การโคลนและการแสดงออกของยีน Na^+/H^+ แอนติพอร์เตอร์จากสาหร่ายขนาดเล็ก

นางสาวเกียรติสุดา ดาวุธ

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CLONING AND EXPRESSION OF Na^+/H^+ ANTIPORTER GENE FROM MICROALGAE

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เกียรติสุดา ดาวุธ : การโคลนและการแสดงออกของยีน Na^+/H^+ แอนติพอร์เตอร์จากสาหร่ายขนาดเล็ก (CLONING AND EXPRESSION OF Na^+/H^+ ANTIPORTER GENE FROM MICROALGAE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รุ่งอรุณ วาดิถี สิริศรัทธา, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: โสภณ สิริศรัทธา, 126 หน้า.

Na^+/H^+ แอนติพอร์เตอร์เป็นทรานส์เมมเบรนโปรตีนที่พบทั่วไปและเป็นโปรตีนขนส่งแบบใช้พลังงานทุติยภูมิ ที่พบได้ในสิ่งมีชีวิตทุกชนิด หน้าที่พื้นฐานของเมมเบรนโปรตีนคือควบคุมปริมาณของโซเดียมไอออน นอกจากนี้ยังทำหน้าที่เกี่ยวข้องกับการรักษาสมดุลของพีเอช วัฏจักรเซลล์และการเพิ่มจำนวนเซลล์ มอร์โฟเจเนซิสและเวซิเคิล ทราฟฟิกกิ้ง ปัจจุบัน Na^+/H^+ แอนติพอร์เตอร์ได้รับการศึกษาอย่างกว้างขวางใน แบคทีเรีย พืชชั้นสูงและสัตว์เลี้ยงลูกด้วยนม ในทางตรงกันข้ามมีการศึกษา Na^+/H^+ แอนติพอร์เตอร์ในสาหร่ายขนาดเล็กน้อยมาก ในการศึกษานี้ Na^+/H^+ แอนติพอร์เตอร์ ชื่อ NhaA จากสาหร่ายขนาดเล็กน้ำเค็ม *Ostreococcus tauri* (*OtNhaA*) ถูกโคลนและแสดงออกในมิวแทนท์ที่ไวต่อเกลือ *Escherichia coli* TO114 (Δ*nhaA*Δ*nhaB*Δ*chaA*) โมเดลโทโปโลยีของ OtNhaA ทำนาย 10 ทรานส์เมมเบรน เซกเมนท์กับปลายด้าน N ขนาดยาว ทั้งโปรตีน OtNhaA รูปเต็ม (FL_OtNhaA) และทรังเคชั่นปลายด้าน N (ΔN112_OtNhaA) ถูกสร้าง เวสเทิร์นบลอตติ้ง แสดงให้เห็นว่าโปรตีนเหล่านี้สามารถแสดงออกและประกอบตัวได้ใน *Escherichia coli* TO114 การทดสอบคอมพลีเมนต์ เผยให้เห็นว่า เซลล์แสดงออก FL_OtNhaA และ ΔN112_OtNhaA เพิ่มความทนต่อความเข้มข้นของเกลือโซเดียมคลอไรด์ที่สูงถึง 700 มิลลิโมลาร์ (ที่พีเอช 7) แอนติพอร์เตอร์แอสเสย์ โดยใช้วิธีการอะคริดีนเควนชิ้ง แสดงให้เห็นว่าทั้ง FL_OtNhaA และ ΔN112_OtNhaA แสดงแอนติพอร์เตอร์แอกทิวิตี้ อย่างเด่นชัด สำหรับ Na^+/H^+ และ Ca^{2+}/H^+ และมีค่าดีเควนชิ้งสูงสุดที่พีเอช 8.5 เป็นที่น่าสนใจว่า ΔN112_OtNhaA สามารถแสดงแอกทิวิตี้ Na^+/H^+ และ Ca^{2+}/H^+ สูงกว่าที่ตรวจพบใน FL_OtNhaA การวิเคราะห์จลนพลศาสตร์ แสดงให้เห็นว่า ยีน *OtNhaA* เข้ารหัสให้ Na^+/H^+ หรือ Ca^{2+}/H^+ แอนติพอร์เตอร์ ชนิดสัมพรรคภาพสูงด้วยค่า K_m สำหรับ Na^+ 1.1 ± 0.23 มิลลิโมลาร์ (ที่พีเอช 8.5) และค่า K_m สำหรับ Ca^{2+} 0.3 ± 0.07 มิลลิโมลาร์ (ที่พีเอช 8.5) นี้เป็นรายงานแรกของการศึกษาลักษณะเชิงสมบัติของ Na^+/H^+ แอนติพอร์เตอร์จากสาหร่ายขนาดเล็ก

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KEATISUDA DAWUT: CLONING AND EXPRESSION OF Na^+/H^+ ANTIPORTER GENE FROM MICROALGAE. ADVISOR: ASST. PROF. RUNGAROON WADITEE-SIRISATTHA, Ph.D., CO-ADVISOR: SOPHON SIRISATTHA, Ph.D., 126 pp.

Na^+/H^+ antiporter is ubiquitous transmembrane protein. It is secondary active transporter, found in all living organisms. Fundamental role of this membrane protein is to regulate Na^+. In addition, it is involved in pH homeostasis, cell cycle and proliferation, morphogenesis, and vesicle trafficking. To date, Na^+/H^+ antiporters have been extensively studied in bacteria, higher plants, and mammals. In contrast, little is known on microalgal Na^+/H^+antiporters. In this study, the putative NhaA Na^+/H^+ antiporter from marine microalga *Ostreococcus tauri* (*OtNhaA*) was cloned and expressed in the salt-sensitive mutant *Escherichia coli* TO114 (Δ*nhaA*Δ*nhaB*Δ*chaA*). Topological model of OtNhaA predicted 10 transmembrane segments with a long N terminus. Both full length OtNhaA (FL_OtNhaA) and N –terminal truncation OtNhaA (ΔN112_OtNhaA) proteins were constructed. Western blotting revealed that these proteins could be expressed and assembed in *Escherichia coli* TO114. Complementation test revealed that FL_OtNhaA and ΔN112_OtNhaA expressing cells increased tolerance to high concentration up to 700 mM NaCl (at pH 7). Antiporter assay using acridine quenching method revealed both FL_OtNhaA and ΔN112_OtNhaA predominantly exhibited Na^+/H^+ and Ca^{2+}/H^+ antiporter activities with maximal dequenching at pH 8.5. Interestingly, the ΔN112_OtNhaA could exhibit Na^+/H^+ and Ca^{2+}/H^+ antiporter activities higher than those of FL_OtNhaA. Kinetic analysis revealed that *OtNhaA* encodes a high affinity Na^+/H^+ or Ca^{2+}/H^+ antiporter 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). To our knowledge, this is the first report of functional characterization of Na^+/H^+ antiporter from microalgae.

Field of Study: Biotechnology
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Student's Signature .....
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LIST OF ABBREVIATIONS

°C	Degree Celsius
kDa	Kilodalton
g	Gram
µg	Microgram
µl	Microliter
hr	Hour
l	Liter
mA	Milliampare
min	Minute
ml	Milliliter
mM	Milli Molar
nm	Nanometer
PCR	Polymerase Chain Reaction
rpm	Revolution per minute
NaCl	Sodium chloride
LiCl	Lithium chloride
KCl	Potassium chloride
$CaCl_2$	Calcium chloride
SDS	Sodium Dodecyl Sulphate
$\Delta\mu H^+$	Proton electrochemical gradient

CHAPTER I

INTRODUCTION

Sodium ion (Na^+) is an essential element for living organisms. In mammals, Na^+ is responsible for controlling the total amount of water in body (Dahl & Love, 1954). Na^+ is used against potassium ion to build up charges on cell membranes in animals (Zuber *et al.*, 2005). It has been shown that Na^+ is also required for some plants, particularly halophyte (Yamaguchi & Blumwald, 2005). When living organisms grow under hypersaline environment where the salinity is high (much higher than of seawater 35 practical salinity unit; psu), Na^+ diffuses into the cytoplasm, thus the osmotic pressure is not balance that can toxic to cells. This causes the decrease the growth rate of living organisms and can also inhibit metabolisms of cells (Apse *et al.*, 1999, Waditee *et al.*, 2002). Therefore, extrusion of Na^+ is crucial to balance and maintain sodium content under hypersaline condition (Hagemann, 2011). The major mechanism to maintain Na^+ is Na^+/H^+ antiporter inwhich exchanges Na^+ for H^+ across the membrane in opposite direction.

Na^+/H^+ antiporter is ubiquitous transmembrane protein, found in all living organisms. It plays a primary role in Na^+ regulation and pH homeostasis. This membrane protein is secondary active transporter using the energy stored in pre-existing gradient to drive transport. The major activity is transport Na^+ and H^+ across the membrane in opposite direction using proton electrochemical gradient ($\Delta\mu H^+$). The energy of transport Na^+ is derived from respiratory electron transport (Kozachkov & Padan, 2013,

Slonczewski *et al.*, 2009). In prokaryotic cells, Na^+/H^+ antiporter is only be found in plasma membrane while in eukaryotic cells can be found in various compartments (Brett *et al.*, 2005, Orlowski & Grinstein, 2007). Moreover, it plays a variety of functions such as internal pH regulation, salt tolerance, transepithelial Na^+ movement, control of cell cycle, cell proliferation, vesicle trafficking and biogenesis (Brett *et al.*, 2005, Hamada *et al.*, 2001).

To date, Na^+/H^+ antiporter has been extensively studied in various living organisms. In *E. coli*, three Na^+/H^+ antiporters (NhaA, NhaB and ChaA) are known and their functional characteristics have been well described (Padan *et al.*, 2004). In yeast, the prevacuole membrane (NHX1) and plasma membrane (SOD2) Na^+/H^+ antiporters have been reported (Hahnenberger *et al.*, 1996, Rao-Naik *et al.*, 1998). In cyanobacteria, Na^+/H^+ antiporters are found in plasma and thylakoid membranes (Elanskaya *et al.*, 2002, Waditee *et al.*, 2006). In higher plants, vacuole and plasma membrane types Na^+/H^+ antiporter have been discovered (Gaxiola *et al.*, 2001). These Na^+/H^+ antiporters play crucial role for salt tolerance. In mammalian cells, there are six types of Na^+/H^+ antiporter (NHE1-6) (Orlowski & Grinstein, 2007). By contrast, little is known about Na^+/H^+ antiporter in microalgae.

In this study, I aim to clone Na^+/H^+ antiporter gene from *Ostreococcus tauri* (*OtNhaA*) and express into the salt-sensitive mutant *E.coli* TO114 (***Δ****nhaA*, ***Δ****nhaB*, ***Δ****chaA*) and *Chlamydomonas reinhardtii* 137c.

The objectives of this research:

1. To analyze Na^+/H^+ antiporter genes in microalgal genomes from bioinformatics databases
2. To clone and express of Na^+/H^+ antiporter gene from microalga *Ostreococcus tauri*
3. To functionally analyze the *O. tauri* Na^+/H^+ antiporter

CHAPTER II

LITERATURE REVIEW

2.1 Regulation of sodium ion

Sodium ion (Na^+) is an essential element for living organisms. In mammals, Na^+ is responsible for controlling the total amount of water in body. It is also important for regulating blood volume and maintaining muscle function (Dahl & Love, 1954). Furthermore, Na^+ is used against potassium ion to build up charges on cell membranes. This is essential for maintenance of electrolyte balance and fluid balance (Zuber *et al.*, 2005). In plants, Na^+ is not essential and it can be used in small quantities as similar to micronutrients. However, Na^+ is also required for some specific plants, particularly halophyte that a plant grows in water of high salinity (Yamaguchi & Blumwald, 2005). When living organisms grow under hypersaline environment where the salinity is high (much higher than of seawater 35 psu), Na^+ diffuses into the cytoplasmic and the osmotic pressure is not balance that can toxic to cells. High Na^+ is toxic to cells, thus it can decrease the growth rate of living organisms. It can also inhibit metabolisms of cells (Apse *et al.*, 1999, Waditee *et al.*, 2002). Therefore, extrusion of Na^+ is crucial to balance and maintain sodium content (Hagemann, 2011). The major mechanism to maintain Na^+ is Na^+/H^+ antiporter in which exchanges Na^+ for H^+ across the membrane in opposite direction using $\Delta\mu H^+$ (Alkoby *et al.*, 2014). The energy of Na^+ transport is derived from respiratory electron transport (Kozachkov & Padan, 2013). In addition, other sources of electrochemical driving force are H^+ -ATPase, Na^+-ATPase or Na^+/K^+

ATPase. In case of yeast and higher plants, only H^+ -ATPase has been found. They do not have Na^+-ATPase or Na^+/K^+ ATPase. In contrast, animal cells contain both Na^+-ATPase or Na^+/K^+ ATPase. The plasma membrane performs to alkalinize the cytoplasm by coupling to the Na^+ electrochemical gradient maintained using Na^+-ATPase or Na^+/K^+ ATPase (Fig 2.1). In case of Na^+/K^+ ATPase, this ATPase is also found in nematodes, insects, fish and mammals (Brett *et al.*, 2005, Elanskaya *et al.*, 2002).

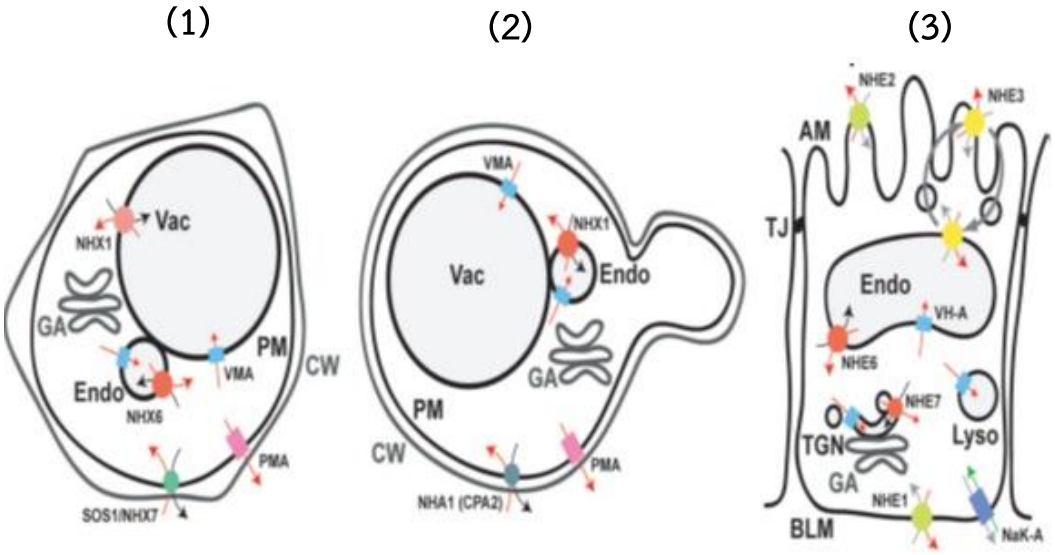


Figure 2.1 Cellular location of cation/proton antiporters (CPA1 transporters) from various organisms. (1) Plant cell, (2) Yeast cell and (3) mammalian epithelial cell. Solid circle represent CPA1 homologs and orthologs. Endosome/TGN (red), SOS1 (dark green), plant vacuolar (pink), NHA1 (CPA2 gene; gray), recycling plasma membrane (yellow) and resident plasma membrane (light green). Filled squares represent ion gradient-providing ATPases. Organellar V-type H^+ -ATPase (VMA or VH-A; light blue), plasma membrane H^+ -ATPase (PMA; pink) and plasma membrane Na^+/K^+ -ATPase (NaK-A; dark blue). Small arrows indicate direction and monovalent cation specificity of transport. Direction of Na^+ and K^+ transport (black), direction of Na^+ transport (gray), direction of K^+ (green) and direction of H^+ (red) (Source from; Brett *et al.*, 2005).

2.1.1 Na^+/H^+ antiporter

Na^+/H^+ antiporter is ubiquitous transmembrane protein found in all living organisms. This membrane protein is secondary active transporter using the energy stored in pre-existing gradient to drive transport. The major activity is transport Na^+ and H^+ across the membrane in opposite direction. The energy of transport Na^+ is derived from respiratory electron transport (Kozachkov & Padan, 2013, Slonczewski *et al.*, 2009). In addition, Na^+/H^+ antiporter is also specific to transport Li^+ for H^+ in some cases (Padan *et al.*, 2005). In prokaryotic cells, Na^+/H^+ antiporter is only be found in plasma membrane while in eukaryotic cells can be found in various compartments. For instance, Na^+/H^+ antiporter is located on vacuole (NHX1), plasma membrane (NHX7) and endosomal (NHX6) in plant cells (Gaxiola *et al.*, 2001). In yeast cells, vacuole membrane (NHX1) and plasma membrane (SOD2) Na^+/H^+ antiporters have been reported (Rao-Naik *et al.*, 1998, Hahnenberger *et al.*, 1996). In mammalian cells, Na^+/H^+ antiporter is found in resident plasma membrane (NHE1-2), recycling plasma membrane (NHE3), endosomal (NHE6) and Golgi apparatus (NHE7) (Brett *et al.*, 2005, Orlowski & Grinstein, 2007).

Fundamental role of Na^+/H^+ antiporter is to regulate Na^+ and pH homeostasis by transport Na^+ and H^+ in opposite direction across the membrane. Furthermore, a variety of functions have been described.

(1) Controlling the conditions inside cells, such as keeping the intracellular pH (Lentes *et al.*, 2014, Paulino & Kuhlbrandt, 2014, Pinner *et al.*, 1993).

(2) Controlling of cell cycle and proliferation such as plasma membrane Na-H exchanger (NHX1) in which regulates intracellular pH homeostasis. It has permissive effect in promoting cell proliferation (Putney & Barber, 2003).

(3) Determining salt tolerance in plant. For example, overexpression of *Arabidopsis thaliana SOS1* gene encoding plasma membrane Na^+/H^+ antiporter confers and improve salt tolerance in transgenic *Arabidopsis thaliana* (Billini *et al.*, 2008, Shi *et al.*, 2003, Yokoi *et al.*, 2002).

(4) Determining salt tolerance in fission yeast *Schizosaccharomyces pombe*. For example, overexpression of plasma membrane Na^+/H^+ antiporter *SOD2* gene increases salt tolerance, whereas loss-of function in Na^+/H^+ antiporter *SOD2* resulted in salt-sensitive phenotype (Jia *et al.*, 1992).

(5) Transepithelial Na^+ movement. In human, Na^+/H^+ exchange (NHE) is essential for albumin absorption in human renal proximal tubule (Zachos *et al.*, 2005).

(6) Vesicle trafficking and biogenesis in yeast *Saccharomyces cerevisiae*. Na^+/H^+ exchange is important for endosomal trafficking. The C-terminal of yeast Nhx1 interacts with Gyp6 (GTPase-activating protein) in endosomal/prevacuole compartment (Ali *et al.*, 2004, Bowers *et al.*, 2000).

(7) Morphogenesis in yeast cells. In *Candida albicans*, deletion of CNH1 encoding Na^+/H^+ antiporter resulted in retardation of growth and elongation morphology of yeast cells (Soong *et al.*, 2000).

2.1.2 Monovalent cation/proton antiporters

In all living organisms, Na^+/H^+ antiporters are crucial for cell energetic and play several crucial roles in homeostasis of intracellular pH as mentioned earlier. To date, various systems have been used for the classification of Na^+/H^+ antiporters. Cation Proton Transport (CPA) is one of good examples for classification. In this system, three families of CPA (CPA1-3) are categorized (Brett *et al.*, 2005, Maser *et al.*, 2001, Morino & Ito, 2012). The CPA1 family is mostly found in bacteria, fungi, metazoa and plants (Fig. 2.2). Most CPA1 transporters are generally electroneutral (i.e. exchange H^+ and Na^+ ions with a 1:1 stoichiometry). The CPA2 family consists of several groups, each of them consists of the prokaryotic members (Fig. 2.2) (Brett *et al.*, 2005, Chanroj *et al.*, 2012). In contrast to CPA1, the CPA2 transporters are electrogenic (i.e. exchange $2H^+$ for $1Na^+$). To date, the roles of plant CPA2 encoding K^+ efflux antiporter (KEA) and cation/H^+ exchanger (CHX) are largely unknown. The best known member of the CPA2 subfamily from prokaryote is the Na^+/H^+ antiporter NhaA from *E. coli* (EcNhaA) (Brett *et al.*, 2005, Padan & Schuldiner, 1994). In addition to CPA1 and CPA2, bacteria have multiple monovalent cation/proton antiporters with a complex structure. They are classified as the CPA3 family. The CPA3 family consists of Mrp-type antiporters (Multiple resistance and pH adaptation). They have been reported to function as hetero-oligomeric monovalent cation/proton antiporters. They could exchange a cytoplasmic monovalent cation (Na^+, Li^+ or K^+) with extracellular H^+. Mrp-type antiporters have been reported to be essential for survival in alkaline or saline environments (Fukaya *et al.*, 2009, Morino & Ito, 2012, Morino *et al.*, 2014, Padan *et al.*, 2005, Swartz *et al.*, 2005, Swartz *et al.*, 2007).

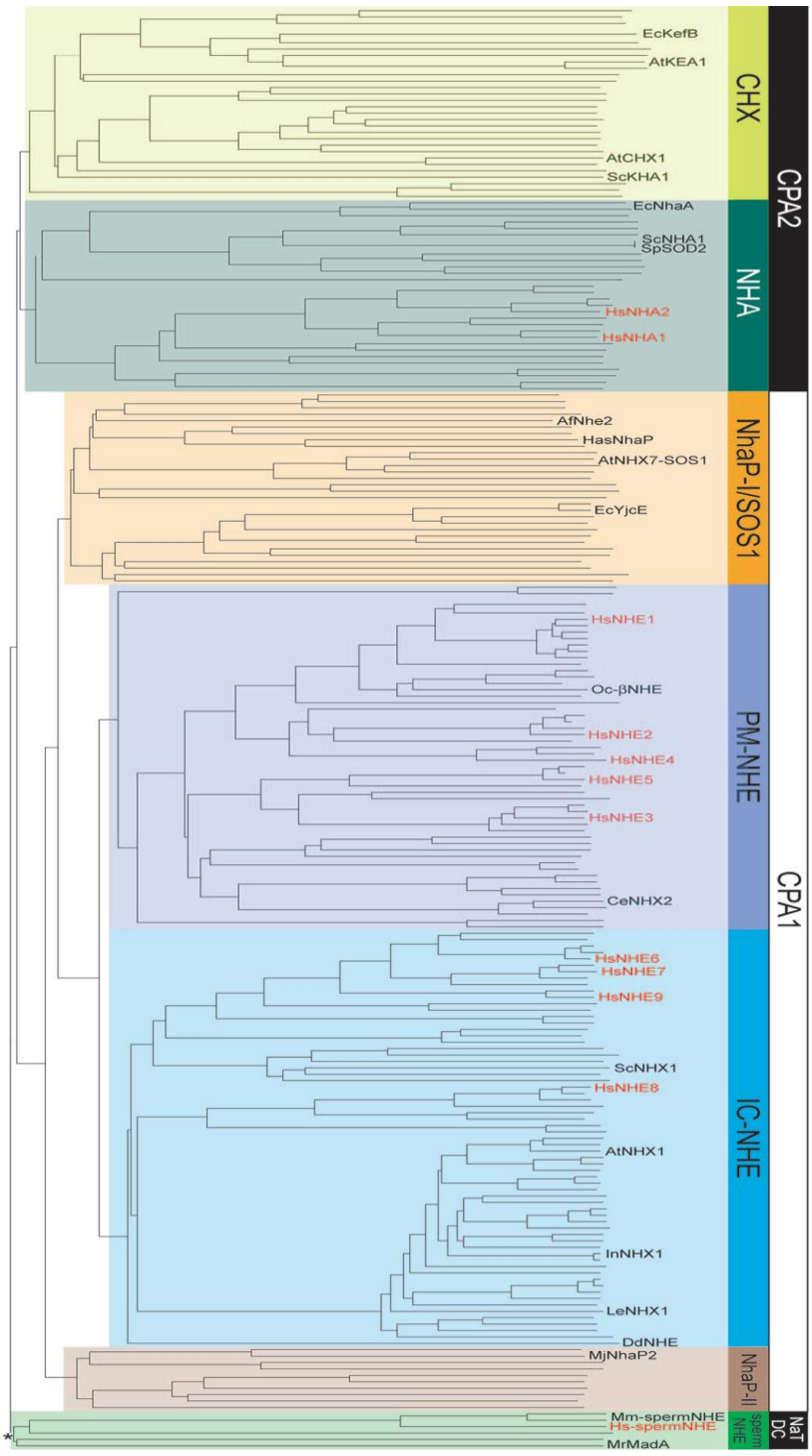


Figure 2.2 Phylogenetic tree of the monovalent cation proton antiporter (CPA), which includes the CPA1 and CPA2 subfamilies (Source from; Brett *et al.*, 2005).

2.1.2.1 Functional property of Na^+/H^+ antiporter in living organisms

To date, Na^+/H^+ antiporter has been functionally characterized in many living organisms. Some examples are described their properties here.

2.1.2.1.1 *Escherichia coli*

In *E. coli,* three Na^+/H^+ antiporters (NhaA, NhaB and ChaA) are known and their functional characteristics have been well described (Padan *et al.*, 2004). NhaA is the first antiporter found in *E. coli*. It is the main Na^+/H^+ antiporter and specifically exchanges between Na^+ or Li^+ for H^+ across membrane. NhaA is indispensable for adaptation to high salinity, resistance to Li^+ toxicity and for growth under alkaline conditions (Dimroth, 1990, Padan *et al.*, 2004). The antiporter activity of NhaA is dependent on pH. It is enhanced at alkaline pH (pH 8.5) and reduced at acidic pH (pH 6.5) (Screpanti *et al.*, 2006). The transcription of NhaA is regulated by protein NhaR. Na^+ is the inducer. The protein NhaR, a member of LysR family, is a regulator of NhaA (Rahav-Manor *et al.*, 1992). *E. coli* NhaA is predicted to have a putative secondary structure consisting of 12 transmembrane segments (TMs) and connected by hydrophilic loop (N and C terminus on the cytoplasmic side of the membrane) (Padan *et al.*, 2004). Furthermore, *NhaA* homologous gene has also been reported in *Helicobacter pylori* (Karasawa *et al.*, 2010, Tsuboi *et al.*, 2003), *Vibrio cholerae* (Herz *et al.*, 2003), *Vibrio parahaemolyticus* (Ganoth *et al.*, 2011), *Yersinia pestis* (Ganoth *et al.*, 2011) and *Salmonella typhimurium* (Lentes *et al.*, 2014).

NhaB is the second antiporter found in *E. coli*, it confers a limited sodium tolerance to bacteria cells. It becomes essential when the lack of NhaA. Furthermore, it is responsible for pH-dependent Na^+/H^+ antiporter (Pinner *et al.*, 1993, Sakuma *et al.*, 1998, Shimamoto *et al.*, 1994).

ChaA is the third antiporter found in *E. coli*, it functions as a Ca^{2+}/H^+ antiporter (Ohyama *et al.*, 1994, Sakuma *et al.*, 1998). It has a physiological role in sodium ion extrusion at alkaline pH (Shijuku *et al.*, 2002). Furthermore, ChaA is not only exchange activity for Ca^{2+}/H^+ but also extrusion activity of Na^+- and K^+ for H^+ (Radchenko *et al.*, 2006).

2.1.2.1.2 *Salmonella typhimurium*

In *S. typhimurium*, Na^+/H^+ antiporter NhaA is crucial for pathogenesis of the causing agent of food-borne human gastroenteritis and typhoid like infections (Lentes *et al.*, 2014). The recombinant NhaA from *S. typhimurium* (STNhaA) was functional characterized both *in vivo* and *in vitro*. Functionality *in vivo* was analyzed using growth complementation of the salt-sensitive phenotype *E. coli* strain EP432. This strain was disrupted two putative Na^+/H^+ antiporter (***Δ****nhaA* and ***Δ****nhaB*). STNhaA functionally complemented the Na^+ or Li^+-sensitive phenotype of *E. coli* strain EP432 at pH 7.5. For NaCl or LiCl, *E. coli* mutant cells transformed with expressing vector *STNhaA* could grow in the presence of 200 mM NaCl or LiCl. The purified STNhaA was reconstituted in proteoliposomes and assayed using solid-supported membrane-based electrophysiological measurement. The antiporter activity of STNhaA is dependent on pH which is similar to *E. coli* NhaA. It has high exchange activity at alkaline pH (pH 9.5)

and low activity at pH 7.0 (Screpanti *et al.*, 2006). Kinetic parameter has shown that, the K_m value of STNhaA for Na^+ was 16 mM at pH 8.5 when the transport rate is maximum, whereas the K_m value for Na^+ was 144 mM at pH 7.0.

2.1.2.1.3 *Helicobacter pylori*

H. pylori is a pathogenic bacterium of gastric inflammation that lives under acidic condition. Functionality of the recombinant NhaA from *H. pylori* (HpNhaA) was analyzed using growth complementation of the salt-sensitive phenotype *E. coli* strains KNabc or HIT**Δ**AB$^-$. The KNabc strain was disrupted three putative Na^+/H^+ antiporter (NhaA, NhaB and ChaA) while HIT**Δ**AB$^-$ strain was disrupted two putative (NhaA and NhaB). HpNhaA functionally complemented the Na^+-sensitive phenotype of two *E. coli* strains at pH 8.0. It also could complemented the Li^+-sensitive phenotype of two *E. coli* strains at pH 7.5 (Inoue *et al.*, 1999). Antiporter activity of HpNhaA was monitored by using ACMA (9-amino-6-chloro-2-methoxyacridine) quenching measurement. The antiporter activity of HpNhaA is pH dependent between 6.5 to 8.5, whereas *E. coli* NhaA is active within the alkaline pH range 7.0 to 8.5. For kinetic measurement, the K_m values of HpNhaA for Na^+ was 101± 5 mM at pH 6.5 and drastically decreased to 7 ± 4 mM at pH 8.0 (Calinescu *et al.*, 2014).

