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Functional characterization of the NhaA Na^+/H^+ antiporter from the green picoalga *Ostreococcus tauri*

Keatisuda Dawut[a,b], Sophon Sirisattha[c], Takashi Hibino[d], Hakuto Kageyama[d,*], Rungaroon Waditee-Sirisattha[a,**]

[a] Department of Microbiology, Faculty of Science, Chulalongkorn University, Pathumwan, Bangkok 10330, Thailand
[b] Program in Biotechnology, Faculty of Science, Chulalongkorn University, Pathumwan, Bangkok 10330, Thailand
[c] Thailand Institute of Scientific and Technological Research (TISTR), Pathum Thani 12120, Thailand
[d] Department of Chemistry, Faculty of Science and Technology, Meijo University, Nagoya, Aichi 468-8502, Japan

A B S T R A C T

Transmembrane ion transport is a critical process in the cellular response to salt stress. Among the known functional membrane transporters that are involved in the salt stress response, Na^+/H^+ antiporters have been extensively studied. These ubiquitous membrane proteins are crucial for salt tolerance and are associated with the regulation of internal pH, cell volume, morphogenesis, and vesicular trafficking. Molecular and functional analyses of Na^+/H^+ antiporters have been characterized among taxa but little is known about algal Na^+/H^+ antiporters. Here, we analyzed putative Na^+/H^+ antiporters from the complete genome sequence of the marine picoalga *Ostreococcus tauri*. At least 10 putative Na^+/H^+ antiporters belonging to the SOS1, NHX, and KEA/Kef families were found. Surprisingly, a bacterial type NhaA sequence (*OtNhaA*) was also found. Topological modeling of OtNhaA predicted 12 possible transmembrane segments with a long N-terminus. The full-length (FL_OtNhaA) and N-terminal truncated (ΔN112_OtNhaA) versions of OtNhaA were constructed, expressed in the salt-sensitive mutant *Escherichia coli* TO114, and functionally characterized. Complementation analysis revealed that FL_OtNhaA- and ΔN112_OtNhaA-expressing cells exhibited increased tolerance to high NaCl concentrations up to 700 mM. Antiporter activity assays showed that both FL_OtNhaA and ΔN112_OtNhaA proteins predominantly exhibited Na^+/H^+ and Ca^{2+}/H^+ antiporter activities at alkaline pH conditions. Intriguingly, the ΔN112_OtNhaA exhibited higher Na^+/H^+ and Ca^{2+}/H^+ antiporter activities compared to FL_OtNhaA. Kinetic analysis revealed that FL_OtNhaA has a high affinity for Na^+ and Ca^{2+} ions with a K_m of 1.1 ± 0.23 mM for Na^+ (at pH 8.5) and a K_m of 0.3 ± 0.07 mM for Ca^{2+} (at pH 8.5). Since NhaA has shown striking diversity among taxa, our results provide insight into the functional properties of the algal NhaA Na^+/H^+ antiporter. These results will contribute to the understanding of Na^+/H^+ antiporters that have various implications in all kingdoms of life.

1. Introduction

Salinity is considered one of the most significant environmental problems facing the world [1]. Elevated salinity results in profound ecological impacts. To survive at high salinity conditions, numerous sophisticated mechanisms of the cellular adaptive response have evolved to maintain intracellular Na^+ ion homeostasis. It has been shown that an increase in intracellular Na^+ occurs immediately after the onset of high salinity in the surrounding environment. Accumulation of Na^+ in the cytoplasm is prevented by limiting both Na^+ influx and Na^+ efflux via the antiporter. In addition, Na^+ accumulation is regulated by monovalent cation/H^+ antiporters [2–4]. Na^+/H^+ antiporters are ubiquitous in nature. They catalyze the exchange of Na^+ for H^+ across membranes, and play a major role not only in sodium homeostasis, but also in internal pH regulation, cell volume control, morphogenesis, and vesicular trafficking [5–8]. The complete genome sequence of numerous organisms revealed that the Na^+/H^+ antiporters are clustered in the following families: the cation/proton antiporter (CPA1-3) superfamilies, the Nha families, and the Pha system [4,8–10].

In mammals, nine Na^+/H^+ exchanger (NHE) isoforms have been

Abbreviations: *O. tauri*, *Ostreococcus tauri*; OtNhaA, NhaA type Na^+/H^+ antiporter from *O. tauri*; TM, trans-membrane

* Corresponding author.
** Corresponding author.
E-mail addresses: kageyama@meijo-u.ac.jp (H. Kageyama), Rungaroon.W@chula.ac.th (R. Waditee-Sirisattha).

