Running Title: Na⁺(K⁺)/H⁺ antiporter of thermotolerant *A. tropicalis* SKU1100

Title: A novel Na⁺(K⁺)/H⁺ antiporter plays an important role in the growth of *Acetobacter tropicalis* SKU1100 at high temperatures via regulation of cation and pH homeostasis

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Abbreviations: AAB, acetic acid bacteria; AO, acridine orange; ISO, inside-out; YPG, yeast extract-peptone-glycerol.

Abstract

A gene encoding a putative Na^+/H^+ antiporter was previously proposed to be involved in the thermotolerance mechanism of *Acetobacter tropicalis* SKU 1100. The results of this study show that disruption of this antiporter gene impaired growth at high temperatures with an external pH >6.5. The growth impairment at high temperatures was much more severe in the absence of Na^+ (with only the presence of K^+); under these conditions, cells failed to grow even at 30°C and neutral to alkaline pH values, suggesting that this protein is also important for K^+ tolerance. Functional analysis with inside-out membrane vesicles from wild type and mutant strains indicated that the antiporter, [At-NhaK2](#) (~~AtNKhA~~) operates as an alkali cation/proton antiporter for ions such as Na^+ , K^+ , Li^+ , and Rb^+ at acidic to neutral pH values (6.5–7.5). The membrane vesicles were also shown to contain a distinct pH-dependent Na^+ (specific) $/\text{H}^+$ antiporter(s) that might function at alkaline pH values. In addition, phylogenetic analysis showed that [At-NhaK2](#) (~~AtNKhA~~) is a novel type of Na^+/H^+ antiporter belonging to a phylogenetically distinct new clade. These data demonstrate that [At-NhaK2](#) (~~AtNKhA~~) functions as a $\text{Na}^+(\text{K}^+)/\text{H}^+$ antiporter and is essential for K^+ and pH homeostasis during the growth of *A. tropicalis* SKU1100, especially at higher temperatures.

Keywords: Acetic acid bacteria, sodium proton antiporter, thermotolerance, [whole bacterial phylogenetic analysis](#).

1. Introduction

Stable and efficient fermentation requires robust microbes to overcome various stressors such as temperature, acids, and alcohols generated during the fermentation process. ~~In particular, recent climate changes require us to create robust strains capable of fermentation under higher temperature conditions; these microbes should be tolerant not only of heat but also of several other stresses such as acids or alcohols produced during the process~~ These stresses can influence transient changes to intracellular pH and consequently trigger important cellular functions including the induction of the heat shock response (Coote et al., 1991; Weitzel et al., 1987). Growth of *Saccharomyces cerevisiae* at sub-lethal temperature revealed increasing activity of membrane ATPase, which is responsible for intracellular pH homeostasis. ~~Such perturbations to the regulation of internal pH could be related to the thermotolerance mechanism~~ (Coote et al., 1991). In addition, the intracellular GTPase Arl1 and organellar cation/H⁺ antiporters (Kha1 and Nhx1) of *S. cerevisiae* were found to be important for survival at nonoptimal pH, temperature and cation concentration (Marešová and Sychrová, 2010). Such perturbations to the regulation of internal pH, therefore could be related to the thermotolerance mechanism. Bacteria must maintain a suitable cytoplasmic pH compatible with the optimal function and structural integrity of the cytoplasmic proteins. *Escherichia coli* has been shown to initiate SOS and heat shock-like responses when the internal pH is altered as a consequence of changes in the external pH (Schuldiner et al., 1986). A large number of adaptive strategies for pH homeostasis have been reported, including Na⁺/H⁺ antiporters, secondary sodium pumps, which play a crucial role by controlling intracellular Na⁺ and H⁺ concentrations in a pH-dependent manner (Häse et al., 2001).

Several families of bacterial Na^+/H^+ antiporters have been reported and characterized in detail (Hunte et al., 2005; Karpel et al., 1991; Mesbah et al., 2009; Minato et al., 2013; Padan et al., 2004; Pinner et al., 1993a; Quinn et al., 2012; Radchenko et al., 2006; Resch et al., 2010). Characterization of these individual antiporters has provided insight into their physiological role and importance in certain growth environments.

Among a group of acetic acid bacteria (AAB), the genera of *Acetobacter* and *Komagataeibacter* are recognized as the most effective vinegar producers used in traditional and industrial vinegar fermentation because of their robust abilities of ethanol oxidation and resistance to acetic acid (Gullo and Giudici, 2008; Stasiak and Błażej, 2009). The mechanism and molecular machinery underlying ethanol oxidation (Yakushi and Matsushita, 2010) and acetic acid resistance (Matsushita et al., 2005; Nakano et al., 2006; Trček et al., 2007) have been extensively studied. In an attempt to perform fermentation at higher temperatures, we have isolated several thermotolerant AAB from flowers and fruits in Thailand. Several strains have been found to exhibit increased growth and effective productivity at 5 to 10°C higher temperatures, compared with the corresponding mesophilic strains (Moonmangmee et al., 2000; Saeki et al., 1997). Popular a previous study, we examined the mechanism underlying this thermotolerant phenotype by randomly knocking out the genes of thermotolerant *Acetobacter tropicalis* SKU1100 by transposon mutagenesis, and obtained several mutant strains exhibiting severe growth defects at higher growth temperature (Soemphol et al., 2011). Based on these thermosensitive transposon mutants and their draft genome sequence information (Matsutani et al., 2011), we have identified 24 genes possibly involved in the thermotolerance mechanism of *A. tropicalis*, which can be classified into several categories such as stress response, cell

division, cell wall and membrane biosynthesis, and membrane transport. ATPR_0071, one of the 24 genes important for growth at high temperatures in *A. tropicalis* SKU1100, is annotated as a Na⁺/H⁺ antiporter. This mutant (No. 3-45) exhibited a defective growth at higher temperature especially at 39°C (Soemphol *et al.*, 2011).