2.1.2.2 Na^+/H^+ antiporter in cyanobacteria

In cyanobacteria, Na^+/H^+ antiporter located in thylakoid and plasma membrane (Elanskaya *et al.*, 2002). From the complete nucleotide sequence of cyanobacterium *Synechocytis* sp. PCC6803, it suggested that this cyanobacterium contains at least five Na^+/H^+ antiporter genes. The functional characterization of cyanobacterium antiporter (SynNhaP) has been reported. SynNhaP shows homology to NhaP from *Pseudomonas aeruginosa*. Furthermore, SynNhaP consist of conserved Asp-138 in transmembrane region and relatively long C-terminal hydrophilic tail (Hamada *et al.*, 2001). The long C-terminal hydrophilic tail is involved in the regulation of transport activity (Waditee *et al.*, 2006).

In fresh water cyanobacterium *Synechococcus elongatus* PCC7942, it consists of seven Na^+/H^+ antiporter genes (*nha1* to *7*). The functional characterization of cyanobacterium antiporters (nha 1 to 7) have been studied. Only *nha3* complemented the Na^+-sensitive *E. coli* TO114. Furthermore, *Δnha3* cells showed high salt and alkaline pH-sensitive phenotype while ***Δ****nha2* cells showed low salt and alkaline pH-sensitivity (Billini *et al.*, 2008).

2.1.2.3 Na^+/H^+ antiporter in higher plants

Salinity is a limiting factor for plant growth in large terrestrial areas of the world. The most area of cultivation is affected by high salinity. It is one of the most important factor to decrease crop yield. In high salinity, excessive Na^+ in saline soil is toxic to plants when it accumulates in the cytoplasm. Therefore, extrusion of Na^+ is critical to balance and maintain osmotic pressure inside cell. In higher plants, the first identified Na^+/H^+ antiporter was reported in *Arabidopsis thaliana* SOS1/NHX7 (salt overly sensitive) (Apse *et al.*, 1999). The SOS1 (AtNHX7) encoded plasma membrane Na^+/H^+ antiporter. The expression of SOS1 is up-regulated by salt stress, particularly in root (Shi *et al.*, 2003). The activity of SOS1 is regulated by protein kinase SOS2 which is in turn activates calcium-binding protein SOS3. In addition, *Arabidopsis thaliana* contains other six Na^+/H^+ antiporter genes (AtNHX1- 6) and many uncharacterized CPA genes (Brett *et al.*, 2005).

2.1.2.4 Na^+/H^+ antiporter in microalgae

Microalgae are photosynthetic organisms, typically found in all habitats from freshwater to marine systems. They are unicellular species which exist individually. Microalgae have short life cycle compare to higher plants. To date, little is known about functional property of Na^+/H^+ antiporter in microalgae. Complete genome sequencing of microalgae from NCBI (http://www.ncbi.nlm.nih.gov/) and KEGG (http://www.genome.jp/kegg/genes.html) databases have been announced recently. These are *Ostreococcus tauri, Ostreococcus lucimarinus, Micromonas pusilla* CCMP1545, *Micromonas* sp. RCC299, *Bathycoccus prasinos* and *Aureococcus anophagefferens*. Among these microalgae, *O. tauri* is one of unique microalga since it

is the smallest eukaryotic organism with a size less than 1 μm. *O. tauri* genome sequencing database revealed the presence of 10 putative Na^+/H^+ antiporters. One of them is the putative NhaA Na^+/H^+ antiporter.

In this research, I aim to clone Na^+/H^+ antiporter gene from *Ostreococcus tauri* (*OtNhaA*) and express into the salt-sensitive mutant *E.coli* TO114 (**Δ***nhaA,* **Δ***nhaB*, **Δ***chaA*) and *Chlamydomonas reinhardtii* 137c. Functional complementation and stress tolerance were performed.

The objectives of this research:

1. To analyze Na^+/H^+ antiporter genes in microalgal genomes from bioinformatics databases
2. To clone and express of Na^+/H^+ antiporter genes from microalga *Ostreococcus tauri*
3. To functionally analyze the *O. tauri* Na^+/H^+ antiporter

CHAPTER III

MATERIALS AND METHODS

Materials

3.1 Instruments

Autoclave: Model HA 30, Hirayama Manufacturing Cooperation, Japan

Autopipette: Pipetteman, Gilson, France

French pressure cell: SIM-Aminco Spectronic Instrument, USA

Gel imaging: Model Gel Doc EZ Imager, Biorad, USA

Illuminated/Refrigerated orbital: Sanyo, England

Incubator: Haraeus, Germany

Incubator shaker: Psyco-them, New Bruncwick Scientific Supply, USA

Lamina flow BVT-124: International Scientific Supply, Thailand

Microcentrifuge: Kubota, Japan

NanoDrop 2000 UV-Vis Spectrophotometer: Thermo scientific, USA

pH meter: PHM 83 Autocal pH meter, Radiometer, Denmark

Refrigerated centrifuge: Model J-21C, Beckman Instrument Inc, USA

Spectrofluorophotometer RF-5300PC: Shimadzu, Japan

Spectrophotometer UV-240: Shimadzu, Japan

Vortex: Model K-550-GE: Scientific Industries, USA

3.2 Chemicals

Acridine orange: Katayama Chem, Japan

Ammonium chloride: Katayama Chem, Japan

Ampicillin: Katayama Chem, Japan

Bacto Tryptone: Merck Ag Darmstadt, Germany

Bacto Yeast extract: Merck Ag Darmstadt, Germany

Biotin: Katayama Chem, Japan

β-mercaptoethanol: Katayama Chem, Japan

Calcium chloride: Katayama Chem, Japan

Choline chloride: Katayama Chem, Japan

Dipotassium phosphate: Katayama Chem, Japan

DDT (Dithiothreitol): Katayama Chem, Japan

EDTA (Ethylenediaminetetraacetic acid): Sigma, USA

Glacial acetic acid: Katayama Chem, Japan

Iron (II) chloride: Katayama Chem, Japan

Lithium chloride: Katayama Chem, Japan

Magnesium chloride: Merck Ag Darmstadt, Germany

Magnesium sulfate: Merck Ag Darmstadt, Germany

Manganese (II) chloride: Katayama Chem, Japan

Potassium chloride: Merck Ag Darmstadt, Germany

Potassium dihydrogen phosphate: Merck Ag Darmstadt, Germany

Sodium chloride: Merck Ag Darmstadt, Germany

Sodium nitrate: Sigma, USA

Streptomycin: Sigma, USA

Sucrose: Katayama Chem, Japan

SYBR safe DNA gel stain: Life technologies, USA

Thiamine HCl: Katayama Chem, Japan

Tris-hydrochloride: Katayama Chem, Japan

Tris (hydroxymethyl) aminomethane: Katayama Chem, Japan

Vitamin B_{12}: Katayama Chem, Japan

3.3 Kits

DNeasy Plant mini kit: Qiagen, Germany

Gel extraction kit: Invitrogen, USA

PureLink Quick Plasmid Miniprep kit: Invitrogen, USA

3.4 Restriction enzymes

*Xba*I: Biolabs, USA

*Nde*I: Biolabs, USA

*Sal*I: Takara, Japan

*BamH*I: Takara, Japan

*Nco*I: Takara, Japan

3.5 Bacterial strains and plasmids

Strains and plasmids	Descriptions	Sources/references
Ostreococcus tauri NIES-2673	Marine green alga	National Institute for Environmental Studies, Japan (NIES)
Chlamydomonas reinhardtii 137c	Unicellular green alga	Life technologies, CA, USA
Escherichia coli DH5α	(φ80d *lacZ*ΔM15 *recA1endA1 gyrA96 thi-1 hsdR17*(r_k^- m_k^+) *supE44 relA*1 *deoR*Δ (lacZYA-argF)*U169)*	Invitrogen, USA
Escherichia coli TO114	(W3110 *nhaA*::Km^r *nhaB*::Em^r *chaA*::Cm^r)	Waditee *et al*., 2001
FL_OtNhaA/pMK	1.6 kb *OtNhaA* coding region in pMK vector	Life technologies, CA, USA
FL_OtNhaA/pTrcHis2C	1.6 kb *OtNhaA* coding region cloned into pTrcHis2C vector	This study
Δ*N112_OtNhaA*/pTrcHis2C	1.2 kb *OtNhaA* truncation cloned into pTrcHis2C vector	This study
FL_OtNhaA/pChlamy_3	1.6 kb *OtNhaA* coding region cloned into pChlamy_3 vector	This study
pTrcHis2C	Expression vector	Invitrogen, USA
pChlamy_3	Expression vector	GeneArt®*Chlamydomonas* Engineering Kits

3.6 Primers

Primers	5'-3'	Base pairs
FL_OtNhaA_F	ATGATCGATGAGGACCGCGT	20 mer
FL_OtNhaA_R	ATGAACGTCTCCGTGAACGT	20 mer
ΔN112_OtNhaA_NcoI_F	CACGGAGACGTCCATGGCGGGCGTG	25 mer
OtNhaA_R	TTACGCCGTCTTCAACTTGGCGTCT	25 mer
OtNhaA_pChlamy3_F	ATGATCGATGAGGACCGCGTG	21 mer
OtNhaA_pChlamy3_R	CGCCGTCTTCAACTTGGCGTC	21 mer
Hygromycin B_F	TGACACAAGAATCCCTGTTAC	21 mer
Hygromycin B_R	AGTACCATCAACTGACGTTAC	21 mer

Methods

3.7 Phylogenetic relationship of NhaA antiporter

3.7.1 Bioinformatics analysis

NhaA Na^+/H^+ antiporter sequences of *O. tauri* (hereafter OtNhaA) and orthologs from bacteria and green algae (Appendix 1) were obtained from National Center for Biotechnology Information (NCBI) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Twenty-one NhaA sequences were derived from bacteria (*Escherichia coli*; accession number CDU40636.1, *Helicobacter pylori*; accession number KFH29266.1, *Yersinia pestis*; accession number KGA52390.1, *Salmonella typhimurium;* accession number NP_459044.1, *Vibrio cholerae*; accession number KFE28035.1, *Shewanella frigidimarina*; accession number WP_011638389.1, *Osedax symbiont* Rs2; accession number WP_020285618.1, *Clostridium* sp. Maddingley MBC34-26; accession number WP_008427652.1, *Dyadobacter fermentans* DSM 18053; accession number ACT94304.1, *Aliivibrio fischeri*; accession number WP_011261552.1,

Vibrio azureus; accession number WP_021710120.1, *Shewanella amazonensis*; accession number WP_011758985.1, *Geobacter* sp. M18; accession number ADW11785.1, *Vibrio ponticus*; accession number GAK84546.1, *Clostridium beijerinckii*; accession number WP_011968778.1, *Vibrio cholerae* HC-7A1; accession number ELT24372.1, *Vibrio ezurae* NBRC 102218; accession number GAD79163.1, *Vibrio proteolyticus*; accession number WP_021705443.1, *Vibrio cholerae* HC-33A2; accession number EHH99586.1, *Vibrio cholerae* HE39; accession number EGQ99624.1, *Edwardsiella piscicida*; accession number GAJ66919.1) and six NhaA sequences were derived from green algae (*Ostreococcus 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, *Aureococcus anophagefferens*; accession number XP_009038116.1). These NhaA sequences were compared and aligned by using Molecular Evolutionary Genetics Analysis (MEGA6) software with Neighbor joining system (bootstrap values from 1,000 replicates).

3.7.2 Topological model prediction

To predict the secondary structure of membrane protein, the hydrophobicity of amino acids of NhaA protein was analyzed. The topological models of Na^+/H^+ antiporters were constructed and analyzed by using THMHMM software (http://www.expasy.org). These topological models represent secondary structures, i.e. numbers, position of transmembrane segments and loop regions.

3.8 Cloning and expression of *NhaA* Na^+/H^+ antiporter from *O. tauri*

3.8.1 Strains and growth conditions

3.8.1.1 *O. tauri* NIES-2673 culture condition

O. tauri NIES-2673 cells were grown in EMS medium (Appendix 2) (Provasoli *et al.*, 1957) and supplemented with 0.3% CO_2. Cell cultures were incubated at 25 °C under continuous fluorescent white light (20 µmol $m^{-2}s^{-1}$) (van Ooijen *et al.*, 2012). The growth of *O. tauri* NIES-2673 was monitored by measuring absorbance at 730 nm with spectrophotometer UV-240 (Shimadzu, Japan).

3.8.1.2 *E. coli* culture condition

E. coli DH5α cells were grown in LB medium (Appendix 3) with shaking 180 rpm at 37 °C and were used as general purpose on cloning. *E. coli* transformed cells were grown under the same condition as wild-type cells and supplemented with 50 µg/ml of kanamycin when they were transformed with 13AA377P_Na_ion_proton2_pMK vector (Appendix 4) (Life technologies, CA, USA). Bacterial cells growth was monitored by measuring absorbance at 600 nm with spectrophotometer UV-240 (Shimadzu, Japan).

E. coli TO114 cells (Δ*nhaA*, Δ*nhaB*, Δ*chaA*) (Waditee *et al.*, 2001) harboring empty vector (pTrcHis2C) or expression vector (*FL_OtNhaA*/pTrcHis2C and *ΔN112_OtNhaA*/pTrcHis2C) were grown in modified LBK medium (NaCl was replaced by KCl) (Appendix 5) and shaking 180 rpm at 37 °C. LBK medium was supplemented with ampicillin, kanamycin, erythromycin and chloramphenicol at final concentration; 100, 50, 50 and 100 µg/ml, respectively. Bacterial cells growth was monitored by measuring absorbance at 600 nm with spectrophotometer UV-240 (Shimadzu, Japan).

3.8.2 Construction of the full length *OtNhaA* Na^+/H^+ antiporter in expression vector

The full length *OtNhaA* Na^+/H^+ antiporter gene from *O. tauri* (Appendix 6) was synthesized by Life technologies, USA. Briefly, the nucleotide sequence was obtained from National Center for Biotechnology Information (NCBI) databases and was designed to introduce *Xba*I restriction site on the 5' end. *BamH*I restriction site and His_6-tagged were added to the 3' end. The full length *OtNhaA* together with introduced restriction sites and His_6-tagged sequences approximately 1.6 kb were cloned into pMK vector (Life technologies, CA, USA).

To construct the full length *OtNhaA* Na^+/H^+ antiporter in expression vector pTrcHis2C, the fragment of full length *OtNhaA* was sub-cloned into cloning vector (pCR2.1). The fragment in pCR2.1 was prepared by double digestion with restriction enzymes *Xho*I and *Hind*III (type2 in schematic construction) and ligated into *Xho*I and *Hind*III sites of pTrcHis2C vector (Figure 3.1) (hereafter *FL_OtNhaA*/pTrcHis2C). The recombinant plasmid *FL_OtNhaA*/pTrcHis2C was transformed into *E. coli* DH5α cells for propagation. For transformation, one hundred microliters of *E. coli* DH5α competent cells were thawed on ice. Then, 100 ng of recombinant plasmid *FL_OtNhaA*/pTrcHis2C was added into *E. coli* DH5α competent cells. The transformation mixture was flicked 2-3 times and stood on ice for 10 minutes. After that, the mixture was heated to 42 ºC for 90 second and stood on ice for 5 minutes. Then the mixture was diluted with 900 µl of LB medium and gently shaking 200 rpm at 37 ºC for 60 minutes. Cell suspension was spreaded and selected on LB agar containing ampicillin at final concentration 50 µg/ml. Several independent single colonies were re-streaked on new LB agar containing ampicillin at final concentration

50 μg/ml. Each colony was checked by colony PCR using specific primers: FL_OtNhaA_F, 5'-ATGATCGATGAGGACCGCGT-3' and FL_OtNhaA_R, 5'- ATGAACGTCTC CGTGAACGT-3'. The positive clones of *FL_OtNhaA*/pTrcHis2C obtained from colony PCR screening were further used for plasmid extraction using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen). Then, the plasmids *FL_OtNhaA*/pTrcHis2C were confirmed the insertion by restriction enzyme analysis.

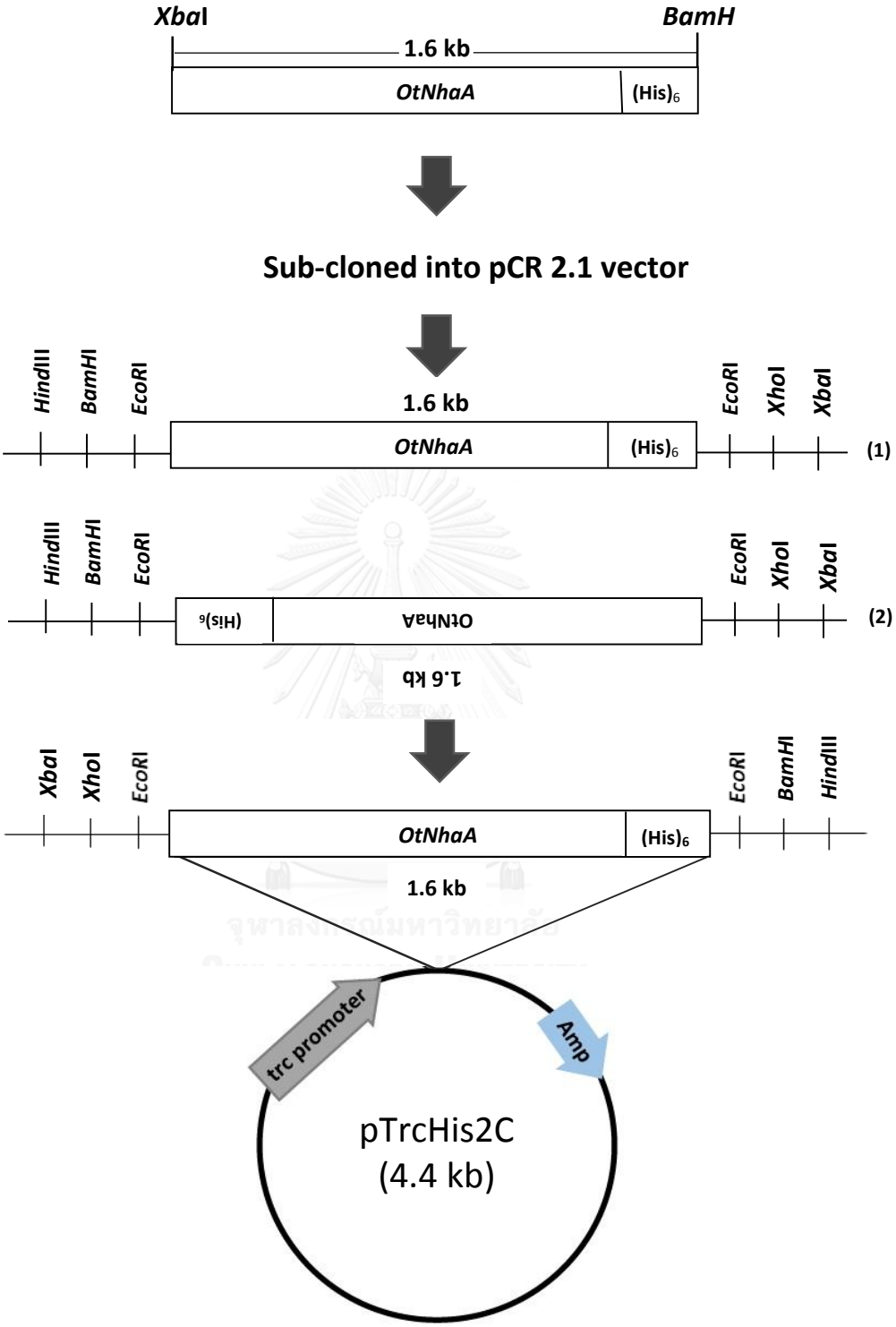


Figure 3.1 Schematic construction for *FL_OtNhaA/*pTrcHis2C.

3.8.3 Construction of N-terminal truncation *OtNhaA* in expression vector

To construct the truncation of *OtNhaA* (deletion 112 amino acids from N-terminus). The fragments were amplified using full length *OtNhaA* as a template. PCR reaction was carried out using specific primers: **Δ**N112_OtNhaA_NcoI_F, 5'-CACGGAGACGTCCATGGCGGGCGTG-3', which introduced *Nco*I restriction site on the 5' and OtNhaA_R, 5'-TTACGCCGTCTTCAACTTGGCGTCT-3'. The PCR products which the size approximately 1.2 kb was cloned into pCR 2.1 vector and sequenced. The DNA fragment in pCR2.1 vector was prepared by double digestion with *Nco*I and *Sal*I and ligated into corresponding sites of pTrcHis2C vector (hereafter ***Δ****N112_OtNhaA*/pTrcHis2C) (Figure 3.2). The recombinant plasmid ***Δ****N112_OtNhaA*/pTrcHis2C was transformed into *E. coli* DH5**α** cells for propagation. For transformation, one hundred microliters of *E. coli* DH5**α** competent cells were thawed on ice. Then, 100 ng of recombinant plasmid ***Δ****N112_OtNhaA*/pTrcHis2C was added into *E. coli* DH5**α** competent cells. The transformation mixture was flicked 2-3 times and stood on ice for 10 minutes. After that, the mixture was heated to 42 ºC for 90 second and stood on ice for 5 minutes. Then the mixture was diluted with 900 µl of LB medium and gently shaking 200 rpm at 37 ºC for 60 minutes. Cell suspension was spread and selected on LB agar containing ampicillin at final concentration 50 µg/ml. Several independent single colonies were re-streaked on new LB agar containing ampicillin at final concentration 50 µg/ml. Each colony was checked by colony PCR using specific primers as described above. The positive clones of ***Δ****N112_OtNhaA*/pTrcHis2C from colony PCR screening were used for plasmid extraction using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen). Then, the plasmids ***Δ****N112_OtNhaA*/pTrcHis2c were confirmed the insertion by restriction enzyme analysis.

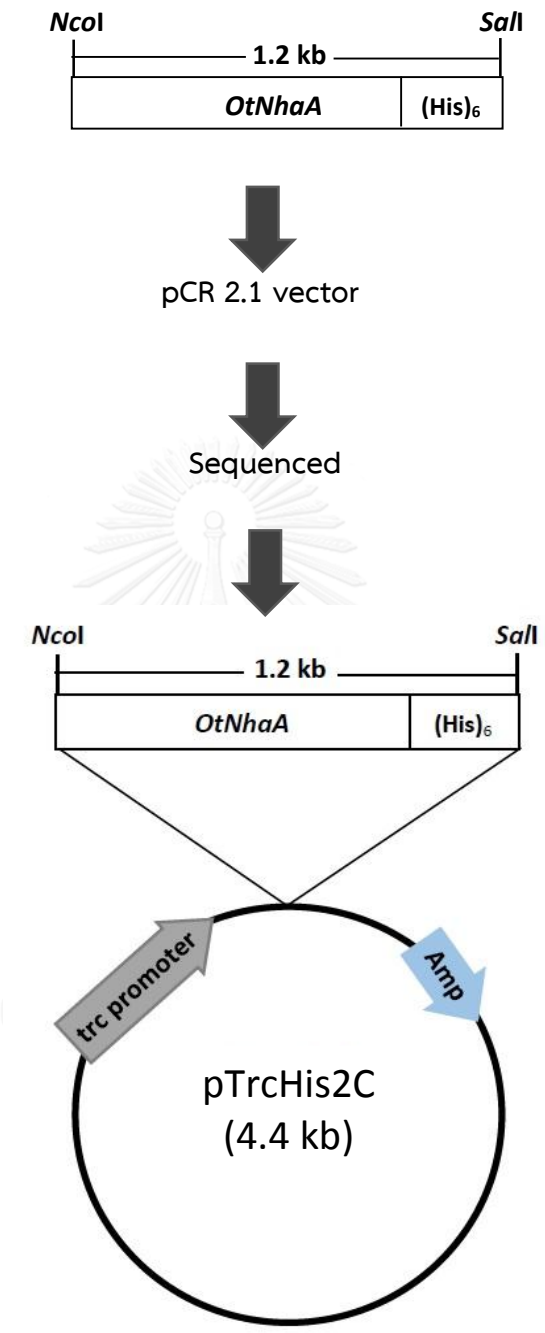


Figure 3.2 Schematic construction for *ΔN112_OtNhaA*/pTrcHis2C.

3.8.4 Expression of *FL_OtNhaA* and *ΔN112_OtNhaA* into *E. coli* TO114

The salt sensitive-mutant *E. coli* TO114 (*ΔnhaA*, *ΔnhaB, ΔchaA*) was used as the host cells for functional complementation. Preparation of competent *E. coli* TO114 cells were done by $CaCl_2$ method. Briefly, salt sensitive-mutant *E. coli* TO114 cells were grown in LBK medium with shaking 200 rpm at 37 ºC until the absorbance at 600 nm reached 0.4. After that, putting the cells on ice for 10 minutes and harvested by centrifugation at 3,500 rpm at 4 ºC for 10 minutes. The cell pellets were resuspended in 0.1 M $CaCl_2$ and incubated on ice 30 minutes. Cell suspension was harvested by centrifugation at 3,500 rpm at 4 ºC for 10 minute. Then, the cell pellets were resuspended in 0.1 M $CaCl_2$ plus 15% glycerol and were kept in – 80 ºC prior the use.

The plasmids *FL_OtNhaA*/pTrcHis2C and *ΔN112_OtNhaA*/pTrcHis2C were transformed into *E. coli* TO114 by chemical transformation. For this, 100 µl of *E. coli* TO114 competent cells were thawed on ice. Then, 100 ng of recombinant plasmid either *FL_OtNhaA*/pTrcHis2C or *ΔN112_OtNhaA*/pTrcHis2C was added into *E. coli* TO114 competent cells. The mixture was flicked 2-3 times and stood on ice for 10 minutes. After that, the mixture was heated to 42 ºC for 90 second and stood on ice for 5 minutes. Then the mixture was diluted with 900 µl of LBK medium and gently shaking 180 rpm at 37 ºC for 60 minutes. Cell suspension was spreaded and selected on LBK agar containing ampicillin, kanamycin, erythromycin and chloramphenicol (final concentration; 100, 50, 50 and 100 µg/ml, respectively) and incubated at 37 ºC for overnight. Several independent single colonies were re-streaked on new LBK agar plate containing ampicillin, kanamycin, erythromycin and chloramphenicol (final concentration; 100, 50, 50 and 100 µg/ml, respectively). Each colony was checked by colony PCR using specific primers as shown in Table 3.6. The positive clones of

FL_OtNhaA/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C from colony PCR screening were used for plasmid extraction using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen). Then, the plasmids *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C were confirmed the insertion by restriction enzyme analysis.

3.8.5 Complementation test

Firstly, the salt-sensitive mutant *E. coli* TO114 harboring empty vector (pTrcHis2C), *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C were grown in LBK medium containing ampicillin, kanamycin, erythromycin, chloramphenicol (final concentrations; 100, 50, 50 and 100 μg/ml, respectively) and shaking 180 rpm at 37 ºC for overnight. These cultures were used as the pre-inoculum. Then, the pre-inoculum cells were inoculated for 1% to new LBK medium with shaking 180 rpm at 37 ºC and supplemented with ampicillin, kanamycin, erythromycin, chloramphenicol (final concentrations; 100, 50, 50 and 100 μg/ml, respectively). The growth of expressing cells was monitored by measuring absorbance at 600 nm with spectrophotometer UV-240 until reached 0.8. After that, 10-fold serial dilutions with distilled water was done. Then, diluted cultures for empty vector, *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C expressing cells were further tested for the ability to grow in LBK agar plates with salts (NaCl and LiCl) at pH ranging from 5.0 to 8.5.

For these complementation tests, 5 μl of each dilution described above were spotted onto LBK agar containing different concentrations of salt at various pH. Then, the cultures were incubated at 37 ºC for overnight and scored the growth. Photographs were taken using SONY Cyber-shot camera.

3.8.6 Na^+/H^+ antiporter activity

3.8.6.1 Preparation of everted membrane vesicles

Everted membrane vesicles were prepared by using *E.coli* TO114 cells harboring empty vector (pTrcHis2C), *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C. Mid-logarithm cells grown in LBK medium or LBK+200 mM NaCl medium with shaking 200 rpm at 37 °C and supplemented with ampicillin, kanamycin, erythromycin and chloramphenicol at final concentrations; 100, 50, 50 and 100 µg/ml, respectively (Optical density at 600 nm approximately 0.6 – 0.8). Two hundred milliliters of each *E.coli* TO114 cells harboring empty vector, *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C were harvested by centrifugation at 5,000 rpm for 10 minutes at 4 °C and then washed with TCDS buffer (Appendix 7). Cell pellets were re-suspended with 10 ml TCDS buffer and then applied to a French Pressure cell (4,000 psi). The cell suspension was centrifuged at 10,000 rpm for 10 minutes at 4 °C to remove cell debris. The supernatant was centrifuged at 30,000 rpm for 60 minutes at 4 °C to pellet everted membrane. The light-yellow pellet was re-suspended gently in 600 µl TCDS buffer. Protein concentration of everted membrane was determined by Lowry's method (Appendix 8).

3.8.6.2 Western Blot analysis

The everted membrane vesicles preparing from step 3.8.6.1 were used for Western Blot analysis. SDS-PAGE was performed by standard protocol (Sambrook 1989) (Appendix 9). The molecular mass of membrane protein was determined by comparing with protein molecular weight marker (Biorad, USA). Western Blot analysis was carried out to confirm the membrane protein was fused in-frame with six histidine tag. For

this, 50 µg of each everted membrane was separated by 12.5% SDS-PAGE and protein band was transferred to nitrocellulose membrane using blotting transfer buffer. Blotting was done at 150 mA/cm^2 for 1 hour and followed by blocking in blocking solution for 2 hours with 100 ml of PBS buffer plus 5% skim milk solution. The nitrocellulose membrane was incubated with primary-antibody (a mouse antibody raised against 6-histidine, 6X-His tag) for 1 hour and membrane was washed with 100 ml of PBS buffer for 15 minutes by gently shaking, for 3 times. After washing, the membrane was incubated with secondary-antibody (an antibody raised against mouse) for 1 hour and washed 3 times with 100 ml of PBS buffer for 30 minutes. The nitrocellulose membrane was detected with substrate for alkaline phosphatase (150 mM Barbital pH 9.6, 0.1% NTB (Nitro Blue Tetrazolium), 1M $MgCl_2$ and 0.5% BCIP (5-bromo-4-chloro-3-indolyl phosphate)) for 30 minutes.

