Heterologous Expression of Na\(^+\)/H\(^+\) Antiporter Gene (CvNHA1) from Salt-Tolerant Yeast Candida versatilis in Saccharomyces cerevisiae Na\(^+\)-Transporter Deficient Mutants

Yasuo Watanabe,\(^1\) Hidenori Akita,\(^1\) Yuka Higuchi,\(^1\) Rie Tsujimatsu,\(^1\) Tsuyoshi Kaneta,\(^2\) and Youichi Tamai\(^1\)

\(^1\)Department of Biological Resources, Faculty of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama, Ehime 790-8566, Japan
\(^2\)Chemistry and Biology, Graduate School of Science and Engineering, Ehime University, 2-5 Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan

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A Na\(^+\)/H\(^+\) antiporter gene (CvNHA1) was cloned from the salt-tolerant yeast Candida versatilis. CvNHA1 encodes an antiporter with a typical yeast plasma membrane Na\(^+\)/H\(^+\) antiporter structure. Transcription of CvNHA1 in C. versatilis cells was dependent on the salinity of the culture. When CvNHA1 was expressed in salt-sensitive Saccharomyces cerevisiae cells, increased salt-tolerance was observed, indicating that CvNha1p possesses an Na\(^+\)/H\(^+\) antiporter function, because the increased salt-tolerance was dependent on the extra-cellular pH. It appears that CvNha1p mediates only the transport of Na\(^+\). In an S. cerevisiae transformant harboring a CvNHA1-EGFP fusion plasmid in which the greater part of the C-terminal hydrophilic region of CvNha1p was deleted by fusion with enhanced green fluorescent protein (EGFP), the Cvnh1-EGFP fusion protein was localized mainly in the plasma membrane, and the NaCl-tolerance of this transformant was greater than that of a strain harboring the entire CvNHA1 gene.

Key words: Na\(^+\)/H\(^+\) antiporter; salt tolerant yeast; Candida versatilis; Zygosaccharomyces rouxi; salt tolerance

Candida versatilis (formerly Torulopsis versatilis) is a yeast that is used in the production of soybean paste (miso) and soy sauce (shoyu), Japanese seasonings.\(^1\) This yeast participates in the formation of the distinct fragrance of miso and shoyu in the later stages of production. The fragrance is due to a complex of phenol components, including 4-ethylguaiacol and 4-ethylphenol.\(^2\) The major acidic components of shoyu and miso are lactic acid and succinic acid, but the acetic acid produced by C. versatilis gives these seasonings a unique flavor.\(^3\)

The salt-tolerant yeast C. versatilis grows in the presence of 3 M NaCl.\(^4,5\) In the presence of high extracellular concentrations of NaCl, yeast cells are subject to osmotic stress. In response to this stress, they accumulate high concentrations of compatible solutes. In C. versatilis cells, the main compatible solutes are glycerol and mannitol, which are maintained at almost equal concentrations.\(^6\) At concentrations below 2 M, the intracellular concentration of Na\(^+\) ([Na\(^+\)]\text{in}) is about 30 mM, and at concentrations of 2.5 to 4.5 M NaCl, [Na\(^+\)]\text{in} is about 150 mM. The corresponding values for [K\(^+\)]\text{in} are about 300 mM and 50 mM respectively.\(^6\) This suggests that the plasma membrane of C. versatilis cells contains an effective Na\(^+\) transporter that extrudes cellular Na\(^+\).

We have studied the relationship between plasma membrane H\(^+\)-ATPase activity in C. versatilis and extracellular NaCl concentration.\(^7\) This enzyme activity was dependent on the concentration of NaCl, but was independent of growth phase and the glucose concentration in the culture medium. In the presence of high concentrations of NaCl, the activity of plasma membrane H\(^+\)-ATPase increased, resulting in the extrusion of large amounts of H\(^+\) by the H\(^+\) pump.

