CHAPTER II

MATERIALS AND METHODS

Materials

2.1 Instruments

ABI310 genetic analyzer: Applied Biosystems, USA

Autoclave: Model HA 30, Hirayama Manufacturing Cooperation, Japan

Autopipette: Pipetman, Gilson, France

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

Digital lux meter FT710: Taiwan

Electrophoresis unit: model Mini-protein II Cell: Bio-Rad, USA

Freeze-dryer: Stone Ridge, New York, USA.

Fraction collector: model 2211 Pharmacia LKB, Sweden

French pressure cell: SIM-Aminco Spectronic Instrument, USA

GeneAmp5700: Perkin Elmer, Japan

Illuminated/Refrigerated orbital: Sanyo, England

Incubator: Haraeus, Germany

Incubator shaker: Psyco-therm, New Brunswick Scientific, USA

Laminar flow BVT-124: International Scientific Supply, Thailand

Microcentrifuge: Kubota, Japan

Microscope: Olympus, USA

Peristatic pump: Pharmacia LKB, Sweden

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

Plastic petri dish (60x15 mm): Costar, USA

Power supply: Pharmacia, England

Scintillation counter: Pharmacia LKB Wallac, Reckbeta 1218, England

Siliga gel plates: Whatman, USA

Spectrofluorophotometer RF-5300PC: Shimadzu, Japan

Spectrophotometer UV-240: Shimadzu, Japan, and Du series 650: Beckman, USA

Ultracentrifuge: Hitachi, Japan

Trans-Blot Transfer Cell: Bio-Craft, Japan

Vacuum dry: Taitech, Japan

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

Water bath: Charles Hearson, England

Water bath shaking: Heto lab Equipment, Denmark

2.2 Chemicals

Acridine orange: Katayama Chem, Japan

Acrylamide: Merck, USA

Ammonium chloride: Katayama Chem, Japan

Ammonium persulfate: Katayama Chem, Japan

An antibody raised against 6-histidine: R & D Systems, USA

An antibody raised against mouse: Biolab, England

Ampicillin: Katayama Chem, Japan

Bacto peptone: Merck Ag Darmstadt, Germany

BCIP (5-bromo-4-chloro-3-indolyl phosphate): Katayama Chem, Japan

Betaine aldehyde chloride: Sigma, USA

Blot absorbant filter paper: BioRad, USA

β-mercaptoethanol: Katayama Chem, Japan

Calcium chloride: Merck Ag Darmstadt, Germany

Chloroform: Katayama Chem, Japan

Choline chloride: Sigma, USA

Coomassie brilliant blue G-250: Sigma, USA

Coomassie brilliant blue R-250: Sigma, USA

Dialysis tube: Sigma, USA

D (+) glucose: Sigma, USA

Dimethyl sulfooxide: Katayama Chem, Japan

Dowex 50W (50x4-200, H^+ from): Sigma, USA

Dowex 1X4 (50x4-200, H^+ from): Sigma, USA

DTT (Dithiothreitol): Sigma, USA

EDTA (Ethylenediaminetetraacetic acid): Sigma, USA

Ethanol: Katayama Chem, Japan

Ethidium bromide: Sigma, USA

Ethylene dichloride(1, 2- dichloroethane): Sigma, USA

Ferric sulfate: Mallinckrodt Chemical, USA

Glycerol: Merck Ag Darmstadt, Germany

Glycine: Sigma, USA

Glycinebetaine: Sigma, USA

HEPES: Sigma, USA

Hydroxyapatite: Sigma, USA

Isoamylalcohol: Katayama Chem, Japan

Lamda DNA: Toyobo, Japan

L-Homocysteine thiolactone: Sigma, USA

Lithium chloride: Katayama Chem, Japan

Magnesium chloride: Merck Ag Darmstadt, Germany

Magnesium sulfate: Merck Ag Darmstadt, Germany

[Methyl-^{14}C]-choline: Amersham, England

Morpholinopropanesulphonic acid (MOPs): Katayama Chem, Japan

N,N'-methylene-bis-acrylamide : Sigma, USA

Noble Agar: BBL, Becton, Dickinson and Company, USA

Nitroblue tetrazolium (NTB): Katayama Chem, Japan

Phenol: Katayama Chem, Japan

POPOP: Sigma, Japan

PPO : Sigma, Japan

Potassium chloride: Merck Ag Darmstadt, Germany

Seakem CTG agarose: Biowhittaker Molecular Application, USA

Sephadex G-200: Pharmacia, England

Sepharose CL-6B: Sigma, USA

Sodium dodecyl sulfate: Sigma, USA

Sodium acetate: Katayama Chem, Japan

Sucrose: Katayama Chem, Japan

Toluene: BDH, England

Tris-hydrochloride: Katayama Chem, Japan

Triton X-100: Packard, USA

2.3 Supplies

Nitrocellulose membrane: Millipore Cooperation, USA

Whatman 3MM paper: Whatman International, England

X-ray film: X-Omat XK-1, Eastman Kodak, USA

2.4 Kit

Gel filtration standard kit: Biorad, USA

Ligation kit version1: Takara, Japan

PCR amplification kit: Applied Biosystems, USA

Standard molecular weight: Sigma, USA

Suprec™-O1 (cartridge for recovery DNA): Takara, Japan

Suprec™-O2 (cartridge for DNA concentration and Primers elimination): Takara, Japan

