Characterization of the *NHA1* gene encoding a Na^+/H^+-antiporter of the yeast *Saccharomyces cerevisiae*

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Abstract **The *NHA1* gene (2958 nt) encoding a putative Na^+/H^+ antiporter (986 aa) in *Saccharomyces cerevisiae* was cloned by selection based on increased NaCl tolerance. The putative protein is highly similar to sodium/proton antiporters from *Schizosaccharomyces pombe* (gene *sod2*), and *Zygosaccharomyces rouxii* (gene *Z-SOD2*). Overexpression of the *NHA1* gene results in higher and partially pH-dependent tolerance to sodium and lithium; its disruption leads to an increased sensitivity towards these ions.**

Key words: Sodium/proton antiporter; Sodium tolerance; Lithium tolerance; (*Saccharomyces cerevisiae*)

1. Introduction

As high intracellular concentration of Na^+ ions is toxic for most organisms, there exists a number of primary (ATPases) and secondary (antiporters and symporters) transport systems eliminating efficiently sodium ions from the cells. Recently, genes coding for sodium antiporters or ATPases in bacteria, animal cells, plants and fungi have been cloned and sequenced (e.g. [1,2]).

Two genes coding for yeast Na^+/H^+ antiporters have been isolated and characterized [3,4]. In the fission yeast *Schizosaccharomyces pombe*, the product of the *sod2* gene is essential for sodium export and its amplification is sufficient to confer sodium and lithium resistance in media at acid or neutral pH [3]. In the osmotolerant yeast *Zygosaccharomyces rouxii*, a similar gene *Z-SOD2* was isolated and sequenced. The expression of *Z. rouxii SOD2* is constitutive, i.e. independent of the presence of NaCl, on the other hand, gene disruption leads to a significant decrease in NaCl tolerance [4].

In the yeast *Saccharomyces cerevisiae*, a family of genes related with Na^+ export has been characterized. The gene *ENA1* was cloned based on its ability to increase the lithium tolerance in a lithium-sensitive strain [5]. The high similarity of the predicted Ena1 protein with P-ATPases suggested that the system was a cation pump, a Na^+-ATPase. The gene *ENA1* (allelic to *PMR2*, [6]) is the first unit of a tandem array of at least four (*ENA1* to *ENA4*) or five (*PMR2a* to *PMR2e*) genes, depending on the strain [6,7]. Whereas *ENA2*, *ENA3* and *ENA4* are expressed constitutively and at low level, the expression of the *ENA1* gene can be induced by Na^+, Li^+ or high pH values [7]. Under high-salt conditions the activation of *ENA1/PMR2* transcription is mediated by calcineurin which antagonizes the negative regulator, cAMP-dependent protein kinase [8].

As the Ena1 ATPase is active mainly at alkaline pH values, the existence of another efflux system, possibly a H^+/cation antiporter, operating at acidic pH values has been predicted for *S. cerevisiae* [9]. This hypothesis was partially confirmed by the observation that the disruption of all four *ENA* genes did not completely eliminate Na^+ and Li^+ effluxes [5]. Besides, the functional expression of *S. cerevisiae ENA1* in *S. pombe sod2*$^-$ mutants restores the Na^+ and Li^+ tolerances of this strain, suggesting that under certain conditions the two Na^+ efflux systems could replace each other [10].

We present here the cloning and characterization of the *S. cerevisiae* gene *NHA1* coding for a putative Na^+/H^+ antiporter, highly similar to those of *S. pombe* and *Z. rouxii*. Overexpression of *NHA1* increases sodium and lithium tolerance mainly at acidic and neutral pH values, and its disruption brings about a significant decrease in salt tolerance.

2. Materials and methods

2.1. Strains and media

Wild type haploid strains FL 100, S288C, and Σ1278b and their respective *ura3* mutants were used. For cloning and phenotype characterization HS100-3C strain (*MATa can1 gap1 lyp1 ura3Δ* [11]) was used. To verify the phenotypes of *NHA1* overexpression or disruption, the strain W303-1A (*MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100*) and its derivative G19 (*MATα leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 ena1Δ::HIS3::ena4Δ*, a gift from A. Rodriguez-Navarro) were used. *Escherichia coli* strain E350 (*leuB, trpC*) was used for selection and amplification of recombinant DNA.