Our finding that a plasma membrane Na\(^+\)/H\(^+\) antiporter plays the most significant role in extrusion of cellular Na\(^+\) in Z. rouxi\(^8,9\)) suggests that this antiporter plays a significant role in the function of Z. rouxi in the production of miso and shoyu. In the present study, we cloned the Na\(^+\)/H\(^+\) antiporter gene (CvNHA1) from C. versatilis, and performed CvNHA1 expression analysis in the presence of various NaCl concentrations, and...
also functional analysis of Cvnh1p in salt-sensitive *Saccharomyces cerevisiae* cells.

## Materials and Methods

### Strains and media.**  
The yeast strains used were *Candida versatilis* NBRC10650 (wildtype), *Saccharomyces cerevisiae* BW31,12) and *S. cerevisiae* W303-1A.13) Strain BW31 lacks Na⁺-transporting activity in its plasma membrane, due to deletion of the Na⁺-ATPase gene and the Na⁺'/H⁺ antiporter gene (ena1-4A nh1A).12) The yeast cells were cultured with shaking in YPAD medium (0.5% w/v yeast extract, 2% w/v peptone, 2% w/v glucose, 0.5% w/v KH₂PO₄, 0.2% w/v MgSO₄·7H₂O, 0.4 mg/ml of adenine) or SC-URA medium (0.67% w/v yeast nitrogen base without amino acids and 0.192% w/v CSM-URA powder) (Sigma, Boston, MA). To induce transcription of genes in plasmids, the transformants were cultivated in SC-URA medium containing 1% (w/v) raffinose and 1% (w/v) galactose.

Nucleotide sequence, amino acid sequence alignment, and hydropathy.  
Nucleotide sequences were determined using a Big Dye Terminator Cycle Sequencing Kit and a PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA). The sequences were determined by the primer walking method with the primers shown in Table 1. Alignment of amino acid sequences was performed using Clustal V software. The hydropathy profile of the Na⁺'/H⁺ antiporter was obtained using DNA Strider 1.1 software and the method of Kyte and Doolittle.14)

Cloning of *CvSOD2* gene.  
The nucleotide sequences of degenerate primers used in degenerate PCR, including inosine, are shown in Table 1. Genomic DNA of *C. versatilis* was prepared using the YeaStar Genomic DNA Kit (Zymo Research, Orange, CA). Degenerate PCR was performed using KOD-plus DNA polymerase and primers for the open reading frame of *CvSOD2* (the *CvNh1* gene-specific 21-mer primers (CvNHA1SQ1 and CvNHA1SQ3/R, Table 1), and KOD-plus DNA polymerase at 24, 28, 32, and 36 cycles. PCR products (318 bp) were analyzed by agarose gel electrophoresis, and the transcription levels of *CvNH1* were estimated from the data obtained at 28 cycles.

Construction of *pESC-URA* harboring *CvNH1* and *CvNH1-EGFP* fusion protein, and estimation of the salt tolerance of the recombinant yeast.  
For expression of the *CvNH1* gene in *S. cerevisiae*, the pESC-URA vector (Stratagene, Tokyo) was used. DNA containing the open reading frame of *CvNH1* was prepared by performing PCR using *C. versatilis* genomic DNA, KOD-plus DNA polymerase, and primers for the restriction enzyme site (NotI or PacI) introduced at the 5'-end of each primer (NotI primer and PacI primer, Table 1). Each vector and PCR product was digested with NotI and PacI, and was then ligated (pCvNH1A). *ZrSOD2* (the Z. rouxii Na⁺'/H⁺ antiporter gene) was inserted into pESC-URA vector at the same restriction enzyme sites.11) Both the *CvNH1* and the *ZrSOD2* gene were expressed under the regulation of *GAL1* promoter and *ADH1* terminator with the same plasmid (pESC-URA vector).