Tagman reverse transcription reagent kit: Perkin Elmer, Japan

2.5 Enzyme and restriction enzyme

*Ase*I: Biolabs, USA

*EcoR*I: Biolabs, USA

*EcoR*V: Biolabs, USA

*Hind*III: Biolabs, USA

*Nco*I: Biolabs, USA

*Sau3A*I: Biolabs, USA

Choline Oxidase from *Alcaligenes* sp.: Sigma, USA

Lysozyme: Sigma, USA

ProteinaseK: Katayama Chem, Japan

RNase: Katayama Chem, Japan

2.6 Bacterial strains and plasmids

Aphanothece halophytica was originally isolated from Solar Lake in Israel. The organism was kindly provided by Dr. T. Takabe of Research Institute of Meijo University, Japan.

E.coli TO114 (Δ*nha*A, Δ*nha*B, and Δ*cha*A) was kindly provided by Dr. T. Takabe of Research Institute of Meijo University, Japan

E.coli DH5α with genotype,φ80d *lacZ*ΔM15 *recA1endA1gyrA96 thi*-1 *hsdR*17($r_k^- m_k^+$) *sup*E44 *relA*1 *deoR* Δ(*lacZYA-argF*)U169

pTrcHis2C , expression vector, Invitrogen, USA (Appendix 1)

pBluescript® II SK^+, cloning vector, Toyobo, Japan (Appendix 2)

Table 1 Primers and probes for isolation and expression of Na^+/H^+ antiporter genes and for detection of their mRNA levels.

A) Primers

Primer/probe	5'----3'	Base pairs
NP-F	TT(T,C)(C,T)TGCC(T,C,G)(A,G,C)(C,T)(C,T)(C,T)T (C,A)(A,T,C)T(A,T,G)(T,G)(T,G) (T,A)GA(A,G)GC	26 mer
NP-R	CC(A,G)TC(A,G)TT(A,G)A(A,G)(C,T)A(T,G)(A,G)CT (T,C)TCCCCTTC	26 mer
ANP-NcoI	CCCCATGGCTATTGAAGCAGCAATGGGA	28 mer
ANP-N-R	TGAGCACAAATTGAATAACGGTTTGC	26 mer
ANP-C-F	AGGACTGACTACCGAGAAGCTAGGCT	26 mer
ANP-HindIII	GTAAGCTTCTCTGGTTTGGCGAGGACTT	28 mer
SNP-N-F	CACCATGGATACAGCGGTCAACG	23 mer
SNP-N-R	AGCCTAGCTTCTCGGTAGTCAGTCCT	26 mer
SNP-C-F	GCAAACCGTTATTCAATTTGTGCTCA	26 mer
SNP-C-R	TCGAATTCGGATGGTTCGGCCACAT	25 mer

Methods

2.7 Isolation and characterization of *A. halophytica* Na^+/H^+ antiporter, *apnha*P

2. 7. 1 *A. halophytica* culture conditions

The basic culture medium for *A. halophytica* cells were grown photoautotrophically in modified BG11 medium, BG11 plus 18 mM $NaNO_3$ and Turk Island salt solution, as previously described (Ishitani et al., 1993; Takabe et al., 1988 and Lee et al., 1997) except that NaCl concentration of the culture medium was adjusted to a range from 0.25 to 2.5 M as desired. Cotton-plugged 500 ml conical flasks containing 200 ml of medium each were used and shaken on a reciprocal shaker without supplementation of condensed CO_2 gas. The culture flasks were incubated at 28°C under continuous fluorescent white light (30 $\mu E\ m^{-2}\ s^{-1}$).

2. 7. 2 Chromosomal DNA extraction

A. halophytica grown in modified BG11 plus 0.5 M NaCl under continuous fluorescent white light was used for extraction chromosomal DNA. Fourteen-day-old culture (mid-exponential growth) was harvested by centrifugation at 4,000 rpm for 15 min at 4°C, washed twice with SET buffer (20% Sucrose, 50 mM EDTA and 50 mM Tris-Cl pH 7.6). Pellet was frozen at –20°C for 2 h, thawed at 65°C for 10 min and resuspended in SET buffer. Cells were lyzed by using lysozyme (final concentration 0.5 mg/ml), incubated at 37°C for 30 min under gentle shaking. Subsequently, SDS and RNase was added at final concentration 0.5% and 0.25 mg/ml, respectively. After an incubation at 37°C for 3 hours, proteinaseK was added at final concentration 0.25 mg/ml and further incubated for 30 min. The mixture was extracted once with equal volume of phenol/chloroform/isoamylalcohol (25: 24: 1) mixed gently and centrifuged at 15,000 rpm for 5 min at 25 °C. The aqueous layer was collected and

reextracted at least three times with equal volume of phenol/chloroform/isoamyl-alcohol High molecular weight DNA was precipitated by adding 2 volume of absolute ethanol and chilled at –20°C for 1-2 h. Chromosomal DNA was collected by centrifugation at 15,000 rpm for 5 min and washed once with 70% ethanol. Chromosomal DNA was allowed to dry under vacuum and suspended with TE buffer pH 8.0 (10 mM Tris-Cl pH 8.0 and 1 mM EDTA). To determine concentration and purity of chromosomal DNA, sample was diluted with TE buffer and checked by measuring the ratio of A260/A280.