In this study, we focus on the physiological function of this putative Na⁺/H⁺ antiporter and its role in thermotolerance. Gene-disrupted and complemented strains were constructed and their growth was compared in order to clarify the crucial role of this antiporter with or without Na⁺ and K⁺ either at normal or high temperatures. In addition, the activity of the Na⁺/H⁺ antiporter was examined by ΔpH measurement in inside-out (ISO) membrane vesicles from the wild type and mutant strains. Finally, the phylogenetic relationship of the Na⁺/H⁺ antiporter family was examined in other bacteria. Our results suggest that this antiporter is a novel enzyme important for cation or pH homeostasis, especially at higher temperatures.

2. Materials and Methods

2.1 Chemicals

Fluorescent dyes, bis (1,3-diethylbarbituric acid) pentamethin oxonol [diBA-C₂(5)] , cyclic guanosine 3', 5'-monophosphate (cGMP), and acridine orange (AO), were purchased from Molecular Probes Inc. (Junction city, OR., U.S.A.), Santa Cruz Biotechnology, Inc. (Dallas Texas, U.S.A.), and Sigma, respectively. All chemicals used were of analytical grade.

2.2 Bacterial strains and culture conditions

A. tropicalis SKU 1100 (NBRC101654) and the mutant strains used in this study

were maintained on potato agar slants, as previously reported (Soemphol et al., 2011). Cultivation was performed in yeast extract-peptone-glycerol (YPG) medium containing 2 % glycerol and 0.5 % each of polypeptone and yeast extract, or in YPGD medium (YPG medium containing an additional 0.5% glucose). *Escherichia coli* DH5 α and *E. coli* S17-1 used for genetic manipulation were cultured in Luria-Bertani (LB) medium at 37°C. Antibiotics were supplemented as indicated at the following concentrations: tetracycline (Tc), 12.5 μ g/ml; kanamycin (Km), 50 μ g/ml; and ampicillin (Ap), 50 μ g/ml. Bacterial growth was monitored using a Klett-Summerson Photoelectric Colorimeter with a red filter.

2.3 Bacterial growth conditions

For liquid cultures, pre-culture was prepared in potato medium, as previously reported (Soemphol et al., 2011), and 1 % of the pre-culture was inoculated into a 500 ml Erlenmeyer flask containing 100 ml of YPG medium and incubated at different temperatures under shaking conditions at 200 rpm (with 25 mm diameter amplitude). For dot plate assays, 5 μ l of the cell suspension grown in YPG medium, adjusted for turbidity, was spotted onto agar plates and incubated at different temperatures for 48 h. The YPG agar medium used for growth at different pH values was prepared by dissolving the medium in a buffer adjusted with 50 mM potassium phosphate or with 50 mM sodium phosphate.

2.4.3 Preparation of inside-out (ISO) membrane vesicles

A. tropicalis SKU1100 was cultured in 100 ml of YPG medium in a 500 ml Erlenmeyer flask at 30°C until it reached the early exponential growth phase

(approximately 250 Klett units). The cells were then collected by centrifugation at 7,700 $\times g$ for 10 min and washed twice with 10 mM Tris-acetate buffer (pH 6.5) containing 5 mM MgSO₄. The cell pellets were then resuspended in the same buffer containing 10 μ g/ml of DNase I to a concentration of 1 g wet weight cells per 4 ml. The cell suspension was passed once through a French press (American Instruments Co., Silver Spring, MD., U.S.A.) at 5,000 psi. Subsequent to the removal of cell debris by centrifugation at 8,000 $\times g$ for 20 min ($\times 2$), the supernatant was centrifuged at 100,000 $\times g$ for 90 min by using an ultracentrifuge (Hitachi). The precipitate was then resuspended with 10 mM Tris-acetate buffer (pH 6.5) containing 5 mM MgSO₄ to a concentration of 1 g wet weight per 5 ml, constituting the ISO membrane vesicles that were stored at -80°C .

The integrity of the ISO membrane vesicles was confirmed by monitoring the fluorescence quenching of the diBA-C₂(5) dye representing the inside positive membrane potential. The reaction mixture (1 ml) contained 10 mM Tris-acetate, 5 mM MgSO₄, 20 mM K₂SO₄, 2 μ M diBA-C₂(5), and the ISO membrane vesicles (300 μ g of protein), and the reaction was carried out at 25 $^{\circ}\text{C}$, with fluorescence emission at 614 nm and excitation at 588 nm. The reaction was initiated by the addition of 10 mM D,L-lactate, followed by the addition of 0.1 μ M nigericin, an ionophore able to catalyze an electroneutral exchange of K⁺ for H⁺, to increase a ~~to stimulate~~ membrane potential ~~generation~~ by dissipating a pH gradient, and 0.4 μ M valinomycin, an ionophore able to catalyze an electrical uniport of K⁺ to dissipate the potential a membrane potential completely (Matsushita et al., 2005).