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identified so far [11]. They are involved in regulating intracellular pH, cellular volume and cellular proliferation [11]. It has been reported that NHE proteins are associated with the pathophysiology of various diseases [12]. In the higher plant *Arabidopsis thaliana*, *AtSOS1* was initially identified as a gene locus required for salt tolerance [13]. Loss of function mutation in *AtSOS1* resulted in extreme salt sensitivity [14]. Another well-characterized plant Na^+/H^+ antiporter is AtNHX1. This vacuolar Na^+/H^+ antiporter is induced by salt and osmotic stress. Overexpression of AtNHX1 generated transgenic plants that were highly tolerant to salt stress [15]. The complete genome sequence of *A. thaliana* showed that this plant contains a number of putative Na^+/H^+ antiporter genes belonging to the *CPA1* and *CPA2* superfamilies [4,8].

The prokaryotic Na^+/H^+ antiporters are less complex compared to those of mammals and plants. One of the most extensively characterized prokaryotic Na^+/H^+ antiporter is the *Escherichia coli* NhaA protein. This main Na^+/H^+ antiporter specifically exchanges Na^+ or Li^+ for H^+ and is indispensable for adaptation to high salinity and resistance to Li^+ toxicity [16]. It has been reported that the antiporter activity of NhaA is pH dependent. It is enhanced at alkaline pH and reduced at acidic pH [17]. Genes homologous to *nhaA* have also been reported and functionally characterized in *Helicobacter pylori* [18,19], *Vibrio cholera* [20], *Vibrio parahaemolyticus* [21], *Yersinia pestis* [22] and *Salmonella typhimurium* [23]. Thus, NhaA Na^+/H^+ antiporters contribute to both Na^+ and Li^+ toxicity resistance and allow for growth under alkaline conditions [17]. In addition, NhaA is crucial for pathogenesis [23].

In the present study, we surveyed putative Na^+/H^+ antiporter genes in the marine picoalga *O. tauri* from a complete genome database. This marine alga is an early diverging class within the green plant lineage and its most striking feature is its minimal cellular organization [24]. It is the smallest photosynthetic eukaryote known to date. Our analysis revealed that its genome contains at least 10 putative Na^+/H^+ antiporters. The majority of these putative Na^+/H^+ antiporter genes resemble those found in higher plants such as *SOS1*, *NHX* and *KEA/Kef*. Interestingly, a bacterial type *nhaA* sequence was found in the *O. tauri* genome database (designated *OtNhaA*). We constructed and functionally characterized the full-length and truncated versions of OtNhaA. Our results revealed that OtNhaA is an enzymatically active antiporter with preferential activity for the exchange of Na^+ and Ca^{2+} for H^+. These activities were observed at alkaline pH conditions. Truncation analysis revealed that the N-terminal cytosolic domain of OtNhaA was associated with the regulation of its antiporter activities. This is the first report describing the functional characterization of a bacterial type NhaA-like Na^+/H^+ antiporter from the genome of a eukaryotic organism.

2. Materials and methods

2.1. Phylogenetic analysis

The putative protein sequences of *O. tauri* NhaA and orthologs from bacteria and green algae were retrieved from the GenBank database and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Twenty-one NhaA sequences were retrieved from bacteria (*E. coli*; accession number CDU40636.1, *H. pylori*; NP_208343.1, *Y. pestis*; KGA52390.1, *S. typhimurium*; accession number NP_459044.1, *V. cholerae*; KFE28035.1, *Shewanella frigidimarina*; WP_011638389.1, *Osedax symbiont* Rs2; WP_020285618.1, *Clostridium* sp. Maddingley MBC34-26; WP_008427652.1, *Dyadobacter fermentans* DSM 18053; accession number ACT94304.1, *Aliivibrio fischeri*; WP_011261552.1, *Vibrio azureus*; WP_021710120.1, *Shewanella amazonensis*; WP_011758985.1, *Geobacter* sp. M18; accession number ADW11785.1, *Vibrio ponticus*; accession number GAK84546.1, *Clostridium beijerinckii*; WP_011968778.1, *V. cholerae* HC-7A1; accession number ELT24372.1, *Vibrio ezurae* NBRC 102218; accession number GAD79163.1, *Vibrio proteolyticus*; WP_021705443.1, *V. cholerae* HC-33A2; accession number EHH99586.1, *V. cholerae* HE39; accession number EGQ99624.1 and *Edwardsiella piscicida*; accession number GAJ66919.1) and six NhaA sequences were retrieved from green algae (*O. tauri*; accession number XP_003075324.1, *Ostreococcus lucimarinus*; accession number ABO94792.1, *Micromonas pusilla* CCMP1545; accession number EEH59182.1, *Micromonas* sp. RCC299; accession number ACO64223.1, *Bathycoccus prasinos*; accession number CCO19648.1 and *Aureococcus anophagefferens*; accession number XP_009038116.1). The phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA6) software. The robustness of the tree was accessed by bootstrap analysis (1000 replicates).

