of BW31 cells expressing *YlNHA1* in media with NaCl is only slightly better than that of cells containing an empty vector. According to these results, it is unlikely that *Yl*Nha1p is efficiently used in the detoxification of surplus $Na^+$ in *Y. lipolytica* cells.

Preloading with 100 mM NaCl at pH 7.0 did not ensure a sufficiently high sodium level in cells producing *Yl*Nha2p. Whereas in control cells or *Yl*Nha1p-producing cells the intracellular concentration of $Na^+$ cations was approximately 90–130 nmol $Na^+$/mg dry weight, cells producing the *Yl*Nha2 antiporter contained less than 30 nmol $Na^+$/mg dry weight after preincubation with 100 mM NaCl at pH 7.0 for 60 min. To improve the experimental conditions for measuring the $Na^+$ efflux caused by *Yl*Nha2p, we compared the sodium content in cells after preloading with 100 or 600 mM NaCl at pH 7.0. For these experiments, after preloading and washing, cells were resuspended in pH 7.0 buffer to minimize $Na^+$ efflux during the withdrawal of cell aliquots. Cells producing the *Yl*Nha2 protein contained only $37.5 \pm 0.7$ nmol $Na^+$/mg dry weight after 1 h of incubation in the presence of 100 mM NaCl (at pH 7.0). This $Na^+$ level was significantly lower than that in control cells containing YEp352 preloaded with NaCl under the same conditions ($93.3 \pm 1.8$ nmol $Na^+$/mg dry weight). The sodium level in cells producing *Yl*Nha2p was significantly increased upon incubation in the presence of 600 mM NaCl at pH 7.0. After this treatment, cells contained $153.8 \pm 4.0$ nmol $Na^+$/mg dry weight. This was still remarkably lower than the

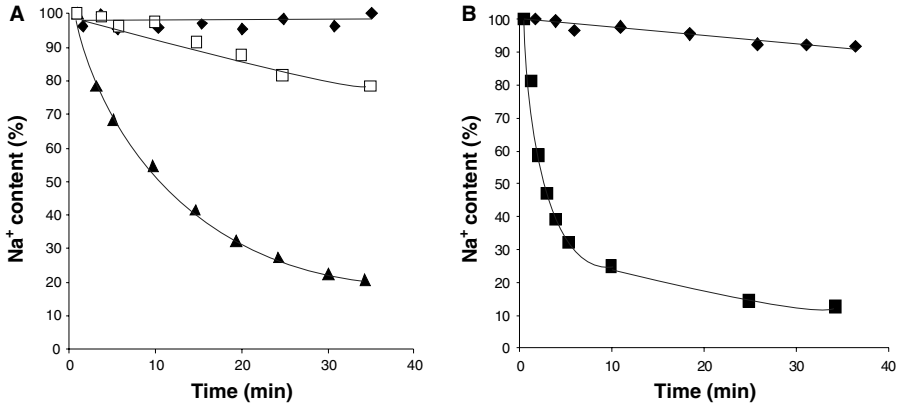


Fig. 3. Loss of $Na^+$ from BW31 cells containing empty vector (◆), producing *Sc*Nha1p (▲), *Yl*Nha1p (□) or *Yl*Nha2p (■). Cells from exponential growth phase were harvested, preloaded for 1 h with 100 mM NaCl (A) or 600 mM NaCl (B) at pH 7.0, washed and resuspended in pH 4.5 incubation buffer. Aliquots of cells were withdrawn over 40 min period, $Na^+$ content was determined according to [7]. Initial concentrations of sodium in cells (upon resuspending in pH 4.5 buffer): (A) 89.2 (◆), 91.4 (▲), 131.7 (□); (B) 220.1 (◆), 133.3 (■) nmol $Na^+$/mg dry weight.

level of sodium in cells transformed with YEp352 (238.5 ± 1.7 nmol $Na^+$/mg dry weight), but was sufficiently high to enable the measurement of $Na^+$ loss. After preloading with 600 mM NaCl, the initial level of $Na^+$ in cells producing *Yl*Nha2p after resuspension in pH 4.5 buffer (to measure $Na^+$ efflux) was slightly lower than the level of $Na^+$ determined when cells were resuspended in pH 7.0 buffer (130 vs. 150 nmol $Na^+$/mg dry weight, respectively). This result suggests that the $Na^+$ efflux caused by *Yl*Nha2p is lower at pH 7.0 than at pH 4.5. *Yl*Nha2p proved to be a very effective exporter of sodium in pH 4.5 buffer; the $Na^+$ content in cells dropped to almost 30% during the first 5 min of experiments (Fig. 3B).

The ability of *Yl*Nha2p to export $Na^+$ from *S. cerevisiae* is remarkable. The small level of $Na^+$ in cells producing *Yl*Nha2p upon preloading the cells with 100 mM NaCl at pH 7.0 suggests that this protein was at least partially functional under the conditions used for preloading. This activity was not observed for *Sc*Nha1p; upon preloading with 100 mM NaCl, the level of sodium in BW31 expressing *ScNHA1* is not significantly different from the sodium level in cells containing the empty vector (approx. 90 nmol $Na^+$/mg dry weight, cf. Fig. 3). In agreement with this finding, *Yl*Nha2p was the most successful among the antiporters tested in providing BW31 cells with tolerance to NaCl at all pH levels, including pH 7.0 (Table 4). Sodium efflux measurements confirmed that the high tolerance to NaCl is provided by the high transport activity of *Yl*Nha2p (Figs. 1 and 3B).