2. 7. 3 Isolation of *A. halophytica* Na^+/H^+ antiporter

A. halophytica genomic DNA prepared from step 2. 7. 2 was used as a template DNA for isolation *apnha*P. Primers used for isolation and expression of Na^+/H^+ antiporter gene was shown in table 1. Partially-degenerate oligonucleotides were designed based on two highly conserved polypeptide regions among several Na^+/H^+ antiporters (Hamada et al., 2001). The forward primer, NP-F, was designed after the polypeptide stretch Phe-Leu-Pro-Pro-Leu-Leu-Phe-Glu-Ala (residues 73 to 81 in SynNhaP). The reverse primer, NP-R, contains the complementary sequence corresponding to the stretch Glu-Gly-Glu-Ser-Leu-Phe-Asn-Asp-Gly (residues 160 to 168 in SynNhaP). Amplified DNA fragments of an expected size (approximately 0.3 kbp) were concentrated and primers were eliminated by Suprec™-O2 followed by ligation into cloning vector, pBluescript® II SK^+, by using ligation kit. Insert fragment of an expected size 0.3 kbp was sequenced. Using the determined nucleotide sequence, the new primers were designed for the amplification of the adjacent unknown regions of DNA by the inverse polymerase chain reaction (PCR) method (Triglia et al., 1988). For this, DNA fragments of *A. halophytica* chromosomal DNA

obtained by partial digestion with *Sau*3AI or complete digestion with *Eco*RI or *Ase*I were size-fractionated by agarose gel electrophoresis and DNA was recovered by using Suprec™-O1. The fragments from 1-5 kbp were self-ligated and used as a template DNA for inverse PCR reaction. The fragment obtained from inverse PCR reaction were primers-eliminated, ligated into pBluescript® II SK^+ and analyzed for nucleotide sequence which covers the whole sequence of Na^+/H^+ antiporter.

2. 7. 4 Construction of expression plasmids (pANhaP and pSNhaP) and complementation test with the salt-sensitive mutant *E.coli* TO114

The coding region of *apnha*P was isolated by the PCR reaction. The forward primer, ANP-NcoI, contains the start codon ATG and *Nco*I site. The reverse primer, ANP-HindIII, contains the *Hind*III restriction site. *Apnha*P was amplified by ANP-NcoI and APN-HindIII. The fragment obtained from PCR reaction was concentrated and primers were eliminated by Suprec™-O2. This was followed by double digestion with *Nco*I and *Hind*III. The cohesive end fragment was ligated into *Nco*I/*Hind*III site of the digested pTrcHis2C expression vector. The resulting plasmid, pANhaP, encoding the ApNhaP fused in frame to six histidines, was transferred first into to *E. coli* DH5α cells by Chang-Miller method (Appendix 3). The transformants was selected on LB agar containing ampicillin at final concentration 50 μg/ml (Appendix 11) after incubation at 37°C for 12-16 h. Single colony of transformant was picked up and inoculated into LB solution containing ampicillin, allowed to grow overnight and used for the plasmid extraction by alkaline lysis method (Appendix 4).

The plasmid, pANhaP, was confirmed from the mobility of agarose electrophoresis of DNA fragments which were double digested with *Nco*I/*Hind*III.

The plasmid pANhaP was reextracted and then transferred to the salt-sensitive mutant, *E.coli* TO114 cells. The transformants were allowed to grow on LBK plus 0.2 M NaCl agar plate containing ampicillin (Appendix 12), at 37°C for 16 h.

The cells expressing pSNhaP were prepared as previously described (Hamada et. al, 2001). Both pANhaP and pSNhaP expressing cells were tested for the ability to grow (complementation test) in LBK plus 0.2 M NaCl, LBK plus 4 mM LiCl (Appendix 12) and TrisE plus 0.1 M $CaCl_2$ (Appendix 13) for sodium, lithium and calcium complementation, respectively. In addition, these transformant cells were examined directly for Na^+/H^+ Antiporter activity as described in step 2. 7. 5

2. 7. 5 Na^+/H^+ antiporter activity

The Na^+/H^+ antiporter activity was examined on everted membrane vesicles (membrane protein of *E.coli*) prepared from the cells grown in LBK as described (Hamada et al., 2001). For this, *E. coli* cells were harvested by centrifugation at 4,000 rpm for 10 min at 4°C and then washed with a suspension buffer TCDS (Appendix 7). The pellet was suspended with 10 ml TCDS buffer and applied to a French Pressure cell (4,000 psi). Unbroken cells were sedimented at 10,000 rpm for 10 min at 4°C and the supernatant was centrifuged at 30,000 rpm for 60 min at 4°C to pellet everted membrane. The pellet was suspended in 600 µl TCDS buffer and protein concentration was determined according to Lowry's method (1951). The antiporter activity was based upon the establishment of ΔpH (TM pH gradient) by addition of salt to the reaction mixture which contained 10 mM Tris-HCl (titrated with HCl to the indicated pH), 5 mM $MgCl_2$, 0.14 M choline chloride, 1 µM acridine orange, and membrane vesicles (50 µg of protein) in a volume of 2 ml. The ΔpH was monitored with acridine orange fluorescence which was obtained by excitation at 492

