ผลของความเครียดจากเกลือต่อการขนส่งโคลีนเข้าสู่ไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica*

นายสุรศักดิ์ ละลอกน้ำ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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EFFECTS OF SALT STRESS ON CHOLINE TRANSPORT INTO HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica*

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สุรศักดิ์ ละลอกน้ำ: ผลของความเครียดจากเกลือต่อการขนส่งโคลีนเข้าสู่ไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica* อ. ที่ปรึกษา: รศ.ดร. อรัญ อินเจริญศักดิ์, อ. ที่ปรึกษาร่วม: Prof. Dr. Teruhiro Takabe, 215 หน้า: ISBN 974-14-2157-5.

สาหร่ายสีเขียวแกมน้ำเงินทนเค็ม *Aphanothece halophytica* เจริญภายใต้ภาวะที่มีความเครียดของเกลือโซเดียมคลอไรด์ 0.5 – 3.0 โมลาร์ พีเอช 6.5 – 10.5 พบว่าเซลล์เจริญได้ดีที่ความเข้มข้นของเกลือโซเดียมคลอไรด์ 0.5 โมลาร์ และ พีเอช 9.5 ตามลำดับ ศึกษาความสัมพันธ์ของการเจริญของเซลล์ ขนาดของเซลล์ สารประกอบภายในเซลล์ องค์ประกอบของกรดอะมิโน และ รูปแบบของโปรตีนภายใต้ภาวะที่มีความเครียดของเกลือ พบว่า การเจริญของเซลล์ลดลงเมื่อความเข้มข้นของเกลือโซเดียมคลอไรด์เพิ่มขึ้น ภายใต้ภาวะที่มีความเครียดของเกลือ พบว่า เซลล์มีขนาดเพิ่มขึ้น มีการสะสมไกลซีนบีเทนเพิ่มขึ้น และ ไอออนโซเดียม โปแตสเซียม แอมโมเนียม และ ไนเตรต มีการเปลี่ยนแปลงอย่างไม่เด่นชัด เมื่อเปรียบเทียบกับภาวะที่ไม่มีความเครียดจากเกลือ กลูตามีนเป็นกรดอะมิโนที่พบมากที่สุดทั้งสองภาวะ และ พบกรดอะมิโนกลูตามีน แอสปาเตต โปรลีน และ กลูตาเมต เพิ่มขึ้นภายใต้ภาวะที่มีความเครียดของเกลือ

ภาวะที่มีความเครียดของเกลือชักนำให้เกิดแถบโปรตีนเพิ่มขึ้นในแฟรกชั่น ไซโตปลาสซึม เพอริปลาสซึม และ เมมเบรน เมื่อทำการวิเคราะห์ด้วย โซเดียมโดเดซิลซัลเฟต พอลิอะคริลิไมด์เจล อิเล็กโตรพอลิซิส แถบโปรตีนที่มีความเข้มสูงสุดของแฟรกชั่น ไซโตปลาสซึม เพอริปลาสซึม และ เมมเบรน มีมวลโมเลกุล 32.0, 35.5, และ 41.5 กิโลดาลตัน ตามลำดับ แฟรกชันเมมเบรนมีสมบัติเป็นโปรตีนที่จับกับโคลีนได้ และถูกยับยั้งสมบัติโปรตีนที่จับโคลีนกับเมมเบรนได้ด้วย ไกลซีนบีเทน ไกลซีนบีเทนอัลดีไฮด์ และ คาร์นิทีน ตามลำดับ เมื่อทำการศึกษาโดยเทคนิค พอลิอะคริลิไมด์เจล อิเล็กโตรพอลิซิส แบบไม่เสียสภาพ ตามด้วย ออโตเรดิโอกราฟ การตกตะกอนโปรตีนที่มีสารรังสี และ เซอร์เฟสพลาสมอน เรโซแนนซ์

ทำการศึกษาจลนพลศาสตร์การนำเข้าโคลีนเข้าสู่เซลล์ และ เมมเบรน เวซิเคิล ของ *A. halophytica* ที่ภาวะปกติ และภาวะที่มีความเครียดของเกลือ 1.0 โมลาร์ และ 2.0 โมลาร์ มีค่า K_m = 246.78, 262.19, และ 299.51 ไมโครโมลาร์ ตามลำดับ และอัตราเร็วสูงสุด V_{max} เป็น 12.12, 24.10, และ 10.71 nmol/min/mg protein, ตามลำดับ การนำโคลีนเข้าสู่เมมเบรน เวซิเคิล พบว่าที่ภาวะปกติโซเดียมคลอไรด์ความเข้มข้น 0.5 โมลาร์ และภาวะที่มีความเครียดของเกลือโซเดียมคลอไรด์ 2.0 โมลาร์ มีค่า K_m = 82.00 and 88.11 ไมโครโมลาร์ ตามลำดับ และอัตราเร็วสูงสุด V_{max} เป็น 4.55 และ 1.82 nmol/min/mg protein ตามลำดับ ที่ภาวะที่มีความเครียดของเกลือ ไกลซีนบีเทน และ ไกลซีนบีเทนอัลดีไฮด์ ยับยั้งการนำเข้าโคลีนได้อย่างสมบูรณ์

เมื่อทำการศึกษาพลังงานที่จำเป็นในการนำโคลีนเข้าสู่เซลล์ และ เมมเบรนเวซิเคิล ของ สาหร่ายสีเขียวแกมน้ำเงินทนเค็ม *A. halophytica* พบว่าผลการทดลองที่ทำทั้งสองมีความคล้ายกัน การนำเข้าโคลีนยับยั้งได้อย่างสมบูรณ์ด้วย คาร์บอนิล ไซยาไนด์ เอ็ม คลอโรฟีนิล ไฮดราโซน และ 2, 4 ไดไนโตรฟีนอล อธิบายได้ว่าการนำเข้าโคลีนขับเคลื่อนโดยโปรตอนโมทีฟฟอร์ซ การศึกษาด้วยการสร้างความต่างศักย์ของความเป็นกรด-ด่าง ระหว่างเมมเบรน (ΔpH) พบว่าเพิ่มการนำเข้าโคลีน เมื่อทำการลดความต่างศักย์ของความเป็นกรด-ด่าง ระหว่างเมมเบรน (ΔpH) พบว่าการนำเข้าโคลีนลดลง และ เมื่อพีเอชภายนอกเพิ่มขึ้น การนำเข้าโคลีนก็เพิ่มขึ้นเช่นกัน เมื่อทำการเพิ่มศักย์ของเมมเบรนอันเนื่องมาจากประจุ ($\Delta\psi$) ไม่มีผลต่อการนำเข้าโคลีน เมื่อทำการศึกษาการลดความต่างศักย์ของเมมเบรนอันเนื่องมาจากประจุ ($\Delta\psi$) ด้วยการใช้วาลิโนไมซิน ไม่มีผลต่อการนำเข้าโคลีนเช่นกันแต่เป็นส่วนหนึ่งที่ให้พลังงานในการนำเข้าโคลีน การนำเข้าโคลีนถูกยับยั้งอย่างสมบูรณ์ด้วย ไนเจอริซิน (ตัวยับยั้งความต่างศักย์ของเมมเบรนอันเนื่องมาจากโปรตอน) อธิบายได้ว่าการนำเข้าโคลีนขึ้นอยู่กับความต่างศักย์ของเมมเบรนอันเนื่องมาจากโปรตอน อย่างไรก็ตาม ความต่างศักย์อันเนื่องมาจากโซเดียมไอออนมีบทบาทในการนำเข้าโคลีน เพราะว่า โมแนนซิน และ อะมิโลไรด์ (ตัวยับยั้งความต่างศักย์อันเนื่องมาจากโซเดียมไอออน) ยับยั้งการนำเข้าโคลีน และทำการศึกษาการนำไนเตรตเข้าสู่เซลล์ สาหร่ายสีเขียวแกมน้ำเงินทนเค็ม *A. halophytica* พบว่าผลการทดลองเหมือนกับการนำโคลีนเข้าสู่เซลล์

ทำศึกษาสมบัติของยีนบีเทนทรานส์พอร์เตอร์ของ *A. halophytica* เนื่องจากไม่สามารถทำการแยกยีนที่เกี่ยวข้องกับการขนส่งโคลีนได้ พบว่ายีนบีเทนทรานส์พอร์เตอร์ของ *A. halophytica* มีความเหมือนสูงกับ OpuD ของ *Bacillus subtilis* และ มีความเหมือนน้อยกับ บีเทน/โปรลีนทรานส์พอร์เตอร์ที่พบในพืช จากนั้นทำการโคลนยีนของบีเทนทรานส์พอร์เตอร์ เข้าสู่ *Escherichia coli* MKH13 ที่ไม่มียีนของระบบการขนส่ง โคลีน บีเทน และ โปรลีน พบว่าสามารถทำให้ MKH13 ที่มียีนบีเทนทรานส์พอร์เตอร์เจริญได้ในความเข้มข้นของเกลือสูงกว่าภาวะปกติ และ มีความจำเพาะต่อ บีเทน และไม่นำเข้าโปรลีน พบว่า โคลีน จีเอบีเอ บีเทนอัลดีไฮด์ ซาร์โคซีน ไดเมทิลไกลซีน และ กรดอะมิโน ไม่มีผลต่อการนำเข้าบีเทน โซเดียมไอออนเร่งการนำเข้าบีเทนโดยไม่เกี่ยวข้องกับโปแตสเซียมไอออน พีเอชที่เหมาะสมในการนำเข้าบีเทนคือ พีเอช 9 จากนั้นทำการโคลนเข้าสู่สาหร่ายสีเขียวแกมน้ำเงินน้ำจืด *Synecococcus* พบว่าสามารถทำให้สาหร่ายสีเขียวแกมน้ำเงินน้ำจืด เจริญเติบโตได้ในน้ำเค็ม และกลไกทางจลนพลศาสตร์ของการนำ บีเทนเข้าสู่เซลล์ *Synecococcus* ที่มียีนบีเทน ทรานส์พอร์เตอร์คล้ายกับ การนำบีเทนเข้าสู่เซลล์ *A. halophytica* จากการค้นพบนี้แสดงว่า *A. halophytica* มี โซเดียมไอออน-บีเทน ซิมพอร์เตอร์ ที่ทนต่อภาวะเครียดของเกลือ และพีเอชที่เป็นด่าง

สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต.....

ปีการศึกษา .....2548..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

##4573854223: MAJOR BIOTECHNOLOGY
KEY WORD: *Aphanothece halophytica*, choline, nitrate, uptake, betaine transporter

Surasak Laloknam: EFFECTS OF SALT STRESS ON CHOLINE TRANSPORT INTO HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica*, THESIS ADVISOR: ASSOC. PROF. Aran Incharoensakdi, Ph.D., THESIS COADVISOR: PROF. Teruhiro Takabe, Ph.D., 215 pp: ISBN 974-14-2157.

A halotolerant cyanobacterium *Aphanothece halophytica* grown under various NaCl concentrations from 0.5 – 3.0 M and pH from 6.5 – 10.5 showed optimal NaCl concentration and optimal pH for cell growth at 0.5 M NaCl and pH 9.5, respectively. We examined the relationships between cell growth, cell size, intracellular solute, amino acid composition, and protein profiles as affected by salt stress. Cell growth was reduced when NaCl was increased while cell size was increased with high accumulation of glycine betaine. Ion contents Na^+, K^+, NH_4^+, and NO_3^- were not so much different under non stress and salt stress conditions. Amino acid glutamine was the major amino acid under both conditions and analysis showed that glutamine, aspartate, proline, and glutamate increased under salt stress condition.

Salt stress induced apparent proteins from all fractions, cytoplasmic, periplasmic, and membrane fraction as determine by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The highest intensity band was observed in cytoplasmic, periplasmic, and membrane fraction showing molecular mass of 32.0, 35.5, and 41.5 kDa, respectively. Membrane fraction showed highest choline binding activity which was strongly inhibited by glycine betaine, glycine betaine aldehyde, and carnitine, respectively when studied by using non-denaturing polyacrylamide gel electrophoresis autoradiograph, radio protein precipitation, and surface plasmon resonance, respectively.

Uptake of [methyl-^{14}C] choline into intact cells and membrane vesicles of *A. halophytica* was studied. The apparent choline uptake for intact cells revealed K_m values of non-stress and salt-stress condition at 1.0 M and 2.0 M NaCl to be 246.78, 262.19, and 299.51 µM, respectively, V_{max} were 12.12, 24.10, and 10.71 nmol/min/mg protein, respectively. The apparent choline uptake for membrane vesicles revealed K_m values of non stress (0.5 M NaCl) and salt stress condition (2.0 M NaCl) to be 82.00 and 88.11 µM, respectively and V_{max} were 4.55 and 1.82 nmol/min/mg protein, respectively. Glycine betaine and glycine betaine aldehyded caused complete inhibition of choline uptake at salt stress condition.

Intact cells and membrane vesicles of the halotolerant cyanobacterium *A. halophytica* were investigated for energetics of choline transport. Both intact cells and membrane vesicles showed almost similar results. Choline uptake was completely inhibited by carbonyl cyanide m-chlorophenylhydrazone and 2, 4 –dinitrophenol suggesting that choline uptake was driven by proton motive force. An artificially-generated pH gradient across the membrane (ΔpH) caused an increase of choline uptake. In contrast, the suppression of ΔpH resulted in a decrease of choline uptake. The increase of external pH also resulted in an enhancement of choline uptake. The generation of the electrical potential across the membrane (Δψ) resulted in no elevation of the rate of choline uptake. On the other hand, the valinomycin-mediated dissipation of Δψ caused no depression of the rate of choline uptake. Thus, it is unlikely that Δψ participated in the energization of the uptake of choline. Choline transport was completely inhibited by nigericin (dissipate proton gradient) suggesting that choline uptake depended on proton gradient. However, Na^+-gradient across the membrane was suggested to play a role in choline uptake since monensin and amiloride, which collapses Na^+-gradient strongly inhibited choline uptake. To study the energetics for nitrate transport of *A. halophytica* showed similar results to choline transport into cells.

Since initial search for choline transporter gene in *A. Halophytica* failed to identify the gene, we then resorted to clone and characteriz a betaine transporter gene (Ap-*betT*) and found that it exhibited high homology to betaine transporters from *Bacillus subtilis* OpuD, but low homologies to betaine/proline transporters from betaine accumulating plants. After heterologous expression of Ap-*betT* in *E. coli* mutant strain MKH13, which lacks choline, betaine, and proline transport systems, only betaine uptake was restored. Sodium, but not potassium markedly enhanced betaine uptake rates, suggesting that ApBetT is a Na^+-betaine symporter. Betaine uptake activities of Ap-BetT were high at alkaline pH with the optimum pH around 9.0. The *Synechococcus* cells overexpressing ApBetT exhibited betaine uptake and enhanced salt tolerance to the extent that the freshwater cyanobacterium could grow in sea water when betaine was included in the growth medium. Kinetic properties of betaine uptake in the *Synechococcus* cells overexpressing ApBet were similar to those in *Aphanothece halophytica* cells. These findings indicate that *A. halophytica* contains a Na^+-betaine symporter that contributes to the salt stress tolerance at alkaline pH.

Field of study.....Biotechnology.....

Academic year.....2005.....

Student's signature.....

Advisor's signature .....

Co-advisor's signature.....

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LIST OF ABBREVIATIONS

A	Absorbance
*apbet*T	*A. halophytica* betaine transporter gene
ApBetT	*A. halophytica* betaine transporter
AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
BT	Glycine betaine
BTA	Glycine betaine aldehyde
CCCP	Carbonyl cyanidetrichloromethoxyphenylhydrazone
Chl	Chlorophyll
Cho	Choline
Ci	Curie
cm	Centimeter
CO_2	Carbondioxide
CP	Cytoplasmic fraction
CPM	Count per minute
DCCD	*N, N*-dicyclohexylcarbodiimide
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
ΔpH	pH gradient or proton gradient
$\Delta\psi$	Membrane potential

ΔNa	Sodium gradient
DNP	2,4 dinitrophenol
EDTA	Ethylenediamine tetraacetic acid
EMB	Ethanolamine buffer
g	Gram
Hepes	N-2-hydroxyethylpiperazine-N-ethanesulfonic
HPLC	High performance liquid chromatography
^{o}C	Degree celsius
kb	Kilo base
KCN	Potassium cyanide
KCl	Potassium chloride
KSCN	Potassium thiocyanate
KF	Potassium fluoride
kDa	Kilo Dalton
KOAc	Potassium acetate buffer
KPB	Potassium phosphate buffer
hr	Hour
l	Litre
mA	Milliampere
min	Minute
ul	Microliter
ml	Milliliter
mg	Milligram
Mg_2SO_4	Magnesium sulfate
mM	Millimolar

MW	Molecular weight marker
µM	Micromolar
M	Molar
MB	Membrane fraction
NaN_3	Sodium azide
NaCl	Sodium chloride
NEM	N-ethylmaleimide
nm	Nanometer
nM	Nanomolar
OD	Optical density
p-Cl-benzoate	p-chloromercurobenzoate
PAGE	Polyacrlamide gel electrophoresis
PCR	Polymerase chain reaction
PMF	Proton motive force
PMS	Phenanzine methanosulfate
PP	Periplasmic fraction
rpm	Revolution per minute
SDS	Sodium dodecyl sulfate
SPR	Surface plasmon resonance
TCA	Trichloroacetic acid
TM	Transmembrane
TLC	Thin layer chromatography
TB	Tris-Cl buffer
TMB	Tris-maleate buffer
UV	Ultraviolet

CHAPTER I

INTRODUCTION

1.1 General introduction

The salinity is one of the physical parameters to determine the ability of organisms to survive in their environment. High osmolarity in hypersaline condition can be deleterious to cells since water is lost to the external medium until osmotic equilibrium is achieved. At high salinity, the bacteria, plant and animal adapt to live in there habitat by using strategy of accumulating a broad spectrum of osmotically active solutes, including ions or osmoprotectants. The increase of osmoprotectants is achieved either by increasing of biosynthesis and/or decreasing of degradation or by transport substances into or out of the cell.

Although the ability to adapt to fluctuations in the external osmolarity is fundamental to the survival of organisms, the mechanisms responsible for osmotic adaptation have been elucidated only relatively recently. There are remarkable similarities between bacteria and plants because organisms from both kingdoms accumulate the same set of cytoplasm solutes upon exposure to conditions of hyperosmolarity. Thus, it is likely that there will be close parallels in the mechanisms that theses organisms employ to regulate response to osmotic stress (Conka and Hanson, 1991).

The type of bacteria can classified as halotolerant or extreme, moderate and slight halophilic bacteria depending on optimal salt concentration of medium for growth rate, the bacteria were classified as non halophiles (grow below 0.2 M NaCl), slightly halophiles (grow at 0.2 to about 1.0 – 1.2 M NaCl), moderately halophiles (grow at 1.0 –

1.2 to 2.0 – 2.5 M NaCl), and extremely halophiles (grow at 2.0 – 2.5 M NaCl or more). A halophilic bacterium is demonstrated by the requirement of a high salt concentration for optimum growth; however halotolerance, qualitatively and quantitatively, describes the ability to grow at a salt concentration higher that optimum.

However, the degree of tolerance of bacteria depends on the composition of the growth medium. The halophilic eubacteria are usually found in numerous saline habitats with different salinities, e.g. hypersaline soils, inland marshes, bottom sediments or other different habitats. They are present in non-saline habitats, in the same manner that non-halophilic bacteria are present in hypersaline environments (Zahran, 1997).

Living cells adapt to water stress by two basic schemes. The first strategy is accumulation of very high intracellular concentration of ions such as Na^+ and K^+ (Ventosa, *et al.*, 1998). Since biological membranes are permeable to water, cells cannot maintain the water activity of their cytoplasm higher than that of the surrounding brine, because this would lead to a rapid loss of water to the environment. Therefore, any microorganism living at high salt concentrations may be expected to keep its cytoplasm at least isoosmotic with the extracellular environment. Build up of a turgor pressure requires a hyperosmotic cytoplasm (Oren, 1999).

Osmotic balance in microbes can be maintained by accumulation of high cytoplasmic concentrations of KCl. As a consequence, the intracellular enzymes and macromolecules must be adapted to high ionic strength. In fact, intracellular enzymes of the microbes using the KCl-strategy are not only fully active at high KCl concentrations, but also require monovalent cations (preferably K^+) for stability. It has been shown that bacteria using this strategy have intracellular proteins with higher proportion of acidic amino acids and lower proportion of nonpolar residues than proteins usually (Lanyi, 1974). The KCl-strategy is typical for halophilic, anaerobic, heterotrophic eubacteria

(*Haloanaerobiales*) (Oren, 1986; Rengpipat *et al.*, 1988; Oren *et al.*, 1997) and extremely halophilic archaebacteria (*Halobacteriales*) (Lanyi, 1974), with the exception of extremely halophilic methanogens. The anaerobic heterotrophic halophiles also accumulate significant amounts of NaCl. Extremely large amounts of KCl can be accumulated by halophilic archaebacteria. Intracellular concentrations of several metal K+ have been reported for some *Halobacterium* strains (Lanyi, 1974).

The second strategy is accumulation of organic solutes to control their internal water activity, maintain the appropriate cell volume and turgor pressure, and protect intracellular macromolecules (Le Rudulier, *et al*., 1984; Galinski, 1994). The compatible solutes, also called osmolytes, include sugars, amino acids and their derivatives, polyols and their derivatives, betaine, and ectoines (Wood *et. al*., 2001). The compatible solutes in general, are low molecular weight organic compounds that accumulate to high intracellular levels under osmotic stress and are compatible with the metabolism of the cell. For example, glycine betaine is compatible solute in halotolerant bacteria, archaea, and cyanobacteria. It is not synthesized by most microorganisms but is taken up from the medium and used for osmoadaptation (Csonka and Hanson, 1991; Booth and Louise, 1999; Wood, 1999).

Exposure of cells to high external osmolarity results in an efflux of water from the interior. The decrease in the internal water content brings about the reduction in the turgor pressure and shrinkage of the cytoplasmic volume. As a consequence of the decrease in the cytoplasmic volume, the concentrations of all the intracellular metabolites increase and thus cause a reduction in the intracellular water activity. In the absence of active osmotic adjustment by the cell, the cytoplasmic volume would shrink until the water activity of interior equaled that of the exterior. Because an elevation in the concentrations of various intracellular molecules may be inhibitory to cellular processes (e.g. inhibitors

of specific enzymes may reach deleterious concentrations or the increase in the concentrations of ions may become toxic) passive alteration of the cell volume is not adequate for adaptation to changes in the osmolarity of the environment. Instead of a passive volume regulation, organisms generally respond to osmotic stress by increasing the concentrations of a limited number of solutes. Thus the water activity of the cell interior can be reduced and consequently cell volume and turgor can be restored near their pre-stress values without an across the board increase in the concentrations of all cytoplasmic component.

A variety of bacteria and archaea have been examined for compatible solute production by means of high performance liquid chromatography and nuclear magnetic resonance methods. The spectrums of compatible solutes used by microorganisms comprise only a limited number of compounds: sugar (e.g. trehalose and sucrose), polyols (e.g. glycerol and glucosylglycerol), free amino acids (e.g. proline and glutamate), and derivatives thereof (e.g. proline betaine, carnitine and dimethylsulfoniopropinate), sulfate ester (e.g. choline-o-sulfate), and *N*-acetylglutaminylglutamine amide).