3.8.6.3 Na^+/H^+ antiporter assay

The Na^+/H^+ antiporter activity assay was based on measurement the change of the vesicular **Δ**pH (transmembrane pH gradient) after addition of salts (NaCl, LiCl, KCl and $CaCl_2$) to the reaction mixture and was measured by acridine orange fluorescence quenching method.

The fluorescence assay was performed in 2 ml reaction mixture containing 10 mM Tris-HCl, 140 mM choline chloride, 2 µM acridine orange and 50 µg of everted membrane vesicles. The fluorescence of acridine orange was monitored by spectrofluorometer using extinction wavelength of 492 nm and emission wavelength of 525 nm. At the first of the experiment, 2 mM Tris-DL-lactate was added to initiate fluorescence quenching (Q) due to respiration. DL-lactate was energized the vesicles

and accumulated H^+ in the vesicles. Upon addition of salt (concentration range of salt was 0.25 to 5.0 mM at pH 7 to 9), the fluorescence level (dequenching) was increased due to excretion of H^+ by antiporter and fluorescence dequenching (**Δ**Q) was monitored. At the end of the experiment, 25 mM NH_4Cl was added to **Δ**pH dissipation (Hamada *et al.*, 2001, Billini *et al.*, 2008). The percent increase of fluorescence upon addition of salt was calculated as follows: **Δ**Q x 100/Q and the apparent kinetic parameters (K_m and V_{max} values) were calculated by GraphPad Prism 6 Software.

3.9 Cloning and Expression *O. tauri* Na^+/H^+ antiporter into *C. reinhardtii* 137c

3.9.1 Strains and growth conditions

3.9.1.1 *C. reinhardtii* 137c culture condition

C. reinhardtii 137c cells were grown photoautotrophically in TAP medium (Appendix 10). The cell cultures were incubated at 25 ºC under continuous fluorescent white light (50 μE $m^{-2}s^{-1}$) and shaking 150 rpm (GeneArt® *Chlamydomonas* Engineering Kits, Life technologies, USA). The growth of *C. reinhardtii* 137c cells were monitored by measuring absorbance at 750 nm with spectrophotometer UV-240 (Shimadzu, Japan).

3.9.1.2 *E. coli* culture condition

E. coli DH5**α** cells were grown in LB medium with shaking 180 rpm at 37 ºC and were used as general purpose on cloning. *E. coli* transformed cells were grown under the same condition as wild-type cells but were supplemented with 50 μg/ml of kanamycin when they were transformed with pMK (kanR) vector (Life technologies, CA, USA). The growth of bacterial cells were monitored by measuring absorbance at 600 nm with spectrophotometer UV-240 (Shimadzu, Japan).

3.9.2 Construction of the full length *OtNhaA* Na^+/H^+ antiporter in expression vector

The full length *OtNhaA* fragment from step 3.8.2 was prepared for expression in pChlamy_3 (GeneArt® *Chlamydomonas* Engineering Kits, Life technologies, USA). The fragment of full length *OtNhaA* was prepared by double digestion with *Xba*I and *Nde*I and ligated into corresponding sites of pChlamy_3 vector (Figure 3.3) (hereafter *FL_OtNhaA*/pChlamy_3). The recombinant plasmid *FL_OtNhaA*/pChlamy_3 was transformed into *E. coli* DH5α cells for propagation. For transformation, one hundred microliters of *E. coli* DH5α competent cells were thawed on ice. Then, 100 ng of recombinant plasmids *FL_OtNhaA*/pChlamy_3 were added into *E. coli* DH5α competent cells. The transformation mixture was flicked 2-3 times and stood on ice for 10 minutes. After that, the mixture was heated to 42 ºC for 90 second and stood on ice for 5 minutes. Then the mixture was diluted with 900 µl of LB medium and gently shaking 200 rpm at 37 ºC for 60 minutes. Cell suspension was spreaded and selected on LB agar containing ampicillin at final concentration 50 µg/ml. Several independent single colonies were re-streaked on new LB agar containing ampicillin at final concentration 50 µg/ml. Each single clones were checked by colony PCR using specific primers: FL_OtNhaA_F, 5'-ATGATCGATGAGGACCGCGT-3' and FL_OtNhaA_R, 5'-ATGAACGTCTCCGTGAACGT-3'. The positive clones of *FL_OtNhaA*/pChlamy_3 from colony PCR screening were used for plasmid extraction using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen). Then, the plasmids *FL_OtNhaA*/pChlamy_3 were confirmed the insertion by restriction enzyme analysis.

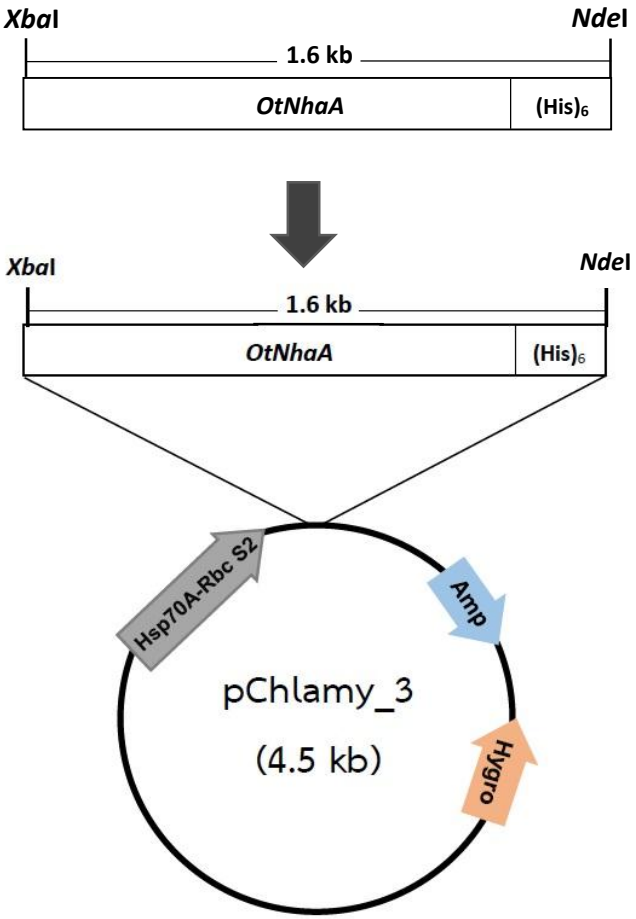


Figure 3.3 Schematic construction for *FL_OtNhaA/*pChlamy_3.

3.9.3 Expression of *FL_OtNhaA* into *C. reinhardtii* 137c

The plasmids *FL_OtNhaA*/pChlamy_3 and empty vector (pChlamy_3) were transformed into *C. reinhardtii* 137c by electroporation (according to the protocol from manufacturer). Fifteen milliliters of *C. reinhardtii* 137c culture cells (OD_{750} approximately 0.4) were harvested by centrifugation at 3,500 rpm for 15 minutes. The cell pellets were re-suspended in 250 µl of TAP-40 mM sucrose medium (Appendix 11). Two micrograms of recombinant plasmid, either *FL_OtNhaA*/pChlamy_3 or empty vector (pChlamy_3) were mixed with 250 µl of cell suspension. After that, the mixtures were transferred into electroporation cuvette and incubated at room temperature for 5 minutes. The electroporation cuvette was transferred into cuvette chamber and set electroporation parameters as follows: Voltage: 600V, Capacity: 50 µF, Resistance: infinity and 0.2 mm cuvette. After electroporation, the cell mixtures were transferred into 12-well plate containing 2 ml/well of TAP-40 mM sucrose medium and incubated at 25 ºC under continuous fluorescent white light (50 µE $m^{-2}s^{-1}$) for 24 hour. The cell mixtures were harvested by centrifugation at 2,500 rpm for 10 minutes. Then, the cell pellets were resuspended in 500 µl of TAP-40 mM sucrose medium and selected on TAP agar supplemented with 10 µg/ml hygromycin. After 10 days, several independent single colonies were re-streaked on new TAP agar containing 10 µg/ml hygromycin. Each single clone was checked by colony PCR using specific primers for *FL_OtNhaA*/pChlamy_3 and hygromycin resistance gene as shown in Table 3.6. The positive clones of *FL_OtNhaA*/pChlamy_3 from colony PCR screening were used for plasmid extraction using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen). Then, the plasmid *FL_OtNhaA*/pChlamy_3 was confirmed the insertion by restriction enzyme analysis.

3.9.4 Stress tolerance

To analyze stress tolerance of transformant and wild-type strain of *C. reinhardtii* 137c, the growth of cells under different concentration of salt were examined. For the growth of transformants *C. reinhardtii* 137c cells harboring *FL_OtNhaA*/pChlamy_3 or empty vector (pChalmy_3), the transformants were grown in TAP agar containing hygromycin at final concentration 10 μg/ml and supplemented with various concentration of NaCl (0-300 mM). For the growth of wild-type strain *C. reinhardtii* 137c was examined using multiwell plate assay, One hundred milliliters of wild-type strain culture cells (OD_{750} approximately 0.4) were harvested by centrifugation at 3,500 rpm for 15 minutes. The cell pellets were resuspended in 5 ml of TAP-40 mM sucrose medium. The cell mixtures were transferred into multiwell plate containing 2 ml/well of TAP-40 mM sucrose medium and supplemented with various concentration of NaCl (0-300 mM). Both wild-type strain and transformant of *C. reinhardtii* 137c cells were incubated at 25 ºC under continuous fluorescent white light (50 μE $m^{-2}s^{-1}$) for 10 days. Then, the growth of wild-type strain of *C. reinhardtii* 137c cells were monitored by measuring absorbance at 750 nm with spectrophotometer UV-240 (Shimadzu, Japan).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Phylogenetic relationship of NhaA antiporter

4.1.1 Bioinformatics analysis

For phylogenetic analysis, 27 sequences annotating as putative NhaA proteins were used. These are 21 protein sequences from bacteria and six sequences from green algae. These NhaA sequences were compared. Phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA6) software with Neighbor joining system (bootstrap values from 1,000 replicates). From our analysis, NhaA antiporter can be divided into 5 clusters. Some of them have functionally characterized such as *E. coli* NhaA, *S. typhimurium* NhaA and *H. pylori* NhaA (Tsuboi *et al.*, 2003, Padan *et al.*, 2004, Lentes *et al.*, 2014). As shown in Figure 4.1, cluster 1 consists of *Escherichia coli*, *Salmonella typhimurium, Osedax symbiont* and *Dyadobacter fermentans* NhaA proteins. They were predicted to have putative secondary structure, consisting of 11 transmembrane segments (TMs). Cluster 2 consists of NhaA proteins from *Shewanella amazonensis*, *Shewanella frigidimarina*, *Aliivibrio fischeri*, *Vibrio cholerae*, *Vibrio ponticus*, *Vibrio proteolyticus*, *Vibrio azureus* and *Yersinia pestis*. These antiporters were also predicted to have 11 TMs. Cluster 3 consists of *Helicobacter pylori*, *Geobacter* sp. M18, *Clostridium beijerinckii*, *Clostridium* sp. Maddingley MBC34-26 and Micromonas pusilla CCMP1545 NhaA proteins. They were predicted to have 10 TMs with a large loop between TM7-8. Next cluster 4, it

consists of *Vibrio cholerae* HE39, *Vibrio cholerae* HC-33A2 and *Vibrio ezurae* NhaA proteins. These antiporters were predicted to have 11 TMs. The last cluster, consists of NhaA proteins from Micromonas sp. RCC299, *Ostreococcus tauri* and *Vibrio cholerae* HC-7A1. NhaA antiporters in this cluster were predicted to have 10 TMs with a long N terminus. For example, *Ostreococcus tauri* NhaA consists of 10 TMs with a long hydrophilic N- terminal region. Moreover, the loop structure between transmembrane 6 and 7 was predicted. Phylogenetic tree suggests that the NhaA antiporter from *Ostreococcus tauri* shows lower similarity to green algae and bacteria (approx. 20 - 28% amino acid levels).

Functional characterization of NhaA antiporter has been extensively studied in bacteria (Padan *et al.*, 2004, Lentes *et al.*, 2014, Tsuboi *et al.*, 2003, Herz *et al.*, 2003). In *Escherichia coli*, three Na^+/H^+ antiporters (NhaA, NhaB and ChaA) are known and their functional characteristics have been described. NhaA is the first-discovered antiporter in *E. coli*. It is the main Na^+/H^+ antiporter and specifically exchange between Na^+ and/or Li^+ for H^+. The *E. coli* NhaA antiporter is indispensable for adaptation to high salinity, resistance to Li^+ toxicity and for growth under alkaline conditions (Padan *et al.*, 2004). In *Salmonella typhimurium*, NhaA antiporter is crucial for pathogenesis of the causing agent of food-born human gastroenteritis and typhoid like infections (Lentes *et al.*, 2014). In *Helicobacter pylori*, NhaA antiporter is involved in both ion transport and pH sensing, which is closely associated with loop 7 and 8 regions. This region is involved in enhancing antiporter activity at alkaline pH (Tsuboi *et al.*, 2003).

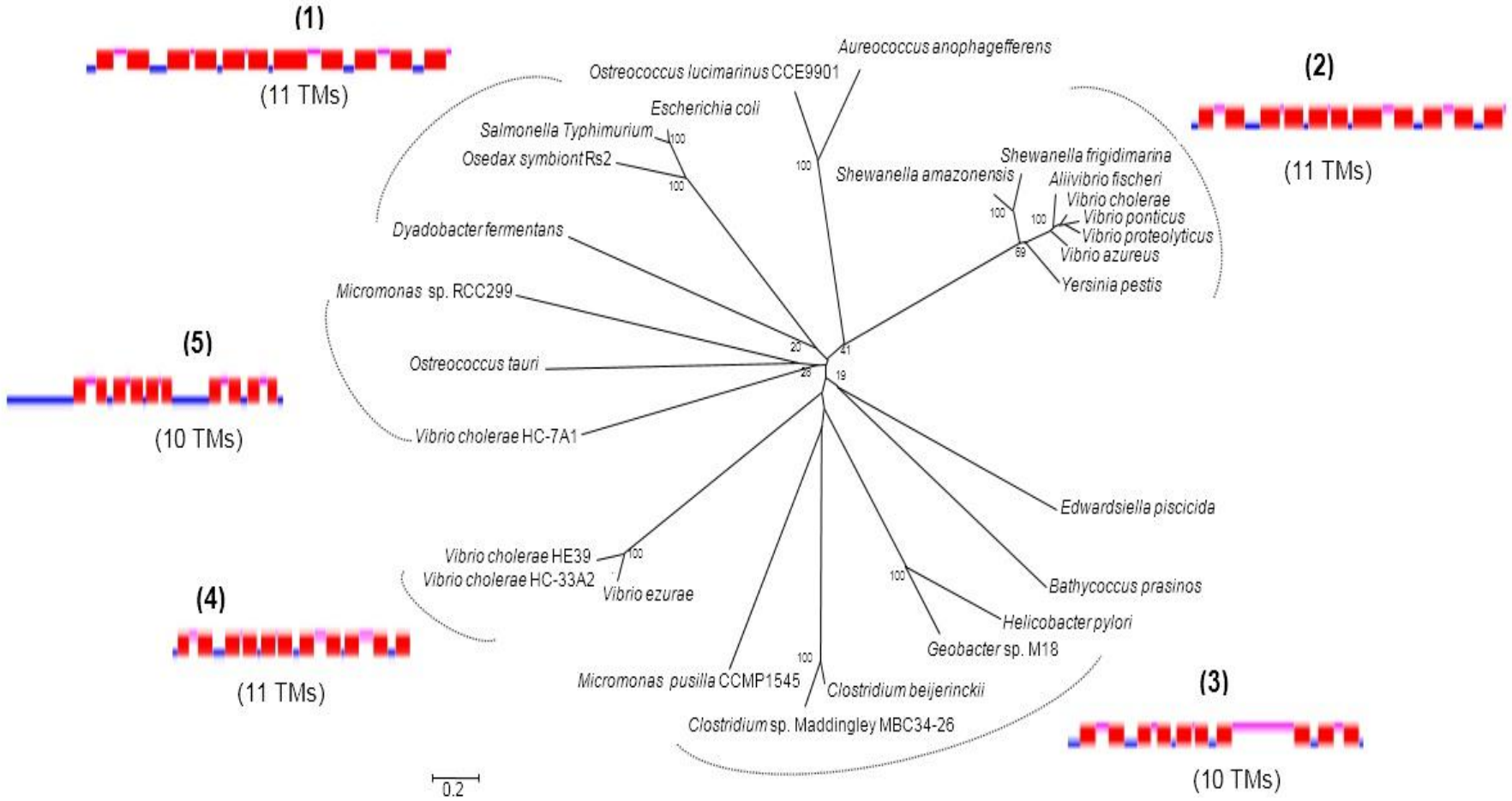


Figure 4.1: Phylogenetic analysis of NhaA antiporters in living organisms. NhaA amino acid sequences were obtained from NCBI and KEGG databases as described in Materials and Methods. These sequences were compared and aligned by using Molecular Evolutionary Genetics Analysis (MEGA6) software with Neighbor joining system (bootstrap values from 1,000 replicates).

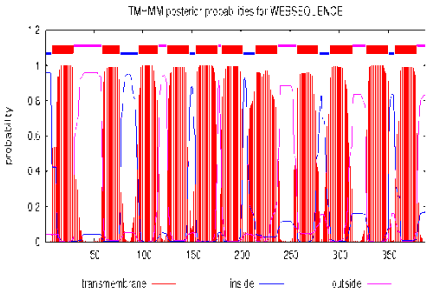
4.1.2 Topological model

The topological model of 27 NhaA proteins were analyzed and constructed using THMHMM software (http://www.expasy.org) (Fig 4.2 to 4.5). According to amino acid hydrophobicity, NhaA antiporters from bacteria generally consist of 9 to 11 putative TMs (Table 1). They lack of hydrophilic cytoplasmic part or extended N-terminus. In *Helicobacter pylori* NhaA, it shows 10 TMs with no hydrophilic part in either N- terminal or C- terminal regions. Furthermore, this NhaA antiporter is involved in ion transport while pH sensing was determined by the loop structure at loop 7 and 8. This region is involved in enhancing antiporter activity at alkaline pH (Tsuboi *et al.*, 2003).

NhaA antiporters from green algae group such as *Ostreococcus tauri*, Micromonas pusilla CCMP1545, Micromonas sp. RCC299 and Bathycoccus prasinos generally contain 9 to 11 putative TMs (Table 1) with a long hydrophilic N- terminal region. Topological model suggests that NhaA antiporter from *Ostreococcus tauri* has 10 putative TMs and it has relatively long hydrophilic N – terminal region.

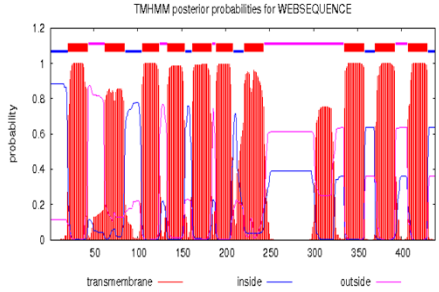
Escherichia coli

(Accession number: CDU40636.1



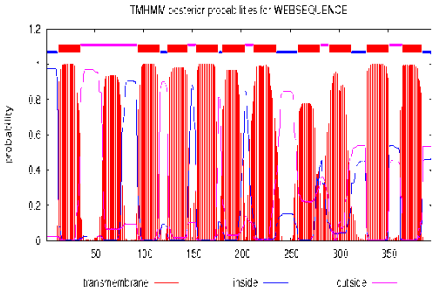
Helicobacter pylori

(Accession number: KFH29266.1)



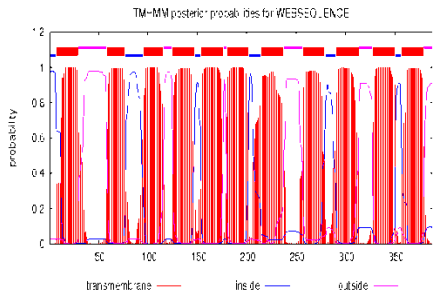
Yersinia pestis

(Accession number: KGA52390.1)



Salmonella Typhimurium

(Accession number: NP_459044.1)

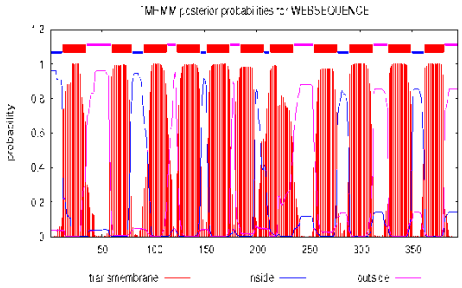


Vibrio cholerae

(Accession number: KFE28035.1)

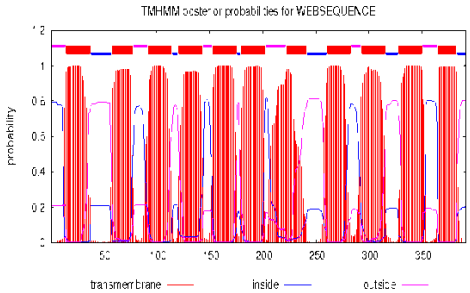
Shewanella frigidimarina

(Accession number: WP_011638389.1)



***Osedax symbiont* Rs2**

(Accession number: WP_020285618.1)



***Clostridium* sp. Maddingley**

(Accession number: WP_008427652.1)

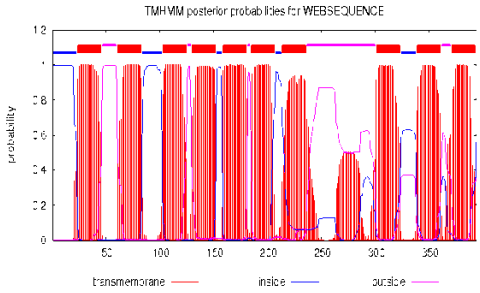
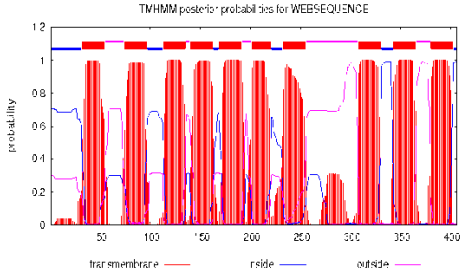


Figure 4.2: Topological models of NhaA proteins for *Escherichia coli*, *Helicobacter pylori*, *Yersinia pestis*, *Salmonella typhimurium*, *Vibrio cholerae*, *Shewanella frigidimarina*, *Osedax symbiont* Rs2 and *Clostridium* sp. Maddingley MBC34-26. NhaA sequences were predicted using THMHMM software (http://www.expasy.org).

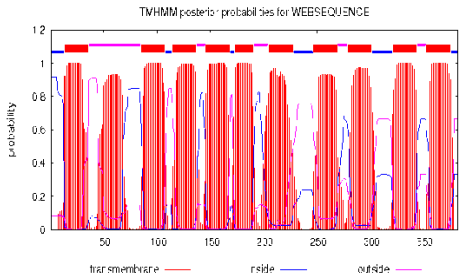
Dyadobacter fermentans **DSM 18053**

(Accession number: ACT94304.1)



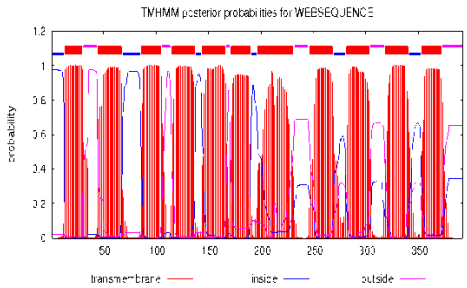
Aliivibrio fischeri

(Accession number: WP_011261552.1)



Vibrio azureus

(Accession number: WP_021710120.1)



Shewanella amazonensis

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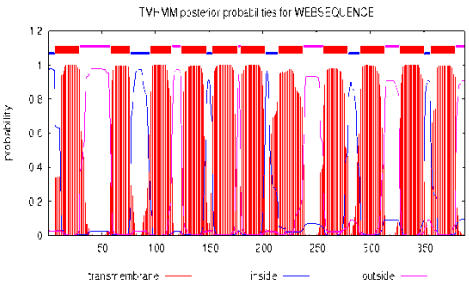
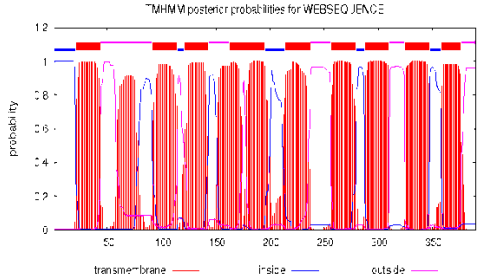


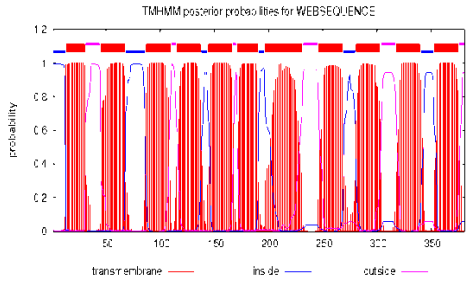
Geobacter **sp. M18**

(Accession number: ADW11785.1)



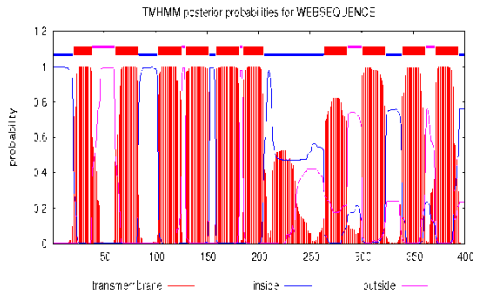
Vibrio ponticus

(Accession number: GAK84546.1)



Clostridium beijerinckii

(Accession number: WP_011968778.1)



Vibrio cholerae **HC-7A1**

(Accession number: ELT24372.1)

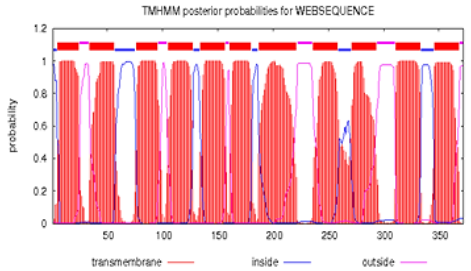
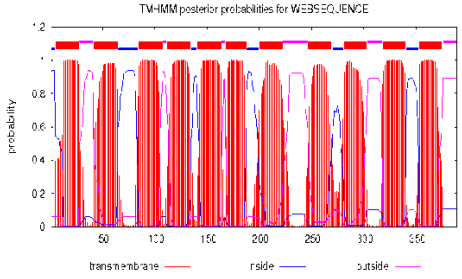


Figure 4.3: Topological models of NhaA proteins for *Dyadobacter fermentans* DSM 18053, *Aliivibrio fischeri, Vibrio azureus*, *Shewanella amazonensis*, *Geobacter* sp. M18, *Vibrio ponticus*, *Clostridium beijerinckii* and *Vibrio cholerae* HC-7A1. NhaA sequences were predicted using THMHMM software (http://www.expasy.org).