nm and emission at 525 nm. Before addition of salt, Tris-DL-lactate (2 mM) was added to initiate fluorescence quenching (Q) due to respiration. Lactate energizes the vesicle and accumulates H^+ intravesiculary which causes the accumulation of dye and fluorescence quenching (Q). Upon addition of salt (5 mM), fluorescence increases due to the excretion of H^+ by antiporters and dequenched fluorescence (ΔQ) was monitored. Then, NH_4Cl (25 mM) was added to dissipate ΔpH.

2. 7. 6 Construction of chimeric gene between *apnha*P (Na^+/H^+ antiporter gene from *Aphanothece halophytica*) and *synnha*P (Na^+/H^+ antiporter gene from *Synechocystis* sp. PCC6803)

Due to the different cation exchange activities between ApNhaP and SynNhaP, a chimeric gene which encodes the N-terminal trans-memebrane region of ApNhaP and the C-terminal cytosolic region of SynNhaP and vice versa were constructed by PCR technique.

A chimeric gene which encodes the N-terminal TM region of ApNhaP (residues Met1-Ile401) and the C-terminal cytosolic region of SynNhaP (residues Gln401-Ser527) was constructed as follows (Fig. 2.1). The nucleotides corresponding to the N-terminal region of ApNhaP and C-terminal region of SynNhaP were amplified by the forward/reverse primer sets, ANP-NcoI/ANP-N-R and SNP-C-F/SNP-C-R, respectively. The template for the former nucleotide was the pANhaP and that for the latter was the pSNhaP (Hamada et al., 2001). The forward primer, SNP-C-F, contains the sequence corresponding to the polypeptide stretches Gln398-Thr-Val-Ile401 of ApNhaP and Gln401-Phe-Val-Leu404 of SynNhaP and the reverse primer, ANP-N-R, is the complementary sequence of SNP-C-F. The reverse primer, SNP-C-R, contains the *EcoR*I site just before the stop codon of pSNhaP. Upon mixing both

PCR products, a chimeric gene *apsynnha*P was amplified using the forward ANP-NcoI and reverse SNP-C-R primers, and ligated into the *NcoI*/*EcoR*I digested sites of pTrcHis2C which generated the plasmid pASNhaP. For the construction of a chimeric gene which encodes the N-terminal region of SynNhaP (residues Met1-Thr400) and the C-terminal region of ApNhaP (residues Glu402-Glu521), the respective nucleotides were amplified by the following forward/reverse primer sets, SNP-N-F/SNP-N-R and ANP-C-F/ANP-HindIII. Here, the reverse primer, SNP-N-R, is the complementary sequence of ANP-C-F. Upon mixing both PCR products, a chimeric gene *synapnha*P was amplified using the forward SNP-N-F and reverse ANP-HindIII primers, and ligated into the *NcoI*/*Hind*III digested sites of pTrcHis2C which generated the plasmid pSANhaP.

Two chimeric Na^+/H^+ antiporters (ASNhaP and SANhaP) were examined for Na^+/H^+ antiporter activity as describe in step 2.7.5

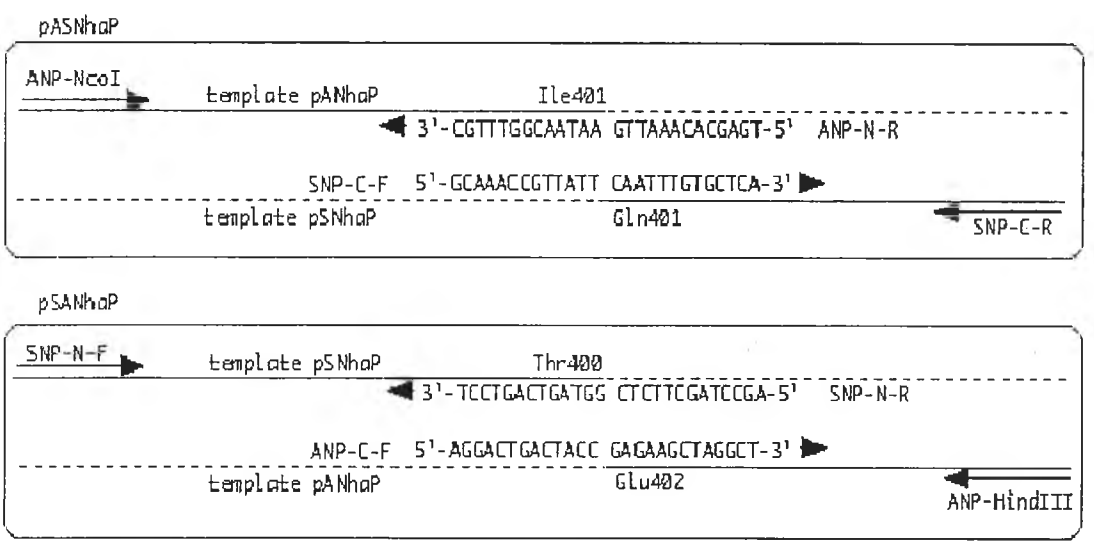

Figure 2.1 Schematic figure for construction of chimeric gene pASNhaP and pSANhaP