Standard media for yeast (YPD, YNB glucose) and *E. coli* (LB) cultures were used. When necessary the yeast media were supplemented with NaCl or LiCl and the pH was adjusted with 20 mM Mes (5.5) or 20 mM Hepes (7.5) prior to autoclaving.

2.2. Genetic and molecular methods

Standard protocols for nucleic acid manipulations were used [12]. The transformation of yeast by electroporation in Bio-Rad equipment was carried out as described previously [13].

2.3. DNA sequencing

DNA sequencing was performed by the dideoxynucleotide termination method [14] using double stranded DNA and T^7 Sequencing Kit (Pharmacia LKB, Sweden). Both strands of the DNA were sequenced and the sequences were analyzed using the UWGCG programs [15] on a VAX11/750 computer.

2.4. Gene disruption

A 2371 bp long *Xba*I-*Bgl*II fragment from pCS1 (see Fig. 1A) was cloned into pUC19. Then the 1187 bp long *Bam*HI-*Bam*HI fragment was replaced by a 1166 bp long fragment containing *URA3* (see pCS1-15, Fig. 1B). The *Xba*I-*Eco*RI fragment (2234 bp) was used for an integrative transformation, and in the obtained clones the disruption of *NHA1* was verified by Southern blots and PCR amplification.

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3. Results and discussion

3.1. Isolation of NaCl-tolerant clones

The tolerance to high concentrations of NaCl (0.5 M) was tested in several *S. cerevisiae* wild type strains and their *ura3* derivatives. Strain HS100-3C (a derivative of Σ1278b) was chosen, since it was relatively most sensitive to NaCl (colonies observed after 11 days of incubation on the plates containing NaCl). The higher sensitivity of this strain to NaCl is most probably due to the fact that Σ1278b harbours only one copy of *ENA/PMR* gene [16]. The HS100-3C strain was transformed with a *S. cerevisiae* genomic DNA library constructed in the multicopy shuttle vector pFL1 with *URA3* marker gene [17], and two clones (CS1 and CS2, containing the plasmids named pCS1 and pCS2) growing on the minimal YNB medium supplemented with 0.5 M NaCl after 3 and 7 days, respectively, were obtained.

To confirm the phenotype of clones harbouring the plasmids pCS1 and pCS2, their tolerance to NaCl at different pH values was assessed. As a control, we used the same strain transformed with 'empty' pFL1. The results (Table 1A) show that the overexpression of pCS1 product brings about a greater sodium tolerance at a more acidic pH value (growth on 0.8 M NaCl at pH 5.5, and on 0.6 M at pH 7.5, respectively), whereas the multiplication of the product encoded by DNA in pCS2 enables cell growth in the presence of high NaCl concentration (0.8 M) at both pH values tested. As lithium is supposed to be a substrate of sodium transport systems, we also checked the Li^+ tolerance or sensitivity. Table 1A shows that the presence of pCS1 confers a greater tolerance to lithium ions, while pCS2 brings about an extremely high Li tolerance.

Plasmids from both clones were characterized first by restriction analysis. The plasmid pCS2 contained a 5.0 kb long insert and the partial sequencing of a subcloned internal part of this fragment (250 nt) showed a 100% identity with the *S. cerevisiae ENA2* gene coding for a Na^+-ATPase [7], thus this plasmid has not been studied further.

3.2. Nucleotide sequence analysis of pCS1 insert region

Plasmid pCS1 contained a 4.6 kb long insert which was entirely sequenced on both strands. The nucleotide sequence revealed a 3521 bp long fragment of chromosome XII in the middle, and two short sequences corresponding to other chromosomes at the extremities of the insert (see Fig. 1A). The presence of fragments from several chromosomes joined by *Sau*3A sites in one plasmid is most probably a consequence of the DNA library construction, when the *Sau*3A-partially digested fragments of genomic DNA were ligated into *Bam*HI-digested pFL1.