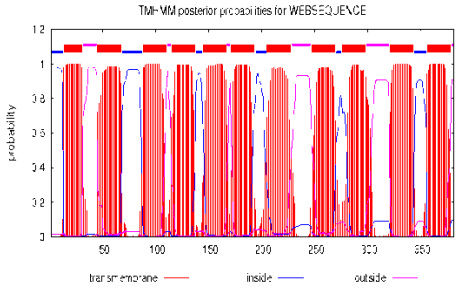
Vibrio ezurae **NBRC 102218**

(Accession number: GAD79163.1)



Vibrio proteolyticus

(Accession number: WP_021705443.1)



Vibrio cholerae **HC-33A2**

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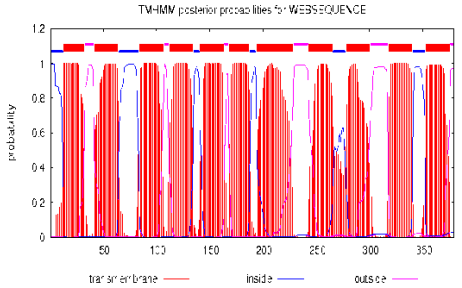
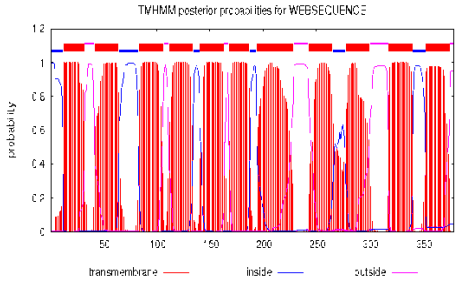


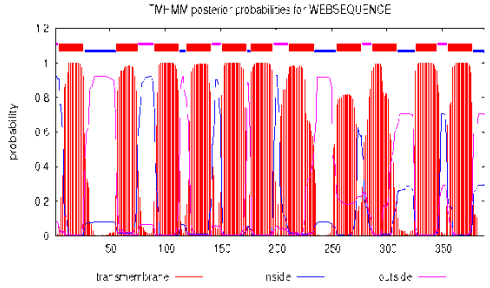
Vibrio cholerae **HE39**

(Accession number: EGQ99624.1)



Edwardsiella piscicida

(Accession number: GAJ66919.1)



Ostreococcus tauri

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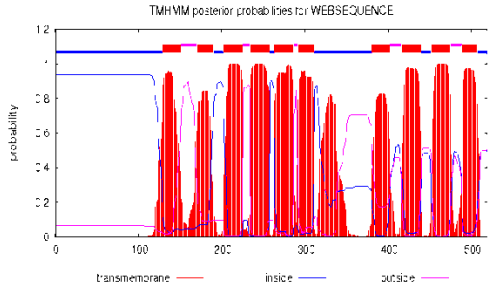
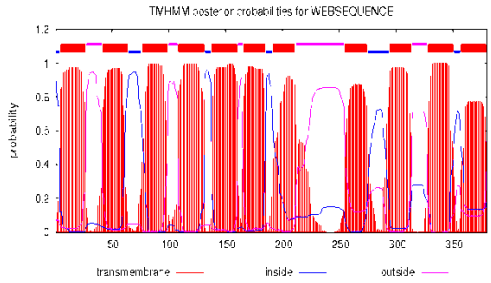


Ostreococcus lucimarinus CCE9901

(Accession number: ABO94792.1)



Micromonas pusilla CCMP1545

(Accession number: EEH59182.1)

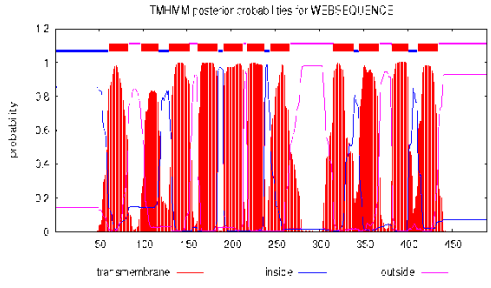
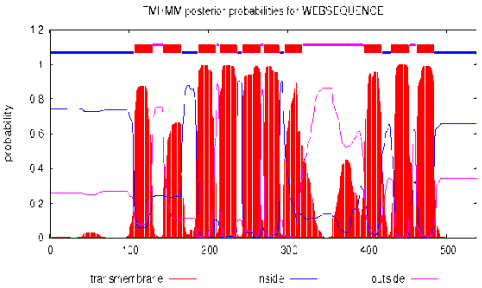
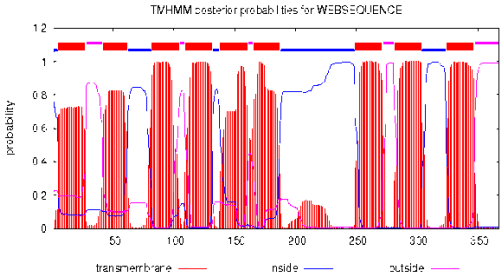


Figure 4.4: Topological models of NhaA proteins for *Vibrio ezurae* NBRC 102218, *Vibrio proteolyticus*, *Vibrio cholerae* HC-33A2, *Vibrio cholerae* HE39, *Edwardsiella piscicida*, Ostreococcus tauri, Ostreococcus lucimarinus and Micromonas pusilla CCMP1545. NhaA sequences were predicted using THMHMM software (http://www.expasy.org).

Micromonas sp. RCC299

(Accession number: ACO64223.1)





Bathycoccus prasinos

(Accession number: CCO19648.1)

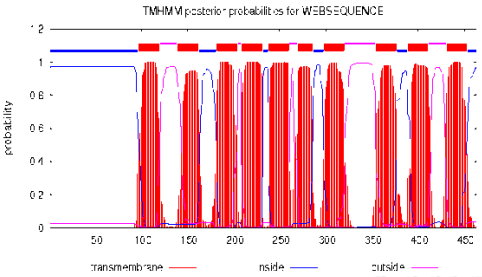


Figure 4.5: Topological models of NhaA proteins for Micromonas sp. RCC299, Bathycoccus prasinos and Aureococcus anophagefferens. NhaA sequences were predicted using THMHMM software (http://www.expasy.org).

Aureococcus anophagefferens

(Accession number: XP_009038116.1)

Table 4.1: Transmembrane segments of NhaA in living organisms

Accession numbers	Organisms	TMs
1. CDU40636.1	*Escherichia coli*	11
2. KFH29266.1	*Helicobacter pylori*	10
3. KGA52390.1	*Yersinia pestis*	10
4. NP_459044.1	*Salmonella Typhimurium*	11
5. KFE28035.1	*Vibrio cholerae*	11
6. WP_011638389.1	*Shewanella frigidimarina*	11
7. WP_020285618.1	*Osedax symbiont* Rs2	11
8. WP_008427652.1	*Clostridium* sp. Maddingley MBC34-26	10
9. ACT94304.1	*Dyadobacter fermentans* DSM 18053	10
10. WP_011261552.1	*Aliivibrio fischeri*	10
11. WP_021710120.1	*Vibrio azureus*	11
12. WP_011758985.1	*Shewanella amazonensis*	11
13. ADW11785.1	*Geobacter* sp. M18	9
14. GAK84546.1	*Vibrio ponticus*	11
15. WP_011968778.1	*Clostridium beijerinckii*	10
16. ELT24372.1	*Vibrio cholerae* HC-7A1	11
17. GAD79163.1	*Vibrio ezurae* NBRC 102218	11
18. WP_021705443.1	*Vibrio proteolyticus*	11
19. EHH99586.1	*Vibrio cholerae* HC-33A2	11
20. EGQ99624.1	*Vibrio cholerae* HE39	11
21. GAJ66919.1	*Edwardsiella piscicida*	11
22. XP_003075324.1	*Ostreococcus tauri*	10
23. ABO94792.1	Ostreococcus lucimarinus CCE9901	11
24. EEH59182.1	Micromonas pusilla CCMP1545	11
25. ACO64223.1	Micromonas sp. RCC299	10
26. CCO19648.1	Bathycoccus prasinos	10

27. XP_009038116.1	Aureococcus anophagefferens	9

4.2 Cloning and expression of *NhaA* Na^+/H^+ antiporter from *O. tauri*

4.2.1 Construction of the full length *OtNhaA* Na^+/H^+ antiporter gene in expression vector

The full length *OtNhaA* Na^+/H^+ antiporter gene from *O. tauri* was synthesized by Life technologies, USA as described in Materials and Methods. The full length *OtNhaA* together with introduced restriction sites and His_6-tagged sequences approximately 1.6 kb were cloned into pMK vector (Life technologies, CA, USA). The fragment of full length *OtNhaA* was sub-cloned into cloning vector (pCR2.1). The recombinant plasmid was confirmed by double digestion with restriction enzymes *Xho*I and *Hind*III. To construct the full length *OtNhaA* Na^+/H^+ antiporter in expression vector pTrcHis2C, the fragment from cloning vector was prepared by double digestion with restriction enzymes *Xho*I and *Hind*III. The fragment was ligated into corresponding restriction sites of expression vector pTrcHis2c and then transformed into *E. coli* DH5α. The insert fragment approximately 1.6 kb was confirmed by double digestion with restriction enzymes *Xho*I and *Hind*III as shown in Fig. 4.6. The plasmid *FL_OtNhaA*/pTrHis2C was extracted and transformed into *E. coli* TO114.

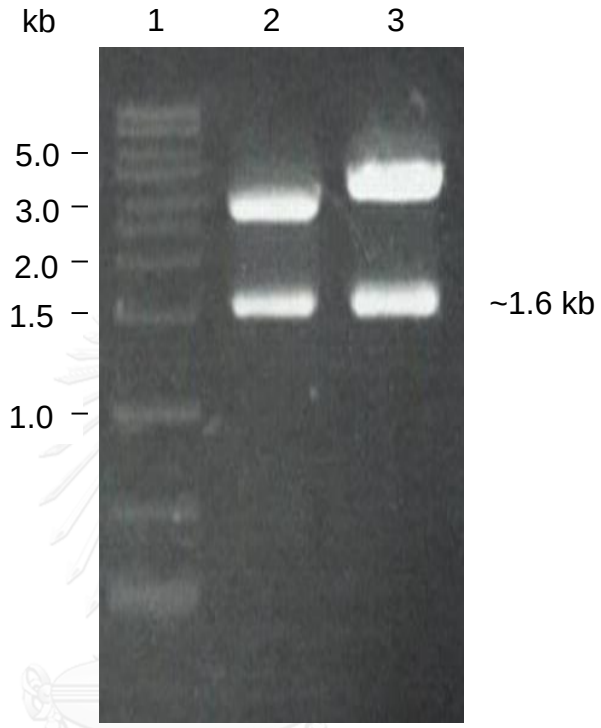


Figure 4.6: Restriction enzyme analysis of *FL_OtNhaA*/pCR2.1 and *FL_OtNhaA*/pTrcHis2C by double digestion *Xho*I and *Hind*III on 1% agarose gel electrophoresis. Lane 1: DNA ladder, lane 2: double digestion of *FL_OtNhaA*/pCR 2.1 with *Xho*I and *Hind*III and lane 3: double digestion of *FL_OtNhaA*/pTrcHis2C with *Xho*I and *Hind*III

4.2.2 Construction of N-terminal truncation *OtNhaA* in expression vector

To construct *OtNhaA* mutant (deletion 112 amino acids from N-terminus), the fragment was amplified by PCR using the full length *OtNhaA* as the template DNA as described in Materials and Methods. The PCR product approximately 1.2 kb was checked by 1% agarose gel electrophoresis (data not shown). PCR products were sub-cloned into cloning vector (pCR 2.1). The recombinant plasmid was confirmed by double digestion with restriction enzymes *Nco*I and *Sal*I. To construct *OtNhaA* N-terminal truncation in expression vector, the fragment from cloning vector was prepared by double digestion with *Nco*I and *Sal*I. The fragments were ligated into corresponding restriction sites of expression vector pTrcHis2c and then transformed into *E. coli* DH5α. The insert fragment approximately 1.2 kb was confirmed by double digestion with restriction enzymes *Nco*I and *Sal*I as shown in Fig. 4.7. The plasmid *ΔN112_OtNhaA*/pTrcHis2C was extracted and transferred into *E. coli* TO114.

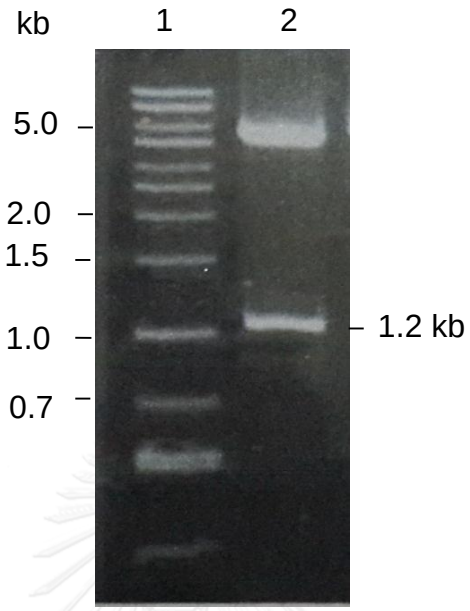


Figure 4.7: Restriction enzyme analysis of ***Δ****N112_OtNhaA*/pTrcHis2C by double digestion *Nco*I and *Sal*I on 1% agarose gel electrophoresis. Lane 1: DNA ladder and lane 2: double digestion of ***Δ****N112_OtNhaA*/pTrcHis2C with *Nco*I and *Sal*I.

4.2.3 Complementation test

The individual *FL_OtNhaA* and ***Δ****N112_OtNhaA* were cloned into expression vector pTrcHis2C as described in Materials and Methods. They were tested for complementation ability using *E. coli* mutant TO114 (Waditee *et al.*, 2001). This mutant was disrupted three putative Na^+/H^+ antiporters (NhaA, NhaB and ChaA), thus it became salt-sensitive phenotype. It cannot grow in the presence of 200 mM NaCl at neutral pH (Ohyama *et al.*, 1994). In this study, functional complementation of the salt-sensitive phenotype of *E. coli* mutant TO114 harboring empty vector (pTrcHis2C), *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C were examined as described in Materials and Methods. Growth was examined in LBK medium with different NaCl and LiCl concentrations at pH 5.0 to 8.5.

For the complementation in LBK medium with different NaCl concentrations (at pH 5.0), there were no significantly different among empty vector, expressing cells harboring *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C (Appendix 12). At pH 6.0, *E. coli* mutant TO114 cells carrying empty vector and expressing cell *FL_OtNhaA*/pTrcHis2C could grow in the presence of 300 mM NaCl while ***Δ****N112_OtNhaA*/pTrcHis2C could grow at higher NaCl concentration, e.g. in the presence of 500 mM NaCl (Fig 4.8). When the complementation test was conducted at neutral pH (LBK pH 7.0), *E. coli* mutant TO114 cells transformed with empty vector could not grow in the presence of NaCl while expressing cells *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C could grow up to 700 mM NaCl (Fig 4.9). At alkaline pH (LBK pH 8.0), *E. coli* mutant TO114 cells transformed with empty vector also could not grow in the presence of NaCl while expressing cells *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C could grow in the presence of 200 and 400 mM NaCl,

respectively (Fig 4.10). At pH 8.5, only ***Δ****N112_OtNhaA*/pTrcHis2C could grow in the presence of 150 mM NaCl (Appendix 13). These results clearly showed that the expressing cells harboring *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C could complement the Na^+-sensitive phenotype of *E. coli* mutant TO114. Furthermore, the expressing cell ***Δ****N112_OtNhaA*/pTrcHis2C could complement the Na^+-sensitive phenotype of *E. coli* mutant TO114 more than *FL_OtNhaA*/pTrcHis2C at pH ranging from 6.0 to 8.5 as the summary in Table 2.

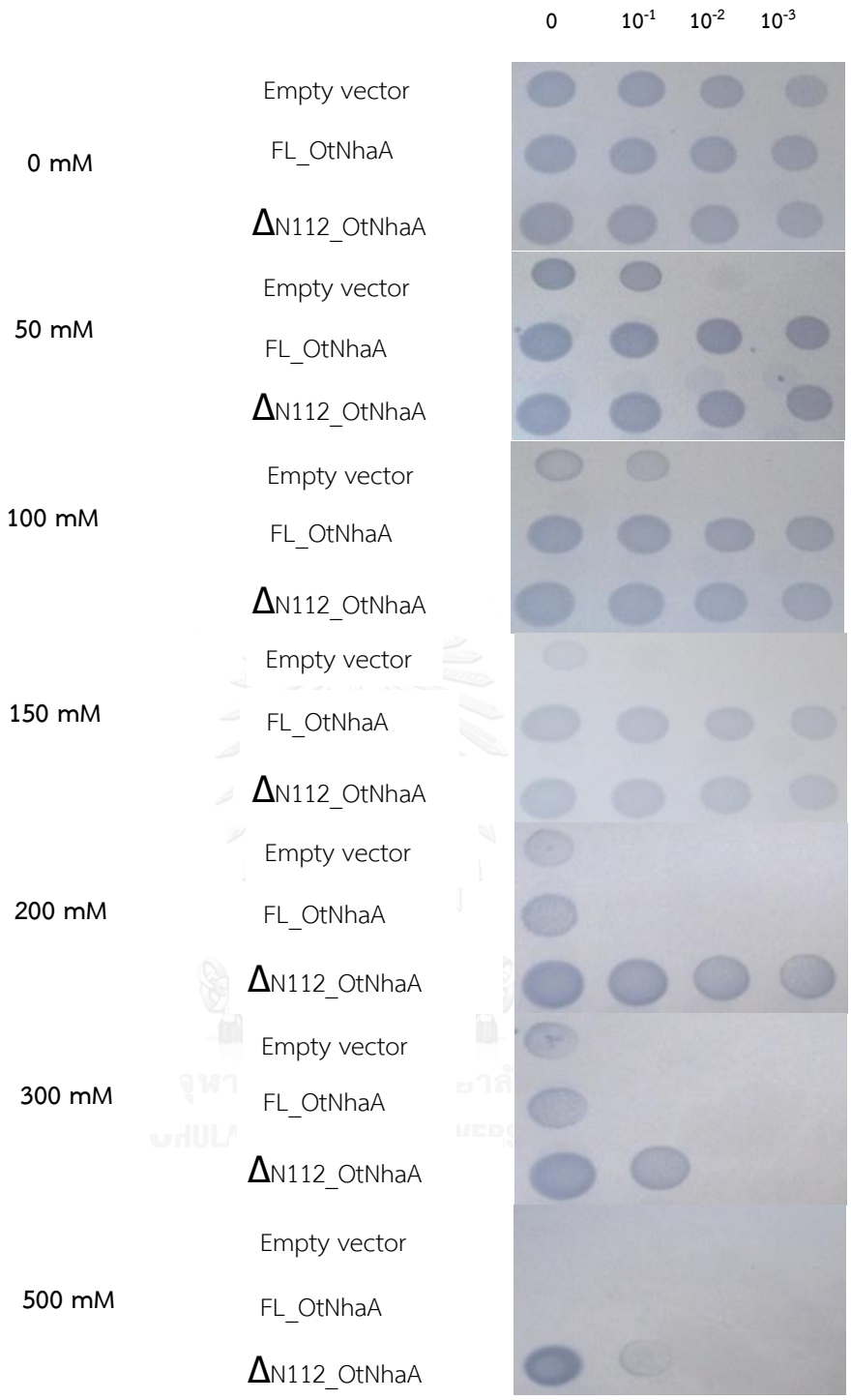


Figure 4.8: Complementation test of *E. coli* TO114 cells harboring empty vector (pTrcHis2C) and *FL_OtNhaA, ΔN112_OtNhaA* in LBK medium with different concentrations of NaCl at pH 6.0. The cultures were incubated at 37 °C for overnight and then scored the growth. Photographs were taken using SONY Cyber-shot camera.

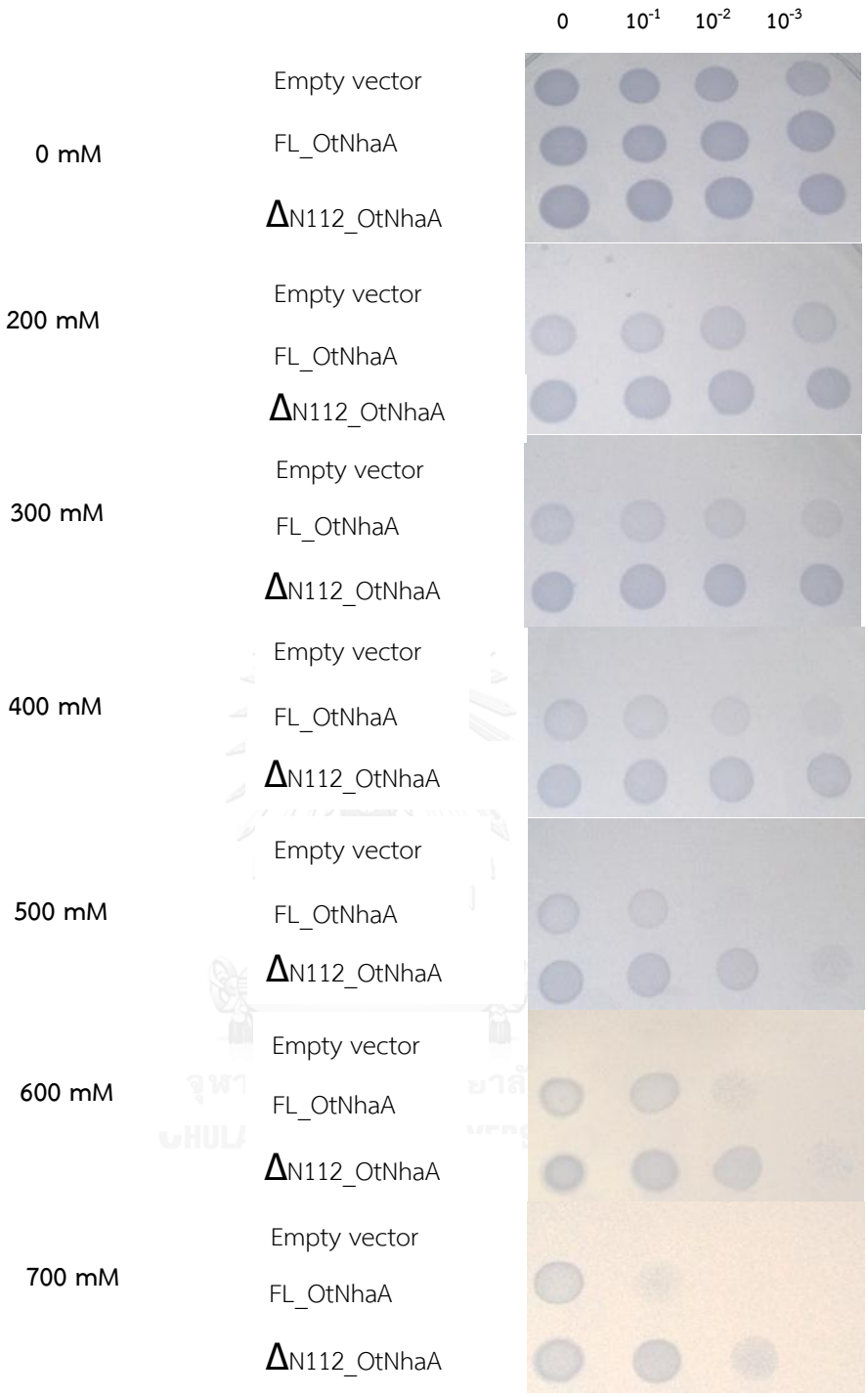


Figure 4.9: Complementation test of *E. coli* TO114 cells harboring empty vector (pTrcHis2c) and *FL_OtNhaA, ΔN112_OtNhaA* in LBK medium with different concentrations of NaCl at pH 7.0. The cultures were incubated at 37 °C for overnight and then scored the growth. Photographs were taken using SONY Cyber-shot camera.

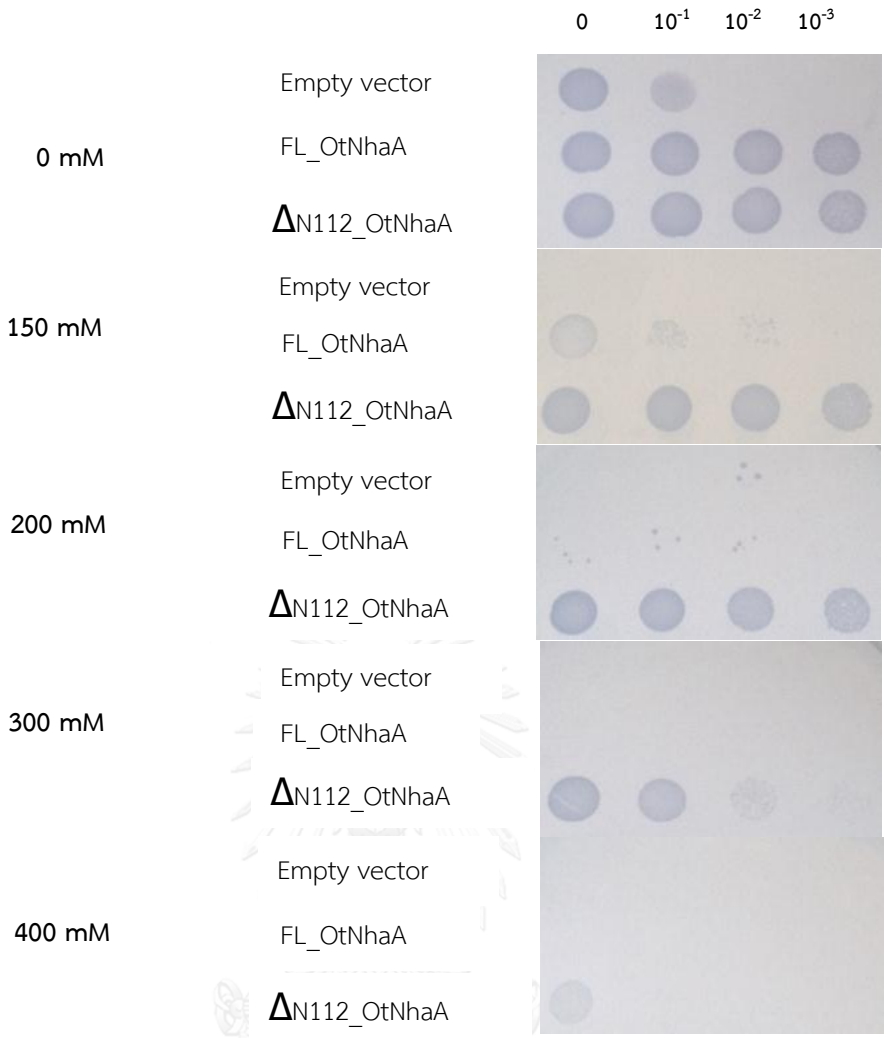


Figure 4.10: Complementation test of *E. coli* TO114 cells harboring empty vector (pTrcHis2c) and expressing cells *FL_OtNhaA, ΔN112_OtNhaA* in LBK medium with different concentrations of NaCl at pH 8.0. The cultures were incubated at 37 °C for overnight and then scored the growth. Photographs were taken using SONY Cyber-shot camera.

Next, ability of complementation with LiCl was examined. At pH 5.0, *E. coli* mutant TO114 cells transformed with empty vector could grow in the presence of 200 mM LiCl while expressing cells *FL_OtNhaA*/pTrcHis2C and **Δ***N112_OtNhaA*/pTrcHis2C could grow even in the presence of 300 mM LiCl (Appendix 14). At pH 6.0, *E. coli* mutant TO114 cells transformed with empty vector could not grow in the presence of LiCl while expressing cells *FL_OtNhaA*/pTrcHis2C and **Δ***N112_OtNhaA*/pTrcHis2C could grow even in the presence of 150 mM LiCl (Fig 4.11). At neutral pH (LBK pH 7.0), *E. coli* mutant TO114 cells transformed with *FL_OtNhaA*/pTrcHis2C could grow at a maximal concentration of 10 mM LiCl while **Δ***N112_OtNhaA*/pTrcHis2C could complement strongly. They could grow even in the presence of 40 mM LiCl. The empty vector had virtually no growth (Fig 4.12). Complementation test revealed **Δ***N112_OtNhaA*/pTrcHis2C could grow even in the presence of 50 mM LiCl. Thus, these results are obvious that **Δ***N112_OtNhaA*/pTrcHis2C had a strong ability for complementation. At alkaline pH (LBK pH 8.0), *E. coli* mutant TO114 cells transformed with empty vector or expressing cell *FL_OtNhaA*/pTrcHis2C could not grow while **Δ***N112_OtNhaA*/pTrcHis2C could grow in the presence of 10 mM LiCl (Fig 4.13). At pH 8.5, *E. coli* mutant TO114 cells transformed with empty vector and expressing cell *FL_OtNhaA*/pTrcHis2C could not grow while **Δ***N112_OtNhaA*/pTrcHis2C could grow in the presence of 10 mM LiCl (Appendix 15). These results showed that the expressing cells *FL_OtNhaA*/pTrcHis2C and **Δ***N112_OtNhaA*/pTrcHis2C could complement the Li^+-sensitive phenotype of *E. coli* mutant TO114. Furthermore, the expressing cell **Δ***N112_OtNhaA*/pTrcHis2C could complement the Li^+-sensitive phenotype of *E. coli* mutant TO114 more than that of *FL_OtNhaA*/pTrcHis2C at pH ranging from 5.0 to 8.5 as the summary in Table 2.

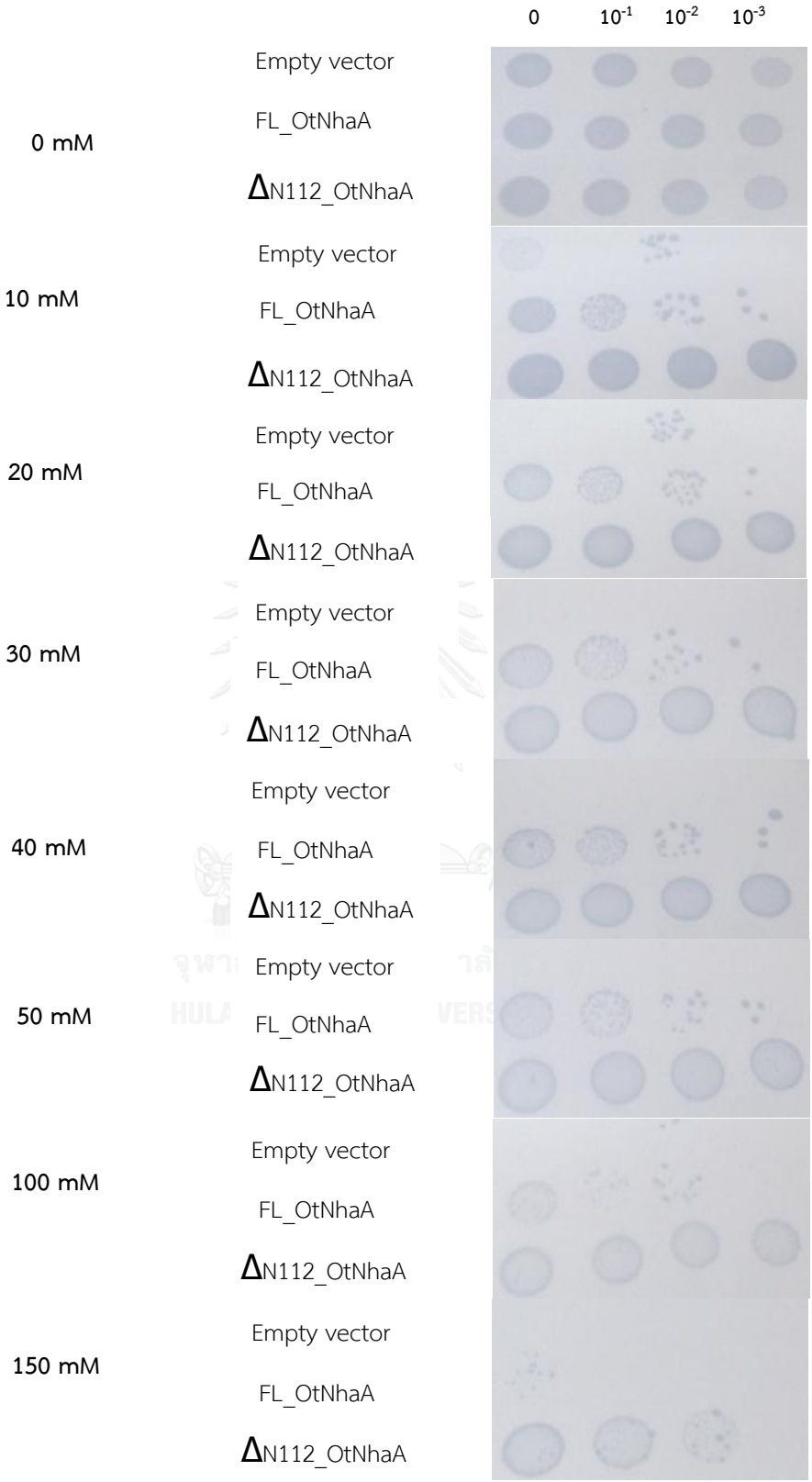


Figure 4.11: Complementation test of *E. coli* TO114 cells harboring empty vector (pTrcHis2C) and *FL_OtNhaA, ΔN112_OtNhaA* in LBK medium with different concentrations of LiCl at pH 6.0. The cultures were incubated at 37 °C for overnight and then scored the growth. Photographs were taken using SONY Cyber-shot camera.