1.2 Compatible solute

In general, compatible solutes (Figure 1) are highly solute molecules and do not carry net charge at physiological pH. In contrast to in organic salts, they can reach high concentrations without disturbing vital cellular functions such as DNA replication, DNA-protein interaction and the cellular metabolic machinery (Strom and Kaasen, 1993). Many microorganisms accumulate compatible solutes as metabolically inert stress compounds, and in those instances where these substances can be metabolized, sensitively balanced regulatory mechanisms ensure their high level accumulation under hypertonic conditions. A spectrum of compatible solutes is usually used by given microorganism for

osmoregulatory purposes, and the composition of the solutes pool can vary in response to growth phase and growth medium (Galinski and Truper, 1982).

The types of organic molecules used for osmotic balance include polyols and derivatives, sugars and derivatives, amino acids and derivatives, betaines, and ectoines and occasionally peptides suitably altered to remove charges (Galinski, 1994). As a general rule of thumb, bacteria and eukaryotes usually accumulate neutral compatible solutes whereas archaea prefer negatively charged solutes (Martin *et al.*, 1999; Robert, 2004). Interestingly, archaea tend to modify many of the same neutral or zwitterionic solutes accumulated by eukaryotes or bacteria to make them negatively charged. Osmolytes can either be synthesized by the cell or transported into the cell from the medium. A key feature of these molecules is that they do not inhibit overall cellular functions, although they may modulate individual enzyme activities. This behavior led to labeling them as “compatible solutes” (Brown, 1976). Their accumulation helps to maintain turgor pressure, cell volume, and concentration of electrolytes all important elements for cell proliferation. It is thought that initial events that trigger osmolyte accumulation could include ion channels or other transmembrane proteins sensing differences in external and internal salt concentration, cell volume changes, and/or turgor pressure changes. However, except for transporters, how these physical changes are translated to increased osmolyte synthesis is not known. Organic osmolytes fall into three general chemical categories: (i) zwitterionic solutes, (ii) noncharged solutes, and (iii) anionic solutes. Structures of these molecules and their occurrence in halotolerant and halophilic microorganisms are presented in Figure 1. Intertwined with these organic solutes are K^+ and Na^+ which also contribute to osmotic balance in cells.

The accumulation of compatible solutes not only allows the cells to withstand a given osmolarity but also expands the ability of microorganisms to colonies ecological

niches that are otherwise strongly inhibitory for their proliferation. Depending on the type, compatible solutes can also protect microorganisms against stresses other than dehydration. An example is the increased cold tolerance conferred by the accumulation of glycine betaine in the food-borne pathogen *Listeria monocytogenes* (Ko *et al.,* 1994).

Compatible solutes serve a dual function in osmoregulating cell. Because microorganisms frequently accumulate them up to molar concentrations, compatible solutes lower the cytosolic osmotic potential and hence make major contributions to the restoration and maintenance of turgor. The free cytoplasmic volume (unbound water, in contrast to water bound by macromolecules) is a key determinant for cell growth (Cayley *et al.*, 1992). Compatible solutes such as glycine betaine and proline increase the cytoplasmic volume and free water content of the cell at high osmolarity, and their accumulation thus permits continued cell proliferation under unfavorable conditions. Compatible solutes also serve as stabilizers of proteins of high ionic strength. This protective property is no fully understood, but according to the preferential exclusion model, osmoprotectants are kept away from the intermediate vicinity of proteins, resulting in a preferential hydration of protein surface. This solvent distribution leads to a situation in which the disruption of water structure in the hydration shell of proteins by local or global unfolding of the polypeptide chain is energetically unfavorable, and hence the native conformations of proteins are stabilized.

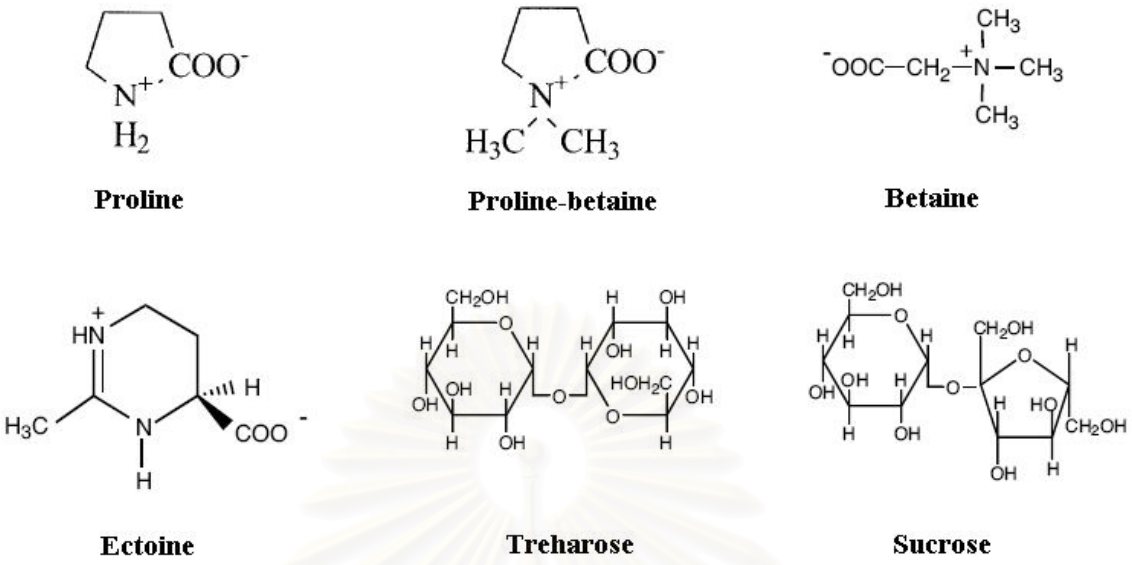


Figure 1 Organic compatible solutes were found in living organisms.

In the most case, microorganisms regulate their osmotic solute content separately from their biosynthetic and energy generating processes. Thus, *Rhizobium* (a bacterium with a relatively low salt tolerance) cans synthesis glycine betaine from choline or takes up glycine betaine from the medium. While glycine betaine can serve as both a nutrient and an osmoprotector, its degradation is repressed at high salt concentration, when it serves as an osmoticum only (Fougere and Le Rudulier, 1990). *Halorhodospira* cells use glycine betaine only as osmotic solute. The compound cannot serve as a nitrogen source, even under the most stringent nitrogen depletion. The physiological basis of this one way only biosynthetic pathway is not understood, but it emphasizes the importance of betaine not only as an inert solute but also as a cytoplasmic protectant. However, nitrogen starvation induces the breakdown of ectoine, mobilizing two nitrogen atoms per molecule; trehalose then replaces ectoine as the osmotic solute (Galinski and Herzog, 1990). The advantage of being able to choose between different osmotic solutes is obvious. Many halophilic and halotolerant microorganisms maintain “cocktails” of osmotic solutes, and the regulation of the synthesis of each of the solutes is optimized according to the needs of the cells (Ventosa *et al*., 1998; Galinski, 1994).

1.3 The role of glycine betaine

Betaine is found in microorganisms, plants, and animals and is a significant component of many foods. Betaine is a zwitterionic quaternary ammonium compound that is also known as trimethylglycine or glycine betaine. It is a methyl derivative of the amino acid glycine with a formula of $(CH_3)_3N\text{-}CH_2COOH$ and a molecular weight of 117.2, and it has been characterized as a methylamine because of its 3 chemically reactive methyl groups. The physiologic function of betaine is either as an organic osmolyte to protect cells under stress or as a catabolic source of methyl groups via transmethylation

for use in many biochemical pathways. The principle role for betaine in plants and microorganisms is to protect cells against osmotic inactivation. Exposure to drought, high salinity, or temperature stress triggers betaine synthesis in mitochondria, which results in its accumulation in the cells. Betaine is a compatible osmolyte that increases the water retention of cells, replaces inorganic salts, and protects intracellular enzymes against osmotically induced or temperature induced inactivation. For example, spinach is grown in saline soil, and betaine can accumulate in amounts of up to 3% of fresh weight. This enables the chloroplasts to photosynthesize in the presence of high salinity.

A very important quarternary ammonium compound commonly used in cellular osmoregulation is glycine betaine (*N*, *N*, *N*-trimethylglycine), a derivative of amino acid glycine (Figure 2), is evidence in a number of diverse microbial systems, including enteric bacteria (Andersen *et al.*, 1988), soil bacteria (Smith *et al.*, 1988), halophilic bacteria (Galinski and Truper, 1982), cyanobacteria (Mackey *et al.*, 1984) and metanogenic archaea bacteria (Robertson *et al.*, 1990). In higher plant, some plants in families *Chenopodaceae*, *Amaranthaceae* and *Gramineae*, for example, accumulate glycine betaine in response to salt stress or drought (Brouquisse *et al.*, 1989; Ishitani *et al.*, 1995). Hanson *et al* (1985) reported that glycine betaine synthesis occurs in chloroplasts of spinach leaves. Subsequently, Robinson and Jones (1986) reported the accumulation of glycine betaine up to 0.3 M in spinach chloroplast provide osmotic adjustment during salt stress.

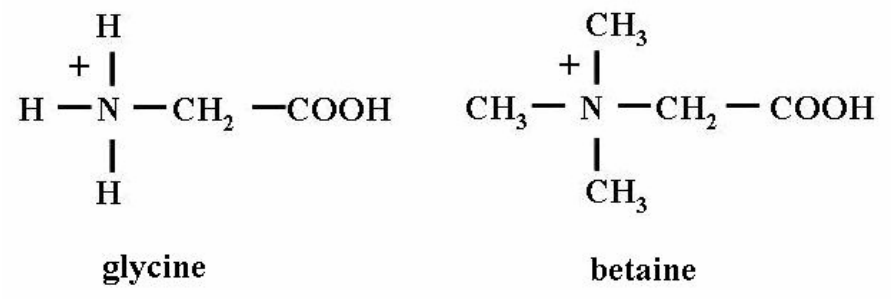


Figure 2 Structure of glycine and betaine.

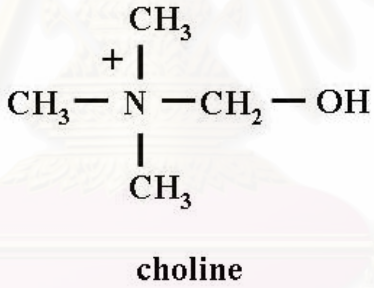


Figure 3 Structure of choline.

Sakaguchi (1960) first reported that exogenous glycine betaine can stimulate the respiration rate of a halophilic bacterium, Ba-1, in media of elevated NaCl concentration. Subsequent, Le Rudulier and Bouillard (1983) observed that this compound is a potent osmoprotectant for members of family *Enterobacteriaceae*. Perround and Le Rudulier (1985) found that the intracellular concentrations of glycine betaine maintained by *E. coli* were proportional to the osmolarity of the medium. The transport of glycine betaine was observed to be stimulated by osmotic stress in *Lactobacillus acidophilus* (Hutchins *et al.*, 1987), *Listeria monocytogenase* (Ko *et al.*, 1994), *Rhodobacter sphaeriodes* (Abee *et al.*, 1990) and *Staphylococcus aureus* (Pourkomailian and Booth, 1994).

Glycine betaine also accumulates in the halophilic, photosynthetic bacterium *Ectothiorhodospira halochloris* and in several strains of cyanobacteria (Shkedy-Vinker and Avi-Dor, 1975; Reed *et al*., 1984). In cyanobacterium *Synechocystis* PCC 6803, glycine betaine was effective both in the stimulation and in the protection of the oxygen evolving machinery and the synthesis of ATP. In *Aphanothece halophytica*, a halotolerant cyanobacterium, glycine betaine was a major osmoticum accumulating inside the cells in response to changes in external salinity. In this organism, glycine betaine acts as an osmoprotectant (Reed *et al.*, 1984).

High concentrations of salts have been reported to inhibit the activity of many enzymes in both prokaryote and eukaryote. Glycine betaine is also known to protect enzymes from inhibitory effects of high salt concentration. Higher plants are able to compartmentalize the accumulated salts, Na^+ and Cl^- in particular, in the vacuole and thereby prevent the inhibition of enzyme activities. In contrast, the soluble enzymes in prokaryotic organism as well as cytoplasmic enzymes are directly exposed to any osmoregulatory substances (Matoh *et al*., 1987). Incharoensakdi *et al* (1986) reported that glycine betaine masks inhibitory effect of Cl^- on the enzyme activity of ribulose 1, 5-

bisphosphate carboxylase/oxygenase and prevents the enzyme dissociation into constituent subunits in *A. halophytica.* Glycine betaine is also shown to specifically protect glucose-6-phosphate dehydrogenase activity to retain full activity in the presence of high salt in *Spirulina subsalsa.*

Furthermore, glycine betaine protects photosystem II complex from the salt induced dissociation of extrinsic proteins and inactivation of the oxygen evolved in not only osmoregulation but also stabilization of enzymes in the cells grown in high salinities. The function of glycine betaine as an osmolyte has also been reported in the mammalian renal system as well (Bagnasco *et al*., 1986). Besides this physiological role as an glycine betaine also functions in the general metabolism where methyl groups derived from it are incorporated into alkaloids in plants (Byerrum *et al.*, 1956), into methionine in mammals (Skiba *et al*., 1982) and microorganisms, and into cabolamine (Vitamin B_{12}) in microorganisms (White and Demain, 1971). Furthermore, glycine betaine can be used as a carbon and nitrogen source by some microorganisms (Pocard *et al.*, 1997). Methyl groups liberated by glycine betaine catabolism in these microbial systems may be assimilated oxidize via one-carbon via metabolism pathway.

1.4 Function of choline and metabolism

Choline [$(CH_3)_3N\text{-}CH_2CH_2OH$] is a methylated nitrogen compound that is need for synthesis of the phospholipids in the cell membranes, methyl metabolism, cholinergic neurotransmission, transmembrane signaling, lipid cholesterol transport and metabolism (Zeisel and Blusztajn, 1994). Choline can be acethylated, phosphorylated, oxidized or hydrolyzed. There is no doubt that cells absolutely require choline and die by apoptosis when derived of these nutrients (Albright, 1996). The debate as to whether the human diet must contained choline arises because there is a pathway (most active in the liver) for the

de novo biosynthesis of the choline moiety via the sequential methylation of phosphatidylethanolamine using S-adenosylmethionine as the methyl donor.

The ability to form choline moiety de novo means that some of the demand for choline can, in part, be met by using methyl groups derived from one carbon metabolism (via methyl-folate and methionine). Because of this metabolic interrelationship of choline, methionine, folate and vitamine B_6 and B_{12}, investigators need to show that, when the other nutrients are available in amounts sufficient to sustain normal growth and function, de novo synthesis rates are not adequate to meet the demand for choline before the designation of choline as essential is made (Zeisel, 2000). Only small amounts of choline are synthesized in the brain and absorbed from the diet, must be transported into the brain (Murakami *et al*., 2000). Choline is widely distributed in foods; the requirement is generally satisfied by both dietary and endogenous source, although choline deficiency has been reported. The most common signs of choline deficiency are fatty liver and hemorrhages kidney necrosis. Evidence for free radical activity in liver with choline deficiency is reported and this may be related to the carcinogenesis process (Panfili *et al.,* 2000).

In many plant species, notably member of *Gramineae* (barley) and *Chenopodiaceae* (Sugar beet and spinach), choline also serve as a precursor for the synthesis of quaternary ammonium compound, glycine betaine [$(CH_3)_3{}^+NCH_2COO^-$]. More recently, choline has been shown to be the precursor for the synthesis of choline-O-sulfate in salinized *Limonium* (Plumbaginaceae) (Hanson *et al*., 1991). Glycine betaine and choline-O-sulfate are believed to be the compatible cytoplasmic osmolytes whose accumulation plays a role in the adaptation of many plants to conditions of drought or salinity (Wyn Jones *et al.,* 1977). Choline synthesis can be viewed not only as having an essential and ubiquitous role in plant phopholipid metabolism but as playing a

fundamental role in the adaptation of many plants toward osmotic stress (Summer *et al*., 1993).

The fate of choline in aquatic system is of interest because it contains nitrogen (C/N = 5) and is precursor of glycine betaine, one of the most potent osmoprotectants known (Csonka and Hanson, 1991; Le Rulier *et al*., 1984; Yancy *et al.*, 1982). A variety of different bacteria including *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Rhizobium meliloti*, *Rhodobacter sphaeroides* and *Vibrio costicola* oxidatively convert choline to glycine betaine (Abee *et al.*, 1990; Boch *et al.*, 1994; Choquet *et al*., 1991; Lanfald and Storm, 1986; Pocard *et al*., 1989). Choline is oxidized to glycine betaine in a two step process with betaine aldehyde as an intermediate. In *Alcaligenes* app., a soluble choline oxidase can carry out both steps of choline oxidation (Ohta-Fukuyama *et al*., 1980), while in *E. coli* and other bacteria; a membrane bound choline dehydrogenase is primarily responsible for oxidation to aldehyde, which is further oxidized by a soluble betaine aldehyde dehydrogenase (Boch *et al.*, 1994; Choquet *et al*., 1991; Lanfald and Storm, 1986). The overall reaction requires NAD^+ and produces H_2O_2 in addition to glycine betaine.

A supply of exogenous choline, and its subsequent conversion to glycine betaine, has been shown to confer osmotolerance to bacteria when cells are grown at other inhibitory osmolarities (Lanfald and Strom, 1986; Le Rulier *et al*., 1984). On the other hand, choline has no osmoprotectant effects in mutants that are defective in their ability to convert choline to glycine betaine (Styrvold *et al*., 1986), indicating that conversion to glycine betaine is required for choline to be an osmoprotectant. Furthermore, choline uptake and oxidation activities are under osmotic control in a number of bacteria, with enhanced transport at high osmolarities (Boch *et al*., 1994; Choquet *et al.*, 1991; Lanfald and Strom, 1986).

The genetic basis for choline uptake and dehydrogenation has been elucidated for *E. coli* (Lamark *et al*., 1991) and recently for *B. subtilis* (Boch *et al*., 1996). In *E. coli*, a gene cluster comprises the genes encoding a choline transporter (BetT), two dehydrogenases, and an NADH-dependent glycine betaine aldehyde dehydrogenase (BetB), and an $FADH_2$-dependent choline dehydrogenase (BetA), which responsible for conversion of choline to glycine betaine aldehyde. In addition, a regulatory protein, BetI, is encoded by the *E.coli bet* gene cluster; BetI binds to the DNA region between the *betBA* operon and *betT* gene and is responsible for the choline dependent regulation of *bet* transcription (Lamark *et al.*, 1996). In *B. subtilis*, an operon encodes two dehydrogenases, a glycine betaine aldehyde dehydrogenase (GbsA) that shows similarity to glycine betaine aldehyde dehydrogenase found in various other organisms, and a choline oxidase (GbsB) that belongs to a family of alcohol dehydrogenase and thus represents a novel type of choline dehydrogenating enzyme involved in glycine betaine biosynthesis. In contrast to *E. coli*, no genes for a choline transporter or a regulatory protein have been identified in the *gsb* locus (Rosenstein *et al*., 1999).

1.5 The glycine betaine pathway

Glycine-betaine (betaine) is a trimethylamine produced by two different general pathways for synthesizing betaine. The oxidative pathway can react with a one enzyme to change choline to betaine (choline oxidase in Gram-positive soil bacteria; *Actinopolyspora halophila*) or require two soluble enzymes to oxidize choline to betaine-aldehyde as intermediate (choline monooxygenase and betaine-aldehyde dehydrogenase in higher plants and choline dehydrogenase and betaine-aldehyde dehydrogenase in marine invertebrates and bacteria including *E. coli*).

This ubiquitous solute, glycine with the primary amine methylated to form a quaternary amine, is found in halophilic bacteria. However, the organism does have a gene that codes for the oxidation of choline to betaine. Several microorganisms can also generate betaine by successively methylating glycine. GSMT (glycine sarcosine methyltransferase) and SDMT (sarcosine dimethylglycine methyltransferase) in *Halorhodospira halochloris* and *A. halophila* transfer the methyl group of S-adenosylmethionine to two different types of amines. Betaine synthesis from glycine in a halotolerant photosynthetic organism *A. halophytica* was also carried out by GSMT and DNMT activities. Only one methanogen, *Methanohalophilus portucalensis,* has been shown to synthesize betaine de novo. The last result suggests that betaine accumulation is likely regulated by the internal K^+ concentration in these cells. In most cells where it is accumulated as an osmolyte, the betaine is actively transported from complex medium. In contrast to the large number of bacteria that transport betaine into the cell for use as an osmolyte.

Despite the widespread occurrence of glycine betaine, the osmotic regulation of it well understood only in several microbial systems and certain plant. Glycine betaine biosynthesis was started to oxidize of choline via two-step reaction with betaine aldehyde as the intermediate (Figure 4). This series of reactions may be catalyzed by three-enzymatic system. In microorganisms and mammals a membrane bound choline dehydrogenase (EC 1.1.99.1) is employed in conjugation with a soluble betaine aldehyde dehydrogenase (EC 1.2.1.8) (Haubrich and Gerber, 1981; Lanfals and Strom, 1986; Nagasawa *et al*., 1976; Rothschild and Barron, 1954; Lin and Wu, 1986). Plants utilize a soluble choline monooxygenase in combination with betaine aldehyde dehydrogenase (Brouquisse *et al*., 1989).

The glycine betaine synthesis has been studied in several bacteria, higher plants and marine animals (Lanfald and Strom, 1986; Reed *et al*., 1984). In most organisms, glycine betaine was formed by the oxidation of choline to betaine aldehyde, which is then oxidized to glycine betaine (Figure 4). Different enzymes such as FAD catalyzes the first step (choline to betaine aldehyde) linked choline dehydrogenase (EC 1.1.99.9) in inner rat liver mitochondria membrane (Zhang *et al*., 1992). Choline oxidase (EC 1.1.3.17) is a new type of flavoprotein enzyme and was found in *Athrobacter globiformis* (Ikuta *et al*., 1977). In all organisms studied, the final step in the synthesis of betaine from choline (betaine to glycine betaine) is catalyzed by a NAD^+-dependent betaine aldehyde dehydrogenase (EC 1.2.1.8).