Table 1
Maximal sodium and lithium concentrations tolerated by different derivatives of the HS100-3C strain

	Na^+ (mM)		Li^+ (mM)	
	pH 5.5	pH 7.5	pH 5.5	pH 7.5
A				
[pFL1]	400	200	5	5
[pCS1]	800	600	20	10
[pCS2]	800	800	200	200
B				
nha1::URA3 (clone 1)	200	<200	2	2
nha1::URA3 (clone 2)	300	200	<5	<5

Serial 10-fold dilutions of saturated cultures were spotted onto YNB-glucose plates (pH 5.5 or 7.5) supplemented with different concentrations of NaCl (100–800 mM) or LiCl (1–200 mM). Growth was recorded after 5 days.

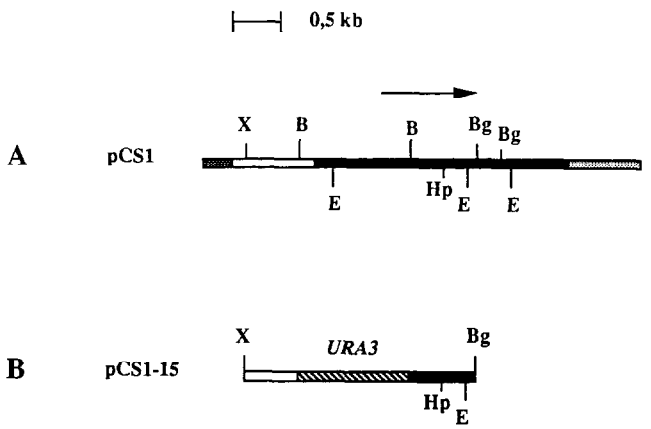

Fig. 1. Restriction maps of (A) DNA insert of pCS1. White and black regions correspond to promoter and *NHA1* ORF (chromosome XII), respectively, and the dotted regions to *Sau*3A-joined fragments of other chromosomes; (B) DNA insert in pCS1-15 constructed for integrative *NHA1* disruption and/or deletion. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; Hp, *Hpa*I; X, *Xba*I.

Nucleotide sequence analysis revealed an ORF starting at position 857 of the chromosome XII fragment. In the promoter region two repeats of CCCCT motifs (stress-responsive elements, STRE [18]) were found at positions −255 and −463, respectively, upstream from the ATG codon.

Unfortunately, the ORF was interrupted after 888 codons (2664 bp) by a *Sau*3A site in which the fragment of chromosome IV was connected, and thus an 'artificial' stop codon was introduced three codons after the *Sau*3A site. A search in the GenEMBL nucleotide sequence data library revealed a 100% identity of our sequence with one ORF of unknown function from the recently submitted part of the right arm of chromosome XII (Delius, H., unpublished, accession no. X91258). From the sequence in the database we could conclude that the entire ORF has 986 codons (2985 bp), so it is 98 amino acids longer than the ORF in pCS1.

3.3. Deduced primary structure of Nha1 protein

Fig. 2 shows the deduced primary structure of the Nha1 protein (Sc-Nha1). It contains 986 amino acid residues, its calculated M_r is 109.4 kDa, overall charge 23 and isoelectric point 5.26. The hydropathy profile was calculated with a window of 19 amino acids using the algorithm of Kyte and Doolittle [19], and 10–12 possible membrane-spanning domains were found. Depending on the criteria of hydrophobicity used, the fourth and the fifth very hydrophobic transmembrane domains can be also considered as one very long membrane-spanning segment, and on the other hand, the level of hydrophobicity of the sixth domain is very low. The last membrane spanning domain ends at Ser-440, leaving a very long (546 residues, i.e. 55.4% of the protein) hydrophilic, highly charged C-terminal. Six possible N-linked glycosylation sites were found, but none of them seems to be accessible