### 3.7. $K^+$ loss from cells producing Yl*Nha proteins*

For potassium loss measurements, no preloading with KCl is necessary as cells naturally contain a sufficiently high potassium level in the cytoplasm. *Yl*Nha production did not substantially affect the potassium content of BW31 when compared to cells containing an empty vector. Cells transformed with YEp352 contained 693.6 ± 26.1 nmol $K^+$/mg dry weight at the beginning of the experiment (the value corresponding to 100% in Fig. 4); the differences in $K^+$ content between BW31 cells producing *Yl*Nha proteins and cells containing YEp352 never exceeded 32 nmol $K^+$/mg dry weight, i.e., were always lower than 5%.

As Fig. 4 shows, *YlNHA1* expression resulted in potassium loss from BW31 cells although the level of exported potassium

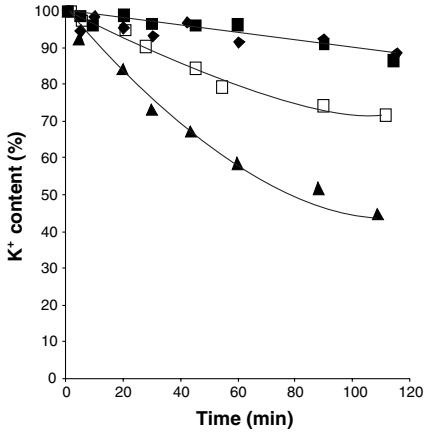


Fig. 4. Loss of $K^+$ from BW31 cells containing empty vector (◆), producing *Sc*Nha1p (▲), *Yl*Nha1p (□) or *Yl*Nha2p (■). Cells from exponential growth phase were harvested, washed and resuspended in the incubation buffer of pH 4.5. Aliquots of cells were withdrawn over 120 min period, $K^+$ content was determined according to [7].

(in 120 min) was lower than from cells producing *Sc*Nha1p. The *Yl*Nha1 antiporter mediated an efflux of approximately 20% of the internal $K^+$ in 1 h, whereas cells producing *Sc*Nha1p lost almost half of their potassium content. Although the observed potassium efflux from cells producing *Yl*Nha1p was significantly lower than the efflux caused by *Sc*Nha1p, the $K^+$ efflux mediated by *Yl*Nha1p was sufficient to enable the long term growth of cells in the presence of high KCl concentrations, similarly to *Sc*Nha1p (Fig. 1). Such a discrepancy between tolerance to $K^+$ and the effectiveness in potassium extrusion has already been observed in several mutant versions of the *Z. rouxii* antiporter with the ability to export potassium [20].

*Yl*Nha2p production did not cause significant $K^+$ efflux from BW31 cells in any experiment. This result, together with the high capacity of *Yl*Nha2p to export $Na^+$ from BW31 cells suggest that *Yl*Nha2p might mainly serve for the detoxification of *Y. lipolytica* cells from toxic $Na^+$ ($Li^+$) cations, whereas the *Yl*Nha1p's function might consist of the maintenance of optimal intracellular potassium concentration.

## 4. Conclusions

In this work, two genes of *Y. lipolytica* encoding alkali metal cation/$H^+$ antiporters were isolated and their products characterized upon heterologous expression in the model yeast *S. cerevisiae*. *Y. lipolytica* antiporters are, together with the *S. pombe* antiporter sod2, phylogenetically very distant from other characterized yeast alkali metal cation/$H^+$ antiporters.

As mentioned in Section 1, yeast plasma membrane $Na^+/H^+$ antiporters can be divided into two subfamilies based on their substrate specificity and putative functions in the maintenance of $K^+$ homeostasis, regulation of cell volume and intracellular pH and/or in the detoxification of surplus $Na^+$ ($Li^+$) [8]. According to our results, *Y. lipolytica* is the first example of a yeast species possessing two different plasma membrane alkali metal cation/$H^+$ antiporters that significantly differ in their length, sequence, and probably play different roles in the physiology of *Y. lipolytica* cells. While *Yl*Nha2p is a very efficient system for the extrusion of toxic sodium cations and might be involved in the sodium tolerance of *Y. lipolytica*, *Yl*Nha1p could take part in the maintenance of *Y. lipolytica* cell homeostasis thanks to its ability to export $K^+$ from cells and thus regulate the potassium content, intracellular pH and cell volume. Both *Yl*Nha proteins might play an important role in the high salt tolerance of *Y. lipolytica* to alkali metal cations.

*Acknowledgments:* We thank S. Casaregola and B. Janderova for providing wild-type yeast strains. The help of O. Zimmermannova and V. Korunova with cation loss measurements is highly appreciated. This study was supported by Grants GA CR 204/03/H066 and 206/05/0035, AV0Z 50110509 and MSMT LC 531.

## Appendix A. Supplementary data

Multiple sequence alignment of *Sc*Nha1p, *Sp*sod2p and *Y. lipolytica* Nha antiporters. Sequence analysis was performed using CLUSTAL W software (www.ebi.ac.uk/clustalw). (*) residues identical in all sequences, (:) conserved substitutions, (·) semi-conserved substitutions. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.02.064.

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