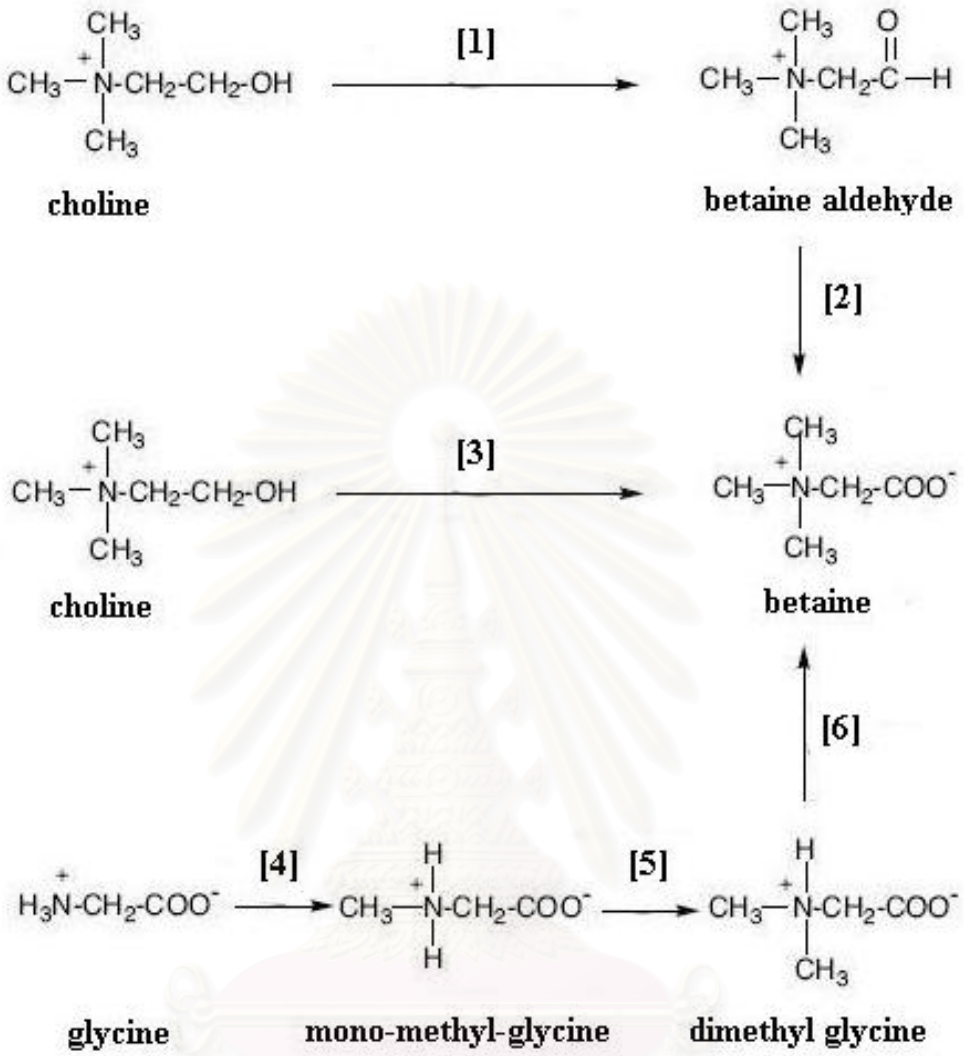


Figure 4 glycine betaine pathways (1 = choline dehydrogenase (bacteria) or choline monooxygenase (plants), 2 = glycine-betaine aldehyde dehydrogenase, 3 = choline oxidase, 4 = glycine-sarcosine methyl transferase, 5, 6 = dimethyl glycine methyl transferase)

Choline dehydrogenase was strongly induced and betaine aldehyde dehydrogenase less strongly induced by choline. The formation of these enzymes was also regulated by NaCl concentration of growth medium, increasing with increasing NaCl concentrations. Intracellular glycine betaine concentration also increased with increasing choline and NaCl concentration in the medium. This increase was almost completely blocked by chloramphenicol, which does not block the increase in salt tolerant active transport on transfer from a low to high salt concentration (Choquet *et al*., 1991; Lanfald and Strom, 1986). The oxidation of choline to betaine aldehyde in rat liver mitochondrial preparation required no addition cofactors, suggesting that mitochondria contained an endogenous electron acceptor. Oxygen might have been the ultimate electron acceptor because cyanide (an inhibitor of cytochrome oxidase) totally inhibited the formation of [methyl-^{14}C] betaine aldehyde from [methyl-^{14}C] choline. This inhibition could, however, be overcome by the addition of phenazine methosulfate (PMS). This would be excepted if the electrons from choline dehydrogenase were shunted to PMS.

1.6 Transport of compatible solutes

In addition to accumulating compatible solutes by endogenous synthesis, a large number of bacteria and archaea have developed the ability to acquire performed osmoprotectants from exogenous sources. These compounds are released into ecosystems by primary microbial producers from osmotically downshocked cells; by decaying microbial, plant and animal cells; by root exudes and by mammals in their excretion fluids (Galinski, 1994; Ventosa *et al.,* 1998). Transporters for osmoprotectants (Table 1) developed to meet the special demands imposed by their biosynthetic precursors are varying and generally very low, with concentrations usually in the nanomolar or micromolar range (Kiene *et al.*, 1998). Therefore, osmoprotectant transporters commonly

exhibit very high affinity for their major substrates, and their capacity is geared to permit accumulation of compatible solutes to molar concentration. In addition, they function effectively at high osmolarity and at high ionic strength, condition that frequently impair the performance of transporters for nutrients. To take advantage of the spectrum of osmoprotectants available in their habitat, microorganisms often posses several transport system, some of which exhibit broad substrate specificity. Transporters for osmoprotectants have been most fully investigated at the molecular level in the gram-negative enteric bacteria *E. coli* and in the gram-positive soil bacteria *B. subtilis*.

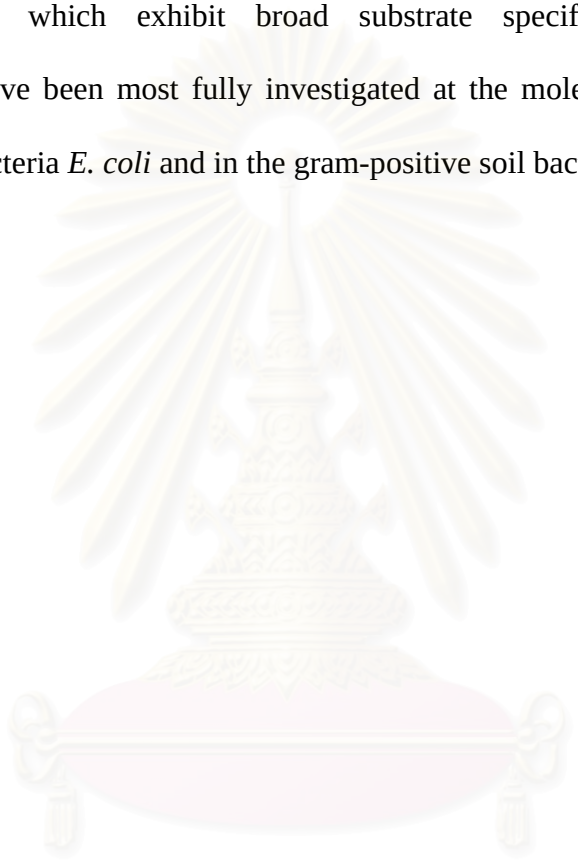


Table 1 Properties of osmoprotectant transport systems (Kempf and Bremer, 1998)

Organism	System	Type of transporter[a]	Components	Osmotic stimulation of osmoprotectant transport by		Substrates
				Gene expression	Transport activity	
Escherichia coli	ProP	Secondary transporter	ProP	+	+	GB, PB, Pro, Car, Ect, PIP, DMP, DMG, DMSP, HB, BB
Escherichia coli	ProU	ABC transporter	ProV, ProW, ProX	+	+	GB, PB, Pro, Car, DMP, HB, BB, Cho
Escherichia coli	BetT	Secondary transporter	BetT	+	+	Cho
Corynebacterium glutamicum	BetP	Secondary transporter	BetP		+	GB
Bacillus subtilis	OpuA	ABC transporter	OpuAA, OpuAB, OpuAC	+	nd	GB, PB, DMSA, DMSP
Bacillus subtilis	OpuB	ABC transporter	OpuBA, OpuBB, OpuBC, OpuBD	+	nd	Cho
Bacillus subtilis	OpuC	ABC transporter	OpuCA, OpuCB, OpuCC, OpuCD	+	nd	GB, PB, Car, DMSA, DMSP, Cho, BB, CB, Ect, Choline-*O*-sulfate
Bacillus subtilis	OpuD	Secondary transporter	OpuD	+	+	GB, DMSA, DMSP
Bacillus subtilis	OpuE	Secondary transporter	OpuE	+	–	Pro
Erwinia chrysanthemi	OusA	Secondary transporter	OusA	+	–	GB, Pro, Ect, PIP

[a] ABC transporter, ATP binding cassette transporter, (*BB* g-butyrobetaine, *Car* carnitine, *CB* crotonobetaine, *Cho* choline, *GB* glycine betaine, *DMG* dimethylglycine, *DMSA* dimethylsulfonioacetate, *DMSP* dimethylsulfoniopropionate, *Ect* ectoine, *HB* homobetaine, *PB* proline betaine, *PIP* L-pipecolate, and *nd* not determined)

The challenge posed by changing environmental osmolarity is vividly illustrated by the common habitat of *B. subtilis*; the initial response of this bacterium to sudden increases in the external osmolarity is the uptake of large quantities of K^+ to counteract the immediate outflow of water from the cell. Two K^+ transport systems (KtrAB; KtrCD) were identified that mediate rapid K^+ uptake subsequent to an osmotic up-shock (Figure 5). There is no K^+ uptake systems showed that cells can not growth when they face a sudden hyperosmotic medium. Hence, K^+ uptake is required for both the initial as well as for the sustained stress response of *B. subtilis* to hyperosmotic environments.

However, because prolonged high intracellular concentration of K^+ is detrimental to many physiological functions for example protein synthesis, *B. subtilis* replaces in the second step of its osmoadaptation process part of the accumulated K^+ with the compatible solute proline through de novo synthesis. Through this stress responsive synthesis pathway, *B. subtilis* is able to produce very large quantities of proline and can thereby partially offset the detrimental consequences of high salinity and osmolarity. *Bacillus* species can synthesize the tetrahydropyrimidine ectoine instead of proline as their dominant compatible solute. In addition to the de novo synthesis of proline, *B. subtilis* can also use exogenously provided proline as an osmoprotectant. The high-affinity transport system was identified, OpuE (Figure 5) (Opu denotes osmoprotectant uptake) that is responsible for the acquisition of proline for osmoprotective purposes. *B. subtilis* can not only use exogenously provided proline for osmoprotection but also as an effective source for carbon and nitrogen. When proline is acquired for nutritional purposes, it is taken up through the PutP transporter and is then degraded to glutamate via the PutB and PutC enzymes (Figure 5). In addition to the synthesis of proline for osmoprotection, *B. subtilis* can acquire a broad spectrum of preformed compatible solutes from the environment through transport processes.

These osmoprotectants are synthesized by plants and are thus brought into the habitats of *B. subtilis* by root exudates and decaying plant tissues. Their availability is likely to vary considerable in the soil and other habitats of *B. subtilis*, and consequently, effective mechanisms are required for the acquisition via transport from the environment. We found that the uptake of osmoprotectants from exogenous sources involve fife osmoregulated and high-affinity transport systems (Figure 5). Three of these (OpuA, OpuB, OpuC) are multi-component transporters and are members of the ATP-binding cassette (ABC) superfamily and two (OpuD, OpuE) are single-component transporters. Each member of the Opu-family of transporters is osmoregulated at the level of transcription. They differ however in their kinetic properties and substrate specificity (Figure 5).

With the exception of proline, the compatible solutes used by *B. subtilis* (Figure 5) are all metabolically inert and they can not be synthesized de novo by this soil bacterium. However, the potent osmoprotectant glycine betaine can be produced by *B. subtilis* provided that the precursor choline is present in the growth medium. Choline uptake is mediated by two evolutionarily closely related ABC transporters: OpuC, a transporter with broad substrate specificity, and the highly substrate-specific transport system OpuB (Figure 5). The choline taken up by the cells is oxidized by the GbsA and GbsB (Gbs denotes glycine betaine synthesis) enzymes to the metabolically inert compatible solute glycine betaine (Figure 5). Both the *opuB* and *gbsAB* operons are up-regulated in response to the presence of choline in the growth medium and this genetic control of *opuB* and *gbsAB* transcription is mediated through the repressor protein GbsR.

The very same compounds that protect *B. subtilis* from the detrimental effects of high osmolarity become a threat to its survival when the cells are exposed to hypoosmotic conditions since this will trigger a rapid influx of water into the cell. The cell must

therefore rid itself rapidly of ions and organic osmolytes to curb the increase in turgor. *B. subtilis* accomplishes this via mechanosensitive channels exhibiting different levels of conductances. We identified two such channels: MscL and MscS (Figure 5). *B. subtilis* mutants lacking the MscL and MscS proteins can not survive severe osmotic down-shocks. The uptake and synthesis of osmoprotectants confers a considerable degree of osmotolerance to *B. subtilis.* The pathways for the production and transport of compatible solutes will serve in future studies as useful model systems to unravel the mechanisms of osmosensing in this soil bacterium and to further study the cellular adaptation response of *B. subtilis* to a changing environment.

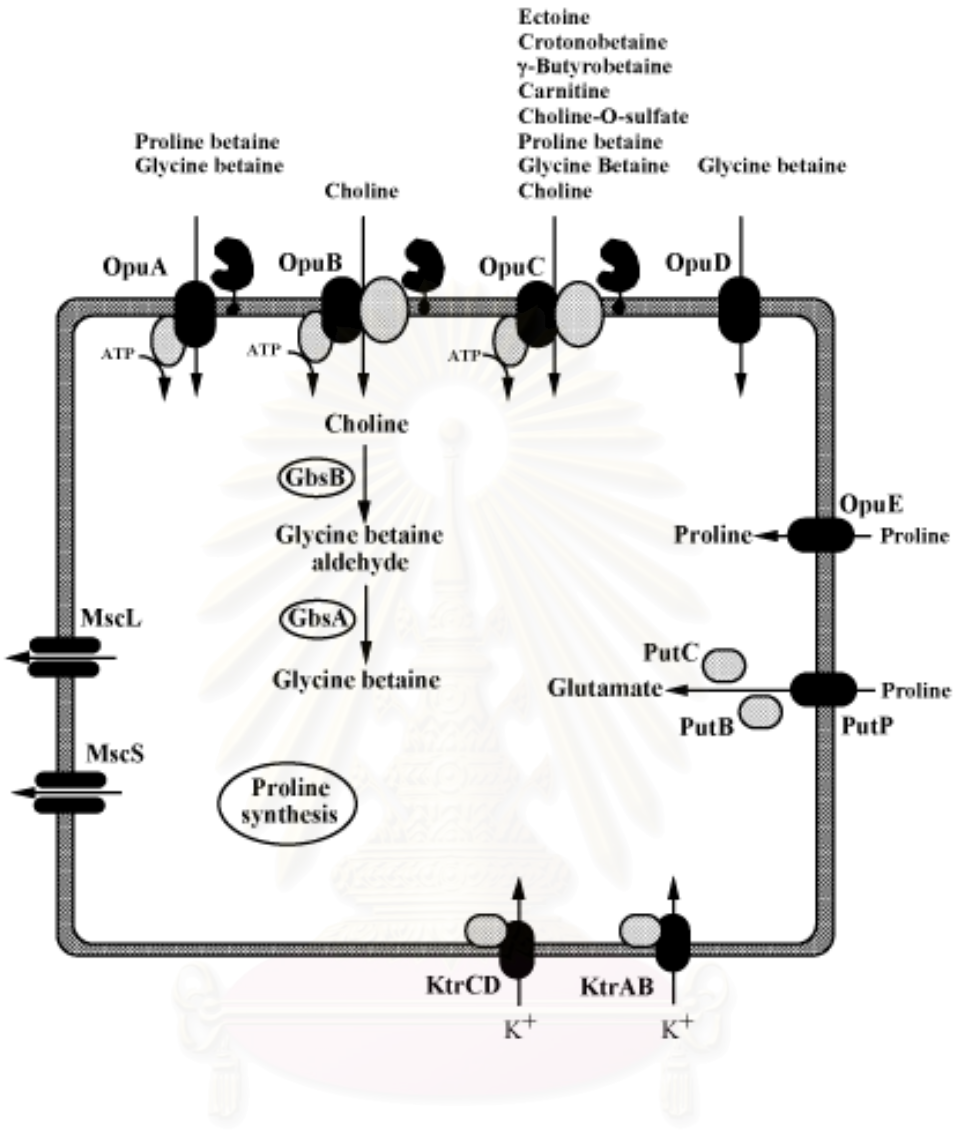


Figure 5 The osmostress response of *B. subtilis* (Kempf and Bremer, 1998)

1.7 Accumulation of glycine betaine in cyanobacterium

Cyanobacteria, the oxygenic photosynthetic prokaryotes from which plastids of photosynthetic eukaryotes (algae and plants) derive, inhabit a variety of environments including those in which extreme conditions such as high temperatures and/or high salinity are found. Cyanobacteria that are able to grow in high salt concentration environment maintain their cell turgor by accumulation of potassium ions and by synthesis and accumulation of low molecular weight organic osmoprotectant (Nomura *et al.*, 1995). A close correlation between the major solute accumulated and the range of salt tolerance has been shown by analyzing osmoprotective compounds synthesized in about 130 strains (Mackay *et al*., 1994; Reed *et al.*, 1984). Three salt tolerance groups have been established. Strains with low tolerance (max 0.7 M) synthesize sucrose or trehalose, strain with moderate salt tolerance (max 1.8 M) synthesize glucosylglycerol and strains with high salt tolerance synthesize betaine and glutamate betaine (Reed and Stewart, 1988). Osmoregulation involves the accumulation of both organic and inorganic solutes. However, a key factor in the adjustment of many cyanobacteria to hypersaline environment is the increase in the cellular level of a species-specific low molecular weight organic solute. The role of low molecular weight carbohydrates in the maintenance of osmotic balance has been shown for several cyanobacteria as well as in many higher plants. There have been few studies on uptake of osmoprotective compounds by cyanobacteria, as is the case for other transport mechanisms of these photoautotropic organisms. An active transport system for glycine betaine has been found in cyanobacteria able to synthesize this compound (Moore *et al.*, 1987).

Glycine betaine was first shown to be the major osmoticum in a halotolerant cyanobacterium *Synechcytis* DUN52 (Mohammad *et al.*, 1983). The unicellular, *A. halophytica* is a highly halotolerant organism that can grow at high external NaCl

concentration up to 3 M (Garlick *et al*., 1997; Reed *et al*., 1984). It was demonstrated that glycine betaine is accumulated as the major osmoticum inside *A. halophytica* cells in response to changes in external salinity (Reed *et al*., 1984). High concentration of salts has been reported to inhibit the activity of many enzymes of both eukaryotic and prokaryotic origin. It has also previously been reported that salt inhibits enzyme activity of RuBisCo from *A. halophytica,* glycine betaine protects the enzyme against salt inhibition (Incharoensakdi *et al*., 1986) and is not harmful to the metabolic activities of the cell even at high concentration.

1.8 The substrate-binding protein

The binding proteins are the most thoroughly analyzed of these transport components for obvious reason. Bacterial active transport systems can broadly be into two classes, those which require the function of specific substrate-binding proteins and those which consist solely of membrane-bound components. These two classes of transport system were originally distinguished by the sensitivity of binding protein-dependent system to cold osmotic shock and by the nature of energy coupling to transport.

Periplasmic, binding protein-dependent transport systems each require the function of several cytoplasmic membrane proteins. In gram-negative bacteria, a number of substrates (such as maltose and maltodextrins, histidine, oligopeptides, phosphates, etc.) are concentrated into the cell by high affinity transport systems which include several proteins, and a common organization. A central component of these systems is a binding protein (e.g. MalE for maltose and maltodextrins in *E. coli*, HisP for histidine in *Salmonella typhimurium*, OppA for oligopeptides in *S. typhimurium*) which is located in the periplasm between the outer and inner membranes. These water soluble “periplasmic binding proteins” presents high affinities for their specific substrates. Their concentration

in the periplasm can reach very high value so that they increase the availability of substrates in the vicinity of the inner membrane. Upon specific interactions of the liganded periplasmic binding proteins with a complex of inner membrane proteins, the substrate is transferred to the complex and further reported into the cytoplasm (Gilson *et al.*, 1988).

One of the major stress proteins accumulated in salt-adapted cells of the cyanobacterium *Synechosistis* sp. PCC 6803 has been identified as flavodoxin (Fluda and Hagemann, 1995). These previous investigations on salt stress proteins have been performed on total cell extracts. However, the cyanobacterial cell is composed of different compartments: periplasm, cytoplasm and lumen of thylakoids. The periplasm is particularly responsive to changes in salinity. The periplasmic of gram-negative bacteria contains proteins of several functions, such as for electron transport, substrate-binding, transport, degradation, cell wall biosynthesis and detoxification. Some of these proteins may be anchored to the cytoplasmic membrane or to the outer membrane; most are believed to be water-soluble and can be osmotic shock (Neu and Happel, 1965). Among the periplasmic proteins that are members of the osmotic stimulation in *E. coli*, the glycine betaine-binding protein of the ProU transport system (OsmY) and a periplasmic trehalose (TreA) have been identified (Gutierrez *et al.*, 1989; Yim and Villarejo, 1992).

1.9 Review of our group's work

Cyanobacteria are photoautotrophic organisms that perform oxygen-evolving photosynthesis typical of higher plant systems using carbon dioxide as carbon source. All cyanobacteria are capable of utilizing nitrate as their sole nitrogen source. However, cyanobacteria usually utilize reduced nitrogen sources such as ammonia and urea in preference to nitrate (Flores and Herrero, 1994). The active transport of nitrate is thought

to be the rate-limiting step for nitrate assimilation. After entry into the cell, nitrate is reduced to ammonium by the sequential action of nitrate reductase and nitrite reductase before being fixed into amide nitrogen of glutamine. Nitrate assimilation with respect to the uptake of nitrate was recently shown to decrease in *A. halophytica* grown under salt-stress condition (Incharoensakdi and Wangsupa, 2003). In addition, it was also shown that nitrate uptake is Na^+-dependent in cells grown under both non–stress and salt-stress conditions.

Nitrate uptake has been studied extensively in plants (Crawford and Glass, 1998). Three kinetically distinct nitrate transport systems are found in plant roots, i. e. two high affinity transport systems (HATS), one is constitutive and the other is inducible, and the third is constitutive low affinity transport system (LATS). The energy for nitrate uptake in plants is provided by the proton gradient or the proton motive force (Crawford and Glass, 1998) and nitrate uptake is mediated by $2H^+/ 1NO_3^-$ symport mechanism (Meharg and Blatt, 1995; Miller and Smith, 1996). Only recently, nitrate uptake in the marine higher plant *Zostera marina* L. was shown to be mediated by a high affinity Na^+-symport system (Garcia-Sanchez *et al*., 2000). Until now the study on the nitrate uptake in cyanobacteria with regard to energy requirement has been very scarce. Most studies in cyanobacteria have dealt mainly with the characterization and regulation of genes for nitrate assimilation (Flores and Herrero, 1994).