Plasmid-based construction of the *CvNH1-EGFP* fusion protein was performed by the gene replacement method,16) with modifications by Iizasa and Nagao.17) PCR was performed using two 40-nt flanking target primers (5' 40-nt *CvNH1* sequence + 20-nt *yEGFP* sequence-3'; *CvNH1-EGFP* F and *CvNH1-EGFP* R/R, Table 1 and Fig. 1), and using a pUG35 plasmid18) harboring the *yEGFP* gene as the template DNA. The DNA produced by this PCR was purified and then mixed with pCvNH1A cut with BglII. This mixture was used to transform *S. cerevisiae* BW31. The presence of a plasmid harboring the *CvNH1-EGFP* fusion protein (pCvNH1-EGFP) in a yeast clone was confirmed by yeast colony PCR,16) using the set of primers shown in Table 1. Transformation of *S. cerevisiae* BW31 using recombinant yeast plasmids was performed according to the Alkali Cation Yeast Transformation Kit manual (Bio 101). The salt tolerance of the recombinant yeast was estimated by measuring the OD at 600 nm (CO8000 Cell
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Underlining (a) shows the sites of restriction enzyme introduced for recombination.
Underlining (b) shows the sequence of the EGFP gene.
Density Meter, WPA Bioware, Cambridge, UK) of culture in SC-URA medium containing various salts. Measurements of cell growth were repeated at least 3 times, and representative data are shown in the figures.

Fluorescence microscopy of transformant cells harboring pCvNHA1-EGFP plasmid. The transformant cells were cultivated in SC-URA medium containing 1% galactose and 1% raffinose. At the stationary phase, cells were collected. The Cvnh1p-EGFP signal was observed using a conventional microscope (Nikon, Tokyo) equipped with epifluorescence illumination. A band-pass 460–500 nm filter was used for excitation, and a band-pass 510–560 nm filter was used as a barrier filter. Digital images were captured using a cooled BS-41L CCD (charge coupled device) camera (Bitran, Saitama, Japan). The images thus obtained were analyzed using Image-Pro Plus version 4.5 image deconvolution software with the Sharp Stack optional module (MediaCybernetics, Buckinghamshire, UK).
Results and Discussion

Amino acid sequences conserved in various plasma membrane Na\(^+\)/H\(^+\) antiporter proteins

Alignment analysis of the Na\(^+\)/H\(^+\) antiporters cloned from *S. cerevisiae* (accession no. Z73310), *Aspergillus fumigatus* (AY080962), Schizosaccharomyces pombe (Z11736), *Pichia farinosa* (PS0496431), *Debaryomyces hansenii* (AJ876409), and *Z. rouxii* (D43629, AB010106, and AJ872201-05), which were available at the start time of the study, confirmed that several peptide motifs (about seven residues) were conserved, viz.

\[ A/CESGCNDG \text{ and } GHFGPIGV. \]

Degenerate primers including inosine were prepared (Table 1). Degenerate PCR produced DNA fragments (about 0.6 kbp) that encoded the central region of \( CvNHA1 \).

Cloning of the \( CvNHA1 \) gene

The 3'0-region of \( CvNHA1 \) was cloned by RT-PCR (about 2.3-kbp of PCR product). The 5'0-region of \( CvNHA1 \) was cloned by Inverse PCR, using self-ligated BamHI-cut *C. versatilis* genomic DNA as a template (about 1 kbp). A single BamHI site was present in a DNA fragment of the central region of the \( CvNHA1 \).
gene (produced by degenerate PCR). We determined
the nucleotide sequence (4 kb) of the DNA segments
connecting the 3 DNA products (accession no.
AB255166). The nucleotide sequence of the CvNHA1
gene and the deduced amino acid sequence of Cvnha1p
are shown in Fig. 1.

Structure of Cvnha1p

The number of amino acid residues that composed
Cvnha1p was 1,113 (Table 2). The number of amino
acids that composes the yeast plasma membrane Na+/H+
antiporter proteins ranges from 468 residues in S. pombe Spsod2p19) to 985 residues in S. cerevisiae
Scnha1p (Table 2). Our previously cloned antiporters,
Zrsod2p16) and Zrsod22p,10) comprised 791 and 808
residues respectively (Table 2). Homology over the
entire sequence of the Na+/H+ antiporter was low (33%
to 38%), but from the N-terminus to the 500th residue
(corresponding to the transmembrane domains shown
below), the homology was 51% to 60%. From the
501st residue to the C-terminus, the homology was low
(16% to 20%).