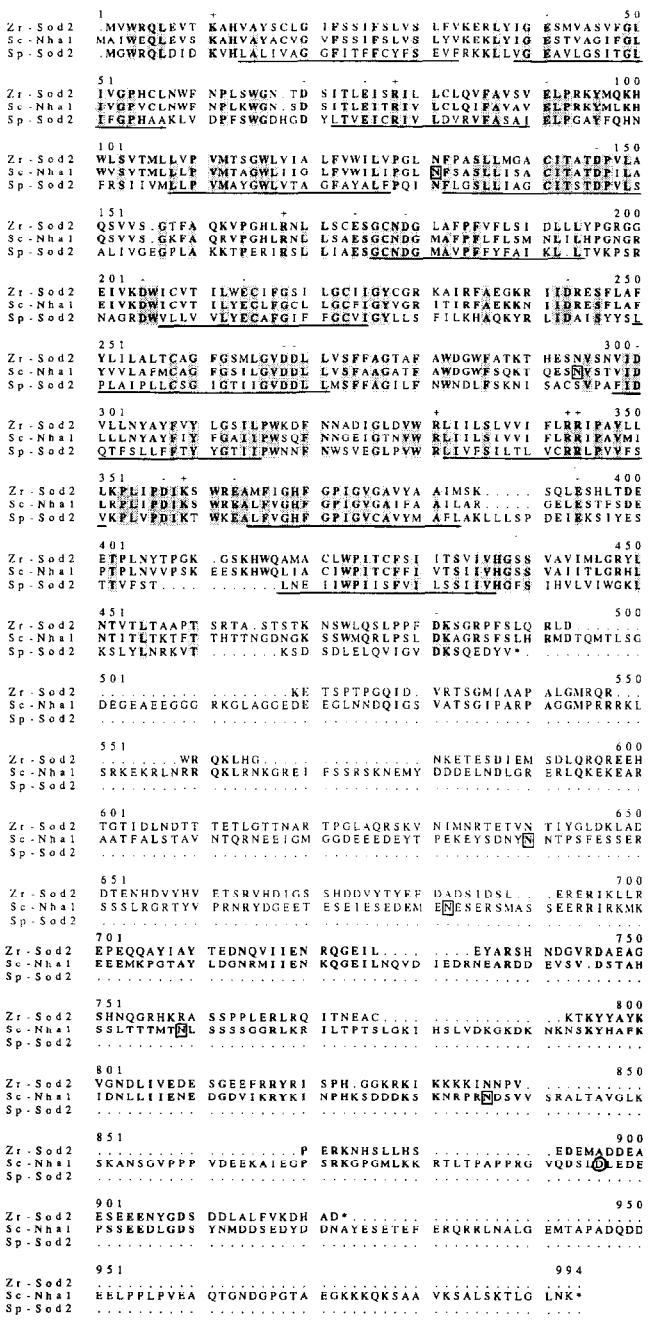

Fig. 2. Alignment of protein sequences of Na^+/H^+ antiporters of *Zygosaccharomyces rouxii* (Zr-Sod2), *Saccharomyces cerevisiae* (Sc-Nha1), and *Schizosaccharomyces pombe* (Sp-Sod2). Amino acid residues identical in all three proteins are shadowed; residues conserved in two proteins are marked in bold. Putative membrane-spanning segments are underlined; potential *N*-glycosylation sites of Nha1p are boxed. The last amino acid from the protein encoded by the pCS1 plasmid is encircled.

from the periplasmic site of the plasma membrane, if we presume the N- and C-termini face the cytosol.

3.4. Similarity of Nha1p with other Na^+ /H^+ antiporters

A search in the GenEMBL nucleotide sequence database revealed a very high level of similarity with *S. pombe* Na^+/H^+ antiporter gene *sod2* [3], and the gene *Z-SOD2* coding for a putative sodium antiporter in the yeast *Z. rouxii* [4].

Table 2
Sodium tolerance of different derivatives of W303-1A and G19 strains

	NaCl (mM)
W303-1A (*ENA1-ENA4*)	1500
[pFL1]	1500
[pCS1]	1500
nha1::URA3 (clone 1)	1500
nha1::URA3 (clone 5)	1200
G19 (*ena1Δ::HIS3::ena4Δ*)	200
[pFL1]	200
[pCS1]	500
nha1::URA3 (clone 1)	80

Saturated cultures were diluted 10^5-fold with water and plated onto YNB-glucose plates (pH 5.5) supplemented with different concentrations of NaCl (50–1500 mM). Growth of single colonies was recorded after 5 days.