Aphanothece halophytica is a halotolerant cyanobacterium that can grow in a wide range of salinity conditions from 0.25 to 3.0 M NaCl. *A. halophytica* accumulates an osmoprotectant glycine betaine at high salinity. The DnaK protein of *A. halophytica* has been shown to contain a longer C-terminal segment than other DnaK/Hsp70 family members and exhibit extremely high protein folding activity at high salinity. Transformation of tobacco with the DnaK from *A. halophytica* was shown to enhance the

tolerance for salt. Previously, it was shown that the expression of genes for synthesis of betaine and catalase could confer salt tolerance on the freshwater cyanobacterium *Synechococcus* sp. PCC 7942. However, the expression of a Na^+/H^+ antiporter from *Vibrio alginolytica* NhaAv resulted in a Na^+-sensitive transformant. Recently, we isolated a eukaryotic Na^+/H^+ antiporter gene (*apnha*P) from a halotolerant cyanobacterium *A. halophytica* and showed that ApNhaP exhibits high Na^+/H^+ exchange activity over a wide range of pH with novel ion specificity. Therefore, it was interesting to examine the effects of expression of ApNhaP and genes for the synthesis of betaine, catalase, and a chaperone (DnaK) and their combinations.

By using ApNhaP, bet, catalase, DnaK and their combination genes eight kinds of transformants has been constructed for the freshwater cyanobacterium *Synechococcus* sp. PCC 7942 and tested for salt tolerance. It was found that compared with the overproduction of betaine, catalase, and DnaK the expression of ApNhaP drastically improved the salt tolerance of the freshwater cyanobacterium and suggested that among the several functions the exclusion of NaCl by the plasma membrane Na^+/H^+ antiporter is a key factor for salt tolerance. Therefore, it was of interest to examine whether *A. halophytica* contains a unique betaine transporter. Here, we isolated betaine transporter gene and transformed to salt-sensitive *E. coli* MKH13, we also transformed *A.* betaine transporter to freshwater cyanobacterium that can grow in medium containing NaCl concentration up to 0.5 M.

The main work of the present study is to investigate the effect of salinity on transportation of compatible solute into *A. halophytica*. In addition, the proteins and genes that control compatible solutes uptake in *A. halophytica* will be investigated. Nitrate is a nitrogen source for growth in many organisms including cyanobacteria. Nitrate was changed to nitrite and ammonium by action of nitrate reductase and nitrite reductase,

respectively (Guerrero *et al.*, 1990). The product, ammonium is in corporate into glutamate and to form glutamine which can be transformed to other amino acids. Amino acids and their derivatives are compatible solutes, for example, proline, ectoine, betaine, respectively. Incharoensakdi and Wangsupa (2003) reported that *A. halophytica* grown under salt-stress conditions showed a reduction in nitrate uptake rate compared with under non-stress condition. This suggests that nitrate uptake plays little role in salt stress resistance of *A. halophytica*. However, the transport of nitrate which is an energy dependent process may also affect the transport of compatible solute. It would be desirable to investigate the energetics aspect of nitrate transport in comparison with that of the compatible solute transport. In this study, choline was chosen as a compatible solute because it can be converted to final product, namely glycine betaine. Incharoensakdi and Karnchanatat (2003) reported that the uptake of choline increased with the detection of a periplasmic choline binding protein in cells grown under high salinity condition. Compatible solute uptake system in many living cells almost used membrane-bound protein (transporter) to transport osmolytes in to the cells such as ProU in *E. coli* and OpuC in *B. subtilis* which were characterized as ABC transporters for glycine betaine, proline betaine, and proline (Kempf and Bremer, 1995; Kapper *et al*., 1996; Jebbar *et al*., 1997; Kiene, 1998). BetT in *E. coli* and OpuB in *B. subtilis* were involved in choline transport activity (Lamark *et al.*, 1996). However, there has been no study on choline transport protein in *A. halophytica*. The periplasmic, cytoplasmic, and membrane fraction were prepared and compared the protein patterns when cell growth with or without salt stress. Characterization of choline transport will be done by whole cells or membrane vesicles. Many research groups used membrane vesicles to determine and characterize choline transport activity by using radiochemical reagent ([methyl-^{14}C] choline). Membrane vesicles will be used to study the choline uptake rate using

radiochemical reagents and some inhibitors to block transport activity (Kapper *et al*., 1996; Vyas and O'Regan, 1985; Knipper *et al*., 1991).

1.10 Objectives:

To this study, the unicellular halotolerant cyanobacterium, *A. halophytica* (This alga is classified into Chroococcales order, Chroococcacean cyanobacteria subgroup) (Geitler, 1932; Stanier *et al*., 1971) was selected as a source for the study of (i) effect of salinity on cell growth, organic and ionic compound accumulation, and protein profiles (ii) uptake of nitrate and choline by *A. halophytica* under non-stress and salt-stress condition with respect to their energetics (iii) to detect the choline binding protein activity under non-stress and salt-stress condition (iv) to study choline transport activity under non-stress and salt-stress condition by *A. halophytica* membrane vesicles (v) isolation of genes from *A. halophytica* for further characterization.

CHAPTER II

MATERIALS AND METHODS

2.1 Instrument

ABI310 genetics analyzer: Applied Biosystems, USA

Autoclave: Model HA 30, Hirayama Manufacturing Cooperation, Japan

Autopipette: Pipetman, Gilson, France

Betascanner BS -4125: Shimadzu, Japan

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

Digital Lux meter FT710: Taiwan

Electrophoresis unit: model Mini-protein II cell: Biorad, USA

Flight mass spectroscopy: KOMPACT MALDI IV tDE, Shimadzu, Japan

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

Glass Microanalysis filter holder: Millipore, USA

High performance liquid chromatography: Model Hewlette Packard series 1050, Japan

Illuminated/Refrigerated orbital: Sanyo, England

Incubator: Haraeus, Germany

Incubator shaker: Psyco-therm, New Bruncwick Scientific Supply, Thailand

Laminar flow BVT-124: International Scientific Supply, Thailand

Microcentrifuge: Kubota, Japan

Microscope: Olympus, USA

Peristaltic pump: Pharmacia LKB, Sweden

Personal ion analyzer: Applied Biosystems, Foster city, CA, USA

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, Reckbet 1218, England

Siliga gel plates: Whatman, USA

Spectrofluorophotometer RF-5300PC: Shimadzu, Japan

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

Surface Plasmon Resonance: AutoLab, Germany

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

Acrylamide:Merck, USA

Amiloride: Sigma, USA

Amino acid and derivatives: Sigma, 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, Japan

Bacto peptone: Merck Ag Darmstadt, Germany

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

Betaine aldehyde chloride: Sigma, USA

Blot absorbent filter paper: Biorad, USA

Beta-mercaptoethanol: Katayama Chem, Japan

Calcium chloride: Merck Ag Darmstadt, Germany

Carbonyl-cyanide trifluoromethoxyphenylhydrazone (CCCP): Sigma, USA

Chloramphenicol: Sigma, USA

Chloroform: Katayama Chem, Japan

Choline chloride: Sigma, USA

Cobalt chloride: Fluka, Switzerland

Coomasie brilliant blue G-250: Sigma, USA

Coomasie brilliant blue R-251: Sigma, USA

Dialysis tube: Sigma, USA

D (+) glucose: Sigma, USA

Dimethyl sulfoxide: Katayama Chem, Japan

3-(3,4-dichlorophenyl)-1, 1-dimethyl urea (DCMU): Sigma, USA

2, 4 dinitrophenol: Sigma, USA

Dowex 50W (50x4 – 200, H+ form): Sigma, USA

Dowex 1x4 (550x4 – 200, H+ form): 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

Gramicidin D: Sigma, USA

HEPES: Sigma, USA

Hydroxyapatite: Sigma, USA

Isoamylalcohol: Katayama Chem, Japan

L-lactate: Sigma, USA

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

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

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

Nigericin: Sigma, USA

Monensin: Sigma, USA

Morpholinopropanesulphonic acid (MOPs): Katayama Chem, Japan

N-ethylmaleimide, Sigma, USA

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

N, *N*'-diclyclohexylcarbodiimide (DCCD): Sigma, USA

Noble Agar: BBL, London, Dickinson and company, USA

Nitroblue tetrazolium (NTB): Katayama Chem, Japan

Ouabain: Sigma, USA

p-chloromercurobenzoate: Sigma, USA

Phenol: Katayama Chem, Japan

POPOP: Sigma, USA

PPO: Sigma, USA

Potassium chloride: Merck Ag Darmatadt, Germany

Seakem CTG agarose: Biowhittaker Molecular Application, USA

Sodium dodecyl sulfate: Sigma, USA

Sodium acetate: Katayama Chem, Japan

Sodium arsenate: Sigma, USA

Sodium azide: Sigma, USA

Sodium fluoride: Sigma, USA

Sodium nitrate: Sigma, USA

Sodium cyanide: Sigma, USA

Sorbitol: BDH, England

Sucrose: Katayama Chem, Japan

Toluene: BDH, England

Tris-hydrochloride: Katayama Chem, Japan

Triton X-100: Packard, USA

Valinomycin: Sigma, USA

Vanadate: Sigma, USA

2.3 Supplied

Nitrocellulose membrane: Millipore Cooperation, USA

Whatman 3MM paper: Whatman International, England

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

2.4 Kit

Ligation kit version 1: Takara, Japan

PCR amplification kit: Applied Biosystems, USA

Standard molecular weight: Sigma, USA

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

SuprecTM-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

*Bam*HI: Biolabs, USA

*Eco*RI: Biolabs, USA

*Eco*RV: Biolabs, USA

*Hind*III: Biolabs, USA

*Nco*I: Biolabs, USA

*Sal*I: Biolabs, USA

Lysozyme: Sigma, USA

ProteinaseK: Katayama Chem, Japan

RNase: Katayama Chem, Japan

2.6 Bacterial strains and plasmids

A. halophytica was originally isolated from Solar Lake in Israel (Geitler, 1932; Stainer *et al.*, 1971). The organism was kindly provided by Professor Dr. Teruhiro Takabe of Research Institute of Meijo University, Japan.

E.coli MHK13 (Haardt *et al*., 1995) was kindly provided by Professor Dr. Teruhiro Takabe of Research Institute of Meijo University, Japan.

E. coli DH5α (Higashitani *et al.*, 1995) was kindly provided by Professor Dr. Teruhiro Takabe of Research Institute of Meijo University, Japan.

Synechococcus PCC7942 (Waditee *et al*., 2001) was kindly provided by Professor Dr. Teruhiro Takabe of Research Institute of Meijo University, Japan.

pBluescript® II SK^+, cloning vector, Toyobo, Japan (Appendix 12)

pTrcHis2C, expression vector, Invitrogen, USA (Appendix 13)

pUC303-Bm (Waditee *et al*., 2002), cloning vector, Toyobo, Japan (Appendix 14)

2.7 Methods

2.7.1 Culture conditions.

A. halophytica is a short cylindrical shape cyanobacterium surrounded with mucous membrane which multiplies by binary fission as shown as shown in Figure 6. Cells were grown photoautotrophically in BG_{11} medium plus 18 mM $NaNO_3$ and Turk Island salt solution as described Appendix 1 except that the NaCl concentration of the culture medium was adjusted to a range from 0.25 to 3.0 M as desired. Cotton-plugged 500-ml conical flasks containing 200 ml of medium 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 ($30\mu Em^2s^{-1}$).

2.7.2 Isolation of periplasmic space, membrane, and cytoplasmic fraction

Cells grown under non-stress and salt-stress conditions were collected by centrifugation (8,000xg, 10 min) and washed twice with 10 mM Tris-HCl buffer, pH 7.6 containing either 0.5 M or 2.0 M NaCl. Periplasmic space fraction was released by cold osmotic shock according to Neu and Heppel (1965). Cells are resuspended in plasmolysis buffer (10 mM Tris-HCl buffer, pH 7.6 containing 0.5 M or 2.0 M NaCl, 1.0 M sorbitol and 1 mM EDTA) and shaken gently for 30 min at room temperature. Cells were collected after centrifugation and resuspended in cold deionized water, frozen at –80 ^{o}C for 30 min. After centrifugation the supernatant was recentrifuged once more to remove remaining cells and the supernatant represented periplasmic fraction.

The remaining cells were washed twice with 50 mM Hepes-NaOH buffer, pH 7.5. Cell pellets are suspended with grinding buffer (50 mM Hepes-NaOH buffer, pH 7.5, 10% glycerol, 10 mM EDTA, and 5 mM DTT) containing 2 mg/ml lysozyme and stirred at 4 ^{o}C for 90 min. After lysozyme treatment, the mixture was centrifuged at 19,000 rpm, 20 min to remove whole cells and large fragment. The supernatant was then centrifuged at 44,000 rpm for 60 min. The resulting supernatant represented cytoplasmic fraction and the pellet represented membrane fraction. Membrane fraction was washed once with grinding buffer and recentrifuged at same speed.

Solubilized membranes were obtained by dissolving the final membrane pellet in grinding buffer containing 0.3% Triton X-100 (Lanfald and Strom, 1986). The solution was stirred for 60 min at 4 ^{o}C and the undissolved material removed by centrifugation (19,000 rpm, for 20 min). The solution containing periplasmic fraction, cytoplasmic, and membrane fraction was assayed for content of protein and characterized for protein pattern by polyacrylamide gel electrophoresis.

Figure 6 Microscopic picture of *A. halophytica* grown in Turk Island Salt Solution plus modified BG_{11} medium at day 14 (x2250).

2.7.3 Polyacrylamide gel electrophoresis (PAGE)

The periplasmic fraction, cytoplasmic, and membrane fraction was characterized by non-denaturing and sodium dodecyl sulfate PAGE according to Laemmli (Laemmli, 1970). All fractions were stained by coomasie brilliant blue for protein and choline binding activity was detected by non-denaturing PAGE (10%) using 50 μg protein sample, mixed with 10 μM [methyl-^{14}C]choline with an incubation at 20 ^{o}C for 30 min. The gels were quickly dried on Whatman 3 MM paper and autoradiographed with X-OMAT S Kodak films for 14 days (Appendix 2).

2.7.4 Surface Plasmon Resonance (SPR) technique

Surface plasmon resonance (SPR) has been determined the interactions of a variety of biopolymers with various ligands and membranes, including protein:ligand, protein:protein, protein:DNA and protein: membrane binding. In a typical SPR biosensing experiment, one interactant in the interactant pair (membrane bound protein) is immobilized on an SPR-active gold-coated glass slide which forms one wall of a thin flow-cell, and the other interactant (effectors for example betaine, choline or amino acids) in an aqueous buffer solution is induced to flow across this surface, by injecting it through this flow-cell. When light (visible or near infrared) is shined through the glass slide and onto the gold surface at angles and wavelengths near the so-called “surface plasmon resonance” condition, the optical reflectivity of the gold changes very sensitively with the presence of biomolecules on the gold surface or in a thin coating on the gold. The high sensitivity of the optical response is due to the fact that it is a very efficient, collective excitation of conduction electrons near the gold surface. The extent of binding between the solution-phase interactant and the immobilized interactant is easily observed and

quantified by monitoring this reflectivity change. An advantage of SPR is its high sensitivity without any fluorescent or other labeling of the interactants.

To prepare membrane bound protein for immobilization, covalent linkage of membrane bound protein to the matrix was achieved by amine coupling. Activation of the carboxyl groups on the matrix was performed by adding a mixture to activate COOH groups as N-hydroxysuccinimide active esters, upon reaction with N-hydroxysuccinimide (NHS) in the presence of N-ethyl-N'-(dimethylaminopropyl)carbodiimide (EDC), in water. N-hydroxysuccinimide (NHS, 11.5 mg/ml in deionized water) and carbodiimide (EDC, 75 mg/ml in deionized water) to form active esters that react spontaneously with amine groups on the membrane bound protein. The degree of membrane bound protein immobilization was achieved through auto injection of 5 µg/ml membrane bound protein pulses followed by continuous flow of 50 mM sodium phosphate buffer, pH 7.6 at 10 µl/min. Non-covalently bound membrane bound protein was generally eluted within 5 minutes, with the difference in sensorgram baseline between pre and post-membrane bound protein injection representing the membrane bound protein immobilization level. After the desired level of immobilization was achieved, a 1 M ethanolamine solution was injected to deactivate remaining active esters. A total of 4 different binding surfaces or flow channels were prepared on each chip. Flow channel 1 (FC1) was activated with NHS/EDC and blocked with ethanolamine but no membrane bound protein was conjugated. The resultant FC1 was used as a negative control for non-specific interactions between analyte and sensor surfaces. Remaining flow channels contained different immobilization levels of membrane bound protein. Twenty thousand RU of membrane bound protein (where 1,000 RU equals to 1 ng/mm^2 of membrane bound protein on the surface) was used to establish the presence and specificity of peptide binding. The high immobilization levels were designed to generate large sensorgram deviations following

binding by the relatively small analytes. Ten thousand RU of membrane bound protein was used for the kinetic analysis of analyte binding reactions and to establish the specificity of membrane bound protein binding. Two hundred fifty RU of membrane bound protein was immobilized for kinetic analysis of membrane bound protein-analyte binding reactions. Sensorgrams readily returned to baseline in the dissociation phase of the membrane bound protein-analyte binding reactions, obviating the need for a sensor surface regeneration step. Binding reaction analysis Signal changes on the activated/blocked control channel were subtracted from the membrane bound protein binding interactions using in-line reference and the subtracted sensorgrams were analyzed. Since membrane bound protein has only a single analyte binding cleft per molecule, curves generated with serial analyte concentrations were applied globally to the 1:1 Langmuir binding model with or without correction for baseline drifting depending on baseline status. Plots of residuals indicate the difference between the experimental and reference data for each point in the fit (Martin *et al*., 2004) (Appendix 3).

2.7.5 Detection of ATP by HPLC

To determine ATP content in the reaction, cells were centrifuged at 10,000 rpm, room temperature, washed twice, and resuspended with 0.22 M phosphate buffer, pH 6.8 after that cell suspension were sonicated for 1 min. Debris was removed by centrifugation and supernatant was determined for ATP by HPLC. The isocratic reverse-phase HPLC with a water Resolve C18 (5-um, spherical) column (Millipore, Milford, MA) was used and mobile phase was 0.22 M phosphate buffer, pH 6.8. The column was monitored by absorption at 259 nm, and flow rate was 1 ml/min. ATP content was compared with standard ATP (Appendix 4) (Sandeep *et al*., 1997).

2.7.6 Preparation of membrane vesicle

Membrane vesicles were prepared by osmotic shock lysis procedure essentially as described previously by Otto *et al.* (1982). The cells were washed twice with 50 mM Hepes-NaOH buffer, pH 7.5. Cell pellets were suspended with grinding buffer (50 mM Hepes-NaOH buffer, pH 7.5, 10% glycerol, 10 mM EDTA, and 5 mM DTT) containing 2 mg/ml lysozyme and stirred at 4 ^{o}C for 90 min. After lysozyme treatment, the mixture was centrifuged at 19,000 rpm, 20 min to remove whole cells and large fragment. The supernatant was then centrifuged at 44,000 rpm for 60 min. The supernatant was removed and pellet was resuspended in the same buffer. Membrane vesicles were checked by Transmission Electron Microscopy (TEM) (Appendix 5) as shown in Figure 6. Membrane vesicles were stored in -80 ^{o}C before used.

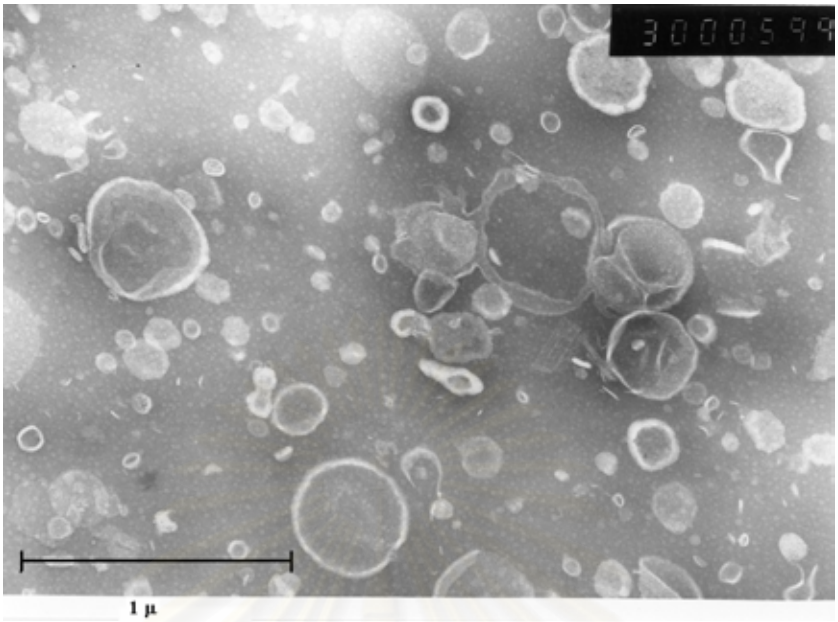


Figure 7 The membrane vesicle was detected by TEM (x45,000)

2.7.7 Transport assays

Nitrate uptake

Log-phase cells were washed twice in 25 mM Hepes-KOH buffer, pH 8.2, containing 10 mM $NaHCO_3$ and 0.5 M sorbitol, and are suspended in the same buffer at a chlorophyll (ChlA) concentration of 25 μg/ml determined as described by Mackinney (1941). The reaction was started by the addition of 100 μM $NaNO_3$ to the suspension and kept at 30 ^{o}C in the light with an irradiance of 60 $\mu Em^{-2}s^{-1}$. Samples were removed at various time intervals and rapidly filtered through a 0.45 μm membrane filter. The nitrate content remaining in the filtrate was determined by anion-exchange high performance liquid chromatography (Hypersil-10 Sax column, 250 mm x 4.6 mm). Nitrate content was compared with standard nitrate (Appendix 6). Chlorophyll content was determined follow Appendix 7.

choline uptake

Cells at log phase were harvested by centrifugation with speed 8,000 x g for 10 min at 4 ^{o}C, washed twice with 50 mM Hepes-NaOH buffer, pH 7.5, and suspended to a concentration of 0.1 mg cell protein/ ml in the same buffer containing either 0.5 M NaCl (non-stress) or 2.0 M NaCl (salt-stress). The uptake experiment was initiated by adding [methyl- ^{14}C] choline with a specific activity of 0.1 μCi/μmol at a final concentration of 50 μM or otherwise stated. The cells suspension was withdrawn at 1-min intervals and rapidly filtered through HAWP cellulose nitrate filters (0.45 μm pore size; Millipore). The filters were washed twice with 3 ml of buffer (same osmolarity as the assay buffer) and the radioactivity trapped in the cells was added with scintillation fluid (Appendix 8) and determined by counting with scintillation counter. ^{14}C content was

determined follow Appendix 9. Protein was determined by method of Bradford (1976) using bovine serum albumin as a standard (Appendix 15).

Membrane vesicles containing about 25 mg/ml of protein were allowed to equilibrate at room temperature before being added to the transport medium. Choline transport was assayed as described above. The membrane vesicles were withdrawn at time intervals and rapidly filtered through HAWP cellulose nitrate filters (0.22 μm pore size; Millipore). The filters were washed twice with 3 ml of buffer (same osmolarity as the assay buffer) and the radioactivity trapped in the cells was determined by counting with scintillation counter. Protein was determined by method of Bradford (1976) using bovine serum albumin as a standard.

2.7.8 Characterization of nitrate and choline transport energetics.

Proton motive force driven uptake.