2. 7. 7 Quantitation of ApNhaP mRNA expression level in intact *A. halophytica* upon the salinity change by Real-time Quantitative RT-PCR

2. 7. 7. 1 *A. halophytica* culture condition

It is interesting to test whether the expression level of Na^+/H^+ antiporter in *A. halophytica* changes upon the change of salinity. The level of mRNA for ApNhaP was examined. For this, *A. halophytica* was initially grown in modified BG11 plus 0.5 M NaCl (for upshock experiment) and 1.5 M NaCl (for downshock experiment). Fourteen-day-old of culture was harvested by centrifugation at 4,000 rpm for 15 min at 4°C. For upshock experiments, pellet of culture grown in modified BG11 in the presence of 0.5 M NaCl was resuspended in the medium in which NaCl concentration was increased to 1.5 M NaCl and cells were collected at 0, 30, 60, 120 and 180 min, respectively. For downshock experiments, pellet of culture grown in the presence of 1.5 M NaCl was resuspended in the medium in which NaCl concentration was decreased to 0.5 M NaCl. Total RNAs were extracted from cell pellets at various times and used for the quantitation of mRNA level.

2. 7. 7. 2 Total RNAs extraction

Total RNAs were extracted from *A. halophytica* cells treated as descrived in 2.7.7.1 by using SDS-phenol method. For this, fresh weight of *A. halophytica* approximately 100 mg was washed twice with SET buffer and then resuspended in extraction buffer (Appendix 6) containing 2.8 M β-mercaptoethanol. Cell suspension was lysed by lysozyme (final concentration 0.5 mg/ml) and incubated under mechanical vortex for 1 h at 4°C. The suspension was extracted with phenol/chloroform/isomylalcohol (25: 24: 1) and the aqueous phase was collected by centrigation at 8,000 rpm for 15 min at 25 °C. The aqueous phase was extracted with

phenol/chloroform/isomylalcohol for several times until no protein was found at the interphase. Total aqueous phase was pooled to which 5 M NaCl and absolute ethanol were added at 0.02 and 2.5 fold of total volume, respectively and allowed to precipitate at –20 °C for 1-2 h. Then, the solution was centrifuged at 8,000 rpm for 15 min at 4 °C. Pellets were dried under vacuum and resuspended in TE buffer (pH 8.0). The solution in TE buffer was added with 10 M LiCl at 0.33 volume and further incubated on ice for 12-16 h. Then, the pellet was collected by centrifugation at 15,000 rpm for 15 min at 4 °C and resuspended in TE buffer. The solution was added with 5M NaCl and absolute ethanol at 0.05 and 2.5 fold of total volume, respectively and incubated at –20 °C for 2 h. Total RNAs were collected by centrifuge at 15,000 rpm for 15 min at 4 °C and washed once with 70% ethanol, dried under vacuum and suspended in small amount of Milli-Q. The purity and concentration of RNA were measure by spectrophotometer at the ratio of absorbance 260/280. Contamination of polysaccharide was check from the absorbance ratio 260/230. About 3 μg of total RNAs was analyzed by formaldehyde-containing 1.2 % agarose gel (Appendix 5).

2. 7. 7. 3 Quantitation of ApNhaP mRNA expression level by TaqMan Fluorescent Chemical Analysis Method

2. 7. 7. 3. 1 General principle

The principle of Taqman Fluorescent Chemical Analysis Method provides a real-time detection and quantitation of the polymerase chain reaction (PCR) process. This method is based on the 5' nuclease activity of Taq polymerase to cleave a nonextendable dual-lable fluorogenic hybridization probe during the extension phase of PCR. The probe is labeled with a reporter fluorescent dye [FAM (6-carboxy-fluorecein)] at the 5' end and a quencher-fluorescent dye

[TEMRA (6-carboxy-tetramethylrhodamin)] at the 3' end. When the probe is intact, the reporter dye emission is quenched owing to the physical proximity of the reporter and quencher fluorescent dyes. During the extension phase of the PCR cycle, however, the nucleolytic activity of the DNA polymerase cleaves the hybridizaton probe and releases the reporter dye from the probe. The resulting relative increase in reporter fluorecent dye emission is monitored in real time during PCR amplification using a sequence detector. The sequence detector is a combination of thermal cycler, laser, and detection and software system that automates 5' nuclease-base detection and quantitation of nucleic acid sequences. Fluorescence intensity produced during PCR amplification is monitored in real time (Heid et al., 1996; Gibson et al., 1996 and Yajima et al., 1998).

2. 7. 7. 3. 2 Quantitation of ApNhaP mRNA expression level

Quantitation of ApNhaP mRNA expression level was carried out using a TaqMan fluorescent chemical analysis method. The synthesis of TaqMan flourescent probe, Taq probe, was performed by Perkin-Elmer, Japan. The clone specific primers, Taq-F and Taq-R were used for amplification.