2.5.4 Measurement of ΔpH with ISO membrane vesicles

Antiporter activity was measured by the fluorescence quenching of AO, a permeable cationic dye to cause its accumulation in an acidic vesicular compartments, ~~fluorescence with excitation at 486 nm and emission at 510 nm in with some~~ modifications of the previous report (Mesbah et al., 2009). The reaction mixture consisting of 10 mM Tris-acetate buffer, 5 mM MgSO₄, 140 mM choline-chloride, 1.5 mM AO, and 300 µg of membrane protein. Buffer pH was adjusted to values of 6.0–9.0. The development of antiporter activity was monitored by quenching of AO fluorescence with excitation at 486 nm and emission at 510 nm (Fig. 4A). Respiration-dependent acidification was initiated by the addition of 4 mM D, L-lactate, which leads the accumulation of AO in the membrane vesicles and resulting in self quenching of this fluorophore, as can be seen in Fig 4A. After the line reach to steady state the reaction was followed by the addition of NaCl or KCl to observe the dequenching of AO fluorescence, representing cation influx-dependent proton efflux (inside alkalization) in the ISO membrane vesicles. Finally, the remaining ΔpH was completely dissipated by the addition of 20 mM NH₄Cl, which reverted the fluorescence to the baseline. ΔpH was defined as the percentage of dequenching at the steady state after adding the respective cation from total (100%) of ΔpH generated from onset reaction above. To determine the effective cation concentration (apparent K_m), the concentration of NaCl or KCl was varied from 0.3 mM to 60 mM.

2.6.5 DNA manipulation

Restriction enzyme digestion, DNA ligation, and other DNA modifications were performed according to the manufacturer's recommendations. Plasmid DNA was prepared using *E. coli* strains and other general molecular biology techniques, as

previously described (Soemphol et al., 2011). PCR reactions were performed using the PuReTaqTM Ready-To-GoTM PCR Beads kit (GE Healthcare, Buckinghamshire, UK). DNA fragments obtained by PCR were isolated by agarose gel electrophoresis, purified with a MagExtractor Kit (Toyobo Co Ltd., Osaka, Japan), and then cloned into the pGEM-T EasyTM vector (Promega Corporation, Madison, WI, USA) and transformed into *E. coli* DH5 α .

2.76 Disruption and complementation of the putative Na⁺/H⁺ antiporter

To construct the mutant strain in which the putative Na⁺/H⁺ antiporter (ATPR_0071) of *A. tropicalis* SKU1100 ~~AtNKha~~ was disrupted, the gene was amplified using primers exp3-45F: 5' GGATGAAGGCAGCTATGAGG 3' and exp3-45R: 5' CACTGCGCCAGAGTGAGAAC 3'. The 3.4-kb PCR product was then ligated into the pGEM-T easy vector, resulting in plasmid pT3-45. A 0.9-kb non-polar kanamycin resistance gene digested with *EcoRV* was then inserted into pT3-45 to generate plasmid pT3-45::Km. The resulting plasmid was then transformed into *A. tropicalis* SKU1100 wild type strain using the electroporation method. The recombinant strain (~~AtNKha~~ mutant the antiporter disruptant) was confirmed by PCR and designated as 3-45::Km.

To complement this mutated gene, pT3-45 was digested with *Bam*HI and ligated into *Bam*HI-digested pCM62 to generate plasmid pCM3-45. This plasmid was then transformed into *E. coli* S17-1 prior to transformation into mutant strain 3-45::Km using conjugation methods, as previously reported (Soemphol et al., 2011). An over-expression strain was also prepared similarly by transforming the same plasmid into wild type strain.

2.87 Sequence data retrieval and phylogenetic analysis

The 704 complete genome sequences (one genus per one genome) were downloaded from the NCBI FTP website at <ftp.ncbi.nlm.nih.gov/>. For phylogenetic analysis, we performed a BLASTP search against all protein-coding sequences in the 704 genomes using amino acid sequences of six Nha type antiporter homologs of *A. tropicalis* SKU1100 genome sequence (GenBank acc. No.: [BABS01000001-BABS01000773](#)) (locus tag id: ATPR_0071, ATPR_0984, ATPR_1229, ATPR_2368, ATPR_2573, and ATPR_3013) and 12 functionally validated protein sequences, NhaA of *Helicobacter pylori* (O26076), NhaA of *Vibrio parahaemolyticus* (Q56725), NhaA of *E. coli* (P13738), NhaB of *E. coli* (P0AFA7), NhaB of *V. parahaemolyticus* (Q87N04), NhaP of *Pseudomonas aeruginosa* (G3XD29), NhaP2 of *V. parahaemolyticus* (Q87KV8), NhaP2 of *V. cholerae* (A5F4U3), Nha1 of *S. cerevisiae* (Q99271), Sod2 of *Schizosaccharomyces pombe* (P36606), Sod22 of *S. pombe* (O14123), and NhaK of *Bacillus subtilis* (O32212) as the query (Altschul et al., 1997). The six Nha type antiporter homologs encoded in *A. tropicalis* SKU1100 genome sequence was manually selected by BLASTP searching against all amino acid sequences using At-NhaK2 (ATPR_0071). The homologous set was selected with a BLASTP filtering expectation value (e-value) $\leq 10^{-10}$ and sequence overlap $\geq 70\%$. All hits were collected and aligned using MUSCLE v.3.8.31 with amino acid sequence level, and used for phylogenetic construction (Edgar, 2004a, b) (Edgar, 2004; Edgar, 2004). Pfam version 26.0 was downloaded from the Pfam FTP site at <ftp.sanger.ac.uk/pub/databases/Pfam/> and used for the motif search analysis (Finn et al., 2010). The motif conserved in C-terminal domain of antiporter homologs were searched using the HMMER version 3.0 package with e-value $\leq 10^{-4}$ (Eddy, 2008).