Cells or membrane vesicles were incubated for 30 min in the presence or absence of ionophores. The proton motive force was modified by artificially imposed ΔpH, $\Delta\psi$ or both.

EDTA-treated cells.

Prior to incubation with monensin or valinomycin, the cells were incubated 15 min at 30 ^{o}C in 100 mM Tris-HCl pH 7, supplemented with 1 mM EDTA, and washed twice with the assay buffer in absence of EDTA. This treatment suppressed impermeability of cell membrane for these ionophores (Ekiel *et al.,* 1985).

Uptake assays in absence of potassium were performed using 100 mM Tris-maleate buffer for pH 5.5 and 100 mM Tris-HCl buffer for pH 7.0 or 8.2. Assays

having potassium in the uptake medium were performed with 100 mM potassium phosphate buffer for the three pH values.

Artificial generation and suppression of pH gradient (ΔpH) and membrane potential (Δψ).

The ΔpH was generated in acetate loaded cells (80 mM potassium acetate buffer at pH 5.5 or 7.0), the supernatant was then removed and cells were diluted in 100 mM Tris-maleate buffer (pH 5.5) or Tris-HCl buffer (pH 7.0). ΔpH was generated at pH 8.2 in ethanolamine-HCl loaded cells instead of acetate loaded cells. The ΔpH was suppressed using 80 mM potassium acetate buffer at pH 5.5 and pH 7.0, 80 mM ethanolamine-HCl buffer at pH 8.2 (Ekiel *et al.*,1985; Espie and kandasamy, 1994).

The artificial Δψ was imposed using 20 mM KSCN or 3 μM valinomycin with EDTA-treated cells in absence of K^+ using 100 mM Tris-maleate buffer (pH 5.5) or 100 mM Tris-HCl buffers (pH 7.0 or 8.2). Δψ term was suppressed using 3 μM valinomycin and EDTA-treated cells with 100 mM potassium phosphate buffer in the medium.

2.8 Isolation and characterization of *A. halophytica* betaine transporter

2.8.1 Isolation of *A. halophytica* Betaine Transporter.

A. halophytica genomic DNA was isolated and used as template for amplification of the *apbetT* gene. The oligonucleotides were designed based on sequence analysis of *A. halophytica* genome, determined by Shimadzu Company. Primers used for isolation of betaine transporter gene were shown in Table 2. The forward primer, ApBetTNco-F1, was designed as start codon of the gene having *Nco*I restriction enzyme, The reverse primer, ApBetTSal-R1, was designed at stop codon of the gene having *Sal*I

restriction enzyme,. Amplified DNA fragments of an expected size (approximately 1.7 kbs) were concentrated and primers were removed by Suprec^{TM}-02 followed by ligation into cloning vector, pBluescript® II SK^{+}/ *Eco*RV by using ligation kit. The recombinant DNA was transformed into *E. coli* DH5α. Insert fragment of an expected size 1.7 kbs were sequenced. The nucleotide sequence of the entire 1.7 bps was shown in Appendix 27.

Table 2 Primers

Primer	5′ ---------- 3′	Base pairs
ApBetTNco-F1	TTCCATGGTTAAACAATCAAAACGT	25 mer
ApBetTSal-R1	CAGTCGACTTCATCTTGGGCAAATCG	26 mer
ApBetTproNco-F	AGCCATGGAAGCGGTGCATTACATG	25 mer
ApBetTproNco-R	AACCATGGAATATTTTCTTTGAAAAGA	27 mer
ApNhBm-R	GTGGATCCTCAATGATGATGATG	23 mer

2.8.2 Construction of expression vector with the salt-sensitive mutant of *E. coli* MKH13.

pBluescript® II SK^{+} containing *apbetT* was double digested by *Nco*I/*Sal*I and was analyzed by agarose gel electrophoresis. 1.7 kb fragment of betaine transporter gene was eluted and purified. The purified fragment of *apbetT* was ligated with pTrcHis2C that was doubled digested with *Nco*I/*Sal*I. The ligation mixture was transformed into *E. coli* DH5α and spread on LB agar (Appendix 10) containing ampicillin at final concentration 50 µg/ml. The transformants containing *apbetT* were analyzed by the PCR reaction using ApbetT-F and reverse primer for pTrcHis2C containing *Bam*H1. Positive clones were selected and grown in liquid media to extract plasmid DNA.

The plasmid pApbetT was transformed to the salt-sensitive mutant MKH13 cells. The transformants were allowed to grow on minimal medium A (MMA) (Appendix 11) plus 0.2 M NaCl agar plate containing ampicillin, at 37 ^{o}C for 16 h. Both pTrcHis2C (negative control) and ApBetT expressing cells were tested for the ability to grow (complementation test) in MMA plus 0 – 1.5 M NaCl.

2.8.3 Complementation Test.

For the complementation test on agar plates, *E. coli* MKH13 cells transformed with pTrcHis2C and pApBetT were grown overnight at 37 °C in minimal medium A (pH 6.7) containing 0.2% glucose and ampicillin (50 µg/ml). Cells were then spread on a 1.5% agar plate containing 0.7 M NaCl, 1 mM IPTG, and 1 mM betaine or 1 mM proline and incubated at 37°C for the indicated times.

2.8.4 Transport Assays.

E. coli MKH13 cells transformed with pTrcHis2C and pApBetT were grown overnight at 37 °C in minimal medium A (pH 6.7) containing 0.2% glucose and ampicillin (50 µg/ml) and were inoculated into the same fresh medium with an absorbance at 620 nm (A_{620}) of 0.05. IPTG (1 mM) was added to the mid log-phase cells (A_{620} between 0.6 and 0.8). After 3-h incubation, cells were harvested, washed twice, and suspended to an A_{620} of 1.0 in the same medium. Subsequently the cells were incubated with shaking for 5 min at 37°C, and transport was initiated by the addition of 0.1 mM [1-^{14}C]betaine or L-[U-^{14}C]proline. For K*m* and V*max* determinations, the concentrations of betaine or proline were varied from 0.01 to 5 mM. Glucose was added to a final concentration of 5 mM to energize the cells, and where indicated, salt (NaCl or KCl) or sucrose was added to the indicated concentrations. Cells were collected on 0.2-µm-pore-

size cellulose nitrate filters (Advantec MFS, Chiba, Japan). Filters were washed with 3 ml of buffer (same salinity as the assay buffer), and the radioactivity trapped in the cells was measured with a liquid scintillation counter (model 3200C, Aloka Instruments Co., Tokyo, Japan). Competitions for betaine uptake and proline uptake were performed in the presence of a 100-fold molar excess (10 mM) of competitors.

2.8.5 Construction of Expression Vectors.

An expression plasmid for ApBetT that contains its own promoter was constructed. *A. halophytica* genomic DNA used as a template DNA for isolation *apbetTpro*. Primers used for isolation and expression of glycine betaine transporter gene were shown in Table 2. The oligonucleotides were designed based on genome sequence of *A. halophytica* determining by Shimazu Company. The forward primer, ApBetTproNco-F, was designed at upstream of start codon of the gene and contained restriction enzyme, *Nco*I. The reverse primer, ApBetTproNco-R, was designed at start codon of the *apbetT* gene and contained restriction enzyme, *Nco*I. Amplified DNA fragments of an expected size (approximately 500 bps) were concentrated and primers were eliminated by Suprec^{TM}-02 followed by ligation into cloning vector, pBluescript® II SK^{+}, by using ligation kit (pBluescript® II SK^{+} was digested by *Eco*RV). The recombinant DNA was transformed into *E. coli* DH5α. Insert fragment of an expected size 500 bps were sequenced. pBluescript® II SK^{+} containing *A. halophytica* promoter was digested with *Nco*I and analyzed by agarose gel electrophoresis. Betaine transporter promoter gene fragment was purified and ligated with pTrcHis2C containing betaine transporter digested by *Nco*I. A recombinant DNA plasmid was transformed into *E. coli* DH5α and grown onto LB agar containing ampicillin final concentration 50 μg/ml. The pTrcHis2C containing betaine transporter gene and promoter was used as a template

DNA for isolation betaine transporter gene with promoter containing His2C. The forward primer, ApBetTproNco-F and the reverse primer, ApNhBm-R (designed containing His2C and *BamH*I) was used for amplification. Amplified DNA fragments of an expected size (approximately 2.2 kbs) were concentrated and primers were eliminated by Suprec^{TM}-02 followed by ligation into cloning vector, pBluescript® II SK^{+}, by using ligation kit (pBluescript® II SK^{+} was digested by *Eco*RV). The recombinant DNA was transformed into *E. coli* DH5α.

2.8.6 Construction of recombinant DNA with pUC303 and complementation test with the fresh water cyanobacterium *Syneccococcus elongatus* PCC7942.

pBluescript® II SK^{+} containing betaine transporter gene with promoter containing His2C was digested with *BamH*I analyzed by agarose gel electrophoresis. The expected fragment was purified and ligated with pUC303 (shuttle vector) digested by *BamH*I. A recombinant DNA plasmid was transformed into *Syneccococcus elongatus* PCC7942 by electroporation and grown onto BG_{11} agar containing streptomycin at a final concentration of 50 µg/ml.

The transformants were allowed to grow in liquid BG_{11} medium with or without 0.1 – 0.6 M NaCl, at 30 ^{0}C for 10 days. Both pUC303 and pUC303 containing betaine transporter expressing cells were tested for the ability to grow for complementation test. *Synechococcus* cells were subcultured at 30°C under continuous fluorescent white light (30µE $m^{-2}s^{-1}$) in BG_{11} liquid medium supplemented with 10 μgm^{-1} streptomycin and bubbled with air. The cultures were incubated for several days. During these periods, the growth of cells was monitored by measuring the absorbance at 730 nm with a Shimadzu UV-160A spectrophotometer.

2.8.7 Computer Analysis and Other Methods.

The hydropathy profile of the deduced amino acid sequence was predicted according to the method of Kyte and Doolittle. The possible transmembrane (TM) segments of the AmT1 sequence were deduced by a computer program TopPredII. SDS-PAGE and immunoblotting were carried out as described previously. Betaine was extracted as described previously and measured after esterification with time of flight mass spectroscopy (KOMPACT MALDI IV tDE, Shimadzu/Kratos) using d11-betaine as an internal standard. Cellular ions were determined with a Shimadzu PIA-1000 personal ion analyzer. The nucleotide sequences were determined using an ABI310 genetic analyzer (Applied Biosystems, Foster City, CA). Protein concentration was determined by the method of Bradford (1976). An antibody raised against a six-histidine (His6) tag was obtained from R&D Systems, Inc.

CHAPTER III

RESULTS

3.1 Energetic aspect of nitrate uptake

3.1.1 Energy-dependent nitrate uptake

To determine whether nitrate uptake was energy-dependent, the effects of some inhibitors on nitrate uptake were investigated. As shown in Table 3 transport uncouplers such as carbonyl cyanide chlorophenylhydrazone (CCCP), 2,4-dinitrophenol (DNP), and gramicidin D, which dissipate proton motive force (Kroll and Booth, 1981), could effectively inhibit nitrate uptake to a similar extent by about 70-75%. Sodium azide, an inhibitor of cytochrome oxidase, which also dissipates proton motive force strongly inhibited the uptake. All these results indicate that nitrate uptake by *A. halophytica* was energized by proton motive force. Interestingly, monensin which collapses Na^+-electrochemical gradient also caused drastic inhibition of nitrate uptake. *N*,*N*'-dicyclohexylcarbodiimide (DCCD), an ATPase inhibitor, also inhibited nitrate uptake with strong inhibition being observed at 100 μM DCCD. This suggests the involvement of ATP hydrolysis in the uptake of nitrate.

Table 3 Effect of metabolic inhibitors on nitrate uptake[a]

Inhibitor	Concentration	Nitrate uptake (%)
None		100
CCCP	20 µM	50
	40 µM	26
DNP	1 mM	60
	2 mM	28
Gramicidin D	10 µg/ml	34
	20 µg/ml	24
NaN_3	10 mM	50
	30 mM	16
Monensin	20 µM	46
	30 µM	25
DCCD	40 µM	60
	100 µM	35
KF	15 mM	101
	30 mM	100

[a]Cells were preincubated with inhibitors in the dark for 30 min before the addition of 100 µM $NaNO_3$ to initiate the uptake as described in Materials and Methods.

3.1.2 Effect of different energy sources on nitrate uptake

To assess the roles of ATP and proton motive force in energizing the uptake of nitrate, *A. halophytica* cells were starved to deplete endogenous energy sources. Nitrate uptake was then monitored after reenergization with glucose or lactate (Table 4). Both glucose and lactate could energize the uptake of nitrate in the starved cells. These results indicate that nitrate uptake is an energy-dependent process and depends on the proton motive force. The respiratory inhibitor, KCN, strongly inhibited nitrate uptake, either alone or together with glucose or lactate.

Table 4 Effect of energy sources on nitrate uptake[a]

Addition	Nitrate uptake (%)
None	100
Glucose (20 mM)	128
Lactate (10 mM)	116
KCN (20 mM)	24
Glucose + KCN	28
Lactate + KCN	33

[a]Cells were starved by suspending cells in the growth medium lacking carbon and nitrogen source in the dark for 24 h. The starved cells were then assayed for nitrate uptake in the presence of different energy sources or respiratory inhibitor. Starved cells were preincubated with the tested compound(s) in the dark for 30 min before the addition of 100 μM $NaNO_3$ to initiate the uptake as described in Materials and Methods.

3.1.3 Role of ΔpH on nitrate uptake

The proton motive force, which is known to energize a number of active transport processes, has two components namely the electrical potential ($\Delta\psi$) and the chemical hydrogen ion concentration gradient (ΔpH) across the membrane. To test whether ΔpH played an energetic role in the uptake of nitrate, we artificially generated pH as well as suppressed ΔpH and followed the extent of nitrate uptake at three different pHs. At pH 5.5, ΔpH was generated by diluting acetate-loaded cells (pH 5.5) into a solution containing Tris-maleate as a less permeable anion (pH 5.5). This would cause the diffusion of acetate across the membrane in its protonated form. As shown in Figure 8A, an increase in nitrate uptake by such treatment was clearly evident. In contrast, the suppression of ΔpH imposed with 80 mM potassium acetate buffer resulted in a reduction of nitrate uptake. Similar results were obtained when the experiments were done at pH 7.0 (Figure 8B) and pH 8.2 (Figure 8C).

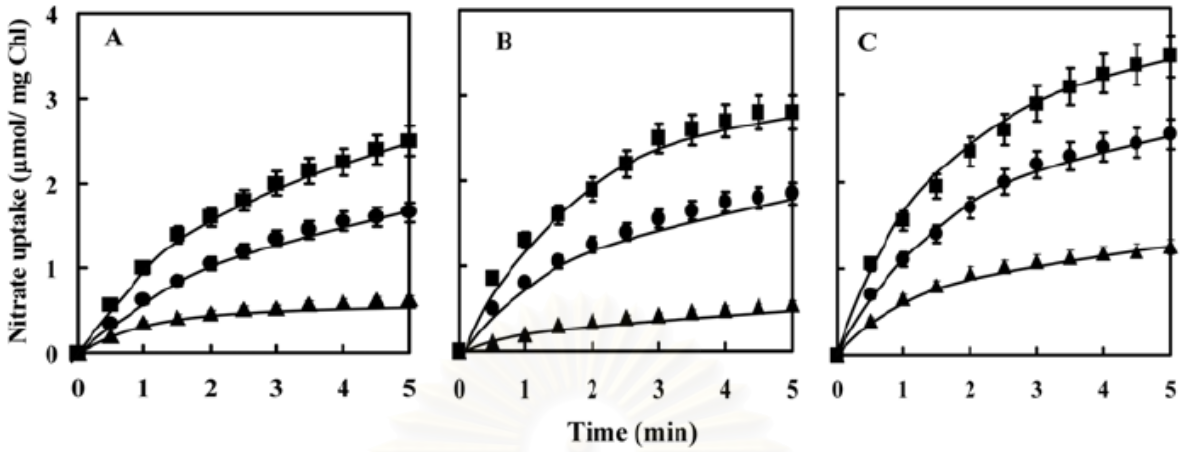


Figure 8 Effect of ΔpH on nitrate uptake at three pH values of 5.5 (A), 7.0 (B) and 8.2 (C). Artificial generation and suppression of ΔpH were done as described in Materials and Methods. Symbols are ●: control, ■: ΔpH generation, ▲: ΔpH suppression. Data are means from three independent experiments with vertical bars representing standard errors of the means.

3.1.4 Role of Δψ on nitrate uptake at different pHs

To investigate whether another component of the proton motive force, Δψ, contributed the driving force for nitrate uptake, we artificially generated Δψ either by supplementation of KSCN or by valinomycin in the absence of K^+ and then monitored the uptake of nitrate. Table 5 shows that at the three pHs tested, the rates of nitrate uptake did not increase under the influence of Δψ by the addition of 20 mM KSCN to the medium (cell inside is more negative than the control). Indeed, a slight decrease of nitrate uptake was observed. Likewise, the generation of Δψ by an imposition of an outwardly-directed, valinomycin-mediated (in the absence of K^+) potassium diffusion gradient caused no increase of the rates of nitrate uptake at the three pHs. On the other hand, the dissipation of Δψ by the addition of valinomycin in the presence of K^+ resulted in a modest stimulation of the rates of nitrate uptake at the three pHs assayed (Table 5). Taken together, these results indicated that Δψ constituted little or no contribution for the energization of nitrate uptake in *A. halophytica*.

Table 5 Effect of Δψ on nitrate uptake at different pHs[a]

pH	Nitrate uptake (μmol · min^{-1} · mg^{-1} Chl)			Nitrate uptake (μmol · min^{-1} · mg^{-1} Chl)	
	Control	Δψ generated by		Control	Δψ dissipated by
		KSCN	Valinomycin (-K$^+$)		Valinomycin (+K$^+$)
5.5	0.79 ± 0.06	0.71 ± 0.05	0.78 ± 0.05	0.84 ± 0.06	0.92 ± 0.06
7.0	0.94 ± 0.07	0.83 ± 0.06	0.86 ± 0.06	0.90 ± 0.06	0.99 ± 0.07
8.2	1.12 ± 0.08	0.97 ± 0.07	0.99 ± 0.07	1.03 ± 0.07	1.14 ± 0.08

[a]For KSCN and valinomycin (-K$^+$) experiments, 100 mM Tris-maleate buffer was used at pH 5.5 and 100 mM Tris-HCl buffer was used at pH 7.0 and 8.2. For valinomycin (+K$^+$) experiments, 100 mM potassium phosphate buffer was used for the three pHs. Data are means ± standard errors of the means (n = 3).

3.1.5 Effect of external pH on nitrate uptake

The data presented above indicate that the main driving force for nitrate uptake was contributed by ΔpH. We therefore further tested the influence of external pH on nitrate uptake. Figure 9 shows the uptake of nitrate as a function of time under different pHs of the assay medium. The uptake of nitrate apparently increased with the increase of the external pH. Within 5 min nitrate uptake appeared to reach maximum for all pHs tested. The initial rates of nitrate uptake at pHs 5.5, 7.0, and 8.2 were calculated to be 0.63 ± 0.04, 0.84 ± 0.06, and 1.16 ± 0.08 μmol.min^{-1}.mg^{-1}Chl, respectively.

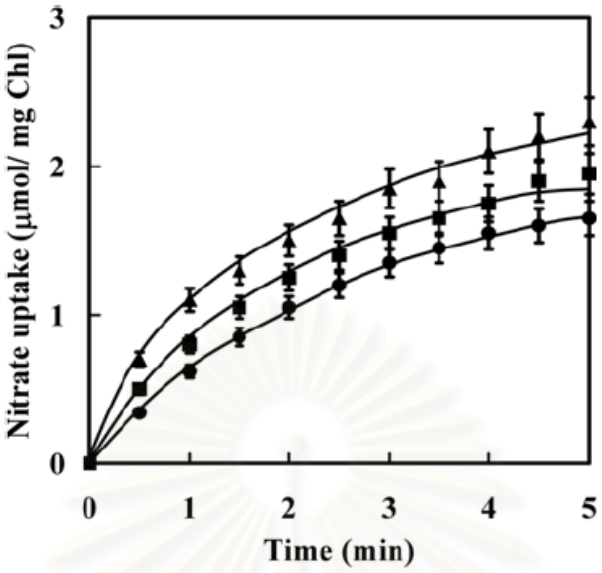


Figure 9 Dependence of nitrate uptake on external pH. Nitrate uptake assay was done as described in Materials and Methods with the modification where 100 mM Tris-maleate buffer was used at pH 5.5 (●) and 100 mM Tris-HCl pH 7.0 (■) or pH 8.2 (▲). Data are means from three independent experiments with vertical bars representing standard errors of the means.

3.2 Growth experiment

3.2.1 Growth of *A. halophytica* at various NaCl concentrations

A. halophytica was able to grow in BG_{11} plus 18 mM $NaNO_3$ and Turk Island Salt Solution plus modified BG_{11} medium under photoautotropically at 30 ^{o}C and varied salt concentration from 0.5 M up to 3.0 M NaCl (Figure 10A). Although the specific growth rate was monitored at day-7 and showed reduced growth profile when the NaCl concentration was increased (Figure 10B). The cell morphology at 0.5 M NaCl (normal condition) and 2.0 M NaCl (stress condition) were photographed (Appendix 5) as shown in Figure 11. We examined the changes in shape of cells during incubation with 0.5 M NaCl (Figure 11A) and a significant increase in the diameter of cells was observed at 2.0 M NaCl (Figure 11B). Shape of cells was grown in stress condition showed longer and flatters than cells were grown in normal condition. The maximum grow rate of *A. halophytica* at normal condition (0.5 M NaCl) and stress condition (2.0 M NaCl) was pH 9.5, data as shown in Figure 12.

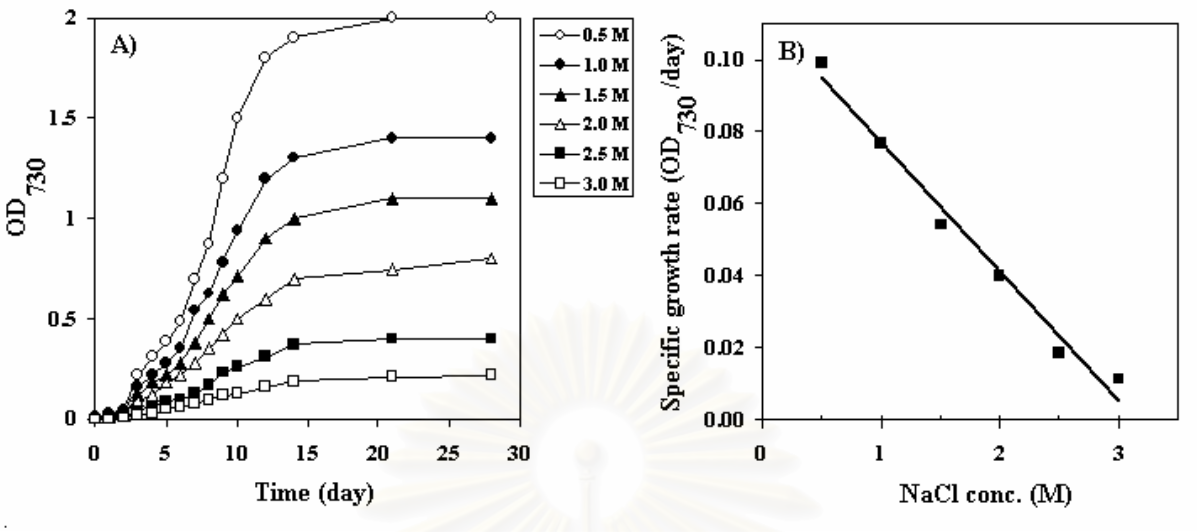


Figure 10 Growth curve of *A. halophytica* was grown in medium containing various NaCl concentrations from 0.5 – 3.0 M (A) and specific growth rate was monitored at day-7 (B).