Figure 4.12: Complementation test of *E. coli* TO114 cells harboring empty vector (pTrcHis2C) and *FL_OtNhaA, ΔN112_OtNhaA* in LBK medium with different concentrations of LiCl at pH 7.0. The cultures were incubated at 37 °C for overnight and then scored the growth. Photographs were taken using SONY Cyber-shot camera.

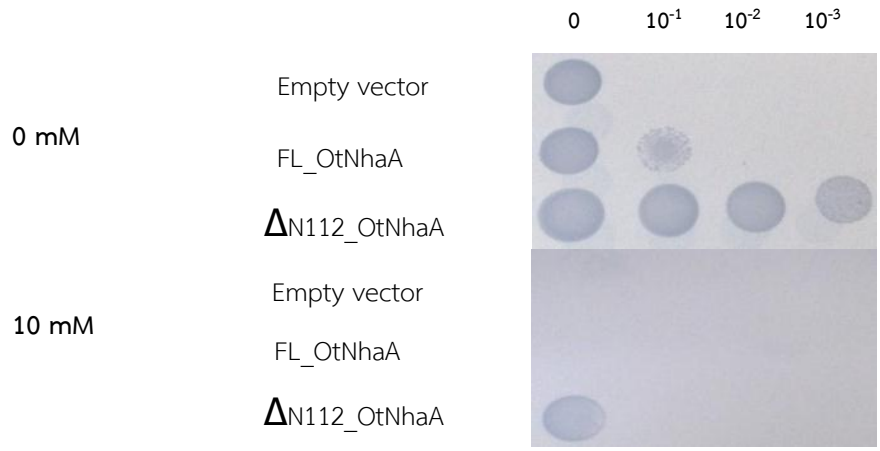


Figure 4.13: Complementation test of *E. coli* TO114 cells harboring empty vector (pTrcHis2C) and *FL_OtNhaA, ΔN112_OtNhaA* in LBK medium with different concentrations of LiCl at pH 8.0. The cultures were incubated at 37 °C for overnight and then scored the growth. Photographs were taken using SONY Cyber-shot camera.

Table 4.2: Maximal concentrations of NaCl and LiCl for the growths of empty vector, expressing cells *FL_OtNhaA* and *ΔN112_OtNhaA* in LBK medium with different concentrations of salts (NaCl and LiCl) at pH ranging from 5.0 to 8.5. Ability of complementation was recorded after the cells were spotted on tested media and incubated at 37 ºC for 24 hr.

	NaCl (mM)					**LiCl (mM)**				
	pH 5	pH 6	pH 7	pH 8	pH 8.5	pH 5	pH 6	pH 7	pH 8	pH 8.5
Empty vector	500	300	0	0	0	200	0	0	0	0
FL_OtNhaA	500	300	700	200	0	300	150	10	0	0
Δ*N112_OtNhaA*	500	500	700	400	150	300	150	40	10	10

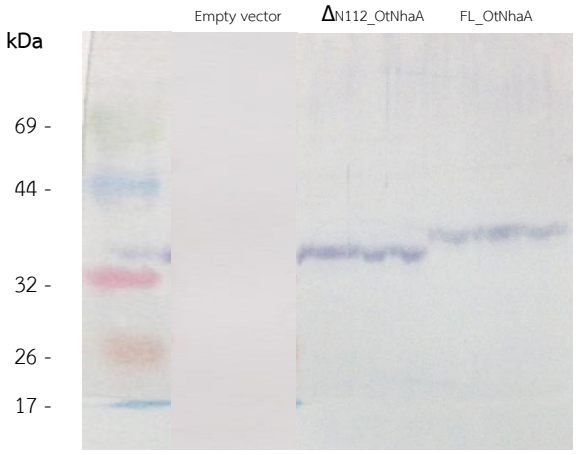
Functional complementation suggests that the expressing cells *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C could complement the Na^+-, Li^+-sensitive phenotype of *E. coli* mutant TO114. The expressing cell harboring ***Δ****N112_OtNhaA*/pTrcHis2C had a strong ability for complementation at pH 5.0 to 8.5 as the summary in Table 2. This is the first study of functional analysis for NhaA Na^+/H^+ antiporter from microalgae. To date, functional analysis of NhaA Na^+/H^+ antiporter has been mostly reported in bacteria. In *Escherichia coli*, the expressing cells NhaA could complemented the Na^+-sensitive phenotype of *E. coli* mutant TO116. For NaCl, *E. coli* expressing cells were able to grow in the presence of 300 mM NaCl at pH 7.2 and also at alkaline pH (pH 8.5) (Sakuma *et al.*, 1998). In *Salmonella typhimurium*, the expressing cell STNhaA could complemented the Na^+, Li^+-sensitive phenotype of *E. coli* mutant EP432 (***Δ****nhaA* and ***Δ****nhaB*). For NaCl or LiCl, *E. coli* mutant cells transformed with *STNhaA* could grow in the presence of 200 mM NaCl or LiCl at pH 7.5 (Lentes *et al.*, 2014). In *Yersinia pestis*, the expressing cell harboring *Y. pestis* NhaA could complement the Na^+, Li^+-sensitive phenotype of *E. coli* mutant KNabc (***Δ****nhaA*, ***Δ****nhaB* and ***Δ****chaA*). For NaCl, *E. coli* mutant cells transformed with *Y. pestis NhaA* could grow in the presence salt, up to 300 mM NaCl. In case of LiCl, the expressing cell could grow at maximum concentration of 150 mM LiCl (Ganoth *et al.*, 2011). These data indicate that the expressing cells harboring *FL_OtNhaA* and ***Δ****N112_OtNhaA* in this study were different (in terms of complementation ability) from the other expressing cells harboring bacteria NhaA as described above (Ohyama *et al.*, 1994, Sakuma *et al.*, 1998, Ganoth *et al.*, 2011, Lentes *et al.*, 2014). At neutral pH, the expressing cells *FL_OtNhaA* and ***Δ****N112_OtNhaA* could complement the Na^+-sensitive of *E. coli* mutant

much better than the other NhaA expressing cells from bacteria. Furthermore, the expressing cells ***Δ****N112_OtNhaA* could complement Na^+-sensitive of *E. coli* mutant more than *FL_OtNhaA*. The plausible reasons to explain are as follows. N-terminus may hinder antiporter activity. Expression level of FL_OtNhaA and **Δ**N112_OtNhaA proteins may not be equally expressed in *E. coli*. Thus the expressing cells ***Δ****N112_OtNhaA* had a strong ability for complementation. The ability of NaCl tolerance is maximum at 700 mM NaCl. On the other hand, the expressing cells *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C could complement Li^+-sensitive of *E. coli* mutant but lower than the other expressing cells NhaA from bacteria as described above.

4.2.5 Western Blot analysis

The everted membrane vesicles of empty vector, *FL_OtNhaA*/pTrcHis2C and *ΔN112_OtNhaA*/pTrcHis2C in *E. coli* TO114 were prepared as described in Materials and Methods. These membranes were used for immunoblotting with the antibody raised against 6X-His tag (Fig 4.14A). The first lane, *E. coli* cells transformed with empty vector alone did not show any cross-reacted band. The second lane, *E. coli* cells transformed with *ΔN112_OtNhaA*/pTrcHis2C exhibited a single cross-reacted band approximately 35 kDa (Fig 4.14A). This result was in agree with theoretical molecular mass of ΔN112_OtNhaA (35 kDa) (Fig 4.14B). The last lane, *E. coli* cells transformed with *FL_OtNhaA*/pTrcHis2C exhibited a single cross-reacted band approximately 40 kDa (Fig 4.14A), whereas the theoretical molecular mass of FL_OtNhaA was 47 kDa. The results from western blotting showed the smaller size of protein than theoretical calculation. This might be from a high hydrophobicity of membrane proteins (Hamada *et al.*, 2001, Lentes *et al.*, 2014). Hydrophobicity generally effects the migration of membrane protein so the detected size on SDS-PAGE would different and/or lower than theoretical calculation. Anyway, Western Blot analysis is positive. Thus, these results indicate that the expressing cells *FL_OtNhaA*/pTrcHis2c and *ΔN112_OtNhaA*/pTrcHis2c could be expressed and assembled in *E. coli* membranes.

A)



B)

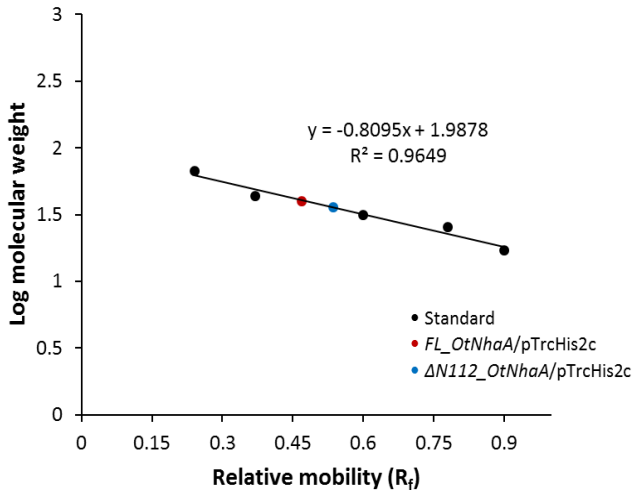
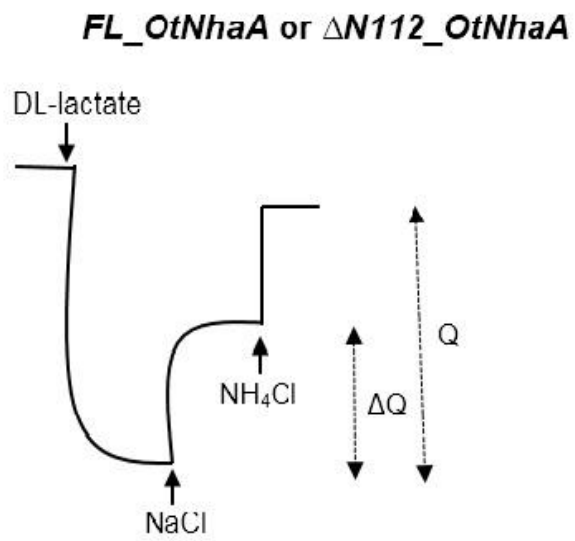


Figure 4.14: A) Western Blotting analysis for everted membrane vesicles preparing from *E. coli* TO114 cells harboring empty vector, *FL_OtNhaA* and *ΔN112_OtNhaA*. Antibody raised against 6X-His tag and antibody raised against mouse were used as primary and secondary antibodies, respectively. The cross-reacting band was detected with substrate for alkaline phosphatase as described in Materials and Methods. Lane 1: control (pTrcHis2C) (50 µg membrane protein), lane 2: *ΔN112_OtNhaA*/pTrcHis2C expressing cell (50 µg membrane protein) and lane 3: *FL_OtNhaA*/pTrcHis2C expressing cell (50 µg membrane protein). B) Standard curve of log molecular weight and relative mobility (R_f) of each protein.

4.2.6 Na^+/H^+ antiporter activity

To examine the antiporter activity of the expressing cells carrying *FL_OtNhaA*/pTrcHis2C and **Δ***N112_OtNhaA*/pTrcHis2C, the everted membrane vesicles were prepared as described in Materials and Methods. Antiporter activity assay was performed based on measurement the change of the vesicular **Δ**pH (transmembrane pH gradient) after addition of salts to the reaction mixture. These antiporter activities were monitored by acridine orange fluorescence quenching method upon addition of salts (NaCl, LiCl, KCl and $CaCl_2$) at pH 7 to 9. Firstly, DL-lactate was added to initiate fluorescence quenching (Q) and salt was added to increase fluorescence dequenching (**Δ**Q). Figure 4.12 shows typical dequenching patterns, the dequenching (**Δ**Q x 100/Q) was observed upon addition of NaCl in the expressing cells *FL_OtNhaA*/pTrcHis2C and **Δ***N112_OtNhaA*/pTrcHis2C (Fig. 4.15A), but not in the control cells because it lacks of Na^+/H^+ antiporters (NhaA, NhaB and ChaA). Thus, it has no exchange activity (Fig. 4.15B).

A)



B)

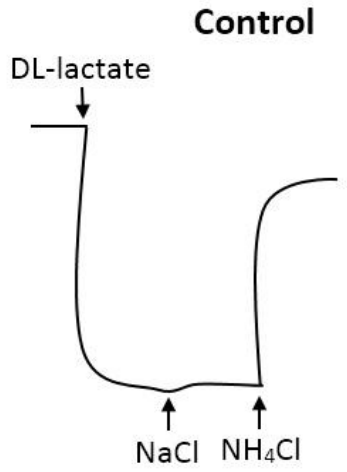


Figure 4.15: Typical quenching patterns A) expressing cells *FL_OtNhaA*/pTrcHis2C, ***Δ****N112_OtNhaA*/pTrcHis2C and B) control cells (pTrcHis2C).

To examine ion exchange activity of the expressing cells *FL_OtNhaA*/pTrcHis2C, ***Δ****N112_OtNhaA*/pTrcHis2C compared with control ones, the everted membrane vesicles were prepared by using *E. coli* mutant TO114 cells grown in LBK or LBK+200 mM NaCl media as described in Materials and Methods. The dequenching was measured upon addition of different four cations (Na^+, Li^+, Ca^{2+} and K^+) at pH 7.0 to 9.0. Figure 4.16 showed all cation/proton antiporter exchange activities from the vesicle membranes prepared from the cells grown in LBK medium.

For Na^+/H^+ antiporter activity, the expressing cell harboring ***Δ****N112_OtNhaA*/pTrcHis2C performed high exchange activity when compared with *FL_OtNhaA*/pTrcHis2C at all assay pHs. Control cells essentially had no exchange activity (or only small background; less than 5% dequenching) (Fig 4.16). The expressing cell ***Δ****N112_OtNhaA*/pTrcHis2C had maximum exchange activity at pH 8.5 (~67% dequenching). The Na^+/H^+ antiporter activity of expressing cell *FL_OtNhaA*/pTrcHis2C is dependent on pH. It had maximum exchange activity at pH 9.0 (~40% dequenching) while control cell had only small activity (less than 5% dequenching) at all pH observed. Furthermore, expressing cells *FL_OtNhaA*/pTrcHis2C, ***Δ****N112_OtNhaA*/pTrcHis2C and control cells had no exchange activity at pH 7.0. For Li^+/H^+ antiporter activity, both the expressing cells *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C had low exchange activity of Li^+ for H^+ at all pHs. Control cells had virtually no exchange activity.

For Ca^{2+}/H^+ antiporter activity, both the expressing cells *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C had very similar in exchange activity (~25 - 33% dequenching) at pH 7.5 to 8.5. Both expressing cells *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C showed no activity of Ca^{2+}/H^+ antiporter at pH 7.0 or 9.0.

The control cells had small Ca^{2+}/H^+ antiporter activity (less than 12% dequenching) at all pH.

For K^+/H^+ antiporter activity, all control and expressing cells had no exchange activity between K^+ and H^+ at all pH tested.

Taken together, these results indicate that the expressing cells *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C had high Na^+/H^+ or Ca^{2+}/H^+ exchange activity at alkaline pH, but they showed no activity of Ca^{2+}/H^+ antiporter at pH 7.0 and 9.0. The expressing cells *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C both had low Li^+/H^+ or K^+/H^+ exchange activity at alkaline pH. Thus, the expressing cells *FL_OtNhaA*/pTrcHis2C could exhibit pH dependence for Na^+/H^+ exchange activity. Their activity increased with increasing pHs. By contrast, the expressing cells ***Δ****N112_OtNhaA*/pTrcHis2C seem to exhibit pH independence for Na^+/H^+, Li^+/H^+ and Ca^{2+}/H^+ exchange activities.

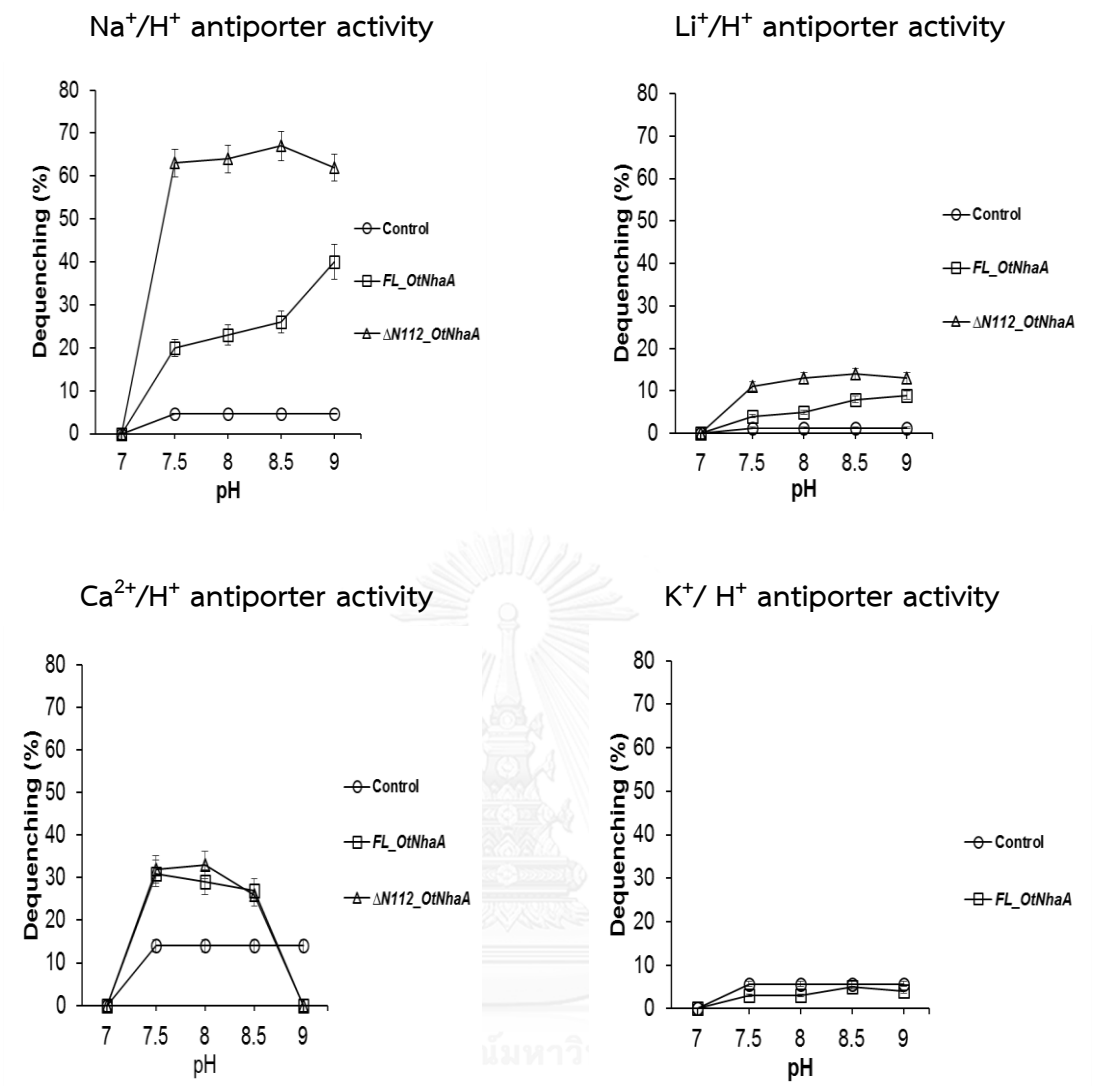


Figure 4.16: The cation/proton antiporter activity measured by acridine orange fluorescence quenching method. The control *E. coli* TO114 (pTrcHis2C) cells and *E. coli* expressing cells *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C were grown in LBK medium. Everted membrane vesicles were prepared from these cells.

Next, the everted membrane vesicle were prepared using *E. coli* mutant TO114 cells grown in LBK+200 mM NaCl medium. We hypothesized that salt stress may stimulate antiporter activity of expressing cells. In Figure 4.17 showed all cation/proton antiporter activities observing from the membrane prepared in LBK+200 mM NaCl medium. For Na^+/H^+ antiporter activity, the expressing cell ***Δ****N112_OtNhaA*/pTrcHis2C had high exchange activity at pH 7.5 to 9.0. *FL_OtNhaA*/pTrcHis2C had Na^+/H^+ exchange activity approx. 30-40% dequenching. Control vesicles membranes showed virtually no exchange activity. The expressing cell ***Δ****N112_OtNhaA*/pTrcHis2C had maximal exchange activity at pH 8.5 (~70% dequenching). The Na^+/H^+ antiporter activity of expressing cell *FL_OtNhaA*/pTrcHis2C is dependent on pH. It has maximal exchange activity at pH 9.0 (~40% dequenching). For Li^+/H^+ antiporter activity, both the expressing cells *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C had low exchange activity (~5 – 15% dequenching) at pH 7.5 to 9.0.

For Ca^{2+}/H^+ antiporter activity, both the expressing cells *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C had no different in exchange activity (~25 - 35% dequenching) at alkaline pH (pH 7.5 to 8.5), whereas these showed no activity of Ca^{2+}/H^+ antiporter at pH 7.0 and 9.0. The control cells had small Ca^{2+}/H^+ antiporter activity at all pHs. For K^+/H^+ antiporter activity, all vesicle membranes had no exchange activities.

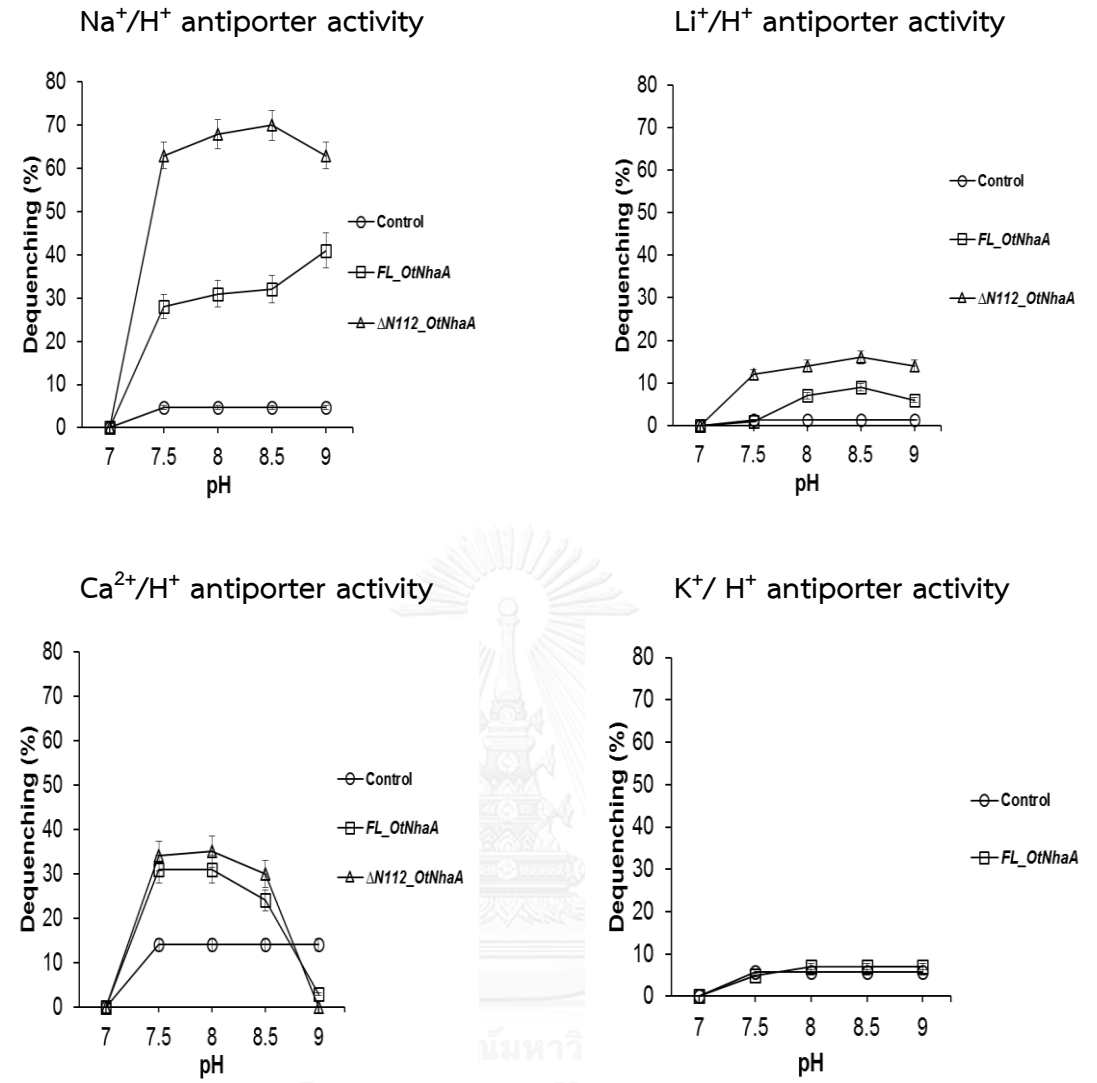


Figure 4.17: The cation/proton antiporter activity measured by acridine orange fluorescence quenching method. The control *E. coli* TO114 (pTrcHis2C) cells and *E. coli* expressing cells *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C were grown in LBK+200 mM NaCl medium. Everted membrane vesicles were prepared from these cells.

For antiporter activity of the expressing cells *FL_OtNhaA*/pTrcHis2C, ***Δ****N112_OtNhaA*/pTrcHis2C and control cells, the everted membrane vesicles were prepared by using *E. coli* mutant TO114 cells in LBK or LBK+200 mM NaCl media. These results suggests that the expressing cells *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C had high Na^+/H^+ or Ca^{2+}/H^+ exchange activity at alkaline pH (up to pH 8.5). On the other hand, the expressing cells *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C had low Li^+/H^+ or K^+/H^+ exchange activity at alkaline pH. Therefore, the expressing cell *FL_OtNhaA*/pTrcHis2C exhibited pH dependence for Na^+/H^+ activity. Their activity increased with increasing pH. For the expressing cell ***Δ****N112_OtNhaA*/pTrcHis2C exhibited pH independence for Na^+/H^+, Li^+/H^+, Ca^{2+}/H^+ and K^+/H^+ exchange activities. All results as shown in Fig 4.16 and 4.17.

To date, NhaA Na^+/H^+ antiporter has been reported in various organisms. In *E. coli,* three Na^+/H^+ antiporters (NhaA, NhaB and ChaA) are known and their functional characteristics have been well described. NhaA is the first-discovered antiporter in *E. coli*. It is the main Na^+/H^+ antiporter and specifically exchange between Na^+ or Li^+ and H^+ (Padan *et al.*, 2004), which is essentially the same ion specificity as that of expressing cells *FL_OtNhaA*/pTrcHis2C and ***Δ****N112_OtNhaA*/pTrcHis2C. NhaA is indispensable for adaptation to high salinity, resistance to Li^+ toxicity and for growth under alkaline conditions (Herz *et al.*, 2003). The antiporter activity of NhaA is dependent on pH. It is enhanced at alkaline pH (pH 8.5) and reduced at acidic pH (pH 6.5) (Screpanti *et al.*, 2006). In *S. typhimurium*, the antiporter activity of NhaA is increase at pH 8.5 and decrease at below pH 7.0 (Lentes *et al.*, 2014). In *V. cholerae*, the antiporter activity of NhaA is increase at pH 8.0 and decrease at pH 7.0 (Herz *et al.*, 2003). Functional characterization of OtNhaA in this study also performed pH dependency for Na^+. It

should be noted that the exchange activity of OtNhaA performed mainly as Na^+/H^+ but not Li^+/H^+. In addition, exchange activity of Ca^{2+}/H^+ was also high with a pH independent profile. These results indicate OtNhaA is distinguishable for exchange activity compared with bacterial NhaA.

4.2.7 Kinetic parameters of FL_OtNhaA and ΔN112_OtNhaA proteins

The kinetic parameters of FL_OtNhaA or ***Δ***N112_OtNhaA proteins were determined at various pHs. For these, the everted membrane vesicles were prepared by using *E. coli* mutant TO114 cells harboring FL_OtNhaA or ***Δ***N112_OtNhaA grown in either LBK or LBK+200 mM NaCl media as described in Materials and Methods. The kinetic parameters were analyzed using Michaelis-Menten kinetics plot from GraphPad Prism 6 Software (www.graphpad.com/guides/prism/6).

As shown in 4.2.6, expressing cell *FL_OtNhaA*/pTrcHis2C had high Na^+/H^+ and Ca^{2+}/H^+ exchange activities (Figs. 4.16 and 4.17). By contrast, it had low Li^+/H^+ and K^+/H^+ exchange activities (Figs. 4.16 and 4.17).