Total RNAs extracted from *A. halophytica* (2. 7. 7. 2) was first used for the generation of cDNA by the reaction of reverse transcriptase (RT) using the TaqMan Reverse Transcription reagents containing 1X TaqMan RT Buffer, 5.5 mM Magnesium chloride, 500 μM deoxyNTPs mixture, 2.5 μM Random hexamer, 0.4 unit/μl RNase inhibitor, 1.25 unit/μl MultiScribe™ Reverse Transcriptase and 100 ng RNA sample. RT reaction was performed under the following conditions; 10 min at 25 °C for incubation step, 30 min at 48 °C for reverse transcription step and 5 min at 95 °C for Reverse Transcriptase Inactivation step.

For quantitation of ApNhaP mRNA, Taq probe and the clone specific primers (Tag-F and Tag-R) were optimized to give the maximum ΔRn. Thermal cycling for primers and probe optimization was performed under the following conditions; 2 min at 50 °C, 10 min at 95 °C for initial step, 15 sec at 95 °C for melting step and 1 min at 60 °C for anneal/extend step (50 cycles). A computer algorithm was used for comparison of the amount of reporter dye emission (R) with the quenching dye emission (Q) during the PCR amplification, generating a ΔRn value as follows: $\Delta Rn = (\Delta Rn^{+}) - (\Delta Rn^{-})$, where ΔRn^{+} is (emission intensity of reporter)/ (emission intensity of quencher at any given time in the reaction tube), and ΔRn^{-} is (emission intensity of reporter)/(emission intensity of quencher before PCR amplification in the reaction tube). The ΔRn value is used for the construction of amplification plots.

2. 7. 8 Immunoblotting of membrane protein of pANhaP expressing cells

Protein content of everted membrane vesicles of *E. coli* prepared from step 2.7.5 was determined according to Lowry's method. The same everted membrane vesicles were used for immunoblotting experiment. Fifty-microgram of membrane protein was separated by 12.5% sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) (Appendix 10) and transferred to nitrocellulose membrane using blotting transfer buffer (Appendix 8). Blotting was done at 150 $mA/inch^2$ for 1 hr followed by blocking in blocking solution (Appendix 8) for 2 h. The nitrocellulose membrane was incubated with primary antibody (an antibody raised against 6-histidine, 6X-His tag) for 1 h and washed with 100 ml of PBS plus 5% skim milk solution for 15 min, 3 times. After washing the membrane with PBS buffer plus 5% skim milk, the membrane was immediately incubated with secondary antibody (an antibody raised

against mouse) for 1 h and washed 3 times with 100 ml of PBS plus 5% skim milk buffer for 15 min. The nitrocellulose membrane was visualized after incubation with the detection reagent for 30 min (Appendix 9).

2. 8 Degradation of glycinebetaine in *A. halophytica*

2. 8. 1 Condition for the induction of betaine-homocysteine methyltransferase (BHMT) in *A. halophytica*

A. halophytica was grown photoautotrophically in modified BG11 medium as described in step 2.7.1 Then, cells were grown in modified BG11 plus 2.0 M NaCl as an extra salt and allowed to grow for 7 days. After harvesting the cells by centrifugation at 4,000 rpm for 15 minutes at 4°C, the pellet was resuspended in the medium containing 0.25, 0.50, 1.00 and 2.00 M NaCl , or suspended in the nitrogen depleting medium, respectively. Cells were collected at various time intervals and kept at -20°C prior to BHMT activity assay and determination of glycinebetaine in the next step.

2. 8. 1. 1 BHMT assay

BHMT activity was assayed as described (Incharoensakdi and Waditee, 2000). The reaction in the final volume of 1.5 ml contained 40 mM potassium phosphate pH 7.5, 7 mM L-homocysteine freshly prepared from L-homocysteine thiolactone (Appendix 15), 7 mM [methyl-^{14}C] glycinebetaine (20,000 dpm) prepared from [methyl-^{14}C] choline using choline oxidase from *Alcaligenes* sp. (Appendix 16). The reaction was started by adding the enzyme extract, prepared from the cells subjected to osmotic shock, to the reaction mixture. Following a 60 min incubation at 37 °C, the reaction mixture was stopped by chilling on ice bath and

subsequently applied to Dowex 1X4 (hydroxyl form) column. The reaction product, [methyl-^{14}C] methionine, was eluted by 3 ml of 1.5 M HCl and quantitated by liquid scintillation counting, one unit of enzyme defined as the rate of nanamol forming [methyl-^{14}C] methionine per hour.

2. 8. 1. 2 Determination of glycinebetaine

The cell pellet was extracted with boiling ethanol (80%, vol/vol) for 5 min and re-extracted with 80% ethanol at room temperature for 18 h before centrifugation at 4,000 rpm for 5 min. The total supernatant was pooled and dried by oven at 65°C. The dried residue was dissolved in distilled water and loaded onto a Dowex 50W (50x4-200, H^+ form) column. After the column was washed with 10 ml of distilled water, glycinebetaine was eluted with 2 M NH_3 and analyzed by tri-iodide assay method (Storey and Wyn Jones, 1977) where the solution of 0.5 ml glycinebetaine was added with 0.2 ml of acid potassium triiodide (7.5 g I_2, 10 g KI in 1 M KCl). The mixture was shaken for 90 min, followed by rapid addition of 2 ml ice-cold distilled water. Five ml of 1, 2- dichloroethane was then added, and the two layers were mixed. The absorbance of the lower organic layer was measured at 365 nm.