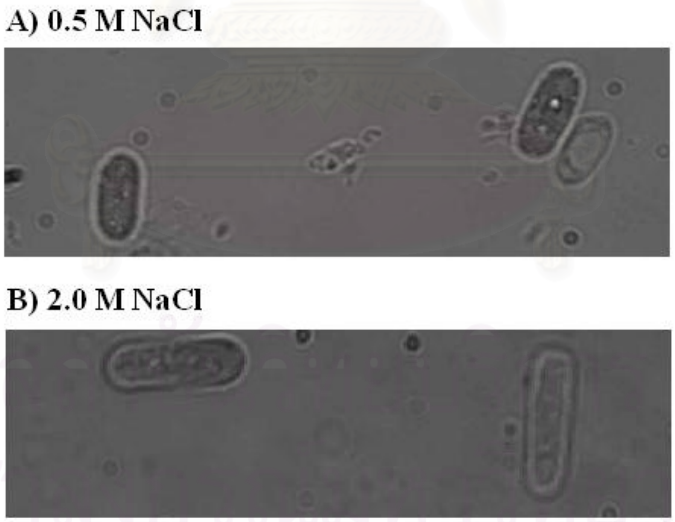


Figure 11 Microscopic picture of *A. halophytica* grown in Turk Island Salt Solution plus modified BG_{11} medium at 0.5 M NaCl (A) and 2.0 M NaCl (B) at day 14 (x2250).

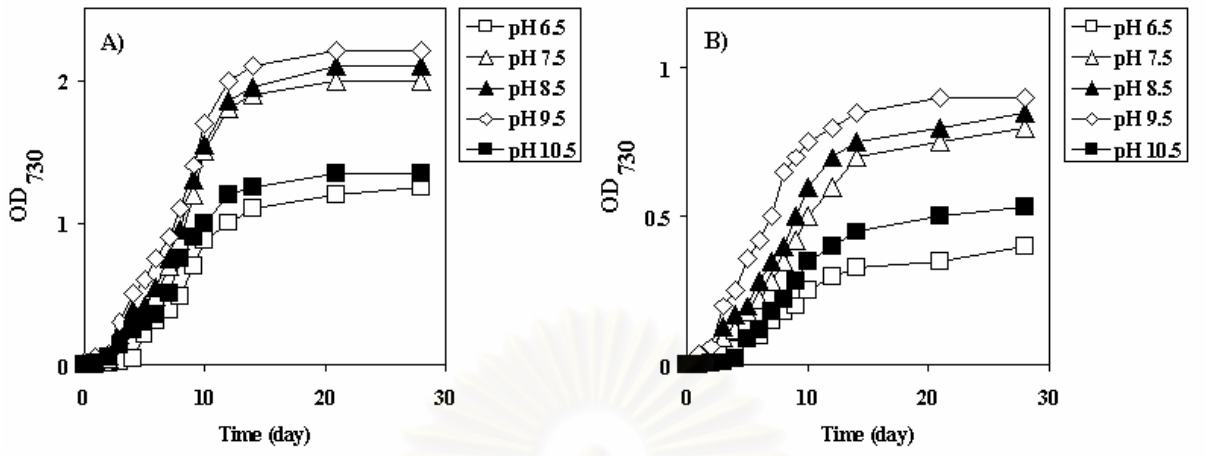


Figure 12 Growth curve of *A. halophytica* was grown in medium containing NaCl concentrations at 0.5 M (A) and 2.0 M (B) and various pH 6.5 – 10.5.

3.2.2 Chemical contents at normal and stress condition

Cells were grown in BG_{11} containing 0.5 M NaCl (normal condition) or 2.0 M NaCl (stress condition) for 7 day and determined for betaine, Na^+, K^+, NO_3^-, and NH_4^+, data as shown in Figure 13. At stress condition; betaine was highest increasing detection, Na^+, K^+, and NH_4^+ was slightly increasing. NO_3^- was slightly reduction about 30% from normal condition.

3.2.3 Amino acid content at normal and stress condition

Cells were grown in BG11 containing 0.5 M NaCl (normal condition) or 2.0 M NaCl (stress condition) for 7 day and determined for amino acid content, data as shown in Figure 14. The result shows that glutamine was the highest amount of amino acid at both conditions. The small figure inside Figure 14 indicated that glutamine slightly increased at stress condition to compare with normal condition. Aspartate, proline, and glutamate were induced about 2 folds and the other hand glycine and arginine was reduced at stress condition when compared with normal condition.

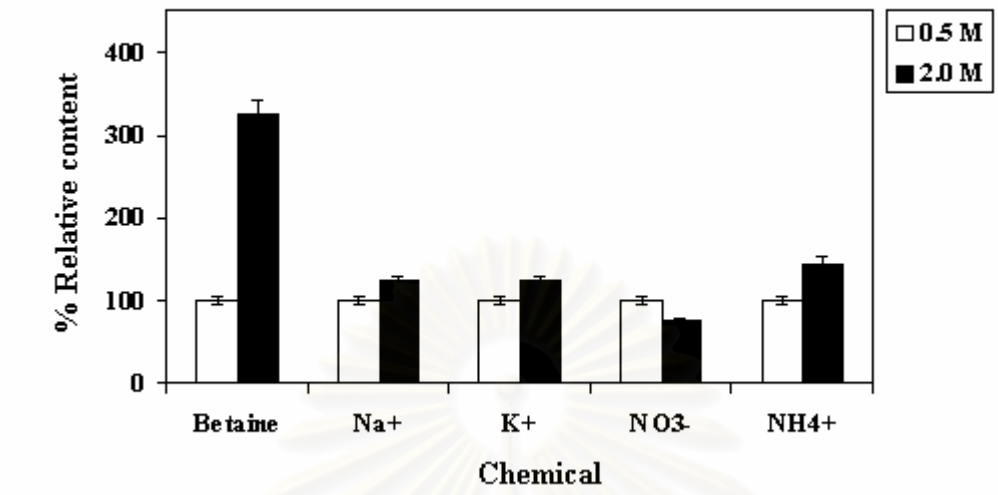


Figure 13 Chemical contents at normal and stress condition. *A. halophytica* was cultured in medium containing 0.5 M or 2.0 M NaCl and checked content of betaine, Na^+, K^+, NO_3^-, and NH_4^+. Data are means from three independent experiments with vertical bars representing standard errors of the means.

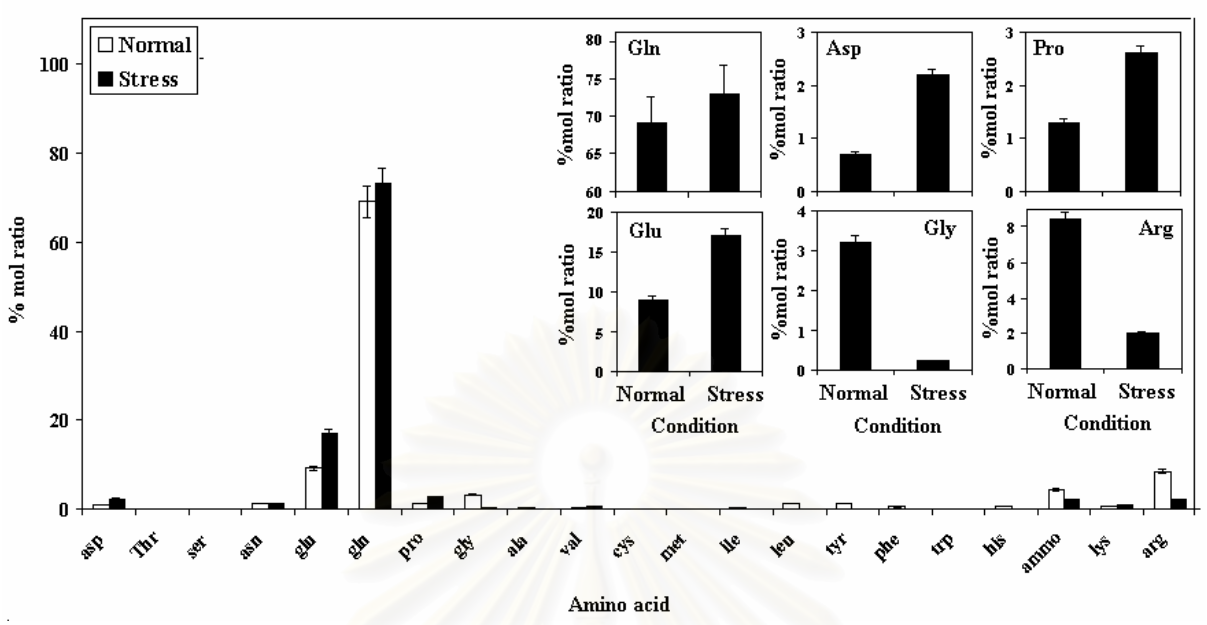


Figure 14 %mol ratio of amino acid at normal and stress condition. *A. halophytica* was cultured in medium containing 0.5 M or 2.0 M NaCl and amino acid was determined. Data are means from three independent experiments with vertical bars representing standard errors of the means.

3.2.4 Effects of glycine betaine and choline on growth rate of *A. halophytica* under various NaCl concentration

Lanfald and Storm, 1986 reported that *E. coli* used glycine betaine as osmoprotectant and needed choline as precursor. 1 mM betaine or choline was added in medium containing various NaCl concentrations to study effects of external glycine betaine and choline on growth rate of *A. halophytica* as shown in Figure 15. The condition lack osmoprotectant shows reduction of growth rate when NaCl was increased. We found that glycine betaine functioned as efficient osmoprotectant when they were added at a concentration of 1 mM to the growth medium and strongly stimulated the growth of osmotically stresses cells. Choline was somewhat less efficient osmoprotectant than betaine (Figure 15A – 15C) and growth rate did not change whereas the medium containing NaCl concentration higher than 2.0 M (Figure 15D – 15F). The specific growth rate was shown in Figure 16.

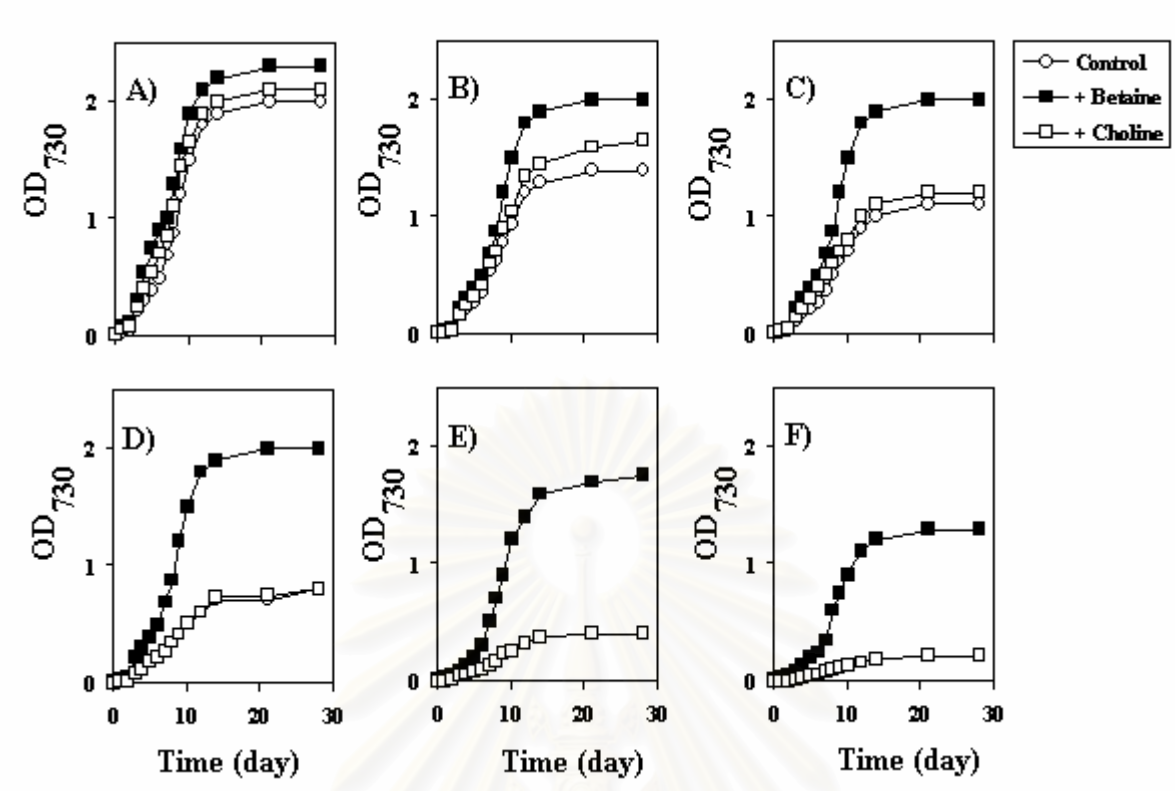


Figure 15 Effects of betaine and choline on growth rate of *A. halophytica* under various NaCl concentrations 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 M (A – F).

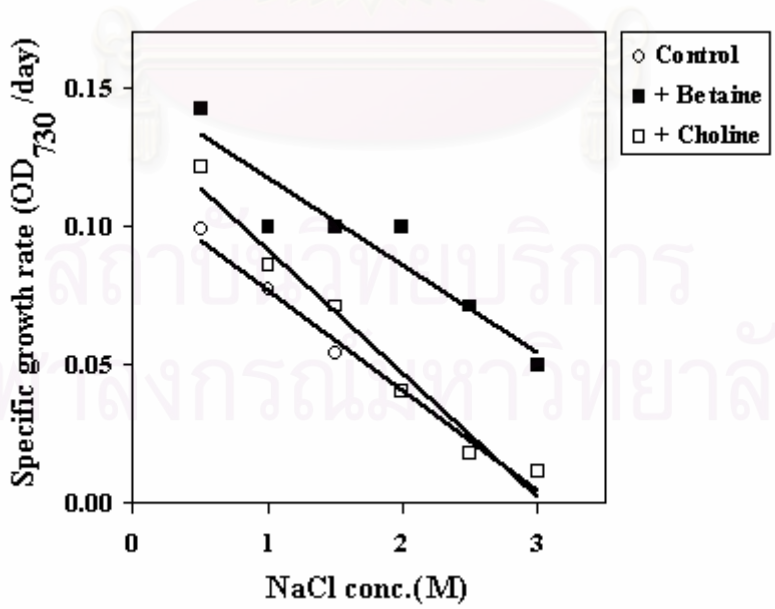


Figure 16 Specific growth rate of *A. halophytica* day-7 at control and with or without 1 mM betaine or 1mM choline.

3.3 Effects of salt stress on protein profiles at various fraction of *A. halophytica*

3.3.1 SDS-PAGE

To study the effects of salt stress on protein profiles at various fraction of *A. halophytica*, SDS-PAGE was used to identify proteins involved in salt stress response in the *A. halophytica*. Detection of proteins whose levels are altered by salt stress was also done by comparing patterns from control and salt-treated conditions. Cells were cultivated at normal condition (0.5 M NaCl), 1.0 M NaCl and 2.0 M NaCl with or without 1 mM betaine or choline. After 10 day, cells were collected and separated for periplasmic, cytoplasmic and membrane fraction by follow materials and methods. All fractions with condition containing 1 mM betaine or 1 mM choline showed similar pattern of condition without osmoprotectants (data not shown). Comparing the protein profiles in control and salt treated cells using SDS-PAGE revealed that salt treatment did induce significant changes in the pattern of proteins (Figure 17, 18, 19).

It was found that the intensity of the CP1 – CP8 protein band in cytoplasmic fraction (Figure 17A), the PP1 – PP4 protein bands in periplasmic fraction (Figure 18A) and the MB1 – MB8 protein bands in membrane fraction (Figure 19A) with condition with increasing NaCl concentration (1.0 and 2.0 M NaCl) were more higher intensity band than in normal condition (0.5 M NaCl). Furthermore, the enhanced expression of these proteins, which also existed in the control cells, were specifically increased and clearly observed in cells grown under saline condition. These results revealed that these proteins were expressed in specific regions of fractions adapted to salt stress.

The molecular weight of increasing intensity band under salt stress condition was determined by comparing with relative mobility (Rf) of molecular weight marker as shown in Figure 17B, 18B, and 19C, respectively. CP1 – CP8 of cytoplasmic fraction was calculated as 77.5, 47.0, 41.5, 38.5, 36.5, 32.0, 29.0, and 21.0 kDa. MW of PP1 – PP4 from

periplasmic fraction was 53.5, 35.5, 33.0, and 30.5 kDa. Membrane fraction showed MB1 – MB8 with MW 61.0, 41.5, 38.0, 27.5, 26.0, 23.5, 18.7, and 16.4 kDa, respectively.

3.3.2 Non-denaturing PAGE

The three fractions were analyzed by non-denaturing PAGE. When stained with protein staining (Figure 20), all of fractions showed increasing intensity band (arrow) when NaCl concentration was increased from 0.5 M NaCl to 1.0 M and 2.0 M NaCl (Figure 16A, 16B, 16C). Lane 5 – 8 showed autoradiogram of all samples by all fractions was incubated with hot choline for 1 hour at room temperature after that samples was analyzed by native PAGE and exposed on X-ray film for 10 days. Cytoplasmic fraction (Figure 20A lane 5 – 8) could not found signal band, one faint band was detected on periplasmic fraction (Figure 20B lane 5 – 8), and one major band was represented on membrane fraction (Figure 20C lane 5 – 8). Major band of membrane fraction showed high intensity when NaCl concentration was increased in the medium from 0.5 M to 1.0 M (Figure 20C lane 6, 7) and did not changed when concentration was increase from 1.0 M – 2.0 M NaCl (Figure 20C lane 7, 8). The faint band of periplasmic fraction (Figure 20B lane 6 – 8) might be appeared from contamination of membrane fraction. These results showed that protein from membrane fraction containing choline binding protein activity.

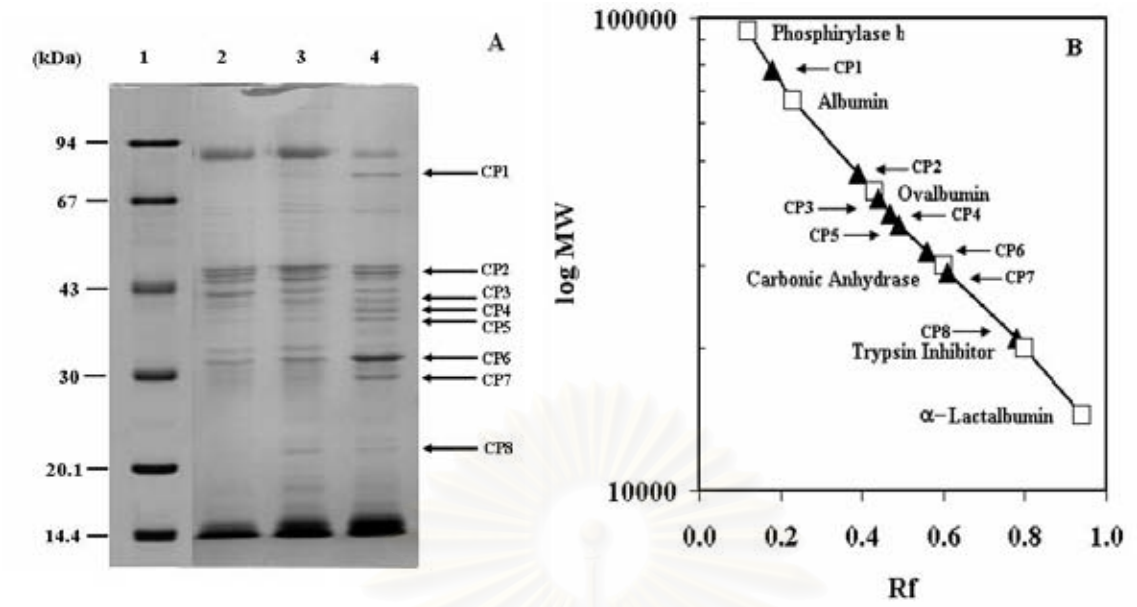


Figure 17 Effects of salt stress on protein profiles in cytoplasmic fraction of *A. halophytica*.

A) SDS-PAGE;

Lane 1 = molecular weight marker.

Lane 2, 3, and 4 = cytoplasmic fraction under medium containing 0.5 M, 1.0 M, and 2.0 M NaCl, respectively. CP1, CP2, CP3, CP4, CP5, CP6, CP7, and CP8 indicated the position of 77.5, 47.0, 41.5, 38.5, 36.5, 32.0, 29.0, and 21.0 kDa, respectively (20 µg protein/Lane).

B) Molecular weight calibration curve of standard protein on 10% SDS-PAGE.

[CP = cytoplasmic fraction, ▲ = cytoplasmic protein, □ = marker]

Phosphorylase B	MW = 94 kDa
BSA	MW = 67 kDa
Ovalbumin	MW = 43 kDa
Carbonic anhydrase	MW = 30 kDa
Soybean trypsin inhibitor	MW = 20.1 kDa
α-Lactalbumin	MW = 14.4 kDa

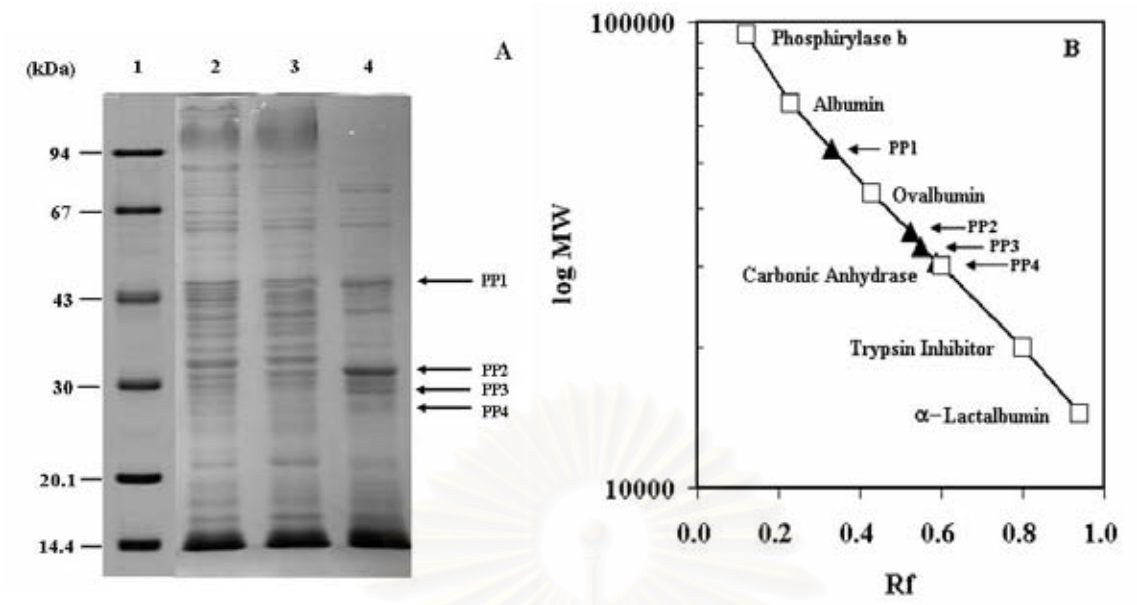


Figure 18 Effects of salt stress on protein profiles in periplasmic fraction of *A. halophytica*.