The apparent K_m of FL_OtNhaA (K_m for Na^+) in LBK medium at various pH was shown in Fig 4.18. The K_m for Na^+ was 2.3 ± 0.33 mM at pH 7.5 and decreased to 1.2 ± 0.34 mM, 1.1 ± 0.23 mM and 0.5 ± 0.04 mM at pH 8.0, 8.5 and 9.0, respectively. In terms of V_{max} (represented in this study as % dequenching), our data showed that the V_{max} of FL_OtNhaA was about 20% at pH 7.5 and increased to 23, 26 and 40% at pH 8.0, 8.5 and 9.0, respectively (Fig 4.18 A-D).

The apparent K_m of FL_OtNhaA was also observed using membrane vesicles prepared from growing under salt-stress condition (LBK+200 mM NaCl). It was found that K_m (for Na^+) in all pHs observed were similar (0.8, 0.8, 0.7 and 0.2 mM) (Fig 4.18 E-H). The V_{max} of FL_OtNhaA was 28% at pH 7.5 and increased to 31, 32 and 41% at pH 8.0, 8.5 and 9.0, respectively. These results indicated that kinetic parameters of FL_OtNhaA were different, depending on conditions for membrane vesicles preparation. It was found that FL_OtNhaA everted membranes preparing under LBK+200 mM NaCl medium exhibited much lower K_m at all pH tested, i.e. 2.3 ± 0.33

mM vs 0.8 ± 0.37 mM at pH 7.5 (everted membrane preparing from cells grown in LBK and LBK+200 mM NaCl medium). Although the promoter used in this study (*trpB* + *lacUV5* hybrid promoter) was salt-independent, but K_m was different. One of reasons could be due to the activation of FL_OtNhaA protein by salt (Waditee *et al.*, 2007). Another reason for this could be due to the allosteric regulation of FL_OtNhaA activity by salt (Calinescu *et al.*, 2014).

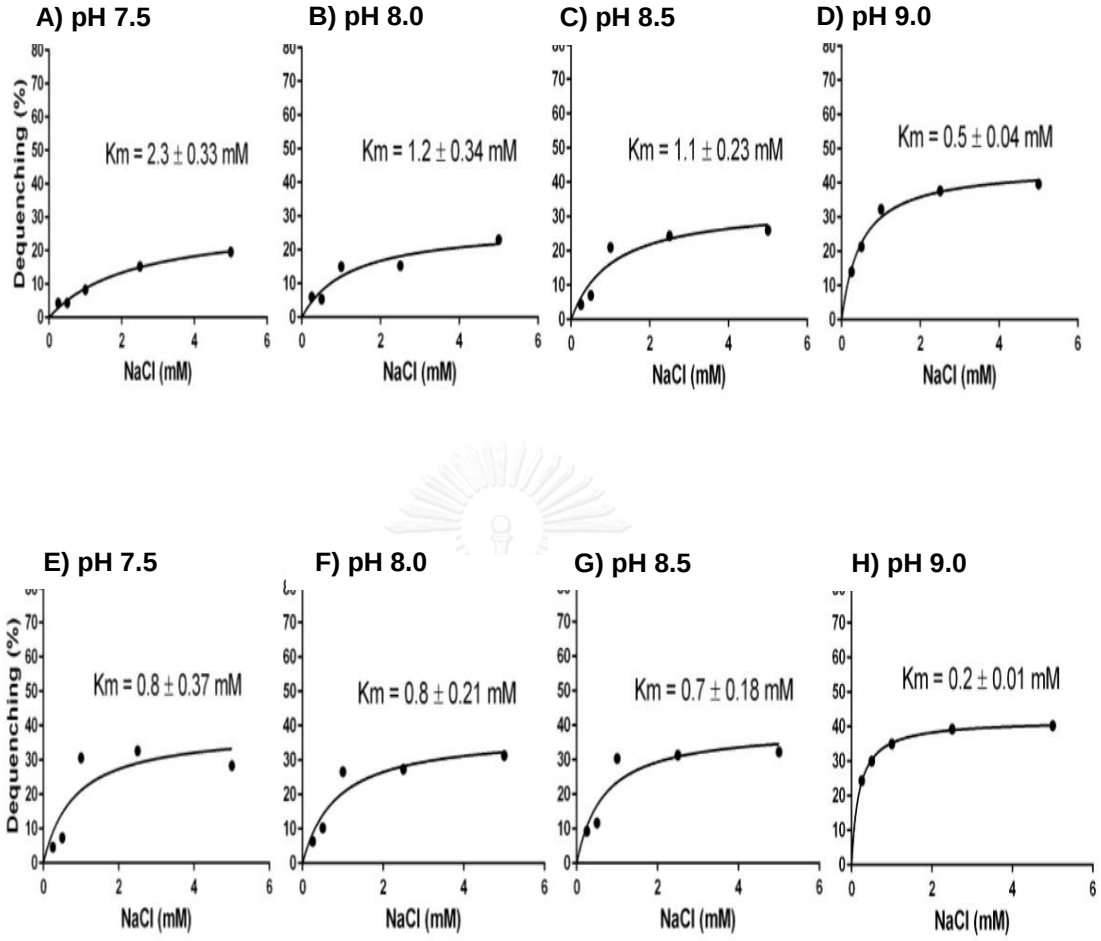


Figure 4.18: kinetic parameters for sodium ion of FL_OtNhaA. A - D) using membranes prepared from cells grown in LBK medium and E – H) using membranes prepared from cells grown in LBK+200 mM NaCl medium at pH 7.5 to 9.0. The kinetic parameters were analyzed using Michaelis-Menten kinetics plot from GraphPad Prism 6 Software (www.graphpad.com/guides/prism/6).

The apparent K_m of FL_OtNhaA for Ca^{2+} in LBK medium at pH 7.5 to 8.5 was shown in Fig. 4.19. The K_m was 0.1 ± 0.02 mM at pH 7.5 and decreased to 0.2 ± 0.03 mM and 0.3 ± 0.07 mM at pH 8.0 and 8.5 respectively, while the V_{max} was 31% at pH 7.5 and decreased to 29% and 27% at pH 8.0 and 8.5, respectively (Fig 4.19 A-C). At pH 9.0, the K_m and V_{max} values of FL_OtNhaA could not be determined due to no dequenching (Fig 4.16). Apparent K_m of FL_OtNhaA for Ca^{2+} using membranes prepared from LBK+200 mM NaCl medium was also tested. The K_m value was 0.2 ± 0.02 mM at pH 7.5 and decreased to 0.1 ± 0.01 mM, 0.1 ± 0.03 mM at pH 8.0 and 8.5, respectively. Furthermore, the V_{max} of FL_OtNhaA was similar FL_OtNhaA everted membrane preparing in LBK medium (Fig 4.19 E-G). These results indicated that Ca^{2+}/H^+ exchange activity of FL_OtNhaA (everted membrane preparing from cells grown in LBK and LBK+200 mM NaCl medium) had similar affinity at alkaline pH.

Since the exchange activity of Li^+/H^+ and K^+/H^+ exchange activity observed in FL_OtNhaA were very low, thus determination of K_m and V_{max} was not determined in this study.

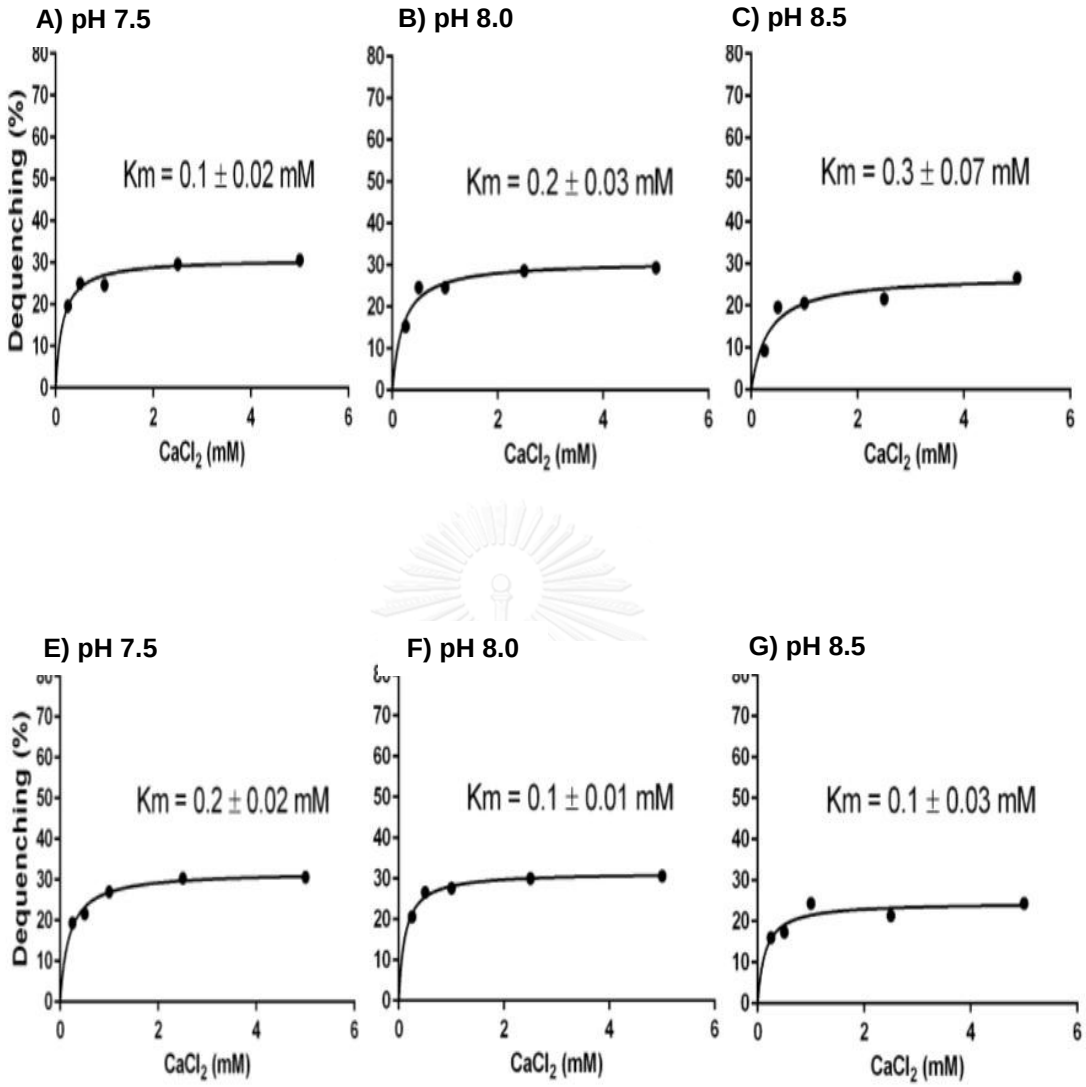


Figure 4.19: kinetic parameters for calcium ion of FL_OtNhaA. A - C) using membranes prepared from cells grown in LBK medium and E – G) using membranes prepared from cells grown in LBK+200 mM NaCl medium at pH 7.5 to 8.5. The kinetic parameters were analyzed using Michaelis-Menten kinetics plot from GraphPad Prism 6 Software (www.graphpad.com/guides/prism/6).

The data described in kinetic measurement section suggested that Na^+/H^+ exchange activity of FL_OtNhaA (everted membrane preparing from cells grown in LBK and LBK+200 mM NaCl medium) had high affinity at alkaline pH. In case of Ca^{2+}/H^+ exchange activity of FL_OtNhaA, it had similar affinity at all pH tested.

Next, the kinetic parameters of truncation FL_OtNhaA (deletion 112 amino acids from N-terminus) were carried out. The apparent K_m of **Δ**N112_OtNhaA (K_m for Na^+ in LBK medium at pH 7.5 to 9.0 was shown in Fig 4.20. The K_m for Na^+ was 2.0 ± 0.25 mM at pH 7.5 and decreased to 1.1 ± 0.07 mM, 0.6 ± 0.07 mM and 0.3 ± 0.03 mM at pH 8.0, 8.5 and 9.0, respectively. In term of V_{max} (represented in this study as % dequenching), the V_{max} of **Δ**N112_OtNhaA was about 63% at pH 7.5 and increased to 64, 67 and 62% at pH 8.0, 8.5 and 9.0, respectively (Fig 4.20 A-D).

The apparent K_m of **Δ**N112_OtNhaA was also observed using membrane vesicles prepared from growing under salt-stress condition (LBK+200 mM NaCl). It was found that K_m (for Na^+) in all pHs observed was 1.7 ± 0.13 mM at pH 7.5 and decreased to 0.9 ± 0.07 mM, 0.4 ± 0.03 mM and 0.3 ± 0.01 mM at pH 8.0, 8.5 and 9.0, respectively. The V_{max} of **Δ**N112_OtNhaA was 63% at pH 7.5 and increased to 68, 70 and 63% at pH 8.0, 8.5 and 9.0, respectively (Fig 4.20 E-H). These results indicated that kinetic parameters of **Δ**N112_OtNhaA were different, depending on conditions for membrane vesicles preparation. It was found that **Δ**N112_OtNhaA everted membranes preparing under LBK+200 mM NaCl medium exhibited much lower K_m at all pH tested, i.e. 2.0 ± 0.25 mM vs 1.7 ± 0.13 mM at pH 7.5 (everted membrane preparing from cells grown in LBK and LBK+200 mM NaCl medium). Although the promoter used in this study (*trpB* + *lacUV5* hybrid promoter) was salt-independent, but K_m were different. One of reasons could be due to the activation of FL_OtNhaA protein by salt (Waditee *et al.*,

2007). Another reason for this could be due to the allosteric regulation of FL_OtNhaA activity by salt (Calinescu *et al.*, 2014).

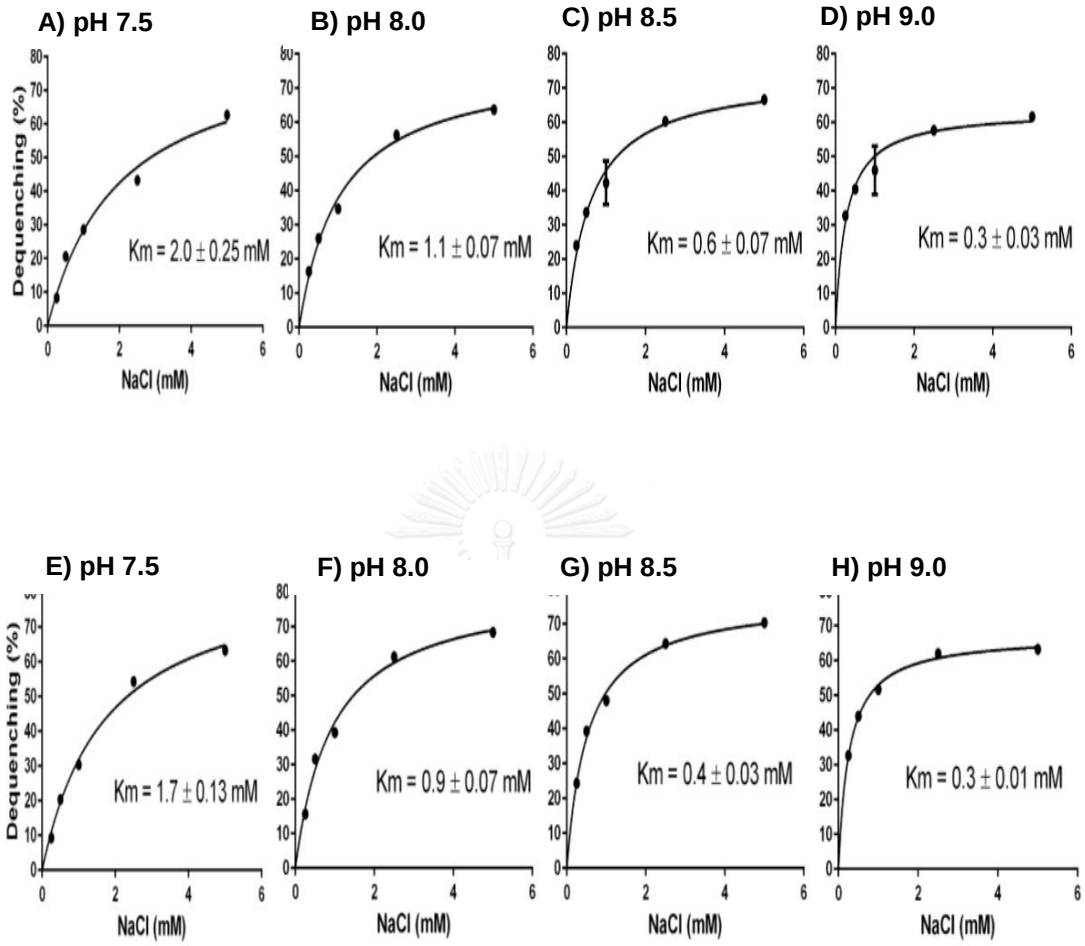


Figure 4.20: kinetic parameters for sodium ion of **Δ**N112_OtNhaA. A - D) using membranes prepared from cells grown in LBK medium and E – H) using membranes prepared from cells grown in LBK+200 mM NaCl medium at pH 7.5 to 9.0. The kinetic parameters were analyzed using Michaelis-Menten kinetics plot from GraphPad Prism 6 Software (www.graphpad.com/guides/prism/6).

The apparent K_m of **Δ**N112_OtNhaA for Ca^{2+} in LBK medium at various pH was shown in Fig. 4.21. The K_m was 0.2 ± 0.03 mM at pH 7.5 and increased to 0.2 ± 0.04 mM and 0.3 ± 0.02 mM at pH 8.0 and 8.5 respectively, while the V_{max} was 32% at pH 7.5 and decreased to 33 and 26% at pH 8.0 and 8.5, respectively (Fig 4.21 A-C). At pH 9.0, the K_m and V_{max} values of **Δ**N112_OtNhaA could not be determined due to no dequenching (Fig 4.16).

For the apparent K_m of **Δ**N112_OtNhaA for Ca^{2+} using membrane from cells grown in LBK+200 mM NaCl medium, K_m was 0.2 ± 0.02 mM at pH 7.5 and increased to 0.2 ± 0.02 mM, 0.3 ± 0.05 mM at pH 8.0 and 8.5, respectively. And V_{max} of *Δ*N112_OtNhaA was similar to FL_OtNhaA (Fig 4.21 E-G). These results indicated that Ca^{2+}/H^+ exchange activity of **Δ**N112_OtNhaA had similar affinity at all pHs, regardless the conditions for membrane preparation.

Since the exchange activity of Li^+/H^+ and K^+/H^+ exchange activity observed in **Δ**N112_OtNhaA were very low, thus determination of K_m and V_{max}, both parameter were not determined in this study.

To date, the K_m values for Na^+ of NhaA antiporter has been reported in various cation/proton antiporter proteins. In *E. coli,* the K_m values for Na^+; 178 mM at pH 7.0 and 7.3 mM at pH 9.0 (Padan *et al.*, 2004). In *S. typhimurium*, the K_m values for Na^+; 144 mM at pH 7.0 and 16 mM at pH 8.5 (Lentes *et al.*, 2014). In *V. cholerae*, the K_m values for Na^+; 0.65 mM at pH 8.5 (Herz *et al.*, 2003). In *H. pylori*, the K_m values for Na^+; 0.2 mM at pH 8.5 (Tsuboi *et al.*, 2003). In *Y. pestis,* the K_m values for Na^+; 3.5 mM at pH 8.0 (Ganoth *et al.*, 2011). Our FL_OtNhaA exhibited much lower K_m values for Na^+ than data described above. Therefore, it had high affinity at pH tested.

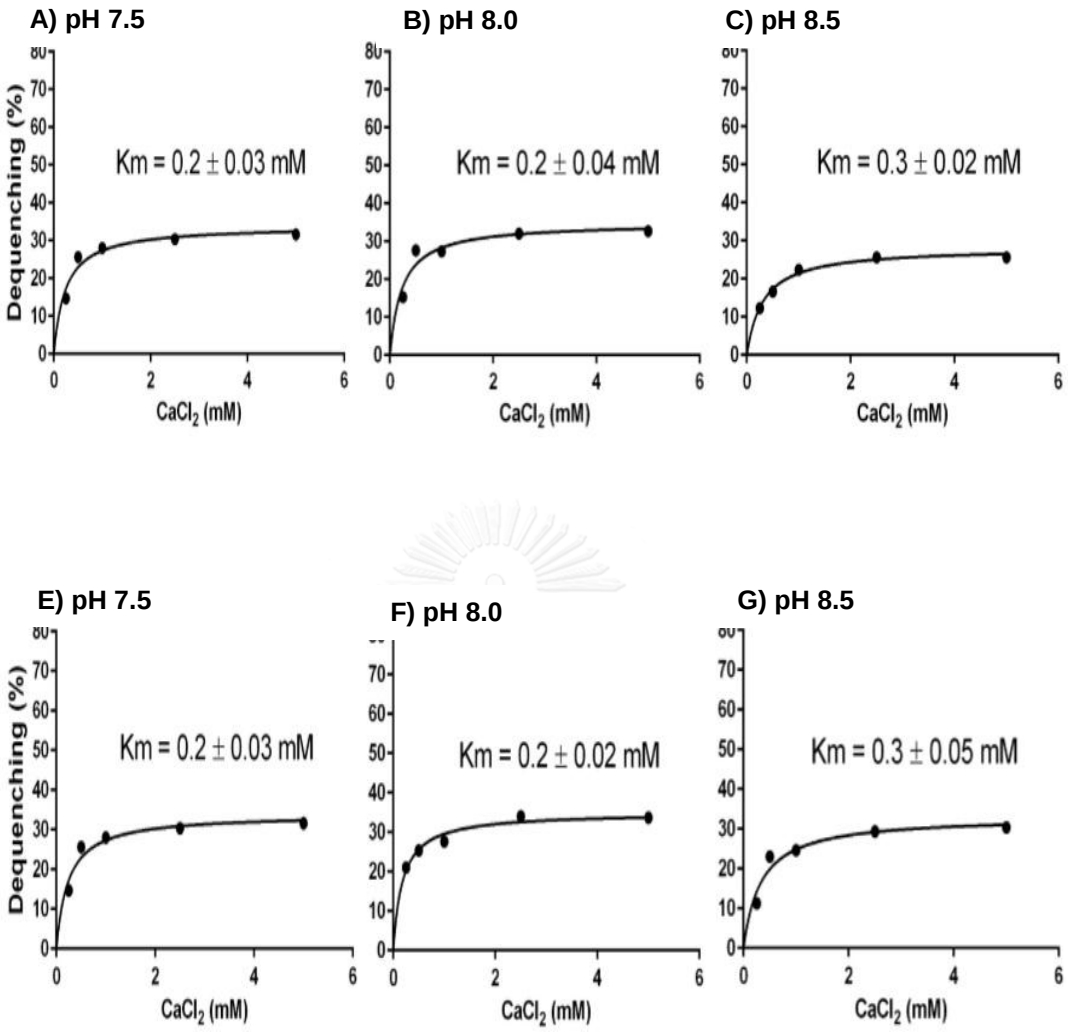


Figure 4.21: kinetic parameters for calcium ion of ΔN112_OtNhaA. A - C) using membranes prepared from cells grown in LBK medium and E – G) using membranes prepared from cells grown in LBK+200 mM NaCl medium at pH ranging from 7.5 to 8.5. The kinetic parameters were analyzed using Michaelis-Menten kinetics plot from GraphPad Prism 6 Software (www.graphpad.com/guides/prism/6).

4.3 Cloning and expression *O. tauri* Na^+/H^+ antiporter in *C. reinhardtii* 137c

4.3.1 Construction of the full length *OtNhaA* Na^+/H^+ antiporter in expression vector

To construct the full length *OtNhaA* Na^+/H^+ antiporter in expression vector pChlamy_3, the full length *OtNhaA* fragment from pMK vector (Life technologies, CA, USA) was prepared by double digestion with restriction enzymes *Xba*I and *Nde*I. The *OtNhaA* fragment approximately 1.6 kb was analyzed by 1% agarose gel electrophoresis. Then, the full length *OtNhaA* fragment was ligated into corresponding restriction sites of expression vector pChlamy_3 at *Xba*I and *Nde*I and further transformed into *E. coli* DH5α. To confirm the insertion, the insert fragment approximately 1.6 kb was confirmed by double digestion with restriction enzymes *Xba*I and *Nde*I (Fig. 4.22). The plasmid *FL_OtNhaA*/pChlamy_3 was extracted and transformed into *C. reinhardtii* 137c.

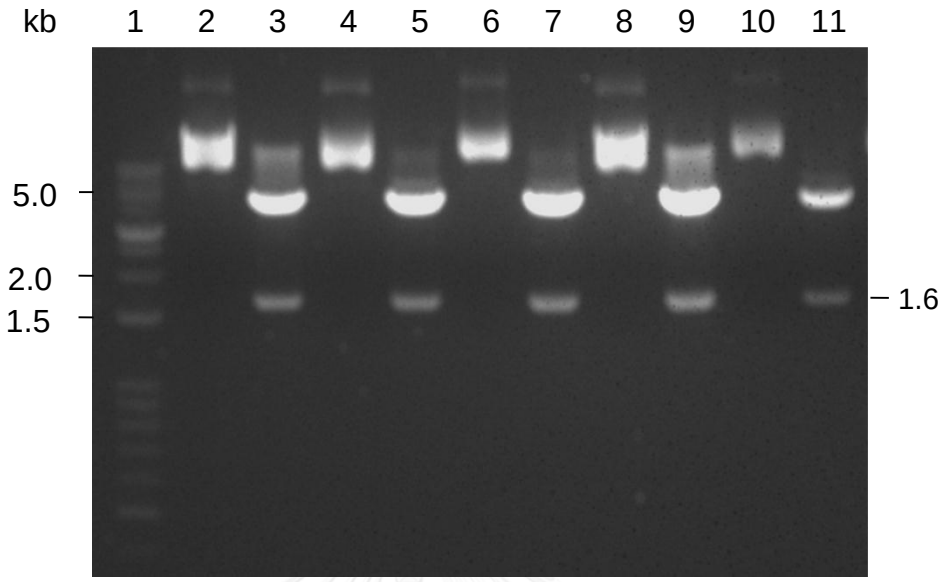


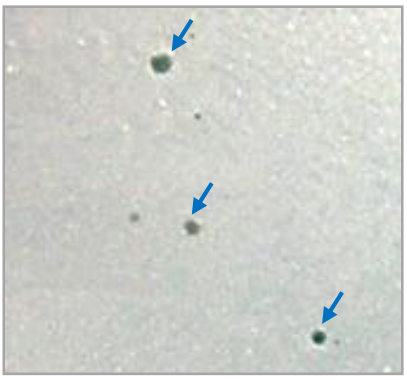
Figure 4.22: Restriction enzyme analysis of *FL_OtNhaA*/pChlamy_3 by double digestion *Xba*I and *Nde*I on 1% agarose gel electrophoresis. Lane 1: DNA ladder, lanes 2, 4, 6, 8 and 10: uncutted of *FL_OtNhaA*/pChlamy_3 and lanes 3, 5, 7, 9 and 11: double digestion of *FL_OtNhaA*/pChlamy_3 with *Xba*I and *Nde*I.

4.3.2 Expression of *FL_OtNhaA* into *C. reinhardtii* 137c

The recombinant plasmid *FL_OtNhaA*/pChlamy_3 and empty vector (pChlamy_3) were expressed into *C. reinhardtii* 137c by electroporation as described in Materials and Methods. After 10 days, the single colony receiving pChlamy_3 or recombinant plasmids (*FL_OtNhaA*/pChlamy_3) and empty vector generally could be observed the ability of growth in the presence of antibiotic (TAP agar containing 10 μg/ml hygromycin) (Fig 4.23). The candidate clones were re-streaked on new TAP agar containing hygromycin 10 μg/ml. Then, each single clone was used to check the insertion by colony PCR using specific primers for *FL_OtNhaA*/pChlamy_3 and hygromycin resistance gene. The electrophoresis result showed in Fig. 4.24A. The expressing cell *FL_OtNhaA*/pChlamy_3 had no band as shown in lanes 2 to 7, whereas the result of PCR product of hygromycin resistance gene had positive hygromycin band signals (Fig. 4.24B).

This result might be from unstable transformation or gene silencing. For manufacturer's instruction (GeneArt®*Chlamydomonas* Engineering Kits) suggests that about 50% of candidate clones should be positive, due to random integration and silencing events in *C. reinhardtii* 137c. But, this study could not obtain any positive clone even more than 60 independent clones were generated.

A)



B)

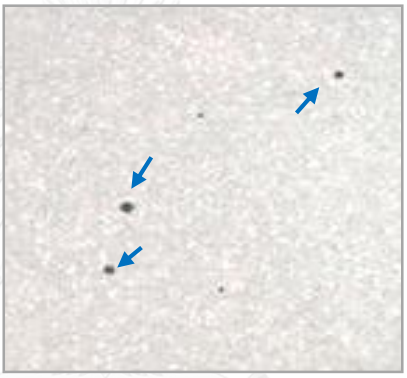


Figure 4.23: Candidate transformants harboring A) *FL_OtNhaA*/pChlamy_3 and B) empty vector (pChlamy_3) on TAP agar containing hygromycin 10 µg/ml (shown by arrow).