2. 8. 2 Determination of intermediate product(s) of BHMT activity

To ascertain that the BHMT was actually operating under the condition shown in 2.8.1(salt downshock), the presence of another product of BHMT activity, namely *N, N*-dimethylglycine, was examined.

2. 8. 2. 1 Labelling *A halophytica* with ^{14}C-glycinebetaine under salt downshock condition

A.halophytica cells grown under the same condition as described in step 2.7.1 was resuspended in modified BG11 containing 2.0 M NaCl

followed by addition of ^{14}C-glycinebetaine (50,000 dpm) and allowed to grow for 7 days. Then, cells were collected by centrifugation at 4,000 rpm for 15 min at 4°C, washed twice with isoosmotic medium and subjected to hyposomotic condition (0.5 M NaCl). Cell pellet was collected at various time intervals, washed twice with the isoosmotic medium and kept at -20°C prior to analysis for radioactive compound (s).

2. 8. 2. 2 Extraction of radioactive compound (s)

Pellet from step 2. 8. 2. 1 was extracted with boiling 80% ethanol for 5 min and re-extracted with 80% ethanol at room temperature for 18 h, then centrifuged at 4,000 rpm for 5 minutes. The total supernatant was pooled and dried by oven at 65 °C, dried pellet was resuspended in small amount of milli-Q. The radioactive compound(s) present in the pellet was separated by thin-layer chromatography (silica gel plate) and visualized by autoradiograph.

2. 8. 2. 3 Identification of radioactive compound(s)

Authentic commercial compounds, *N, N*-dimethylglycine (dimethylglycine) and glycinebetaine from sigma were used as standard. The radioactive compound under hypoosmotic stress condition and standard samples were separated by TLC. The solvent system for the separation of dimethylglycine and glycinebetaine is a mixture containing methanol : 0.88 M ammonium hydroxide (9:1 vol/vol). TLC plate was developed in solvent system for 1.5 hours. Glycinebetaine and dimethylglycine were identified by spraying with Dragendroff ' s reagent (Appendix 14). Radioactive compounds were visualized by autoradiography.

2. 8. 3 Purification of BHMT and its properties

2. 8.3.1 Cell extraction

Approximately 5 g of *A. halophytica* cell pellet upon the downshock condition (2.0 M to 0.5 M NaCl) was suspended in 10 ml of grinding buffer containing 50 mM Hepes-KOH pH 7.5, 10 mM EDTA, 5 mM DTT and 10% glycerol. After additon of lysozyme (final concentration 5 mg/ml), cells were allowed to incubate at 37°C for 60 min to lyse the cell. The suspension was then centrifuged at 14,000 rpm for 15 min at 4°C to collect crude enzyme in the supernatant. Protein content was determined according to method of Bradford (1976). Crude enzyme solution was subjected to hydroxyapatite column chromatography.

2. 8. 3. 2 Hydroxyapatite Column Chromatography

Approximately 15 g of the commercial hydroxyapatite was swollen in 1 mM potassium phosphate buffer pH 7.5 and equilibrated overnight with buffer A (25 mM potassium phosphate buffer pH 7.5 containing 1 mM EDTA and 10 mM 2-mercatptoethanol). The prepared hydroxyapatite was packed into a column (1.8 x 10.0 cm) at the height of 7.0 cm. The column was equilibrated with bufferA for at least 4 to 6 hr at the flow rate 6 ml/hr. Five millilitres of protein solution containing about 230 mg protein was loaded and allowed to be absorbed. Elution was carried out by a linear gradient of 0-500 mM potassium phosphate buffer pH 7.5 containing 1 mM EDTA and 10 mM 2-mercatptoethanol. A flow rate of 6 ml/hr was maintained and 2 ml of each fractions were collected. The protein content of each fraction was monitored by measuring the A280. The BHMT activity was detected as described in step 2. 8. 1. 1. The fraction showing high activity of BHMT in this step was pooled and dialyzed against the buffer B (25 mM Hepes-KOH pH 7.5 containing 1 mM

EDTA and 10 mM 2-mercatptoethanol), concentrated by aquasorb and applied to Sepharose CL-6B.

2. 8. 3. 3 Sepharose CL-6B Column Chromatography

For preparation Sepharose CL-6Bcolumn chromatography, commercial Sepharose CL-6B in buffer B was packed into a column (1.7 x 25.0 cm) at the height of 20.0 cm. The column was equilibrated with buffer B for at least 4 to 6 hr at the flow rate 18 ml/hr. Two millilitres of protein solution containing about 30 mg protein was loaded and allowed to be absorbed. Elution was carried out by a continuous linear gradient of 0-1.0 M KCl prepared in buffer B. A flow rate of 18 ml/hr was maintained and 2 ml fractions were collected. The protein content of each fraction was monitored by measuring the A280. The BHMT activity was detected as described in step 2. 8. 1. 1. The fraction showing high activity of BHMT from this step was pooled, dialyzed against the buffer B, concentrated by aquasorb and applied to Sephadex G-200.

