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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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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 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)/H^+ antiporter(s) that might function at alkaline pH values. In addition, phylogenetic analysis showed that At-NhaK2 is a novel type of Na^+/H^+ antiporter belonging to a phylogenetically distinct new clade. These data demonstrate that At-NhaK2 functions as a $Na^+(K^+)/H^+$ antiporter and is essential for K^+ and pH homeostasis during the growth of *A. tropicalis* SKU1100, especially at higher temperatures.

1. Introduction

Stable and efficient fermentation requires robust microbes to overcome various stressors such as temperature, acids, and alcohols generated during the fermentation 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 (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 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

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

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and resistance to acetic acid (Gullo and Giudici, 2008; Stasiak and Błażejak, 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 5–10 °C higher temperatures, compared with the corresponding mesophilic strains (Moonmangmee et al., 2000; Saeki et al., 1997). In 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-C_2(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). *E. coli* DH5α 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 μg/ml; kanamycin (Km), 50 μg/ml; and ampicillin (Ap), 50 μg/ml. Bacterial growth was monitored using a Klett-Summerson Photoelectric Colorimeter with a red filter.

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 μl 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.

2.3. 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 7700 × *g* for 10 min and washed twice with 10 mM Tris-acetate buffer (pH 6.5) containing 5 mM $MgSO_4$. The cell pellets were then resuspended in the same buffer containing 10 μg/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 5000 psi. Subsequent to the removal of cell debris by centrifugation at 8000 × *g* for 20 min (×2), the supernatant was centrifuged at 100,000 × *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 $MgSO_4$ 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-C_2(5) dye representing the inside positive membrane potential. The reaction mixture (1 ml) contained 10 mM Tris-acetate, 5 mM $MgSO_4$, 20 mM K_2SO_4, 2 μM diBA-C_2-(5), and the ISO membrane vesicles (300 μg of protein), and the reaction was carried out at 25 °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 μM nigericin, an ionophore able to catalyze an electroneutral exchange of K^+ for H^+, to increase a membrane potential by dissipating a pH gradient, and 0.4 μM valinomycin, an ionophore able to catalyze an electrical uniport of K^+ to dissipate a membrane potential completely (Matsushita et al., 2005).

2.4. 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, with some modifications of the previous report (Mesbah et al., 2009). The reaction mixture consisting of 10 mM Tris-acetate buffer, 5 mM $MgSO_4$, 140 mM choline-chloride, 1.5 micromolar 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 alkalinization) in the ISO membrane vesicles. Finally, the remaining ΔpH was completely dissipated by the addition of 20 mM NH_4Cl, which reverted the flu-