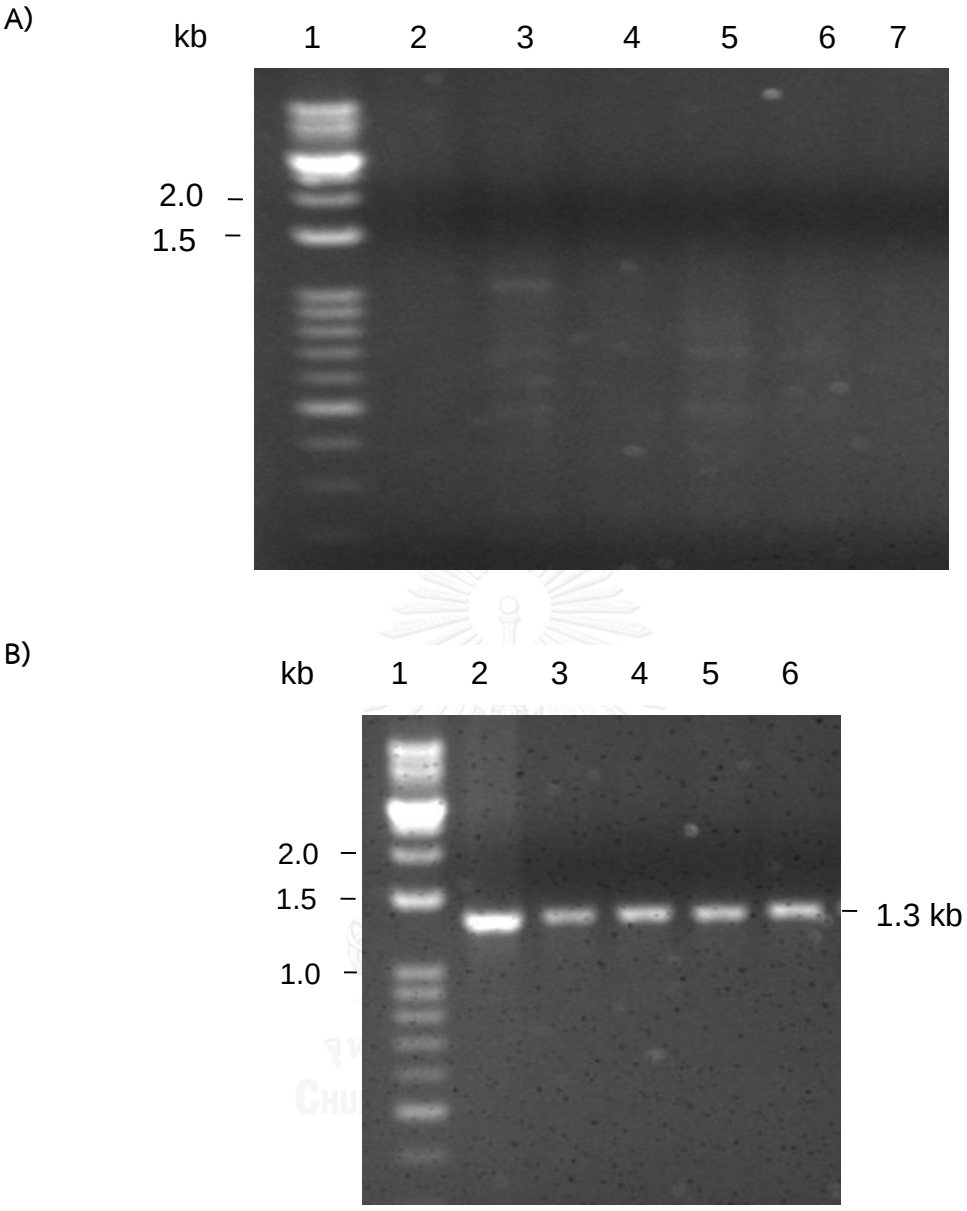
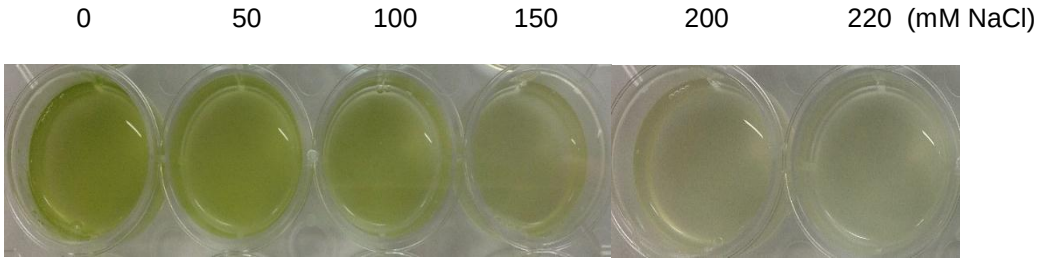


Figure 4.24: A) Colony PCR analysis of expressing cell *FL_OtNhaA*/pChlamy_3 on 1% agarose gel electrophoresis. Lane 1: DNA ladder, lanes 2 to 7: candidate transformants of *FL_OtNhaA*/pChlamy_3 (no band). B) The result of PCR product of hygromycin resistance gene (positive control).

4.3.3 Stress tolerance

To analyze stress tolerance of transformant and wild-type strain of *C. reinhardtii* 137c, the growth of cells under different concentration of salt were measured as described in Materials and Methods. For the growth of transformants *C. reinhardtii* 137c cells harboring *FL_OtNhaA*/pChlamy_3 and empty vector (pChalmy_3), this study has not successfully expressed recombinant plasmid *FL_OtNhaA*/pChlamy_3 and empty vector in *C. reinhardtii* 137c. For the growth of wild-type strain *C. reinhardtii* 137c was examined using multiwell plate assay and supplemented with various concentration of NaCl (0-300 mM). The wild-type strain *C. reinhardtii* 137c could grow up to in the presence of 200 mM NaCl as shown in Fig. 4.25A. This observation was further examined by measuring the growth rate of wild-type strain *C. reinhardtii* 137c cells as shown in Fig. 4.25B. The growth rate of wild-type strain *C. reinhardtii* 137c cells were decreased with increasing concentrations of NaCl.

A)



B)

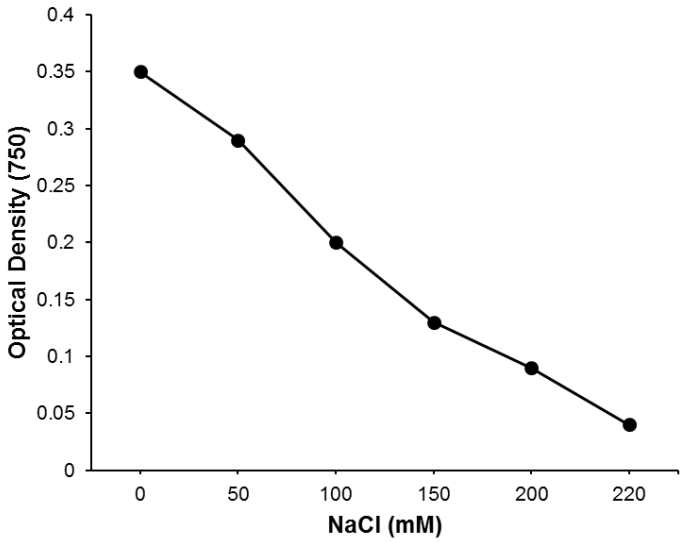


Figure 4.25: A) Multiwell plate assay and B) Growth curve of wild-type strain *C. reinhardtii* 137c in TAP+40 mM sucrose medium and supplemented with various concentration of NaCl (0, 50, 100, 150, 200 and 220 mM).

CHAPTER V

CONCLUSION

This study is the first report for expression and functional characterization of microalgal Na^+/H^+ antiporter. The *Ostreococcus tauri nhaA* encoding OtNhaA protein was successfully expressed in the salt-sensitive *E. coli* mutant TO114.

1. Putative OtNhaA encoded 519 amino acid residues.
2. Topological model of OtNhaA has 10 putative TMs with a long hydrophilic N -terminal region.
3. FL_OtNhaA and ΔN112_OtNhaA were successfully expressed in *E. coli* mutant TO114.
4. Expressing cells FL_OtNhaA and ΔN112_OtNhaA could complement the salt sensitive *E. coli* mutant TO114. They could complement up to 700 mM NaCl at neutral pH, whereas could complement of 300 mM LiCl at acidic pH.
5. Expressing cells FL_OtNhaA and ΔN112_OtNhaA had high Na^+/H^+ or Ca^{2+}/H^+ activity at alkaline pH.
6. The apparent K_m of FL_OtNhaA and ΔN112_OtNhaA for Na^+ had high affinity at alkaline pH (put values).
7. FL_OtNhaA was not successfully expressed in *C. reinhardtii* 137c.

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APPENDICES

Appendix 1

Amino acid sequences of NhaA Na^+/H^+ antiporter

Twenty-one NhaA sequences from bacteria

1. ***Escherichia coli*** **(Accession number: CDU40636.1) 388 AA**

MKHLHRFFSSDASGGIILIIAAILAMIMANSGATSGWYHDFLETPVQLRVGSLEINKNMLLWINDALMAVFFL
LVGLEVKRELMQGSLASLRQAAFPVIAAIGGMIVPALLYLAFNYADPITREGWAIPAATDIAFALGVLALLGSR
VPLALKIFLMALAIIDDLGAIIIIALFYTNDLSMASLGVAAVAIAVLAVLNLCGVRRTGVYILVGVVLWTAVLKSG
VHATLAGVIVGFFIPLKEKHGRSPAKRLEHVLHPWVAYLILPLFAFANAGVSLQGVTLDGLTSILPLGIIAGLLI
GKPLGISLFCWLALRLKLAHLPEGTTYQQIMAVGILCGIGFTMSIFIASLAFGSVDPELINWAKLGILVGSISSA
VIGYSWLRVRLRPSV

2. ***Helicobacter pylori*** **(Accession number: KFH29266.1) 438 AA**

MNIKKTENALSVTLKNFIKSESFGGIFLFLNAVLAMVVANSFLKESYFALWHTPFGFQIGDFFIGFSLHHWID
DVLMALFFLMIGLEIKRELLFGELSSFKKASFPVIAAIGGMIAPGLIYFFLNANTPSQHGFGIPMATDIAFALGVI
MLLGKRVPTALKVFLITLAVADDLGAIVVIALFYTTNLKFAWLLGALGVVLVLAVLNRLNMRSLIPYLLLGVL
LWFCVHESGIHATIAAVILAFMIPVKIPKDSKNVELLELGKRYAETSSGALLTKEQQEILHSIEEKASALQSPLE
RLEHFLAPISGYFIMPLFAFANAGVSVDSSINLEVDKVLLGVILGLCLGKPLGIFLITFISEKLKITARPKGISWW
HILGAGLLAGIGFTMSMFISNLAFTSEHKDAMEVAKIAILLGSLISGIIGALYLFVLDKRAALKK

3. ***Yersinia pestis*** **(Accession number: KGA52390.1) 394 AA**

MTNIIRQFLRQEAAGGLILIIAAAIALLMANSALQGVYQSFLDIPVSIKIASLDISKPLLLWINDGLMAVFFLMIG
LEVKRELMEGSLAGRDKAVFPAIAALGGMLAPALIYLLFNGADEVTRQGWAIPAATDIAFALGVMALLGNRV
PTGLKVFLLALAIIDDLGVIIIIALFYTQQVSLQSLGIAAAAAIALLAYMNWRGVGKTSAYLLVGLVLWVCILKSG
VHATLAGVIVGFMIPLHTQDQRSPSESLEHGLHPWVAYLILPLFAFANAGVSLQGVSLSGLTSLLPMGIATG
LFIGKPLGIFTFSWLAVKLGIAKLPDAINFKQIFAVSVLCGIGFTMSIFIASLAFEGTDIALTTYSKLGILLGSTTA
AVVGYSLLRLVLPARRKAVNVR

4. ***Salmonella typhimurium*** **(Accession number: NP_459044.1) 388 AA**

MKHLHRFFSSDASGGIILIIAAALAMLMANMGATSGWYHDFLETPVQLRVGALEINKNMLLWINDALMAVF
FLLIGLEVKRELMQGSLASLRQAAFPVIAAIGGMIVPALLYLAFNYSDPVTREGWAIPAATDIAFALGVLALLG
SRVPLALKIFLMALAIIDDLGAIVIIALFYTSDLSIVSLGVAAFAIAVLALLNLCGVRRTGVYILVGAVLWTAVLKS
GVHATLAGVIVGFFIPLKEKHGRSPAKRLEHVLHPWVAYLILPLFAFANAGVSLQGVTIDGLTSMLPLGIIAGL
LIGKPLGISLFCWLALRFKLAHLPQGTTYQQIMAVGILCGIGFTMSIFIASLAFGNVDPELINWAKLGILIGSLL
SAVVGYSWLRARLNAPA

5. *Vibrio cholerae* (Accession number: KFE28035.1) 382 AA

MSDMIRDFFKMESAGGILLVIAAAIAMVIANSAMGEGYQAFLHTYVFGMSVSHWINDGLMAVFFLLIGLEVK
RELLEGALKSRETAIFPAIAAVGGMLAPALIYVAFNFNDPAAIQGWAIPAATDIAFALGIMALLGKRVPVSLKV
FLLALAIIDDLGVVVIIALFYSSDLSTIALTIGFIMTGVLFMLNAKHVTKLSIYLVAGLILWIAVLKSGVHATLAGV
VIGFAIPLKGNKGEHSPLKHLEHALHPYVAFAILPVFAFANAGISLQGVSLAGLTSMLPLGVALGLFLGKPLGI
FSFSWAAVKLGVAKLPEGINFKHIFAVSVLCGIGFTMSIFISSLAFGQANEAYDTYARLGILMGSTTAALLGYS
LLRLSLPLKKA

6. *Shewanella frigidimarina* (Accession number: WP_011638389.1) 394 AA

MERAIKNFLSQESAGGILLMVAVALAMILANSPLAGVYQGFLATEVQLRVGDLDIDKPLLLWINDGLMALFF
LLIGLEVKRELLEGALSSVAKASLPSIAAIGGMVFPALFYLAFNYATPETQVGWAIPAATDIAFALGIMALLGN
RVPVALKVFLLALAIIDDLGVIVIIALFYSTDLSMTSLVIAAVSIVLMVALNKKGVSSILPYGLLGFILWVAVLKSG
VHATLAGVIIAFCIPLRAKDGTSPSEHLEHKLHPWSTFMILPVFAFANAGLSLTNMTLDSFAEPITLGIIMGLL
LGKPIGVLLFSYLAVKLKLAELPPGIGWRHIIPVAVMCGIGFTMSVFIASLAFEHSPAAYGDYARLGILTGSLLA
ALIGYFWLAKVLPETGEKHETH

7. *Osedax symbiont* Rs2 (Accession number: WP_020285618.1) 391 AA

MKIIKDFIDNESSSGIILIAVTILALILSNSALSGLYESFLHIPVEIRFGSLSIDKSLYHWVNDGLMAIFFLLIGLEVK
REILQGHLSSVSQIALPGIAAIGGMVVPAAVYLYFNADSEVAVNGWAIPTATDIAFALGILSMLGKRVPVSLKI
FLMALAIIDDLGAIVIIAIFYTSELSTLSITVAAAALSVLVVLNLLGVSKKAAFFVVGAVLWVSVLKSGVHATLA
GVALAFTIPLSAIDEDNRQVSPLKEIEHSLHFWVAFFILPLFAFVNAGVNFQQISISQMSGAVPMGIIMGLFVG
KQVGVFGFSWIAIKLNIANLPKDSSWLQLYGVSLLTGIGFTMSLFIVSLAFEDDSLFQYTDKLAILIGSLLSGVL
GYAILRMGKSKSSE

8. *Clostridium* sp. Maddingley MBC34-26 (Accession number: WP_008427652.1) 394 AA

MNKKFKIKVFNQLSYFFKDESSSGLVLIFCAIIAMIIANSSIAGAYEHLLHKNITIGYKDFSLSMSILHWINDGLM
AIFFLVVGMEIKRELVIGELKSIKKAILPISAAIGGMVMPAVIYLISNYNQPTVGGWGIPMATDIAFALGILSLVG
KNAPKGIVIFLTALAIVDDLGAIIVIAIFYTSQISFSALIVGVVIFILLILANKFKVKFKSAYIIGGIFLWICILKSGIHAT
VAGVLLGMVLPLGKDANESKKSMLHKFEHALSPWSSFVIMPMFALANSGITIDFSSLQRSFFTPVSLGIIFGL
FIGKQIGIFGVSYILVKLKVAKLPSKVTKRHLYGASVLGGIGFTMSLFVSSLSFSDAGVLSIVKISIMISSILAAAA
GMIIYKFIIR

9. *Dyadobacter fermentans* DSM 18053 (Accession number: ACT94304.1) 406 AA

MPVRQFRWIYYIACMAKLINLKPFKEFIQSESLGGVILIVCVIISLIIANSPAAGTFEKWLGTEVGFHSESVYLRY
PILLWINDGLMAVFFLLVGLEIKRELVEGELSSFKKAALPIFAALGGVLAPAAIYFLLNKGTHTAAGWGIPMAT
DIAFAIAIITMLGSKVPSSLKIFLAALAIVDDLMAILVIAIFYSSDLHYNYLLYAAGIFAALLVMNRAGVKNLAAYL

VPGALIWYFIHHSGVHATIAGVLTAFAIPTTPDAQESPLERLEHLLVKPVNFAIMPVFALANTNITFEAEMLEG
LTTSLGLGIILGLVIGKPLGITLLSWLTVKLGISSMPSRAGWAHIIGVGMLGGIGFTMSVFIALLSFPGEHLILSE
AKFSILTGSVLSGILGYAALYAVSRRAAR

10. *Aliivibrio fischeri* (Accession number: WP_011261552.1) 382 AA

MSDVIKNFFKLESAGGILLVIAAAIAMMIANSSLAPMYDTFLHSYIGGMSVSHWINDGLMAVFFLLIGLEVKR
ELLEGALKSKETAIFPAIAAVGGMLAPALVYVAFNMGDPEALSGWAIPAATDIAFALGIMALLGNRVPVSLKV
FLLALAIIDDLGVVVIIAFFYTSDLSVLALVIGFVMTGLLFLLNAKHVTKIRWYLLVGFILWVSVLQSGVHATLA
GVVLGFAIPLKGNKGERSPLKHMEHALHPYVAFAILPVFAFANAGISLEGVSLDSLTTTLPLGVALGLFLGKP
LGIFSFSYLAVKSGVAKLPTGVNMKHIFAVSVLCGIGFTMSIFISSLAFGGVNPEFDKLARLGILMGSTFAAVV
GYALLSISLPKKAA

11. *Vibrio azureus* (Accession number: WP_021710120.1) 392 AA

MNDIVRDFFKMESAGGILLVIAAAIAMTIANSPLGETYQSILHTYVFGMSVSHWINDGLMAIFFLLIGLEVKRE
LLEGALKSKETAIFPAIAAVGGMLAPALIYVAFNSGDSEAMAGWAIPAATDIAFALGIMALLGKRVPISLKVFL
LALAIIDDLGVVVIIALFYTGELSTSALTVGFIMTGLLYVLHTKKVTRLLPYMVVGAILWFAVLKSGVHATLAG
VVIGFAIPLKGKEGQPSPLKHMEHALHPYVAFFILPLFAFANAGISLQGVSLSGLTSMLPLGIALGLLVGKPLG
IFTFSWVAVKAGVAKLPKGVNFMHIFAVSVLCGIGFTMSIFISSLAFAGVSPEFDTYARLGILMGSTTAAILGY
ALLNGSLPKNAIEETAEAKKCS

12. *Shewanella amazonensis* (Accession number: WP_011758985.1) 391 AA

MEKAIRNFLSQESAGGILLLVAVALAMLLANSPLSGLYQGFLNTEMQVRFGALDINKPLLLWINDGLMALF
FLLIGLEVKRELLEGALSSPSKASLPTFAAIGGMLVPAAIYLFFNFDDPVTKVGWAIPAATDIAFALGIMALLG
NRVPVALKVFLLALAIIDDLGVIVIIALFYSTDLSMLSLVIAAIAVTGLVALNRKGVTSLAPYGVLGIILWIAVLKS
GVHATLAGVVIAFCIPLRAKDGSSPSEHLEHSLHPWSNFLILPVFAFANAGVPLGNVGFDSILSPVPVGIALGL
LLGKPIGVLLFSYAAVKLRLAELPKGIGWHQIAPVAVMCGIGFTMSMFIASLAFEHGGELYGDLARIGILLGSL
FAAVIGYFWLSKVLPKAGERI

13. *Geobacter* sp. M18 (Accession number: ADW11785.1) 390 AA

MKRHARKMVNLWTDFIETEKSSGVVLIACTIVSITLANSAIGGRYLDFWHAEVLHHSLQHWINDGLMAVFFL
LIGLEIERELYVGELSDLKSASLPIAAAIGGMVTPAAFHFLLNRGTATAGGAGIPMATDIAFALGILALLGKRAP
LSLKIFVTALAIIDDLGAIVVIALFYVGNFSLPYFAGALAVFALLLLFNRLGVNRLAAYLLPGALMWYLMLQSG
VHATIAGVLLAFAIPFRDGSESSPSYKLQHILHKPAAFVIMPVFALANTGITLGSNWIEGVTSMNSLGIFVGLL
AGKPVGIACFSYLAVKGGLARLPKAVSWRHIAGAGFLGGIGFTMSIFITLLAFEDAAVIEASKLAILLTSALAGT
TGYLMLRAASKHPSPPVGSG

14. *Vibrio ponticus* (Accession number: GAK84546.1) 381 AA

MSDIIRDFFKMESAGGVLLVIAAAIAMTIANSPLNETYQAFLHSYVLGMSVSHWINDGLMAIFFLLIGLEVKRE
LLEGALKSRETAIFPAIAAVGGMLAPALVYLAFNFNDPMAIQGWAIPAATDIAFALGIMALLGSRVPVSLKVF
LLALAIIDDLGVVVIIALFYSGDLSTTALAIGFAMTAALFILNSRKVTKLLPYMIVGAILWVAVLKSGVHATLAG
VVIGFAIPLKGKKGEHSPLKHMEHALHPYVAFGILPLFAFANAGISLEGVSMEGLTSMLPLGIALGLLVGKPL
GIFSFSWVAVKSGIAKLPEGITMFHIFAVSVLCGIGFTMSIFISSLAFGQTNVEFDTYARLGILMGSTTAAILGYF
LLHISLPKNK

15. *Clostridium beijerinckii* (Accession number: WP_011968778.1) 400 AA

MKNKIKNKIKNPFLQFFKNESSSGIVLMFCAIIAIIIANSNFSSMYNNIIHTYITIGYKDFSLSMSILHWINDGLMAI
FFLVVGMEIKREIVFGELKSFKKTILPVSAAIGGMVVPAIIYALFNFNQPTIIGWGIPMATDIAFALGILSLVGKKA
PKGIIIFLTALAIVDDLGAIIVIAIFYTSEISWIALILGLIIFLAIILANKLNVKNKWLYIIFGIALWICFLKSGVHETIAG
VLLGMGLPIGKNMEEFRTSILYRFEHVLTPLSSFIIMPIFALANSGITIDINSLSAAIMNPVSLGIIFGLFIGKQIGIF
GASYILVKLKIAKLPSKVTKRHLYGASVLGGIGFTMSLFVSSLSFTEESALSMAKISIIIASILSAAFGAAIFKIIKFK
NEERV

16. *Vibrio cholerae* HC-7A1 (Accession number: ELT24372.1) 372 AA

MESAGGILLVIAAAIAMVIANSAMGEGYQAFLHTYVFGMSVSHWINDGLMAVFFLLIGLEVKRELLEGALKS
RETAIFPAIAAVGGMLAPALIYVAFNFNDPAAIQGWAIPAATDIAFALGIMALLGKRVPVSLKVFLLALAIIDDL
GVVVIIALFYSSDLSTIALTIGFIMTGVLFMLNAKHVTKLSIYLVAGLILWIAVLKSGVHATLAGVVIGFAIPLKG
NKGEHSPLKHLEHALHPYVAFAILPVFAFANAGISLQGVSLAGLTSMLPLGVALGLFLGKPLGIFSFSWAAV
KLGVAKLPEGINFKHIFAVSVLCGIGFTMSIFISSLAFGQANEAYDTYARLGILMGSTTAALLGYSLLRLSLPLK
KA

17. *Vibrio ezurae* NBRC 102218 (Accession number: GAD79163.1) 389 AA

MLKDFFKMESAGGVILVIAAALAMFVANSPLNEAYQGALHSYVLGMSVSHWVNDGLMALFFLLIGLEVKRE
LLEGALKSRETAIFPAIAAVGGMVAPALVYVLFNMGDPAALSGWAIPAATDIAFALGIMALLGNRVPVSLKVF
LLALAIIDDLGVVVIIALFYSSDLSTLALAVGFAMTGLLFYLNHKKVTSLKWYALVGAILWFAVLKSGVHATLA
GVVIGFAIPLKGNKGEHSPLKHLEHALHPWSAFFILPIFAFANAGVSLEGISLSTLGSTLPMGIALGLLLGKPL
GIFTFSWLAVKSGVAKLPKGIDFRHIFAVSVLCGIGFTMSMFIASLAFTGANADFNTHARLGILMGSSIAAVLG
YFLLSTSLPKKSVVDVQTEAEAK

18. *Vibrio proteolyticus* (Accession number: WP_021705443.1) 381 AA

MSDVIRDFFKMESAGGILLVIAAAIAMTIANSPLNDVYQGFLHSYVFGMSVSHWINDGLMAVFFLLIGLEVKR
ELLEGALKSKETAIFPAIAAVGGMLAPALIYVLFNASDAAAIQGWAIPAATDIAFALGIMALLGKRVPVSLKVF
LLALAIIDDLGVVVIIALFYTGDLSTLALVVGFIMTGALFMLNAKHVTKLTPYMIVGAILWVAVLKSGVHATLA
GVVIGFAIPLKGNEGERSPLKHMEHALHPYVAFGILPLFAFANAGISLEGVSMAGLTSMLPLGVALGLLIGKP
LGIFSFSWAAVKMGVAKLPEGINMKHIFAVSVLCGIGFTMSIFISSLAFGAANADYDTYARLGILMGSTTAAV
VGYILLHLSLPKKA

19. *Vibrio cholerae* HC-33A2 (Accession number: EHH99586.1) 379 AA

MIRDFFKMESAGGILLVIAAAIAMVIANSAMGEGYQAFLHTYVFGMSVSHWINDGLMAVFFLLIGLEVKRELL
EGALKSRETAIFPAIAAVGGMLAPALIYVAFNFNDPAAIQGWAIPAATDIAFALGIMALLGKRVPVSLKVFLLA
LAIIDDLGVVVIIALFYSSDLSTIALTIGFIMTGVLFMLNAKHVTKLSIYLVAGLILWIAVLKSGVHATLAGVVIGF
AIPLKGNKGEHSPLKHLEHALHPYVAFAILPVFAFANAGISLQGVSLAGLTSMLPLGVALGLFLGKPLGIFSFS
WAAVKLGVAKLPEGINFKHIFAVSVLCGIGFTMSIFISSLAFGQANEAYDTYARLGILMGSTTAALLGYSLLRL
SLPLKKA

20. *Vibrio cholerae* HE39 (Accession number: EGQ99624.1) 379 AA

MIRDFFKMESAGGILLVIAAAIAMVIANSAMAEGYQAFLHTYVFGMSVSHWINDGLMAVFFLLIGLEVKRELL
EGALKSRETAIFPAIAAVGGMLAPALIYVAFNFNDPAAIQGWAIPAATDIAFALGIMALLGKRVPVSLKVFLLA
LAIIDDLGVVVIIALFYSSDLSTIALTIGFIMTGVLFMLNAKHVTKLSIYLVAGLILWIAVLKSGVHATLAGVVIGF
AIPLKGNKGEHSPLKHLEHALHPYVAFAILPVFAFANAGISLQGVSLAGLTSMLPLGVALGLFLGKPLGIFSFS
WAAVKLGVAKLPEGINFKHIFAVSVLCGIGFTMSIFISSLAFGQANEAYDTYARLGILMGSTTSALLGYSLLRL
SLPLKKA

21. *Edwardsiella piscicida* (Accession number: GAJ66919.1) 388 AA

MQRFVGSDAFGGVILIIAAALAMILANTDWTSHIYQAFLNTPVEVRVGSLHINKNMLLWVNDALMAIFFLMI
GLEVKRELVCGSLASTRQAAFPVIAALGGMVVPALIYLLFNGQDAVASTGWAIPAATDIAFALGVLALLGNR
VPLALKVFLLALAIIDDLGAIIIIALFYTSDLSILSLAVAGAAIVALALLNLFNVRRVGLYILVGVVLWTAVLKSGV
HATLAGVVIGFFVPLKEQDGQSPARSLEHALHPWVAYMILPLFAFANAGVSLDGVTLSGLFSLLPLGVIAGL
FIGKPLGISLFCWLAVKLKLATLPQGTTFKEIIAIGVLCGIGFTMSIFIASLAFGDADPALAVFSRLGILLGSSLAA
IVGYLLLRRVLPAQKAAA

Six NhaA sequences from green algae

22. *Ostreococcus tauri* (Accession number: XP_003075324.1) 519 AA

MIDEDRVTALVPQGDGNSRTPHSRARAMRTLARAPAARCVSIQRSRDRDVVKNARVATPRATIVPRGFRPN
ARASSRAGVVLDRSRSTVVAKSSSSDEAPRLTRSKTFTETFMTGVDVRAALDAGFGSVALLGATALSLMLA
NSAASGVWASFWHAHIGPAALGLNMSLHHWTNEGLMALFFFAVGLEIKREFVHGSLKSIKQAALPCIGALG
GMIVPMGVYLALNNPAMTASAVVAGWAIPMATDIAFAMGVYNFFKNKLPGGVAAFLLTLATVDDLGAIAVI
AVCFAKSLTMSYIAGAAAATGALFLACKKEVTNMAVYAGLGVALWYCLLQGGINADVAGVIAAFAVPGNAP
APAGSDATPEHEGGEPTLLDHLVHKFAPLSALVIMPLFALANTGVPLDASMIGKVFTEPVGQGIMFGLLLGK
PVGIAGLSWLAVKAKVGTLPNGMNNVHLLIVGLLGGIGFTMCLFLVEMALAGNAAAANTGKLAVLVSSTLA
ALIGAGCMARLPDRDAKLKTA