Comparison of the deduced proteins' primary structures (Fig. 2) showed several almost identical regions, and the most significant difference at the C-termini, where the proteins of *Z. rouxii* and especially *S. cerevisiae* are much longer compared to the product of the *S. pombe sod2* gene. The high level of similarity confirmed our results from NaCl-tolerance tests that the ORF in pCS1 could code for a Na^+/H^+ antiporter. As we could not designate the pCS1 ORF *SOD*, this name being already attributed to the *S. cerevisiae* superoxide dismutase gene, we termed the pCS1 ORF *NHA1* as $\underline{Na}^+/\underline{H}^+$ antiporter.

If the first half of Nha1p containing the putative 12 transmembrane segments is compared with corresponding parts of both Sod2p, there are 166 amino acid residues conserved in all three transporters, which corresponds to 37.8% identity in a 440 amino acid long overlap, and if the conservative replacements are considered, the similarity among three peptides reaches 58%. The most conserved residues are Gly (22×) and Leu (21×). If the comparison is made separately for Sc-Nha1 vs. Zr-Sod2, and Sc-Nha1 vs. Sp-Sod2 the levels of identities are 71.7 and 40.1%, respectively, so the Na^+/H^+ antiporters of *S. cerevisiae* and *Z. rouxii* are much closer to each other than to that of *S. pombe*.

Regarding the putative transmembrane domains, there are several conserved negatively and positively charged residues. Recently, three aspartic residues (D125, and the neighbours D155, D156) from the second and third membrane-spanning regions of *Vibrio alginolyticus* Na^+/H^+ antiporter playing a role in the transporter activity were identified [20]. In the yeast Nha1 and Sod2 proteins, there are several conserved aspartyl residues in putative transmembrane segments, and two of them – the neighbours from the eighth transmembrane domain D266, D267 (see Fig. 2) could also play a similar role in the activity of yeast carriers.

No significant similarity was found when the Nha1p was compared with the products of two genes coding for putative Na^+-transporters which were identified during sequencing of *S. cerevisiae* chromosome II (YBR235w, putative Na^+-(K^+)-Cl^- cotransporter [21]) and chromosome IV (D9461.40, putative Na^+/H^+ antiporter [22]), respectively.

3.5. The role of Nha1 in sodium tolerance

To verify the role of Nha1p in sodium tolerance, two strains used in the studies of Ena systems (W303-1A and G19) were transformed with either the multi-copy pCS1 con-