The detected motif sequences were used for the input of phylogenetic tree construction.

The MEGA version 5.05 package was used to generate the phylogenetic tree to study the phylogenetic relationships using the neighbor-joining (NJ) approach with 1,000 bootstrap replicates (Tamura et al., 2007; Tamura et al., 2011). Functionally validated protein sequences used as the query for BLASTP searches were also included in the phylogenetic tree.

3. Results

3.1 The putative Na^+/H^+ antiporter is required for high-temperature growth of *A. tropicalis* SKU 1100

As previously reported, one of the thermosensitive *Tn10* transconjugants of *A. tropicalis* SKU1100, No. 3-45, was identified to contain a disruption in a gene encoding a putative Na^+/H^+ antiporter (Soemphol et al., 2011), which was named ~~AtNKh~~ **At-NhaK2** based on the results described below.

To examine the physiological function of this antiporter, we constructed a disruption mutant by insertion of a kanamycin resistance cassette, as described in Materials and Methods. As shown in Fig. 1, growth of the mutant (3-45::Km) was impaired at high temperatures (37°C or 39°C), especially in the presence of ethanol, consistent with our previous report that mutation of this gene by transposon insertion resulted in cells with greater sensitivity to ethanol and/or its oxidation product, acetic acid, as well as high temperatures (Soemphol et al., 2011). These growth defects could be restored by complementation with the ~~AtNKh~~ **At-NhaK2** gene (pCM3-45) harbored on a shuttle vector (pCM62), while the empty vector did not affect the growth phenotype. Since this antiporter was hypothesized to play an important role in the regulation of

intracellular ion and/or pH homeostasis, growth ability was also examined by dot test on YPG agar plates, in which the pH was adjusted using sodium (Na^+) phosphate or potassium (K^+) phosphate buffer. As shown in Fig. 2, the antiporter mutant exhibited a dramatic growth defect even at 30°C on YPG medium containing K^+ but not Na^+ at pH 7.0 and 7.5. The growth defect was more significant at 39°C and was observed even at acidic pH conditions (pH 5.6–6.6) in the presence of K^+ , and at pH values >7.0 in the presence of Na^+ . The mutant also exhibited a certain growth impairment when grown in unbuffered liquid YPG (~pH 6.3) with high concentrations of K^+ , even at 30°C (Fig. 3A); this inhibition was observed at KCl concentrations higher than 90 mM (data not shown), but not when NaCl was added up to 100 mM (Fig. 3C). The mutant exhibited a growth defect at 39°C, which could be recovered by the addition of 50 mM or 100 mM NaCl (Fig. 3D), but not by the addition of KCl (Figs. 3B). These results indicate that disruption of the transporter gene prevented the cells from growing at higher temperatures (e.g. 39°C) and increased their sensitivity to high concentrations of K^+ , even at 30°C. The addition of Na^+ partially restores the growth of the mutant strain at higher temperatures.

3.2 Cation- and pH-dependency of *At-NhaK2* ~~*AtNKh2*~~

To determine whether this transporter is able to transport K^+ or Na^+ ions, we examined the internal pH change in ISO membrane vesicle prepared from cells of wild type and mutant *A. tropicalis* SKU1100 strains using AO fluorescence quenching, as described in Materials and Methods. Initially, the integrity (orientation and tightness) of the ISO membrane vesicles prepared was confirmed as described in Materials and Methods, and the vesicles were shown to generate a high and stable inside positive

1 membrane potential by lactate-dependent respiration (data not shown). As shown in
2 Fig. 4B, addition of lactate led to rapid acidification inside the ISO membrane vesicles
3 due to a respiration-dependent H^+ -pump; the acidification was partially reverted by the
4 subsequent addition of NaCl or KCl, indicating the presence of Na^+ or K^+ -dependent
5 H^+ antiporter activity. Therefore, in order to estimate the activity of the actual target
6 antiporter, activity was measured in ISO membrane vesicles from both wild type and
7 mutant strains. As shown in Fig. 4C, ISO membrane vesicles from the wild type strain
8 exhibited a Na^+ -dependent ΔpH change and a lower K^+ -dependent ΔpH change at all
9 pH values examined (pH 6.5, 7.5, and 8.5). In contrast, mutant vesicles exhibited
10 almost no pH change at pH 7.5 and 6.5, while demonstrating a similar Na^+ -dependent
11 ΔpH change (alkalinization) at pH 8.5. However, there was no apparent K^+ -dependent
12 ΔpH change at pH 8.5. Thus, both Na^+ and K^+ -dependent ΔpH change (alkalinization)
13 were absent in the mutant strain at pH 6.5 and 7.5, while only Na^+ -dependent
14 alkalinization was retained at pH 8.5.