The hydropathy of the amino acid sequences of
Cvnha1p was determined using DNA strider software.
Cvnha1p exhibited characteristics typical of plasma
membrane Na+/H+ antiporters, which generally consist
of 12 hydrophobic transmembrane domains and a long
hydrophilic C-terminus (about 880 residues, Fig. 1). The
hydropathy profiles of the 12 yeast plasma membrane
Na+/H+ antiporters indicated that they have similar
hydrophobic transmembrane domains, suggesting that
this region plays a significant role in Na+/H+ antiporter
function. In particular, Spsod2p is the smallest of these
Na+/H+ antiporters. It consists only of the 12 trans-
membrane domains, suggesting that this region is
required for the fundamental function of Na+ transport.
It has been reported that the conserved polar residues
that are important for Na+/H+ antiporter function are
located in the 12 hydrophobic transmembrane domains
of Spsod2p and Scnha1p.19–24) It has been found that
Scnha1p-C-terminus is not important to substrate speci-
city.25,26)

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Table 2. Comparison of Amino Acid Sequence of Cvnha1p with Various Yeast Na+/H+ Antiporters

Fig. 2. Semi-Quantitative PCR Analysis of CvNHA1 in C. versatilis Cells Cultivated in the Presence of NaCl at 0 to 2 M.
For each reverse transcriptase reaction, 1 μg of total RNA was
used. cDNA samples (1 μl) were used in PCR. PCR was run at 24,
28, 32, and 36 cycles, and the DNA products were amplified
exponentially at 28 cycles. DNA samples obtained at 28 cycles were
analyzed by 1.5% agarose gel electrophoresis.

Semi-quantitative RT-PCR

Total RNA was prepared from C. versatilis cells
cultivated in the presence of NaCl at 0, 1, and 2 M until
the mid-exponential phase. Aliquots (1.0 μg) of total RNA were used in the RT reaction. PCR was performed
using 28 thermal cycles, at which point the PCR products (318 bp) were amplified exponentially
(Fig. 2). Transcription of CvNHA1 was found to be
dependent on the NaCl concentration of the culture
medium; few transcripts of CvNHA1 were detected in
cells grown without NaCl, but relatively high levels of
CvNHA1 mRNA were detected in cells grown with 2 M
NaCl. This suggests that there is a significant relationship
between Cvnha1p function and the salt tolerance of
C. versatilis cells.

Expression of the CvNHA1 gene in salt-sensitive
S. cerevisiae BW31

The CvNHA1 gene, contained in the pESC-URA
plasmid, was expressed in the salt-sensitive S. cerevisiae
strains BW31 under the regulation of the GAL1/10
promoter. Strain BW31 lacks Na+-transporting activity
in its plasma membrane, due to deletion of the Na+/H+
ATPase gene and the Na+/H+ antiporter gene (ena1-4Δ
nha1Δ).12) When the CvNHA1 gene was expressed in
this yeast strain, it exhibited partial complementation
of these phenotypes (Fig. 3). In recombinant strain,
this complementation was dependent on the pH of the
culture medium. For example, at pH 5.0, the effect of \(\text{CvNHA1}\) gene expression was significant, but at pH 6.5 it was negligible (Fig. 3). This suggests that the \(\text{CvNHA1}\) gene encodes an \(\text{Na}^+/\text{H}^+\) antiporter protein.

We have characterized the \(\text{Na}^+/\text{H}^+\) antiporter gene (\(\text{ZrSOD2}\), formerly \(\text{Z-SOD2}\)) from the salt-tolerant yeast \(\text{Z. rouxii}\).\(^9-11\) In the present study, \(\text{ZrSOD2}\) was expressed in strains \(\text{BW31}\). \(\text{ZrSOD2}\) expression produced a greater degree of complementation of \(\text{Na}^+\) sensitivity than did \(\text{CvNHA1}\) expression (Fig. 3). Since expression of \(\text{CvNHA1}\) and \(\text{ZrSOD2}\) was induced by the same promoter in the same vector, these results suggest the possibility that \(\text{Zrsod2p}\) transports \(\text{Na}^+\) across the plasma membrane more efficiently than \(\text{Cvnha1p}\).