A) SDS-PAGE;

Lane 1 = molecular weight marker.

Lane 2, 3, and 4 = periplasmic fraction under medium containing 0.5 M, 1.0 M, and 2.0 M NaCl, respectively. PP1, PP2, PP3, and PP4 indicated the position of 53.5, 35.5, 33.0, and 30.5 kDa, respectively (20 µg protein/Lane).

B) Molecular weight calibration curve of standard protein on 10% SDS-PAGE.

[PP = periplasmic fraction, ▲ = periplasmic protein, □ = marker]

Phosphorylase B	MW = 94 kDa
BSA	MW = 67 kDa
Ovalbumin	MW = 43 kDa
Carbonic anhydrase	MW = 30 kDa
Soybean trypsin inhibitor	MW = 20.1 kDa
α-Lactalbumin	MW = 14.4 kDa

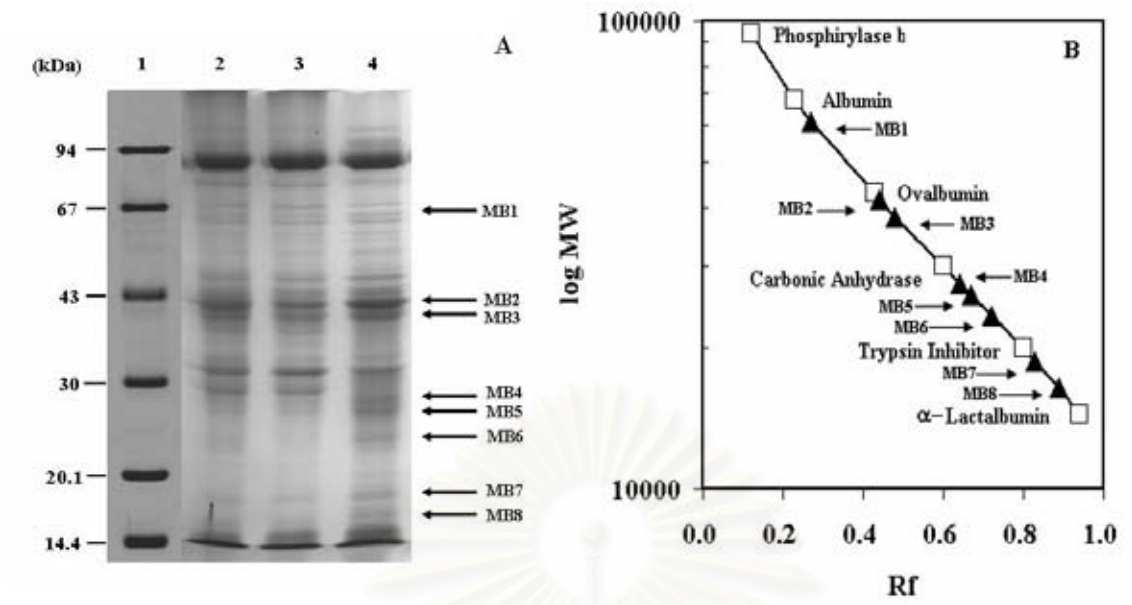


Figure 19 Effects of salt stress on protein profiles in membrane fraction of *A. halophytica*.

A) SDS-PAGE;

Lane 1 = molecular weight marker.

Lane 2, 3, and 4 = membrane fraction under medium containing 0.5 M, 1.0 M, and 2.0 M NaCl, respectively. MB1, MB2, MB3, MB4, MB5, MB6, MB7, and MB8 indicated the position of 61.0, 41.5, 38.0, 27.5, 26.0, 23.5, 18.7, and 16.4 kDa, respectively (20 µg protein/Lane).

B) Molecular weight calibration curve of standard protein on 10% SDS-PAGE.

[MB = membrane fraction, ▲ = membrane protein, □ = marker]

Phosphorylase B	MW = 94 kDa
BSA	MW = 67 kDa
Ovalbumin	MW = 43 kDa
Carbonic anhydrase	MW = 30 kDa
Soybean trypsin inhibitor	MW = 20.1 kDa
α-Lactalbumin	MW = 14.4 kDa

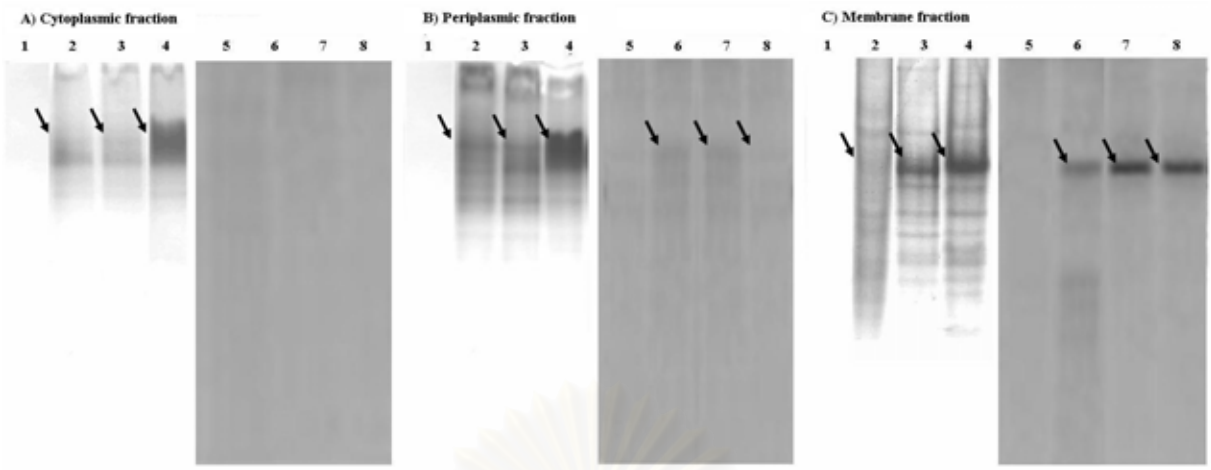


Figure 20 Non-denaturing PAGE and autoradiogram of fractions separated from *A. halophytica* (arrow showed observed band).

A) Cytoplasmic fraction;

Lane 1 no protein, Lane 2, 3 and 4 cells were cultured in medium containing 0.5, 1.0 and 2.0 M NaCl, respectively (20 µg protein/Lane), Lane 5 (autoradiogram) hot choline, and Lane 6, 7 and 8 (autoradiogram) cells were cultured in medium containing 0.5, 1.0 and 2.0 M NaCl, respectively.

B) Periplasmic fraction;

Lane 1 no protein, Lane 2, 3 and 4 cells were cultured in medium containing 0.5, 1.0 and 2.0 M NaCl, respectively (20 µg protein/Lane), Lane 5 (autoradiogram) hot choline, and Lane 6, 7 and 8 (autoradiogram) cells were cultured in medium containing 0.5, 1.0 and 2.0 M NaCl, respectively.

C) Membrane fraction;

Lane 1 no protein, Lane 2, 3 and 4 cells were cultured in medium containing 0.5, 1.0 and 2.0 M NaCl, respectively (20 µg protein/Lane), Lane 5 (autoradiogram) hot choline, and Lane 6, 7 and 8 (autoradiogram) cells were cultured in medium containing 0.5, 1.0 and 2.0 M NaCl, respectively.

3.4 Detection of choline binding activity

3.4.1 Radioisotope technique

Autoradiogram from Figure 20B and 20C showed that periplasmic and membrane fraction containing choline binding activity but membrane fraction represented higher activity than periplasmic fraction. We tested for choline binding activity by using all of fractions was incubated in buffer containing hot choline (20,000 CPM per reaction) and incubated at room temperature for 30 min or 60 min after that one volume of 10% TCA was added, mixed and placed for 30 min. The reaction mixture was centrifuged and pellet was determined by liquid scintillation counter (Figure 21). The membrane fraction showed highest choline binding activity at 30 min (Figure 21A) and 60 min (Figure 21B), activity was increased when NaCl concentration change from 0.5 M to 1.0 M but NaCl was higher than 1.0 M the activity was reduced. A little activity of choline binding activity was found in periplasmic fraction and not so much different when incubation was increased from 30 min to 60 min. The cytoplasmic fraction could not found choline binding activity (Figure 21). The results showed that major choline binding activity found in membrane fraction.

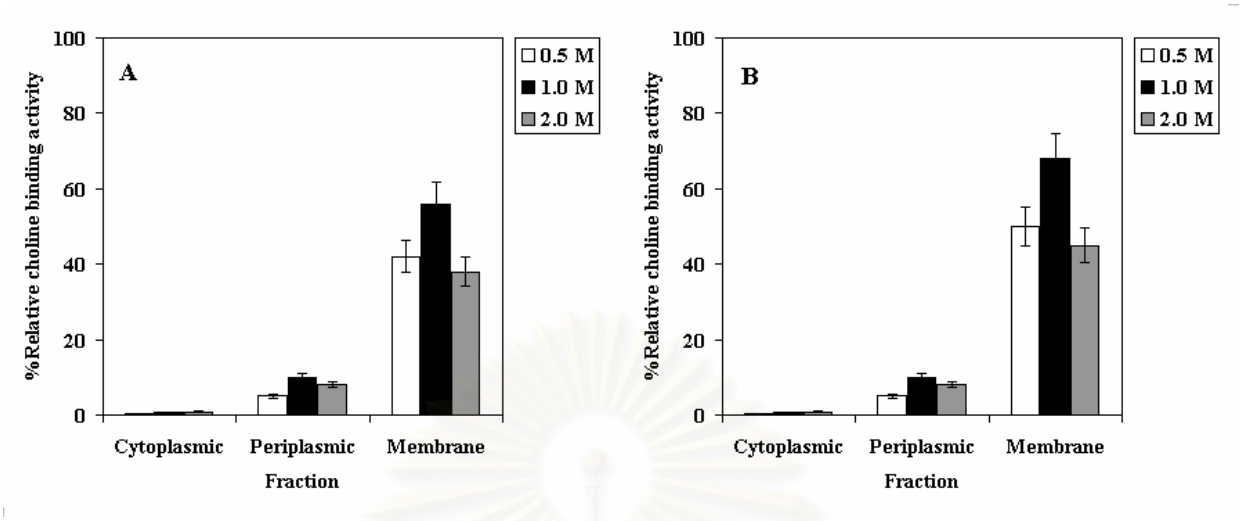


Figure 21 Chromatogram of choline binding activity. The reaction containing 20 µg protein/ ml and 10 µM hot choline (20,000 CPM) was incubated in medium containing 0.5 M, 1.0 M or 2.0 M NaCl at room temperature for 30 min after that one volume of 10% TCA was added, mixed and placed for 30 min (A) or 60 min (B). The reaction mixture was centrifuged and pellet was determined by liquid scintillation counter. Data are means from three independent experiments with vertical bars representing standard errors of the means.

3.4.2 Substrate specific for choline binding activity

The specificity of choline binding activity in membrane fraction of *A. halophytica* was studied by addition of 100-fold excess of various osmoprotectants into the assay medium and % choline binding activity or % inhibition as shown in Table 6. The choline analogue, acetylcholine, acted as an effective competitor for choline whereas phosphorylcholine did not. Betaine aldehyde, differing from choline by having an aldehyde group instead of an alcohol group, showed strong inhibition of choline uptake. However, glycine betaine and glycine with a carboxyl group were poor competitors for choline uptake. Proline, glutamate, aspartate, valine, and histidine were no effect for choline binding activity.

Table 6 Substrate specific for choline binding activity

Osmoprotectant	% Inhibition		
	0.5 M NaCl	1.0 M NaCl	2.0 M NaCl
Control*	0.00	0.00	0.00
Choline	78.66	84.74	71.57
Glycine betaine aldehyde	89.68	94.22	94.87
Betaine	85.71	100	100
Carnitine	64.33	69.79	74.52
Acetyl choline	56.06	43.65	39.78
Phosphoryl choline	52.55	39.49	31.68
Ethanolamine	37.43	29.68	30.20
Trimethylamine	26.74	25.61	25.97
Glycine	4.77	5.55	4.64
Proline	4.72	4.63	4.94
Glutamate	3.77	2.82	3.31
Aspartate	3.52	3.41	2.94
Valine	2.56	2.82	2.14
Histidine	6.59	7.63	7.86
Arginine	8.13	7.35	7.7
Lysine	11.75	10.73	12.57
Cysteine	6.87	7.13	8.52
Phenylalanine	7.68	9.85	8.53
Serine	4.38	3.99	2.74
Alanine	2.57	3.38	3.47

*condition without osmoprotectant

3.4.3 Surface Plasmon Resonance (SPR) technique

Surface plasmon resonance (SPR) has been determined the interactions of a variety of membrane fraction and analytes (for example, betaine, choline, amino acid, and their derivatives). This technique was used to test binding activity by no radioisotope. Figure 22 represents the sensorgram of 100 mM sodium phosphate buffer, pH 7.6 (Figure 22A) and varied analytes at 100 mM of tyrosine (Figure 22B), leucine (Figure 22C), proline (Figure 22D), asparagine (Figure 22E), betaine aldehyde (Figure 22F), carnitine (Figure 22G), betaine (Figure 22H), and choline (Figure 22I). The results show that membrane fraction no respond for buffer because both channels show the equal base line (Figure 22A). Membrane fraction can strongly react with betaine aldehyde, carnitine, betaine, and choline, respectively (Figure 22F – I). The response unit (angle shift) of this assay was shown in Table 7.

To investigate the significance of association between membrane fraction and betaine as well as choline, we varied concentration of analytes from 1 – 500 mM. Surface plasmon resonance detects molecular interactions, because there is a corresponding change in refractive index when a analyte binds to a immobilized membrane on the sensorchip. The response unit was increased when the concentration of analytes was increased, data as shown in Figure 23. The Sensorgram showed binding activity of choline (Figure 23B) or betaine (Figure 23A) on membrane fraction was rapidly association and rapidly disassociation. We plot the relationship between concentration and the rate of binding at highest response unit as shown in Figure 24. Km for betaine, choline, carnitine, and betaine aldehyde is 80, 120, 130, and 160 mM, respectively.

Table 7 The response unit of membrane fraction and varied analytes.

Analyte[a]	Angle shift (m^o)
Control[b]	0
Choline	1,250
Glycine betaine aldehyde	1,100
Betaine	1,250
Carnitine	1,000
Acetyl choline	850
Phosphoryl choline	725
Ethanolamine	545
Trimethylamine	350
Glycine	235
Proline	360
Glutamate	300
Aspartate	225
Valine	220
Histidine	300
Arginine	225
Lysine	250
Cysteine	200
Phenylalanine	350
Serine	300
Alanine	225

[a] All of analytes use 100 mM in 100 mM sodium phosphate buffer, pH 7.6

[b]100 mM sodium phosphate buffer, pH 7.6

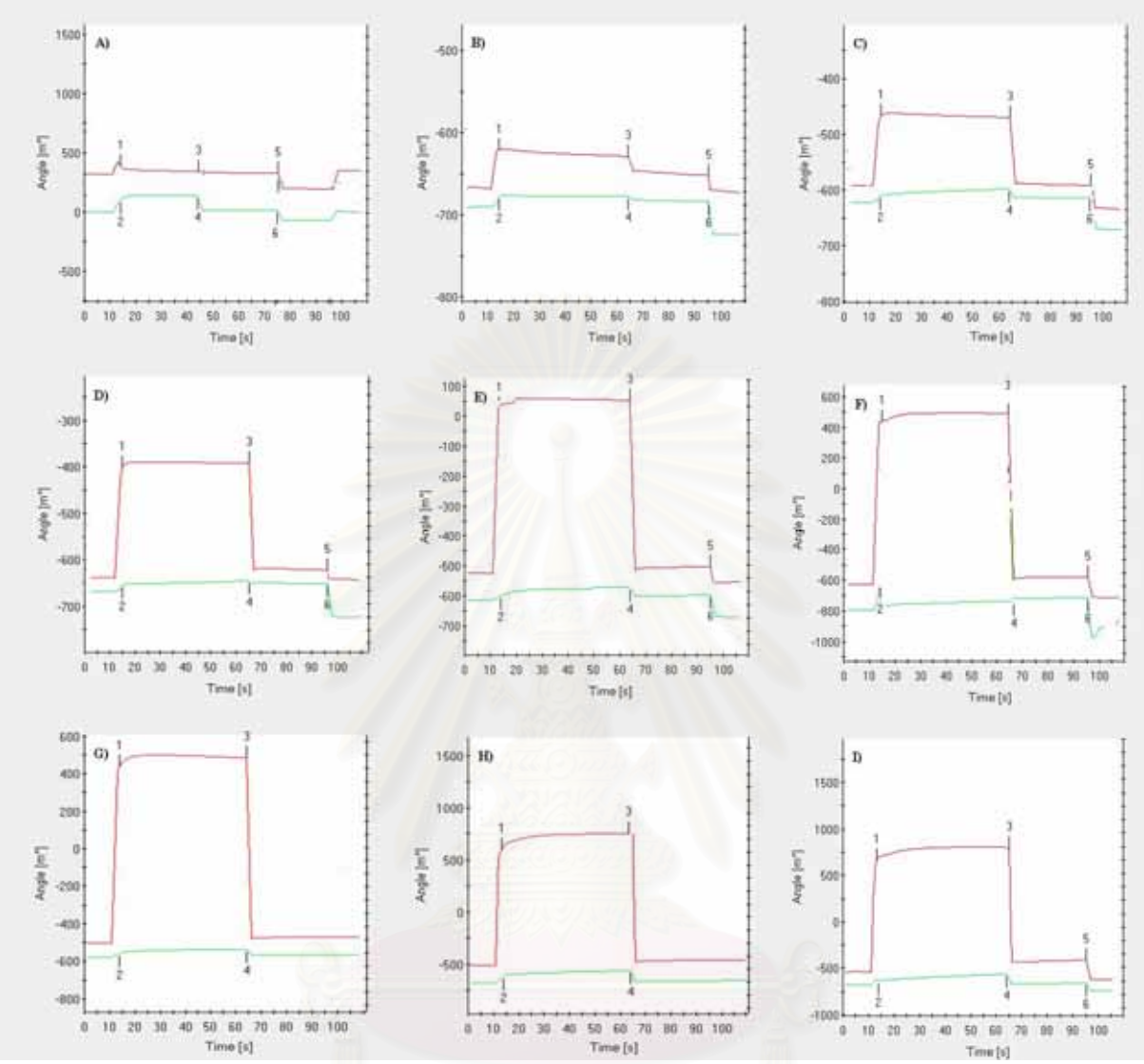


Figure 22 Sensorgrams of binding activity between membrane fraction and analytes. A – I show the results of buffer and 100 mM analytes; tyrosine, leucine, proline, asparagines, betaine aldehyde, carnitine, betaine, and choline, respectively. Top line is channel 1 (control) and bottom line is channel 2 (analyte).

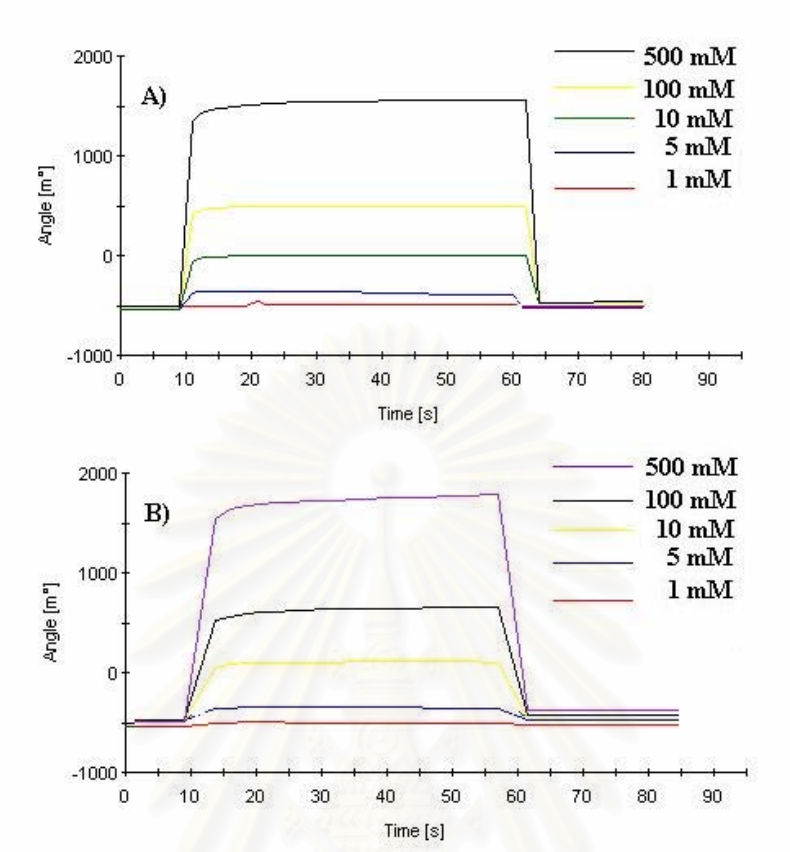


Figure 23 Sensorgrams of binding activity between analytes, betaine (A) and choline (B) on immobilized membrane fraction. The signal lines from bottom to top were various concentrations of analytes 1 – 500 mM.

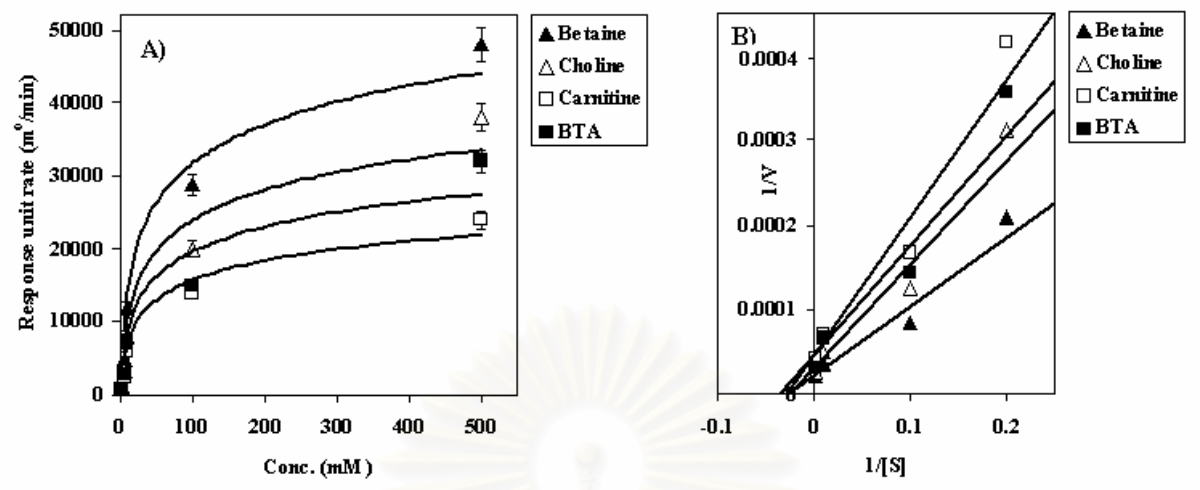


Figure 24 Effects of analyte's concentration on binding protein activity on membrane fraction. A) Saturation curve and B) Lineweaver-Burk plot.