15 To further elucidate the cation selectivity of [At-NhaK2](#) ~~AtNKh~~, we examined the
16 kinetic properties of the antiporter with various concentrations of NaCl or KCl at pH
17 6.5, 7.5 and 8.5 (Fig. 5). Antiporter activity in the wild type was enhanced with
18 increasing KCl concentrations and the highest activity was observed at pH 6.5 (Fig. 5A
19 lower panel), while the highest activity for Na^+ was observed at alkaline pH. In
20 contrast, as described above, antiporter activity was almost undetectable in the mutant
21 strain at pH 6.5 and 7.5, except for Na^+ -dependent activity at pH 8.5 (Fig. 5B upper
22 panel) and K^+ -dependent acidification at pH 6.5. Thus, to determine the actual activity
23 of [At-NhaK2](#) ~~AtNKh~~, the antiporter activity was roughly estimated by subtracting the
24 values of the mutant vesicles (Fig. 5B) from the values of the wild type vesicles (Fig.

5A), as indicated in Fig. 5C. However, as described below, there are several *At-NhaK2* ~~*AtNkha*~~ paralogs in this strain, the expression of which might be altered by the disruption of ~~*AtNkha*~~ *nhaK2*; therefore, it is difficult to accurately ascertain the activity of *At-NhaK2* ~~*AtNkha*~~. Nonetheless, data (Fig. 5C) indicate that *At-NhaK2* ~~*AtNkha*~~ has a slightly higher affinity for Na⁺ (K_m=12~13 mM) than K⁺ (K_m = 24~28 mM) at pH 6.5 and 7.5. Moreover, this antiporter was able to translocate Li⁺ and Rb⁺ as well as Na⁺ and K⁺, as demonstrated in the drastic decrease of antiporter activity in the mutant strain compared with the wild type under the same conditions (Fig. 6). These results indicate that *At-NhaK2* ~~*AtNkha*~~ has broad substrate spectrum.

~~substrate specificity.~~

3.3 Phylogenetic relationship of *At-NhaK2* ~~*AtNkha*~~ among bacterial Na⁺/H⁺ antiporter family

Based on the draft genome sequence of *A. tropicalis* SKU1100, six putative Na⁺/H⁺ antiporter homolog genes (locus ATPR_0071, ATPR_0984, ATPR_1229, ATPR_2368, ATPR_2573, and ATPR_3013) were identified by *in silico* analysis, of which ATPR_0071 corresponded to *At-NhaK2* ~~*AtNkha*~~. The phylogenetic tree of all known bacterial Na⁺/H⁺ antiporters, including the enzymes of the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and the six *A. tropicalis* paralogs, resolved into nine clades (Fig. 7). The NhaA, NhaB, NhaK, NhaP, NhaP2, and Yeast Nha clades included antiporters that had been experimentally validated. *At-NhaK2* ~~*AtNkha*~~ (ATPR_0071) was located in one of three unknown clades (~~unknown-I~~ now *NhaK2*) that contained the antiporters of all major AABs, including *Granulibacter*, as well as a number of α- and β-proteobacteria and an exceptional γ-proteobacteria strain,

Frateuria aurantia, which has been shown to possess a unique AAB enzyme (Trček and Matsushita, 2013) (Trček J and K. Matsushita, 2013) (data not shown). The other five *A. tropicalis* paralogs are present in the NhaK (ATPR_0984), NhaP (ATPR_1229, ATPR_2368 and ATPR_3013), and unknown-I (ATPR_2573) clades, but not in the NhaA, NhaB, or NhaP2 clades (Fig. 7). Besides the unique phylogenetic location, At-NhaK2 was also shown to be structurally unique, as described in Discussion section, and have a unique cyclic nucleotide (cNMP)- binding motif of about 120 residues (Pfam accession No.: PF00027) in the large hydrophilic C-terminal region. Therefore, in order to know whether or not the antiporter activity is regulated by cNMPs, we examined the effect of cyclic adenosine 3', 5'-monophosphate (cAMP) and cGMP on the Na⁺- and K⁺-dependent antiporter activity of At-NhaK2 with the ISO vesicles. However, we could not see any significant effects of these nucleotides on the antiporter activity (data not shown). Then, we searched this motif against 963 antiporters using HMMER package (see Table S2), together with fully characterized cNMP-gated ion-channels (AtCNGC2 and AtCNGC4) of *Arabidopsis thaliana* (Gobelt et al. 2006). The results clearly indicated that the cNMP binding motif is mainly conserved in almost all the antiporters from the clade of “NhaK2”, and exceptional four antiporters from the clades of “NhaP” and “Unknown II” (Table S2), and that the phylogenetic tree was clearly divided into two large clades, of which the one clade includes all antiporters classified into NhaK2 group while another clade does exceptional bacterial antiporters of the NhaP and Unknown II groups together with *A. thaliana* ion-channels (Fig. 8).

4. Discussion

To the best of our knowledge, this study presents the first direct physiological characterization of an AAB Na^+/H^+ antiporter using ISO membrane vesicles. The results clearly indicate that At-NhaK2/AtNkha functions as a cation and H^+ exchanger with broad substrate specificity, and is essential for growth at higher temperatures as well as K^+ tolerance.