2.2. Strains and culture conditions

E. coli DH5α cells were grown in LB medium with shaking (180 rpm) at 37 °C. This strain was used for the routine cloning of plasmids. For complementation analysis, we used *E. coli* TO114 cells (Δ*nhaA*, Δ*nhaB*, Δ*chaA*), which was kindly gifted by professors T. Nakamura (Niigata University of Pharmacy and Applied Sciences) and H. Kobayashi (Chiba University), as previously reported [25,26]. This mutant *E. coli* strain was grown at 37 °C with shaking (180 rpm) in LBK medium (NaCl was replaced with KCl) [26]. Ampicillin, erythromycin, kanamycin and chloramphenicol were used at final concentrations of 50, 150, 30, and 30 μg/ml, respectively. Bacterial cell growth was monitored by measuring the absorbance at 600 nm with a UV-240 spectrophotometer (Shimadzu, Japan).

2.3. Construction of plasmids for expression in E. coli TO114

The plasmid pMK_ORF_OtNhaA harboring the full-length *O. tauri* NhaA was obtained from the algae library of Thailand Institute of Scientific and Technological Research (Thailand). Constructions of the full-length and N-terminal truncated forms were performed using the expression vector pTrcHis2C. The full-length *OtNhaA* sequence was amplified from the plasmid pMK_ORF_OtNhaA (Life Technologies CA, USA) by PCR using the following primers: FL_OtNhaA_F (5′-ATGATCG ATGAGGACCGCGT-3′) and FL_OtNhaA_R (5′-ATGAACGTCTCCGTGAA CGT-3′) (Supplementary Table S1). The PCR products were cloned into pCR2.1. The fragment containing the full-length OtNhaA in pCR2.1 was then prepared by double digestion with XhoI and HindIII and ligated into the corresponding sites of pTrcHis2C (hereafter referred to as pTrcHis2C_FL_OtNhaA). The recombinant plasmid pTrcHis2C_FL_OtNhaA was then transformed into *E. coli* DH5α for propagation and then transformed into *E. coli* TO114. The DNA sequence of the N-terminal truncated form was amplified by PCR using the following primers: ΔN112_OtNhaA_NcoI_F (5′-CACGGAGACGTCCATGGCGGGC GTG-3′) and OtNhaA_R (5′-A TTACGCCGTCTTCAACTTGGCGTCT-3′) (Supplementary Table S1). The PCR products were cloned into pCR2.1 and sequenced. The fragment carrying the N-terminal OtNhaA truncated form in pCR2.1 was prepared by double digestion with NcoI and SalI and ligated into the corresponding sites of pTrcHis2C (hereafter referred to as pTrcHis2C_ΔN112_OtNhaA). The recombinant plasmid pTrcHis2C_ΔN112_OtNhaA was transformed into *E. coli* DH5α for propagation and then transformed into *E. coli* TO114. *E. coli* TO114 cells harboring pTrcHis2C_FL_OtNhaA or pTrcHis2C_ΔN112_OtNhaA were used for complementation analysis and preparation of everted membrane vesicles.

The site-directed mutagenesis of D276 and D277 was carried by overlap-extension PCR. The specific primers are listed in Supplementary Table S1. Two aspartic acids D276 and D277 in OtNhaA correspond to D163 and D164 in EcNhaA were shown in Supplementary Fig. S1. Residues D276 and D277 of OtNhaA were substituted with alanine, which generated the plasmids pTrcHis2C_D276A and pTrcHis2C_D277A, respectively. The change of nucleotide was confirmed by DNA sequencing. These recombinant plasmids were transformed into *E. coli* DH5α for propagation and then transformed into *E. coli* TO114. *E. coli* TO114 cells harboring