23. *Ostreococcus lucimarinus* CCE9901 (Accession number: ABO94792.1) 380 AA

GSIALLGATAVSLALANSAMSGAYLSFWHAKVGPAALALHMSLHHWVNEGLMAIFFFAVGLEIKREFVHGS
LRSLQQAALPCIGALGGMLVPMGVYLACNNPGATAAAVTAGWAIPMATDIAFAMGVYNFFKNKMPGGVA
AFLLTLATVDDLGAIAVIAVCFAKSLTMSYIAGAAAATAALFVACKKEVTNMAVYAALGVALWYCLLQGGIN
ADVAGVIAAFAVPAHAMAPAGSDATPEHEGGEPTLLDHLVHKFAPLSALVIMPLFALANTGVPLDASMVSK
VFTEPVGQGIMLGLLLGKPVGIAGLSWLAVKSKIGKLPSGMNNSHLIIVGLLGGIGFTMCLFLVEMALAGQP
SAANVAKLAVLASSTVAATAGAALM

24. *Micromonas pusilla* CCMP1545 (Accession number: EEH59182.1) 490 AA

MASARAPVALGRSSARRSVIPASSPSSVAVVEGDGEVREKKWSEMDIHELLDQGLGSIMLLGATALSLFLAN
SAMSGGFIGFWEHFHIGPASLGLHLNAHEWVNEGLMAIFFFMVGLEIKREFVFGSLSNVKAALLPCFGALGG
MVAPMGIYLALNSVTGGIPAGWAIPMATDIAFAMGVYNFFKNRMPPAVATFLLTLATVDDLGAIAVIAVCFA
KGIVPAYLAASAGICAVLAICCKKKVSNMIVYSALGVALWFALLKGGINADIAGVVAALAIPADADAPAGSHA
HAFEDGMKVTLIDDLIHFLHPISSMLIMPLFALANCAVVVQASAMAGVFTAPVGQGIMAGLLVGKPLGIAAI
CMAAIKMNLCSFPPGMNLKHMLTVGMLAGIGFTMSLFLIEQALVGMPVASVTAKLAILVSSGIAATVGGFA
MTRFPVYFCEIVCDDDGCAPELFEEAKFKSENDCDEDGCVPKFDEPVSAVTGEAKKEQ

25. *Micromonas* sp. RCC299 (Accession number: ACO64223.1) 537 AA

MASMMISGLAGARVGPAAVSRKSSVRARAGQSIRMKSFPARRAVELATVASLVAPGGAQSGANLRYASISS
AAGKSPRVESIAFNDPTPAPWYAREFDVHIALDKGLGSIMLLGATALSLYLANSGYAHDFIHFWEHFHFGPK
AIGLFLNAHEWVNEGLMAIFFFNVGLEIKREFAFGSLSDIKAALLPCFGALGGMIAPMGIYLALNMVNGGITA
GWAIPMATDIAFAMGVYNFFKNRMPPAVAAFLLTLATVDDLGAIAVIAVCFAKGIVPAYLAASAAITGALFVA

CKKKVTSMAVYGGLGVALWYALLKGGINADIAGVVAALAVPAAAPAPPGSHAHGMEEGMEPTLLDDLIHSL
HPISSLLIMPCFALANCAVPVDASALGGVVGTPVGRGIMAGLLLGKPLGIFALCYGAVKMGICSFPKGMNGK
HLVTVGMLAGIGFTMSLFLIEQALVGMPAAAVSAKLAILCSSGIAAVIGGFAMTRFPVYFCEIVCDEDEGCRP
ELYEEQVFKAENDCDEVACTPKFLTEGEEESA

26. *Bathycoccus prasinos* (Accession number: CCO19648.1) 463 AA

MIASSPFVARLVYTKPQCPGATIARATHPYKLKLLGESCEINGEVAERPLKLSSINFRRVNLASNLSNHQMNS
QRRRLNTLMRVSASSLDKDVHYYLNNGLGSIVLLVATGVALIFANIAKTAPLYELFWSSYIGPKALNLSMTLH
HWVNEGLMALFFFSVGLEIKREFIHGSLSSLKQAILPCFGAVGGMLVPMLFYLVFNLASSNGVMAGWAIPM
ATDIAFAMGIYGFFKNKMPTGVAAFLLTLATVDDLGAILVIAIFFSKTLIKEYVFLAIAVSGVMFSACKRKVTNT
KVYMSLFVLLWYFLLQGGINADIAGVITALAIPGNSLAPIHSKAPPEHEGQSVTLLDHLIHAWSPWTTLLVM
PLFALANTAVLIDRAVFGSIMTTPIGQGIFFGLMLGKPIGIAGISWLAINLRIAKFPEGMSLKHLGIVGLLGGIGF
TMSLFLIEMALSVLHQLYLAVRS

27. *Aureococcus anophagefferens* (Accession number: XP_009038116.1) 367 AA

GAAALLGATAVSLSLANGPSSAWWVGLWESPLGPAIGGHALSARAWVNEGLMGVFFFLVGLEIKEELRHG
ALTSVKKAALPAIAALGGMVTPMAVYAAAQGFLPVAARSLSGLAVPMATDIAFAMAIFGLFRSKMPASSSA
FLLTLATVDDFGAIFVLATCFAANVSLGFLGLASALTAALGWIGCGPRKCNDARVFTAGGVGIWWSLLRAG
VSAEVAGVLAALCVSTTAEGPPGAIEDPLAERMIRRLAPLSTFGIMPLFALANTGIPLMGVMKGVSGPGAGA
AAGIAAGLVLGKPLGIFGFTWLATKLNIADMPTGMGNNHLSIVSMLGAIGFTMCLLLTEVAIPAPIQALPKLS
VLVASTAACV

Appendix 2

ESM medium

Composition per 1 liter

$NaNO_3$	120	mg
K_2HPO_4	5	mg
Vitamin B_{12}	1	µg
Biotin	1	µg
Thiamine HCl	100	µg
Fe – EDTA	259	µg
Mn – EDTA	332	µg
Tris (hydroxymethyl) aminomethane	1	g
Soil extract*	25	ml
Seawater	975	ml

Dissolve all compositions with seawater, adjust the pH to 8.0 with 6 M NaOH. Autoclave at 121 °C, 15 lb/in^2 for 15 minutes.

*** Soil extract**

Add 5 g of soil (soil from undisturbed deciduous woodland is the best) to 500 ml distilled water and heat by autoclaving for 1 hr at 105°C. When cool, heat by autoclaving for 1 hr at 105°C again. Pass the supernatant through a GF/C filter and keep at room temperature.

Appendix 3

LB medium

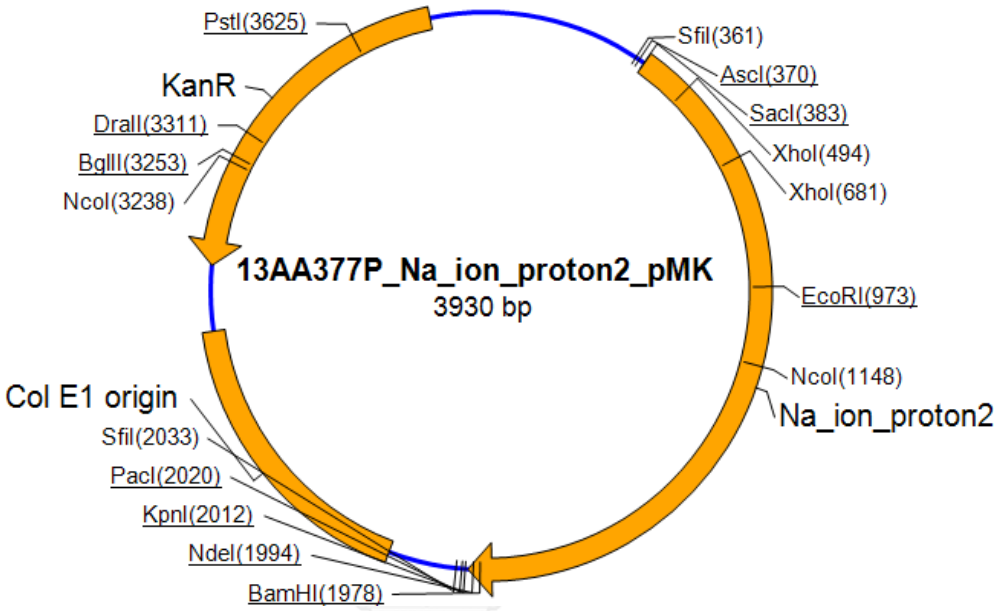
Composition per 1 liter

Bacto Tryptone	10	g
Yeast extract	5	g
NaCl	10	g

Dissolve all compositions with distilled water, adjust the pH to 7.0 with 6 M NaOH. Autoclave at 121 °C, 15 lb/in^2 for 15 minutes. For media containing agar add bactoagar 15 g/1 l.

Appendix 4

13AA377P_Na_ion_proton2_pMK vector



Appendix 5

LBK medium

Composition per 1 liter

Bacto Tryptone	10	g
Yeast extract	5	g
KCl	10	g

Dissolve all compositions with distilled water, adjust the pH to 7.0 with 6 M NaOH. Autoclave at 121 °C, 15 lb/in^2 for 15 minutes. For media containing agar add bactoagar 15 g/1 l.

For LBK+200 mM NaCl medium, NaCl is added to the final concentration of 200 mM after adjusted pH with KOH.

Appendix 6

cDNA sequence of *Ostreococcus tauri*

1560 nt

ATGATCGATGAGGACCGCGTGACCGCACTGGTCCCTCAGGGCGATGGCAACTCGCGAACGCCG
CACTCACGCGCGCGCGCGATGCGCACCCTCGCTCGAGCGCCCGCGGCGCGATGTGTCTCCATC
CAACGCTCTCGTGACCGTGACGTCGTGAAGAACGCGCGCGTGGCGACGCCGCGAGCAACGATT
GTTCCGAGAGGTTTTCGACCCAACGCTCGTGCCTCGTCGCGCGCCGGCGTCGTCCTCGATCGCT
CGCGCTCGACAGTGGTCGCGAAGTCGTCCTCGAGCGATGAAGCTCCCAGGTTGACCCGCTCGA
AAACGTTCACGGAGACGTTCATGACGGGCGTGGACGTGCGCGCGGCGCTCGATGCCGGGTTCG
GGAGCGTCGCGCTGCTCGGCGCTACGGCACTGTCGTTGATGCTGGCGAACAGCGCCGCAAGCG
GGGTATGGGCTTCATTTTGGCACGCCCACATCGGTCCGGCCGCGCTCGGGTTGAACATGTCGTT
GCATCACTGGACGAACGAGGGTTTGATGGCGCTGTTCTTTTTTGCCGTCGGGCTGGAGATAAAA
CGCGAATTCGTCCACGGGTCGCTGAAGAGCATTAAGCAGGCAGCGCTGCCGTGCATCGGTGCT
TTGGGGGGTATGATCGTACCGATGGGGGTGTATTTGGCGCTGAATAACCCGGCGATGACGGCC
TCGGCAGTGGTCGCCGGATGGGCCATTCCGATGGCGACGGATATCGCCTTCGCCATGGGTGTG
TACAATTTTTTCAAGAATAAACTGCCGGGCGGCGTCGCGGCGTTCCTATTGACGCTTGCCACCG
TGGATGACCTCGGTGCGATCGCCGTGATCGCGGTGTGCTTCGCCAAGTCGTTGACGATGTCTTA
CATCGCTGGGGCGGCGGCGGCGACTGGGGCGCTCTTCTTGGCGTGCAAGAAGGAAGTCACCAA
CATGGCCGTCTACGCCGGTCTAGGTGTCGCTCTTTGGTACTGCCTGTTGCAGGGGGGGATCAAC
GCCGATGTCGCGGGCGTGATCGCGGCGTTTGCCGTGCCCGGGAACGCGCCGGCGCCAGCTGGG
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TTCGCCCCGCTCTCAGCGTTGGTCATAATGCCTCTGTTCGCATTGGCGAACACGGGCGTGCCGC
TGGACGCGAGCATGATAGGCAAGGTGTTCACCGAACCCGTCGGCCAAGGCATCATGTTTGGCTT
GCTCTTGGGTAAGCCCGTCGGCATCGCGGGCTTGTCCTGGCTGGCTGTCAAGGCCAAGGTGGG
AACGCTCCCGAACGGGATGAACAACGTGCACTTGCTCATCGTCGGCTTACTCGGCGGTATCGGT
TTCACCATGTGCTTGTTCCTCGTCGAAATGGCACTCGCGGGTAACGCGGCGGCGGCGAACACG
GGCAAGCTCGCCGTTTTGGTCTCGTCCACGCTCGCGGCACTCATAGGCGCCGGGTGCATGGCT
CGACTTCCCGACCGAGACGCCAAGTTGAAGACGGCGCATCATCACCATCACCACTAA

Appendix 7

TCDS buffer

Composition per 1 liter

Tris – Cl, pH 7.0	10	mM
Choline Chloride	140	mM
$MgCl_2$	50	mM
Sucrose	250	mM

Dissolve all compositions with distilled water and keep at room temperature.

Appendix 8

Standard protein

(Lowry method)

1. Stock reagents

Solution A

2% (w/v) Na_2CO_3 in distilled water

Solution B

1% (w/v) $CuSO_4.5H_2O$ in distilled water

Solution C

2% (w/v) sodium potassium tartrate in distilled water

2. 2 N NaOH

3. Folin reagent

Use at 1 N concentration

Appendix 9

Preparation for polyacrylamide gel electrophoresis

1. Stock reagents

30% Acrylamide, 0.8% bis acrylamide, 100 ml

Acrylamide	29.2	g
N, N´ methylene bis acrylamide	0.8	g

Adjust volume to 100 ml with distilled water.

1.5 M Tris Cl pH 8.8

Tris (hydroxymethyl) aminomethane	8.17	g

Adjust pH 8.8 and adjust volume to 100 ml with distilled water.

2 M Tris Cl pH 8.8

Tris (hydroxymethyl) aminomethane	24.2	g

Adjust pH 8.8 and adjust volume to 100 ml with distilled water.

0.5 M Tris Cl pH 6.8

Tris (hydroxymethyl) aminomethane	6.06	g

Adjust pH 6.8 and adjust volume to 100 ml with distilled water.

1 M Tris Cl pH 6.8

Tris (hydroxymethyl) aminomethane	12.1	g

Adjust pH 6.8 and adjust volume to 100 ml with distilled water.

Solution B (SDS - PAGE)

2 M Tris Cl pH 8.8	75	ml
10% SDS	4	ml
Distilled water	21	ml

Solution C (SDS - PAGE)

2 M Tris Cl pH 6.8	75	ml
10% SDS	4	ml
Distilled water	21	ml

2. SDS – PAGE

12.5% separating gel

30% acrylamide solution	3.33	ml
Solution B	2.5	ml
Distill water	5.0	ml
10% Ammonium sulfate	50	ml
TEMED	10	µl

5% stacking gel

30% acrylamide solution	0.67	ml
Solution B	1.0	ml
Distill water	2.3	ml
10% Ammonium sulfate	30	ml
TEMED	5.0	µl

Sample buffer

1M Tris Cl pH 6.8	0.6	ml
50% glycerol	5.0	ml
10% SDS	2.0	ml
2 mercaptoethanol	0.5	ml
1% bromphenol blue	1.0	ml
Distilled water	0.9	ml

4x of sample buffer is mixture sample to 1x. The mixture heated 5 min in boiling water before loading to the gel.

Electrophoresis buffer for 1 liter

Tris (hydroxymethyl) aminomethane	3.0	g
Glycine	14.4	g
SDS	1.0	g

Adjust volume to 1 liter with distilled water (pH 8.3).

Buffer for western blotting

PBS buffer (Phosphate buffer saline)

Final concentration per 1 liter

10 mM sodium phosphate pH 7.4

150 mM NaCl

Blocking buffer

5% (w/v) skim milk and 0.01% Tween20 in 1x PBS buffer

Blotting transfer buffer

Final concentration per 1 liter

39 mM glycine

48 mM Tris base

0.037% SDS

20% methanol

Detection reagent for western blotting

150 mM Barbital pH 9.6	18	ml
0.1% NTB (Nitro Blue Tetrazolium)	2	ml
1M $MgCl_2$	80	µl
0.5% BCIP (5-bromo-4-chloro-3-indolyl phosphate)	200	µl

Detection reagent for western blotting should be freshly prepared and used within 30 min. When the bands are desired intensity, wash the nitrocellulose membrane with distilled water 2-3 times.

Appendix 10

TAP medium

Composition per 1 liter

Tris base	2.42	g
TAP salts	25	ml
Phosphate solution	750	ml
Trace element	1	ml
Acetic acid	1	ml

Dissolve all compositions with distilled water. Autoclave at 121 ºC, 15 lb/in^2 for 15 minutes. For media containing agar add bactoagar 15 g/1 l.

Appendix 11

TAP-40 mM sucrose medium

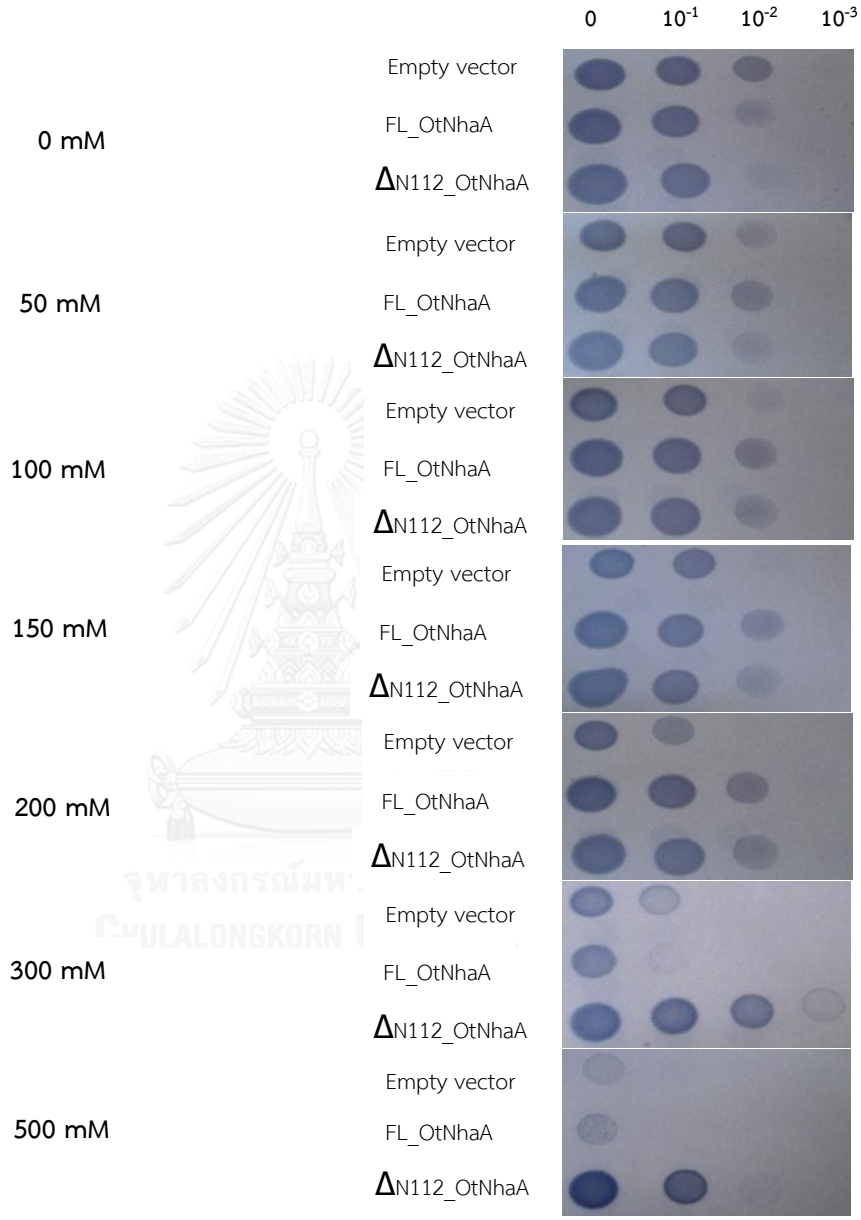
Composition per 1 liter

TAP medium	960	ml
1 M sucrose	40	ml

To prepare TAP-40 mM sucrose, add 40 ml of 1 M sucrose to 1 L of TAP medium.

Appendix 12

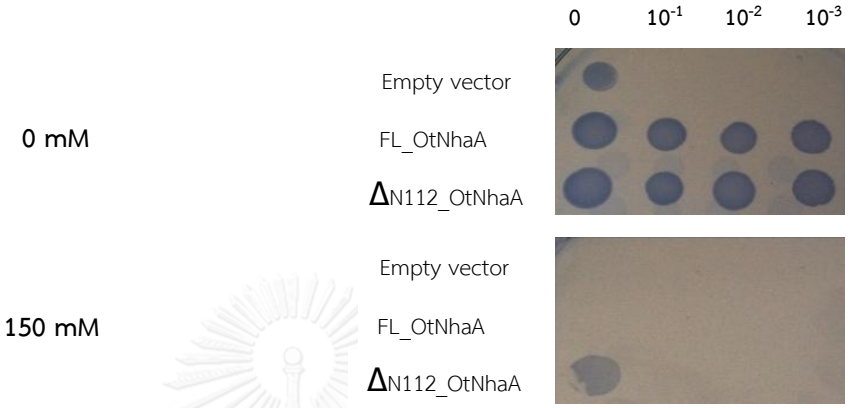
Complementation test



Complementation test of empty vector (pTrcHis2c) and expressing cells *FL_OtNhaA, ΔN112_OtNhaA* in LBK medium with different concentration of NaCl at pH 5.0. The cultures were incubated at 37 ℃ for overnight and then scored. Photographs were taken using SONY Cyber-shot camera.

Appendix 13

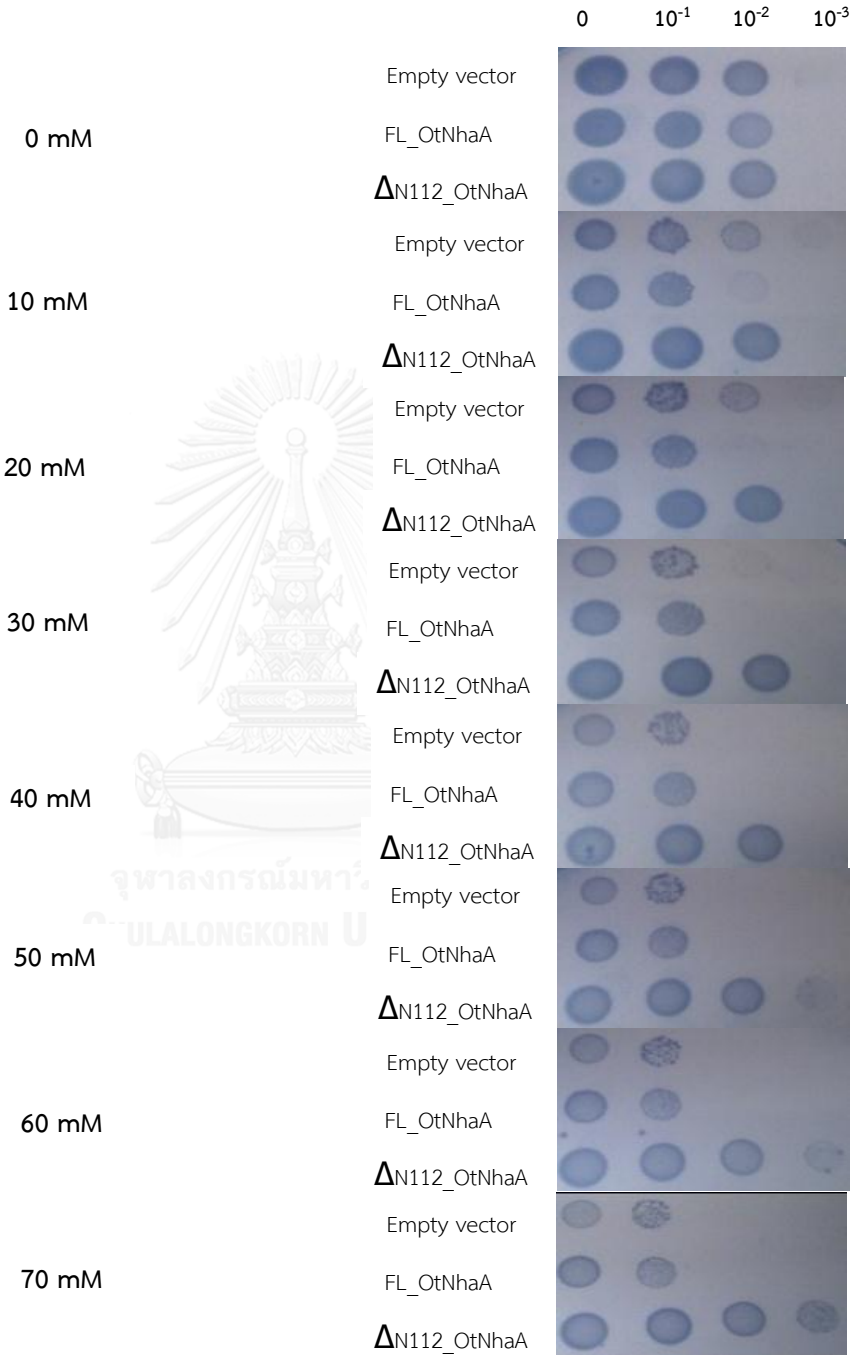
Complementation test

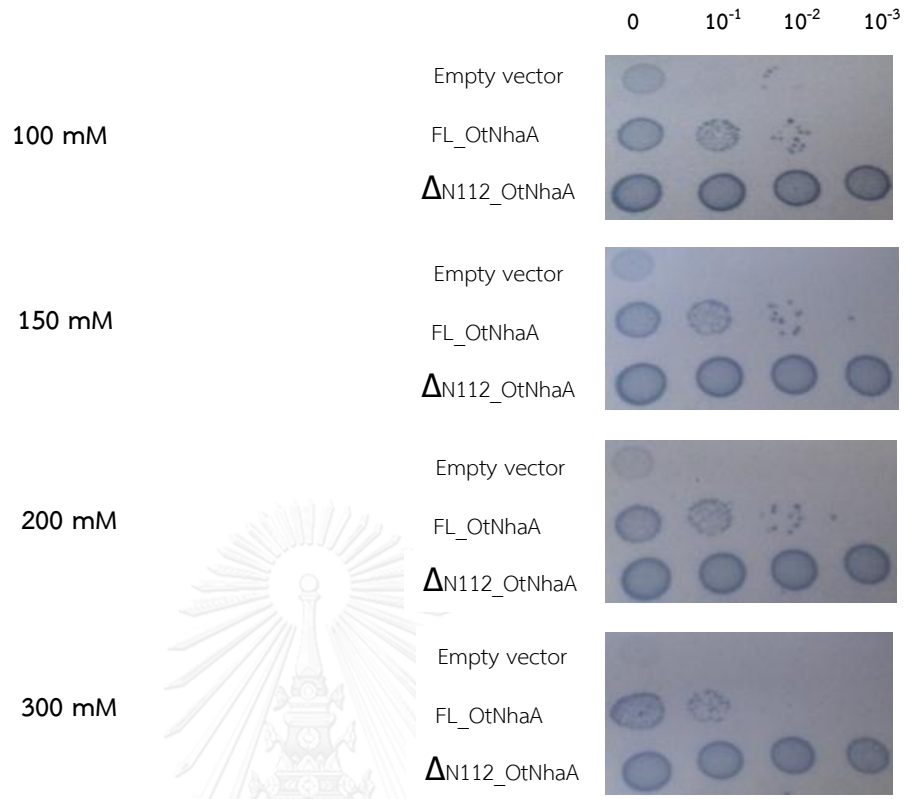


Complementation test of empty vector (pTrcHis2c) and expressing cells *FL_OtNhaA, ΔN112_OtNhaA* in LBK medium with different concentration of NaCl at pH 8.5. The cultures were incubated at 37 ℃ for overnight and then scored. Photographs were taken using SONY Cyber-shot camera.

Appendix 14

Complementation test

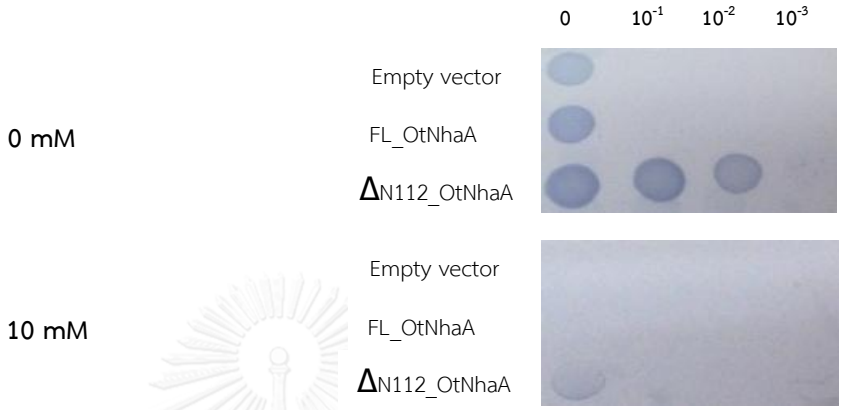




Complementation test of empty vector (pTrcHis2c) and expressing cells *FL_OtNhaA, ΔN112_OtNhaA* in LBK medium with different concentration of LiCl at pH 5.0. The cultures were incubated at 37 ℃ for overnight and then scored. Photographs were taken using SONY Cyber-shot camera.

Appendix 15

Complementation test



Complementation test of empty vector (pTrcHis2c) and expressing cells *FL_OtNhaA, ΔN112_OtNhaA* in LBK medium with different concentration of LiCl at pH 8.5. The cultures were incubated at 37 ℃ for overnight and then scored. Photographs were taken using SONY Cyber-shot camera.

VITA

Miss Keatisuda Dawut was born on September 1, 1989 in Roi-Et, Thailand. She graduated from Department of Microbiology, Faculty of Medical Science, Naresuan University in 2012 with a Bachelor degree of Science (Microbiology). Recently, she has pursued her Master degree of Program in Biotechnology, Faculty of Science, Chulalongkorn University. Some parts of this work were published in The 7th National Conference on Algae and Plankton at Narai Hotel, Bangkok. The topic is Bioinformatics analysis of sodium/proton transport in cyanobacteria.