Not surprisingly, there are many examples of Na^+/H^+ antiporters located in the bacterial membrane that maintain cytosolic ion and pH homeostasis, and several bacterial Na^+/H^+ antiporters responsible for extrusion of Na^+ have been reported. In *E. coli*, NhaA and NhaB play an important role as the major secondary Na^+ -extruding system (Padan et al., 2005; Padan et al., 2001)(Padan et al., 2001; Padan et al., 2005) and exhibit specific $\text{Na}^+(\text{Li}^+)/\text{H}^+$ exchange activity (Pinner et al., 1994; Taglicht et al., 1991)(Taglicht et al., 1991; Pinner et al., 1994). *E. coli* NhaA (EcNhaA) is a pH-dependent Na^+/H^+ antiporter that is more active at alkaline pH, and required for high salinity adaptation and Li^+ resistance (Padan et al., 1989). NhaB seems to play a housekeeping role but is crucial in strains lacking NhaA (Pinner et al., 1993b)(Pinner et al., 1993). Similarly, NhaA and NhaB were shown to confer Na^+ resistance in *Vibrio cholerae* and *Yersinia pestis* (Herz et al., 2003; Minato et al., 2013). The *Pseudomonas aeruginosa* Na^+/H^+ antiporter, NhaP, was also shown to have the ability to exchange H^+ for Na^+ but marginal for Li^+ at alkaline pH (Utsugi et al., 1998). Unlike EcNhaA, *Helicobacter pylori* NhaA (HpNhaA) was shown to be active for Na^+ at acidic to neutral pH (Inoue et al., 1999). In addition to Na^+ extrusion, K^+ transport is also important for cell growth; microorganisms need to maintain an intracellular K^+ concentration higher than that outside the cell, while at the same time, excess extracellular K^+ can be toxic for cells at alkaline pH (Radchenko et al., 2006). NhaP2

of *Vibrio parahaemolyticus* (VpNhaP2) was shown to be specifically involved in K^+/H^+ exchange at alkaline pH, while NhaA and NhaB possess certain K^+ exchange activity in addition to Na^+ transport (Radchenko et al., 2006). Furthermore, NhaP2 of *V. cholerae* (VcNhaP2), which is able to catalyze electroneutral $K^+(Rb^+)/H^+$ exchange with a pH optimum of approximately 7.75, was shown to be essential for resistance to high external K^+ concentrations during growth at pH 6.0 (Resch et al., 2010). Similarly, *Bacillus subtilis* NhaK (BsNhaK) has the ability to extrude several cations, including K^+ , Li^+ , Rb^+ , as well as Na^+ (Fujisawa et al., 2005).

These antiporters could be divided into nine clades, based on the constructed phylogenetic tree (Fig. 7). Of the six paralogs identified in the *A. tropicalis* genome, ATPR_1229, ATPR_2368, and ATPR_3013 are positioned in the NhaP clade, ATPR_0984 in the NhaK clade, while ATPR_0071 and ATPR_2573 are in the unknown clades. At-NhaK2 (ATPR_0071) is unique among the six paralogs; HMMTOP (Tusndy and Simon, 1998, 2001) analysis predicts that it has a very large C-terminal hydrophilic domain (~460 amino acid residues) located inside the cytoplasm following 13 transmembrane helices (~410 amino acid residues). The other five paralogs present in the *A. tropicalis* genome are relatively small (374~562 amino acid residues) and have 11 to 13 transmembrane helices, similar to other bacterial counterparts. Thus, At-NhaK2 (ATPR_0071) seems to be a novel type of antiporter. In addition, we could also find a characteristic cNMP-binding domain in the unique C-terminal hydrophilic portion of At-NhaK2. However, at the same time, we could not detect any functional effect of cAMP and cGMP on the antiporter activity of At-NhaK2. Since the cNMP binding domain of At-NhaK2 was included in one large clade consisting of NhaK2 group

antiporters, which is clearly distinct from another phylogenetical clade including *A. thaliana* ion channels of which the cNMP domain was functionally validated (Fig. 8). Thus, function of the unique domain in At-NhaK2 is not clear at this moment, but it may work with some other molecule(s) for the regulation of At-NhaK2 activity.

~~At-NhaK2~~AtNKha shows unique characteristics of broad substrate specificity for cation exchange, and maximum activity at acidic to neutral pH values. These characteristics are different from those of EcNhaA (Padan et al., 2004b)(Padan *et al.*, 2004) or PaNhaA (Utsugi et al., 1998) both in terms of substrate specificity and optimum pH, but similar to those of HpNhaA in terms of pH optimum (Inoue et al., 1999), and to those of BsNhaK (Fujisawa et al., 2005) in terms of substrate specificity. The activity of both VpNhaP2 (Radchenko et al., 2006) and VcNhaP2 (Resch et al., 2010) is similar to that of AtNKha for K⁺ at alkaline pH and Na⁺ at relatively neutral pH (pH 7.75). Furthermore, yeast antiporters possess properties similar to those of ~~At-NhaK2~~AtNKha; the Nha1 antiporter of *Saccharomyces cerevisiae* can efflux both Na⁺ and K⁺ at acidic pH, suggesting greater importance for K⁺ tolerance (BaAueIos et al., 1998), and *Schizosaccharomyces pombe* Sod2 and Sod22 exhibit broad substrate specificity at acidic pH (Papouskova and Sychrova, 2007). Thus, similar to yeast antiporters, ~~At-NhaK2~~AtNKha seems to function in both Na⁺ and K⁺ efflux at acidic pH, the natural living environment of AAB.