2. 8. 3. 4 Sephadex G-200 Column Chromatography

Commercial Sephadex G-200 about 10 g was swollen in distilled water by boiling in hot water bath for at least 5 h, cooled down at room temperature before packing. The prepared Sephadex G-200 was packed into a column (1.1 x 65.0 cm) at the height of 60.5 cm. The column was equilibrated with buffer B for at least 4 to 6 hr at the flow rate 12 ml/h. Void volume and total bed volume were determined by blue dextran 2000 (3 mg/ml) and potassium dichromate (0.3 mg/ml). These two markers were detected by measuring the A_{620} and A_{410} respectively. The standard protein mixture containing thyroglobulin (670,000 dalton), immunoglobulinG (158,000 dalton), ovalbumin (44,000 dalton), myoglobin (17,000 dalton), and vitamin

B12 (1,650 dalton) was layered on the column. Elution was carried out by the same buffer and the flow rate was maintained at 12 ml/hr. Two ml fractions were collected and the absorbance of each fraction was monitored at 280 nm. The distribution coefficient (Kav) was calculated by :

$$Kav = (Ve-Vo)/(Vt-Vo)$$

Ve(elution volume) = The volumn required to elute protein or enzyme from the column

Vo(void volume) = The volume required to elute blue dextran from the column

Vt(Total bed volumn) = The volume of space occupied by the gel beads which could be determined by the volume required to elute potassium dichromate from the column

Two millilitres of dialyzed BHMT from Sepharose CL-6B column was loaded on the Sphadex G-200 column. The protein was eluted by the buffer B at the flow rate 12 ml/hr. Two ml fractions were collected and the absorbance of each fraction was measured at 280 nm. The molecular mass of the protein (BHMT) was estimated from standard curve plotted between the log molecular mass of protein markers and the Kav. The BHMT activity was detected as described in step 2. 8. 1. 1. The fraction showing high activity of BHMT from this step was pooled and dialyzed against the buffer B and used for the aim of determine the molecular mass of BHMT in denatured form by SDS-PAGE and also for the study of BHMT properties.

2. 8. 3. 5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular mass of BHMT in the denatured state was determined by SDS-PAGE according to Laemmli (1970). For this, sample to be separated was treated with sample buffer for SDS-PAGE and boiled for 5 minutes prior to application to the gel and then loaded to slab gel (10% separating gel and 5.0% stacking gel) (Appendix 10). The electrophoresis was performed at the constant current of 20 mA per slab, on Mini protein cell electrophoresis unit from cathode toward the anode. The gel was stained with coomassie blue. The protein molecular weight markers were bovine serum albumin (66,000 dalton), egg albumin (45,000 dalton), glyceraldehyde-3-phosphate dehydrogenase (36,000 dalton), carbonic anhydrase (29,000 dalton), trypsinogen (24,000 dalton) and soy bean trypsin inhibitor (20,100 dalton). Relative molecular mass for the protein sample was estimated from the standard curve plotted on the semilog scale between the molecular mass of protein marker and the relative electrophoretic mobility (Rf). The Rf was calculated by: the distance of protein migration / distance of tracking dye migration

2. 8. 3. 6 The properties of BHMT

The partially purified BHMT was characterized with respect to pH, temperature, substrate analog inhibition and effect of NaCl.

2. 8. 3. 6. 1 Effect of pH and temperature on BHMT activity

The partially purified BHMT was assayed in the reaction mixture as described in step 2. 8. 1. 1 with appropiate amount of enzyme. In each assay, the pH was adjusted with appropriate buffer, i. e. potassium phosphate buffer for pH 6.0-7.5; HEPES-KOH buffer for pH 7.5-8.0 and Tris-Cl buffer for pH

8.5-9.0. For the temperature effect, the enzyme was incubated for 1 h at 30, 35, 37, 40 and 45°C. The initial velocity of enzyme was expressed in [methyl-^{14}C] methionine forming nmol per hour.

2. 8. 3. 6. 2 The kinetic of BHMT

The kinetics of the BHMT were studied by assaying the enzyme as described in step 2. 8. 1. 1. The reaction was started by the addition of varying concentrations of either glycinebetaine or L-homocysteine (1 mM-5 mM). The Michaelis constant (Km) and maximun velocity (Vmax) were calculated from the intercepts of x-axis and y-axis of the double-reciprocal plot, i. e.,1/[s] vs 1/[v], respectively.

2. 8. 3. 6. 3 The substrate analog inhibition

The partially purified BHMT was assayed as described in step 2. 8. 1. 1. Four substrate analogs (choline, betaine aldehyde, monomethylglycine and dimethylglycine) were used. The reaction was started by addition of substrate analog and incubated at 37°C for 1 h. the inhibition was expressed as the percentage of remaining activity of the enzyme without inhibitor.

2. 8. 3. 6. 4 Effect of NaCl on BHMT activity

The partially purified BHMT was assayed as described in step 2. 8. 1. 1. Various NaCl concentrations of 0-1000 mM were added to the reaction mixture. BHMT activity was expressed as the percentage of remaining activity of the enzyme without addition of NaCl.