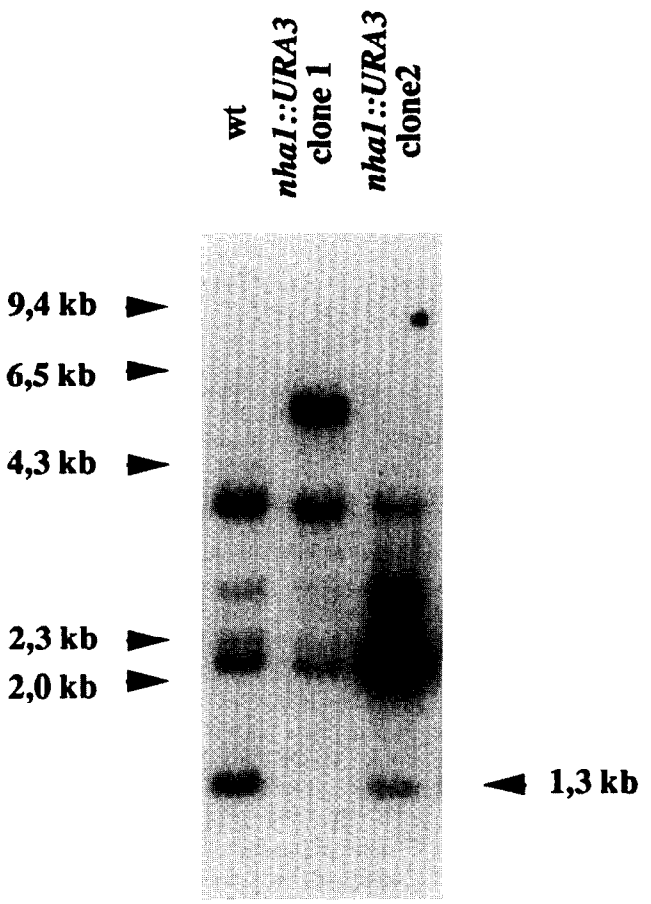

Fig. 3. Southern analysis of genomic DNAs from wild-type and *nha1::URA3* derivatives of the HS100-3C strain. Isolated DNA was digested with *Eco*RI and hybridized with a probe containing *Eco*RI-*Eco*RI fragment (1.3 kb) of *NHA1*.

taining the truncated *NHA1* gene, or pFL1 with no insert, or the integrative fragment of pCS1-15 (Fig. 1B) constructed for *NHA1* disruption.

Test of the growth of resulting transformants in the presence of different concentrations of NaCl (Table 2) showed that the already very high sodium tolerance (growth on 1.5 M, i.e. 8.7% NaCl) of W303-1A, which contains four genes *ENA*, is not increased by the multiplication of *NHA1* gene. On the other hand, if the *NHA1* gene is introduced in a multicopy vector into the strain G19, in which all four *ENA* genes are deleted, the NaCl tolerance increases reaching almost the values observed for the strain HS100-3C containing only one copy of *ENA* gene (cf. Tables 1 and 2).

The increased level of sodium and lithium tolerance in strains containing pCS1 is most probably due to the 'multicopy' character of the plasmid. On the other hand, as the pCS1 encodes a truncated protein (see Fig. 2), we cannot exclude that the presence of the complete *NHA1* gene on the multicopy vector would change the maximal concentrations of sodium and lithium ions tolerated by transformed yeast strains. With the entire transporter the tolerance could either increase, if the missing part of the C-terminal is important for the protein structure and/or activity, or decrease, if the C-terminal plays a similar role in the regulation of transport activity, as was shown for the amino acid permease Bap2p, whose activity increases after shortening the C-terminal [23].

As for the disruption of the *NHA1* gene, in strain G19 it brings about very high sensitivity to sodium, and the cells are not able to grow if the NaCl concentration is higher than 0.08 M (approx. 0.5%, see Table 2). When the *NHA1* gene was disrupted in W303-1A two types of transformants were observed. From the total of 40 disruptants studied, 29 belonged to a group in which no change in the sodium tolerance was observed (e.g. *nha1::URA3* clone 1, Table 2). The other group contains 11 strains with decreased tolerance towards the NaCl (e.g. *nha1::URA3* clone 5, Table 2) which grow very poorly even in the absence of NaCl. The Southern blots and some preliminary PCR-based results confirmed that in both types of disruptants the *URA3* gene of pCS1-15 was integrated into the *NHA* locus, but simultaneously revealed the existence of at least one other copy of the *NHA1* gene.

A detailed characterization of several *nha1::URA3* disruptants of HS100-3C (Table 1B) also revealed two groups with slightly different phenotypes concerning the sodium and lithium tolerance, and different 'profiles' on Southern blots. As shown in Fig. 3, the P^{32}-labelled probe (1.3-kb long *Eco*RI-*Eco*RI fragment of the *NHA1* ORF, see Fig. 1) hybridizes with at least 5 different fragments of the wild-type HS100-3C DNA, and disruption of the *NHA1* gene by *URA3* leads to some characteristic changes in the profiles depending on the clones with different phenotypes (cf. Fig. 3 and Table 1B)

All these results suggested that more than one copy of *NHA1* could exist in HS100-3C and W303-1A strains. As in the physical mapping by the chromoblot technique the probe corresponding to *NHA1* gene hybridized only to chromosome XII (results not shown), it seems that the other copy is located on the same chromosome, although not in the vicinity of *NHA1*, at least in the strain S288C, as the systematic sequencing of chromosome XII has revealed only one copy of *NHA1* thus far.

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