As shown in Fig. 2, the ~~AtNKha~~~~At-nhaK2~~ mutant strain exhibits a severe growth defect at both high temperature and/or alkaline pH conditions, especially in the absence of Na⁺ ions (with only the presence of K⁺), suggesting that ~~At-NhaK2~~AtNKha is involved in K⁺-dependent pH homeostasis at a broad range of pH. High temperature could result in an increase in membrane permeability, allowing for greater diffusion of

Na⁺, H⁺ and K⁺, thus disturbing the internal pH homeostasis (Coote et al., 1991; Konings et al., 2002). In *S. cerevisiae*, two antiporters involved in K⁺ efflux have been reported to participate in the detoxification and/or cell response to various extracellular stress, including non-optimal temperatures (Marešová and Sychrová, 2010). Moreover, in mammalian cells, it has been reported that there is a linear relationship between thermosensitivity and internal pH, which could be mediated by a Na⁺/H⁺ antiporter (Liu et al., 1996a; Liu et al., 1996b) (~~Liu et al., 1996; Liu et al., 1996~~). The results obtained in this study suggest that ~~At-NhaK2~~ *AtNKh*a functions mainly in pH homeostasis by K⁺ efflux or influx at acidic to neutral pH, and that this activity is especially important at higher growth temperatures. In fact, bacteria maintain a much higher intracellular K⁺ concentration ~~than Na⁺~~, which may be disturbed at higher temperatures, as described above; proper concentrations could be maintained by ~~At-NhaK2~~ *AtNKh*a and the other paralogous antiporters at higher temperatures. A recent expression analysis study in another *Acetobacter* species, *A. pasteurianus* SKU1108, has indicated that expression of the ~~At-NhaK2~~ *AtNKh*a (ATPR_0071) and ATPR_1229 orthologs, *APT_0405* and *ATP_2007*, increased by 62% and 76%, respectively, when the growth temperature was increased from 37°C to 40°C (unpublished data).

In addition, the findings that the ~~*nhaK2*-disrupted~~ *AtNKh*a mutant strain could grow in the presence of Na⁺ at alkaline conditions at 30°C (Fig. 2) and that its growth could be partially recovered by the addition of NaCl at 39°C (Figs. 2 & 3) suggest the presence of an additional Na⁺/H⁺ antiporter(s), which might be activated by the proper level of Na⁺, in the cells. Although *A. tropicalis* has five other Na⁺/H⁺ antiporter paralogs (located in the NhaK, NhaP, and unknown-II phylogenetic clades), it is most

likely, based on the phenotypes of previously validated antiporters, that either ATPR_1229, 2368 or 3013, an ortholog of PaNhaP, maintain pH homeostasis with Na⁺ at neutral to alkaline pH. As described above, the expression of these antiporters also increased at higher growth temperatures.

Thus, it is conceivable that [At-NhaK2](#) ~~AtNha~~ plays an important role in K⁺ and pH homeostasis at acidic to neutral pH, which could be greatly disturbed at higher temperatures. However, additional Na⁺-specific antiporters could partially support cell growth at these conditions.

Acknowledgments

This research work was financially supported by the Advanced Low Carbon Technology Research and Development Program (ALCA) funded by the Japan Science and Technology Agency. [This work was also supported partially by a Grant-in-Acid for Scientific Research \(26830126\) from the Ministry of Education, Science, Sports and Culture of Japan to MM.](#) The research collaboration was carried out between Yamaguchi University, Japan and Khon Kaen University, Thailand under the Asian Core Program (ACP). We are deeply grateful to [Aiko Furukawa, Faculty of Agriculture, Yamaguchi University, for her technical assistance and also](#) Masahiro Ito, Faculty of Life Sciences, Tokyo University for his critical reading of this manuscript.

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Figure legends

Fig. 1 Growth comparison of *A. tropicalis* SKU1100 wild type and *nhaK2*-disruptant strains wild type and AtNKha antiporter mutant strains at high temperatures

To confirm the physiological role of At-NhaK2 AtNKha at high growth temperatures, the wild type strain, mutant strain (3-45::Km), complemented strain (3-45::Km [pCM3-45]), and the over-expression strain (wild type pCM3-45) were cultivated with 5 mL of YPGD medium at 30°C for 18 h, collected and resuspended with sterilized water containing 0.85% (w/v) NaCl to the same concentration (approximately 250 of Klett units), and then cells were subjected to tenfold dilutions. 5 µl of serial diluted (10 times each) cells was spotted cultured on YPGD agar plates containing 4% or 6% ethanol (EtOH) and incubated at 30°C, 37°C, and 39°C for 2 days.

Fig. 2 Effect of Na⁺ or K⁺ on the growth of *A. tropicalis* SKU1100 wild type and *nhaK2*-disruptant strains wild type and AtNKha mutant strains at different pH values

A 10-fold serially diluted cell suspension obtained from cells grown to mid-exponential phase by the same way as described in the legend of Fig. 1 was spotted on YPG agar plates prepared by the addition of 50 mM sodium phosphate

(NaPB) or potassium phosphate (KPB) to obtain the indicated final pH (for pH 6.5 and pH 6.0, the actual pH values obtained with NaPB were 6.6 and 6.1, respectively). Cell growth was observed subsequent to two days of incubation at 30°C and 39°C.