**Localization of \(\text{Cvnha1p}\) in the plasma membrane**

The \(\text{Na}^+\) tolerance of the yeast clone harboring the \(\text{CvNHA1-EGFP}\) gene (the \(\text{CvNHA1-EGFP}\) strain) was compared with that of the yeast clone harboring the native \(\text{CvNHA1}\) gene (the \(\text{Cvnha1}\) strain, Fig. 4). When the \(\text{S. cerevisiae}\) \(\text{BW31}\) strain was used as host cells, the \(\text{Cvnha1-EGFP}\) strain had a somewhat higher salt tolerance than the \(\text{CvNHA1}\) strain. This indicates the possibility that the C-terminal hydrophilic region of \(\text{Cvnha1p}\) does not play a significant role in its antiporter function.

To confirm the localization of \(\text{Cvnha1p}\) in the yeast plasma membrane, we constructed a fusion protein...
consisting of CvNha1p and yEGFP. CvNha1p was fused with EGFP protein at the 540th residue of CvNha1p, which is approximately 100 amino acid residues after the end of the 12th transmembrane domain of CvNha1p. Thus most of the long C-terminus hydrophilic domain of CvNha1p was deleted. The results of fluorescence microscopy are shown in Fig. 5. The CvNha1p-EGFP fusion protein localized mainly in the plasma membrane.

Tolerance of transformants to other metal ions

When S. cerevisiae BW31 was used as recipient cells, expression of CvNha1p and Zrsod2p did not result in increased tolerance to Li⁺, Rb⁺, or K⁺ (Fig. 6). Although the CvNHA1-EGFP strain had increased salt-tolerance (Fig. 5), the transformant derived from strain BW31 did not exhibit enhancement of growth ability in the presence of high LiCl concentrations. Also, the increase in LiCl tolerance caused by CvNha1p expression was not affected by deletion of the hydrophilic C-terminal domain of CvNha1p (Fig. 5).

Papouskova and Sychrova27 recently reported that Yarrowia lipolytica is the first yeast species known to have two plasma membrane alkali metal cation/H⁺ antiporters (YNh1p and YNh2p) that differ in their putative functions. They proposed that the main functions of YNh1p and YNh2p are to increase cell tolerance to K⁺ and to transport large amounts of Na⁺, respectively. Snh1p transports at least four metal ions (Na⁺, Li⁺, K⁺, and Rb⁺). YNh1p from Y. lipolytica27 and Dhnh1p from D. hansenii28 have been classified in the same group as Snh1p (Table 3). The Na⁺/H⁺ antiporters in this group are involved in the maintenance of intracellular pH and K⁺ homeostasis, rather than in Na⁺ extrusion. In contrast, Zrsod2-22p and Spsod2p transport Na⁺ and Li⁺. They belong to a group of Na⁺/H⁺ antiporters that transport (extrude) Na⁺ across the plasma membrane (Table 3). Some studies involving site-directed mutagenesis indicate that amino acid residues in the transmembrane regions of yeast Na⁺/H⁺ antiporters are involved in their substrate specificity (Table 3). When, in the Z. rouxii Na⁺/H⁺ antiporter, Zrsod2-22p, Pro145, which is present in the 5th transmembrane region, was replaced with Ser or Thr, the mutation decreased antiporter activity for both Na⁺ and Li⁺.23 Also, a mutation in T141S or S150T in YNh1p of Y. lipolytica27 and Dhnh1p from D. hansenii28 have been classified in the same group as Snh1p (Table 3). The Na⁺/H⁺ antiporters in this group are involved in the maintenance of intracellular pH and K⁺ homeostasis, rather than in Na⁺ extrusion. In contrast, Zrsod2-22p and Spsod2p transport Na⁺ and Li⁺. They belong to a group of Na⁺/H⁺ antiporters that transport (extrude) Na⁺ across the plasma membrane (Table 3). Some studies involving site-directed mutagenesis indicate that amino acid residues in the transmembrane regions of yeast Na⁺/H⁺ antiporters are involved in their substrate specificity (Table 3). When, in the Z. rouxii Na⁺/H⁺ antiporter, Zrsod2-22p, Pro145, which is present in the 5th transmembrane region, was replaced with Ser or Thr, the mutation decreased antiporter activity for both Na⁺ and Li⁺.23 Also, a mutation in T141S or S150T in Zrsod2-22p increased antiporter activity for K⁺.21 Otherwise, in S. cerevisiae Snh1p, mutation of D266N almost completely abolished antiporter activity for Na⁺.210 The present finding that CvNha1p contributes to increased tolerance to only Na⁺ (Figs. 3, 5, 6) suggests that