Fig. 3 Effect of KCl and NaCl on the growth of *A. tropicalis* SKU1100 wild type and *nhaK2*-disruptant strains wild type and *AtNkha*-mutant strains in YPG medium at 30°C and 39°C

One percent of the pre-culture (300 Klett units) was inoculated into 100 ml of YPG medium without additions or with the addition of KCl (A, B) and NaCl (C, D), and then cultivated with shaking at 200 rpm at 30°C (A, C) or 39°C (B, D). Growth of the wild type (filled symbols) and mutant (un-filled symbols) in the absence (●, ○; solid line) or the presence of 50 mM (■, □; broken line) or 100 mM (▲, Δ; solid line) KCl or NaCl was monitored by measuring turbidity using a Klett-Summerson Photoelectric Colorimeter with a red filter.

Fig. 4 Cation/H⁺ antiporter activity in isolated ISO membrane vesicles

(A) Representative assay scheme for antiporter activity in ISO membrane vesicles. The ΔpH across the ISO membrane vesicles obtained from cells grown on YPG medium was determined using acridine orange (AO), as described in Materials and Methods. (B) Comparison of fluorescence dequenching due to the Na⁺(K⁺) /H⁺ antiporter in ISO membrane vesicles prepared from SKU 1100 wild type and 3-45::Km mutant (*nhaK2*-disruptant) strains measured with the addition of 20 mM NaCl (solid line) or 20 mM KCl (dotted line) at pH 7.5. (C) Measurements performed in ISO membrane vesicles from wild type (WT) and mutant (3-45::Km) strains with the

addition of 20 mM NaCl (gray bar) or 20 mM KCl (white bars) at pH 6.5, 7.5, and 8.5.

All experiments were repeated at least three times, and standard deviations are shown.

Fig. 5 Comparison of the kinetic properties of the *At-NhaK2* ~~*AtNKh*~~ antiporter at different pH values

Antiporter activity in wild type (A) and mutant 3-45::Km (B) strains was measured with 0.3–60 mM NaCl (upper panels) or KCl (lower panels) at pH 6.5 (open circles: solid line), 7.5 (gray squares: broken line), and 8.5 (black triangles: dotted line), respectively. The antiporter activity (apparent Km values for Na⁺ and K⁺) of *At-NhaK2* ~~*AtNKh*~~ (C), estimated by subtracting the values of the mutant strain (B) from the values measured in the wild type (A), are 13.7 or 23.6 mM at pH 6.5, and 12.5 or 28.3 mM at pH 7.5.

Fig. 6 Antiporter cation specificity

ΔpH generation in ISO membrane vesicles of wild type (hatched bar) and mutant 3-45::Km (white bar) strains was examined with several cations. The reactions were initiated by the addition of 4 mM Tris-lactate (pH 7.5), followed by the addition of 20 mM each of LiCl, NaCl, KCl, RbCl, CsCl or CaCl₂ and NH₄Cl. Fluorescence quenching of acridine orange (AO) was monitored as described in Materials and Methods.

Fig. 7 Unrooted neighbor-joining phylogenetic tree of Na⁺/H⁺ antiporter proteins from all known bacterial genera and two yeast strains. The phylogenetic tree of 963 antiporter homologs was constructed using MEGA 5.05. The scale bar represents 0.2

substitutions per site. The 6 antiporter homologs of *A. tropicalis* and 12 experimentally validated functional sequences, described in Materials and Methods, are designated by closed and open circles, respectively. The antiporters resolved into 9 clades: Yeast Nha (including Sc Nha1, Sp Sod2, and Sp Sod22), NhaA (Hp NhaA, Vp NhaA, and Ec NhaA), NhaB (Ec NhaB and Vp NhaB), NhaK (Bs NhaK and ATPR_0984), NhaP (Pa NhaP, ATPR_1229, ATPR_2368, and ATPR_3013), NhaP2 (Vp NhaP2 and Vc NhaP2), Unknown-I (ATPR_0071), Unknown-II (ATPR_2573), and Unknown-III (no known candidates). Strain abbreviations are as follows: Bs: *Bacillus subtilis*, Ec: *Escherichia coli*, Hp: *Helicobacter pylori*, Pa: *Pseudomonas aeruginosa*, Sc: *Saccharomyces cerevisiae*, Sp: *Schizosaccharomyces pombe*, Vc: *Vibrio cholerae*, Vp: *Vibrio parahaemolyticus*.

Fig. 8 Neighbor-joining phylogenetic tree of cNMP binding motif conserved in 23 antiporters. The phylogenetic tree was constructed using MEGA 5.05. The scale bar represents 0.2 substitutions per site. More than 50% bootstrap values are shown. Two fully functional validated motif from *Arabidopsis thaliana* (GenBank acc. No.: O65718 and Q94AS9) was used as the reference data set. The tree was divided into NhaK2 group consisting of the antiporters of α - and β -proteobacteria, and another group of the antiporters from two clades “Unknown II” and “NhaP”, and also of *Arabidopsis*.

Figure

Figure 1

Figure 2

Figure 3

C

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8