Table 3. Substrate Specificity of Various Yeast Na⁺/H⁺ Antiporters and Site-Directed Mutagenesis

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Protein</th>
<th>Mutation</th>
<th>Substrate specificity</th>
<th>ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. pombe</td>
<td>Spsod2p</td>
<td>Na⁺, Li⁺</td>
<td>Jia et al. (1992)20</td>
<td></td>
</tr>
<tr>
<td>Z. rouxii</td>
<td>Zrsod2-22p</td>
<td>Na⁺, Li⁺</td>
<td>Kinclova et al. (2002)30</td>
<td></td>
</tr>
<tr>
<td>Y. lipolytica</td>
<td>Ynh1a2p</td>
<td>Na⁺, Li⁺</td>
<td>Papouskova and Sychrova (2006)27</td>
<td></td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>Scnha1p</td>
<td>Na⁺, Li⁺, K⁺, Rb⁺</td>
<td>Banuelos et al. (1998)22</td>
<td></td>
</tr>
<tr>
<td>P. Sorbitophils</td>
<td>Pnha1/2p</td>
<td>Na⁺, Li⁺, K⁺</td>
<td>Banuelos et al. (2002)31</td>
<td></td>
</tr>
<tr>
<td>C. albicans</td>
<td>Cin1p</td>
<td>Na⁺, Li⁺, K⁺, Rb⁺</td>
<td>Kinclova et al. (2001)200228</td>
<td></td>
</tr>
<tr>
<td>D. hansenii</td>
<td>Dhnh1p</td>
<td>Na⁺, Li⁺, K⁺, Rb⁺</td>
<td>Velokova and Sychrova (2006)28</td>
<td></td>
</tr>
<tr>
<td>S. pombe</td>
<td>Spsod22p</td>
<td>Na⁺, Li⁺, K⁺, Rb⁺</td>
<td>Papouskova and Sychrova (2002)32</td>
<td></td>
</tr>
<tr>
<td>Y. lipolytica</td>
<td>Ynh1a1p</td>
<td>K⁺, Li⁺, Rb⁺</td>
<td>Papouskova and Sychrova (2006)27</td>
<td></td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>Scnha1p</td>
<td>Na⁺, Li⁺, K⁺</td>
<td>Ohgaki et al. (2005)39</td>
<td></td>
</tr>
</tbody>
</table>

(a) †, increase; ‡, decrease.

Fig. 6. Effects of CvNHA1 Expression on the Growth of S. cerevisiae BW31 Cells in the Presence of LiCl, KCl, and RbCl.

Recombinant yeast (BW31) cells harboring the empty pESC-URA plasmid (negative), Zrsod2, or and CvNHA1 were cultivated at pH 5.0 (a) in the presence of LiCl for 4–6 d. Recombinant yeast (BW31) harboring the empty pESC-URA plasmid (negative), Zrsod2, or CvNHA1 were cultivated in the presence of KCl (b) or RbCl (c) at pH 5.0 for 4 d. Symbols: ○, negative control; ●, CvNHA1 strain; △, Zrsod2 strain.
CVnha1p participates in the extrusion of intracellular Na\(^+\) in salt-tolerant C. versatilis yeast cells.

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**References**


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