3.5 Determination of [methyl-^{14}C] choline uptake system

3.5.1 Uptake of [methyl-^{14}C] choline into *A. halophytica*

A. halophytica were grown in modified BG_{11} medium containing 0.5 M, 1.0 M or 2.0 M NaCl for 7 days. Cultures were centrifuged and resuspended in buffer containing 0.5 M, 1.0 M, and 2.0 M NaCl, respectively. The uptake of choline was assayed in osmotic stress condition. We therefore measured the initial rates of choline uptake in cells at control and stress conditions at 0.5 M, 1.0 M, and 2.0 M NaCl, using [methyl-^{14}C] choline (final concentration at 50 µM) as a substrate. There was substantial choline uptake of *A. halophytica* in osmotic stress condition, and this choline uptake rate was 1.7, 2.5 and 1.3 nmol/min/mg protein at 0.5 M, 1.0 M and 2.0 M NaCl, respectively (Figure 25).

3.5.2 Effect of various osmoticums and NaCl on choline uptake

A high rate of choline uptake was dependent on energization of cells with osmoticum, such as mannitol, sorbitol, and sucrose. Choline uptake was also stimulated by various osmoticums with the same osmolarity of stress condition. There was hardly any difference in stimulation whether the osmolarity was increased with mannitol, sorbitol and sucrose, indicating that the osmolarity determined the uptake rate (Figure 26). Choline uptake was dependent on Na^+ and 1.0 M NaCl showed maximum uptake rate. Of the concentration of Na^+ tested higher than 1.0 M Na+ represented reduction of choline uptake and at 4.0 M NaCl choline could not uptake.

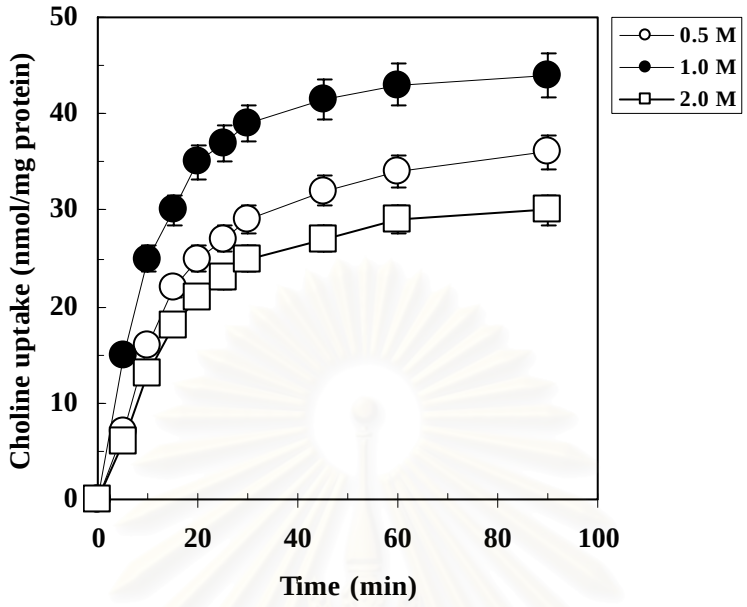


Figure 25 Time course of choline uptake into *A. halophytica* at 0.5 M, 1.0 M, and 2.0 M NaCl. Data are means from three independent experiments with vertical bars representing standard errors of the means.

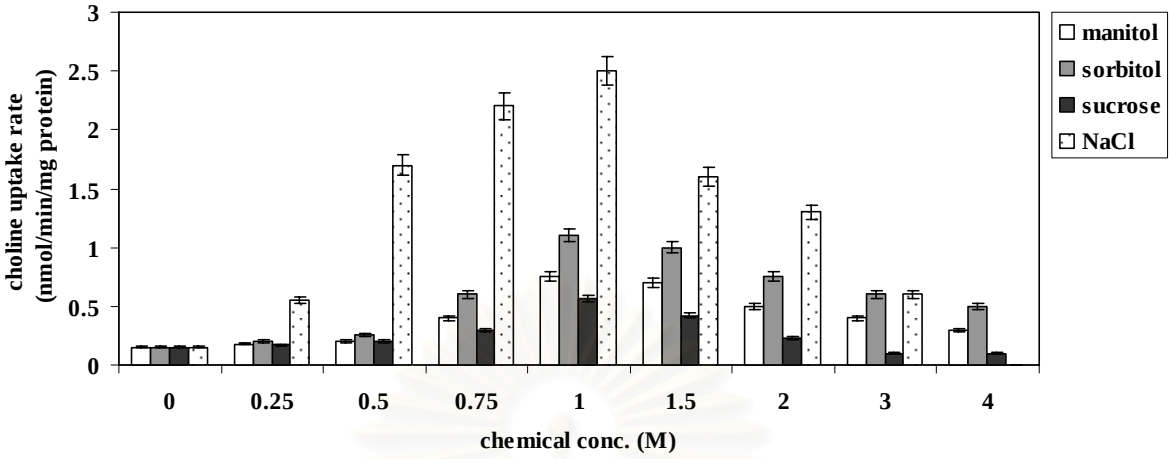


Figure 26 Effect of osmoticum and NaCl on choline uptake. Data are means from three independent experiments with vertical bars representing standard errors of the means.

3.5.3 Kinetics of choline uptake

We also determined the concentration needed to saturate the uptake systems of osmotic stress condition on the rate of choline uptake by *A. halophytica*. When cells were assayed with 50 µM [methyl-^{14}C] choline uptake was linear for 10 min. Initial rates were determined over a wide range of choline concentrations (0 – 5 mM). The choline uptake system was saturable and displayed typical Michalis-Menten type kinetics (Figure 27). The lineweaver-Burk Transformation of the data under these conditions, the line of best fit was performed by using a least squares linear regression, the apparent Km values for control (0.5 M NaCl) and stress condition at 1.0 M and 2.0 M NaCl were 246.78, 262.19, and 299.51 µM, respectively, the maximum velocity (Vmax) was 12.12, 24.10, and 10.71 nmol/min/mg protein, respectively.

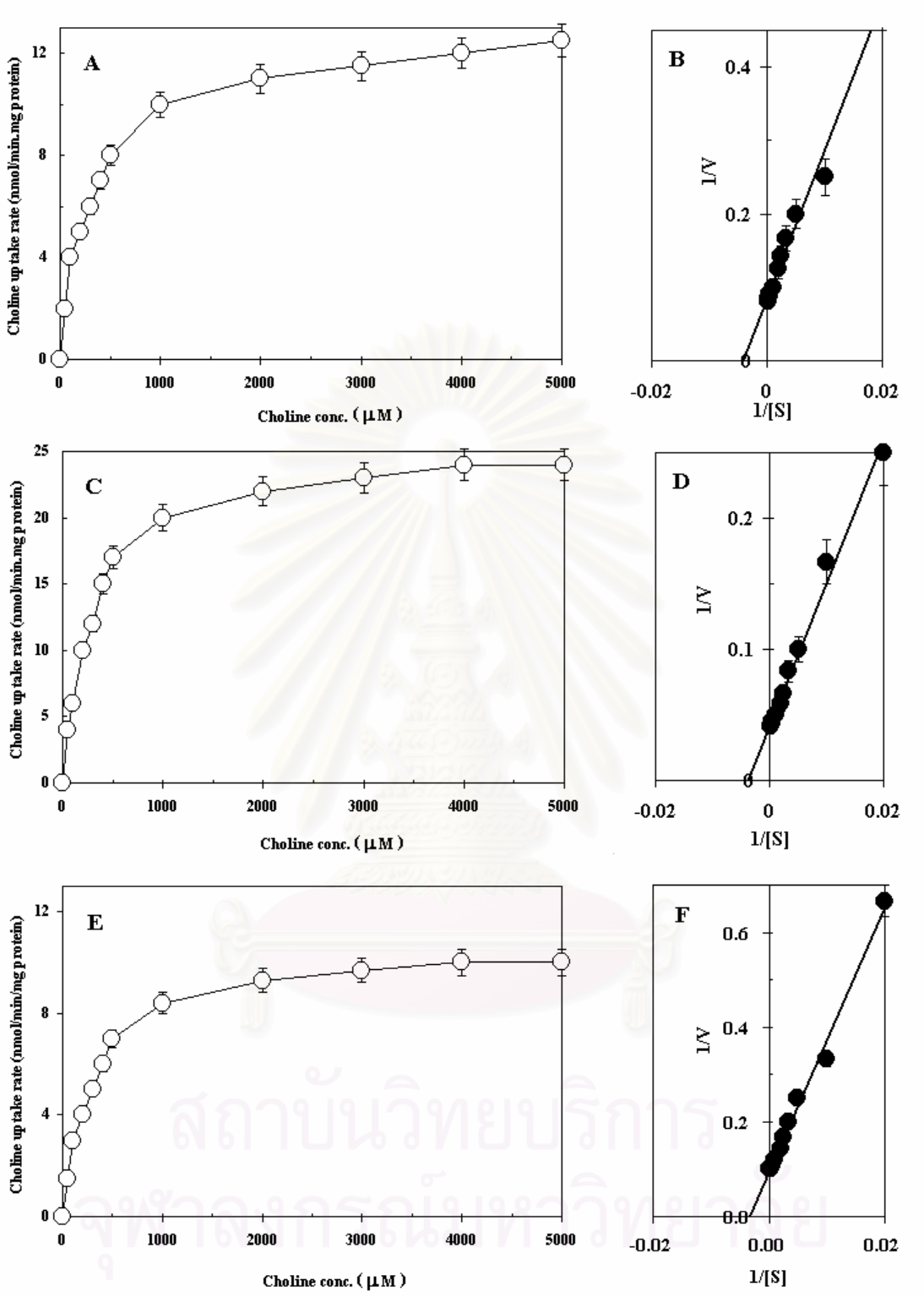


Figure 27 Kinetic of choline uptake. A, C, and E showed saturation curve of choline uptake at 0.5 M, 1.0 M, and 2.0 M NaCl, respectively. B, D, and F showed Lineweaver-Burk plot of choline uptake at 0.5 M, 1.0 M, and 2.0 M NaCl, respectively. Data are means from three independent experiments with vertical bars representing standard errors of the means.

3.5.4 Substrate specificity of choline uptake

The specificity of the system was studied by observing the initial rate of [methyl-^{14}C]choline uptake in the presence of 100 folds excess of unlabeled compounds (Figure 28). At normal condition (0.5 M NaCl), the choline analogue, betaine, betaine aldehyde (intermediate in the oxidation of choline to glycine betaine), carnitine, acetylcholine, was effective competitor for choline uptake. Of the osmoregulatory molecules tested, L-proline did not compete with choline. However, phosphoryl choline, Trimethylamine, and ethanolamine showed compete for choline uptake. Although the basic amino acid (arginine and lysine) or anionic amino acid (glutamate and aspartate) slightly reduced choline uptake rate. At 1.0 M NaCl; choline, betaine, and betaine aldehyde showed completely inhibition for choline uptake and 2.0 M NaCl betaine and betaine aldehyde showed completely inhibition for choline uptake.

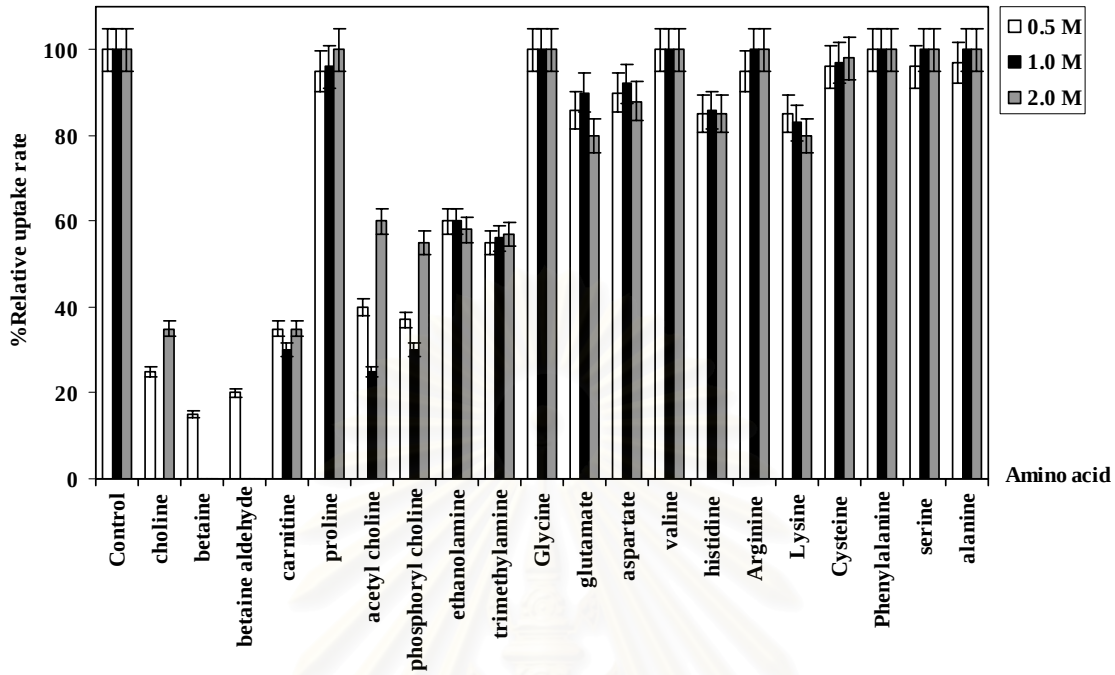


Figure 28 Substrate specific of choline uptake at normal and stress condition. Data are means from three independent experiments with vertical bars representing standard errors of the means.

3.5.5 Effect of cation and anion on choline uptake

Ions and some osmotic chemical were tested for the ability to act as a coupling ion for [^{14}C]choline transport (Figure 29). The ions Rb^+, Li^+, Cs^+, K^+, NH_4^+, Ca^{2+}, and Mg^{2+} were ineffective in driving [^{14}C]choline transport (Figure 29A). Almost anions were not effective for choline uptake except HCO_3^- and CO_3^{2-} had effected on choline uptake (Figure 29B).

3.5.6 Effects of energy source on choline uptake

To assess the roles of ATP and proton motive force in energizing the uptake of nitrate, *A. halophytica* cells were starved to deplete endogenous energy sources. Choline uptake was then monitored after reenergization with glucose or lactate (Table 8). Both glucose and lactate could energize the uptake of choline in the starved cells at normal and stress condition. These results indicate that choline uptake is an energy-dependent process and depends on the proton motive force. The respiratory inhibitor, KCN, strongly inhibited nitrate uptake, either alone or together with glucose or lactate.

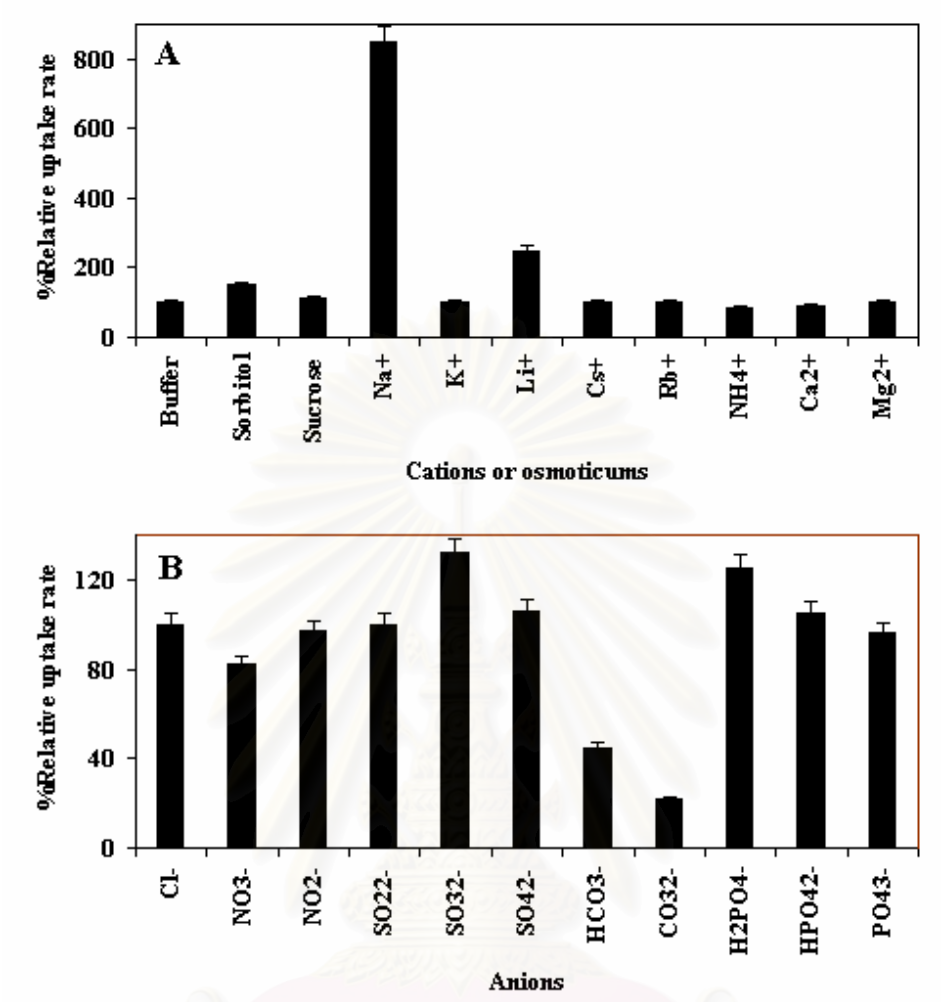


Figure 29 Effect of cation (A) and anion (B) on choline uptake. The reaction mixture contained 50 mM Hepes-OH buffer, pH 7.6 containing 0.5 M cation or anion. For cation experiment, buffer lacked osmoticum or cation was control condition (100%) and for anion experiment, buffer containing 0.5 M NaCl used as control condition (100%). Data are means from three independent experiments with vertical bars representing standard errors of the means.

Table 8 Effect of energy sources on choline uptake[a] and ATP level[b]

Addition	Choline uptake (%)			ATP level (%)		
	0.5 M NaCl	1.0 M NaCl	2.0 M NaCl	0.5 M NaCl	1.0 M NaCl	2.0 M NaCl
None	100	100	100	100	100	100
ATP						
– 50 μM	100	100	100	125	125	125
– 100 μM	100	100	100	144	145	145
Glucose (20 mM)	120	135	105	136	135	130
Lactate (10 mM)	114	126	103	126	123	122
KCN (1 mM)	22	20	17	15	15	15
Glucose + KCN	26	22	20	18	17	17
Lactate + KCN	27	24	22	18	18	17

[a]Cells were starved by suspending cells in the growth medium lacking carbon and nitrogen source in the dark for 24 h. The starved cells were then assayed for choline uptake in the presence of different energy sources or respiratory inhibitor. Starved cells were preincubated with the tested compound(s) in the dark for 30 min before the addition of 50 μM hot choline to initiate the uptake as described in Materials and Methods. 100% control condition means choline uptake rate was 1.7, 2.5 and 1.3 nmol/min/mg proteins at 0.5 M, 1.0 M, and 2.0 M NaCl, respectively and 100% control ATP content was 20.7, 15.3, and 12.4 nmol/mg proteins at 0.5 M, 1.0 M, and 2.0 M NaCl, respectively and

[b]Cells were starved by suspending cells in the growth medium lacking carbon and nitrogen source in the dark for 24 h. The starved cells were then assayed for ATP in the presence of different energy sources or respiratory inhibitor. Starved cells were preincubated with the tested compound(s) in the dark for 30 min after that cells were broken and ATP was determined as described in Materials and Methods

3.5.7 Effects of pH on choline uptake

The rate of choline uptake was studied on pH values. The pH range was varied from 5 – 10. The maximum choline uptake was at pH 8.0 and lowest levels of uptake at pH 5. At 2.0 M NaCl concentration could not uptake choline, data as shown in Figure 30.

3.5.8 Effects of various inhibitor on choline uptake

The effects of various agents that disrupt protein structure, ATPase activity, proton motive force and Na^+ electrochemical gradients on the initial rate of [methyl-^{14}C]choline via osmotic stress condition were studied, data as shown in Figure 31 – 34. The results were shown in Figure 31, the inhibitors KCN (Figure 31A), NaN3 (Figure 31B), NaF (Figure 31C), Na-arsenate (Figure 31D) that affect electron transport chain and ATP synthesis, all inhibited choline uptake and decreased ATP level in both 0.5 M and 2.0 M NaCl in medium. NaF showed slightly inhibition choline uptake and ATP level but strongly inhibited by KCN, NaN_3, and Na-arsenate at both condition. Figure 32 showed that affect of Amiloride (Na^+/H^+ antiporter inhibitor), Monensin (dissipate Na^+ gradient), N-ethylmaleimide and sodium p-chloromercurobenzoate that affect protein structure. The choline uptake and ATP level were decreased about fifty percentages when amiloride and monensin were presented (Figure 32A and 32B). N-ethylmaleimide and sodium p-chloromercurobenzoate were completely inhibited choline uptake and ATP level (Figure 32C and 32D). Gramicidin D (Na^+ gradients) inhibited choline uptake and ATP level in both control and stress systems (Figure 33A). Membrane potential was inhibited by valinomycin in both conditions (Figure 33B). The inhibitors DNP and CCCP showed completely inhibition on choline uptake and ATP level, data as shown in Figure 33C and 33D. We investigated the inhibitors of chloramphenicol on choline uptake by blocking induction with chloramphenicol, a protein synthesis inhibitor. Chrloramphenicol showed reduction of

choline uptake activity (data not shown). The inhibitor of photosystem I, DCMU showed slightly inhibit on choline uptake and ATP level (data not shown). The dissipate proton gradient, nigericin showed completely inhibited choline uptake and ATP level (Figure 34A). DCCD (ATP synthase inhibitor) showed completely inhibit ATP level and strongly inhibited choline uptake in both condition (Figure 34B). The ATPase inhibitors, vanadate and ouabain showed completely inhibit ATP level and inhibited choline uptake (Figure 34C and 34D).

3.5.9. Energy dependent

Cells were suppressed in 100 mM potassium acetate buffer pH 5.5 and 7.0 or ethanolamine buffer pH 8.2 (Figure 35A). The results showed that cells could not uptake choline at all pH and found a little uptake when pH was increased and presented with 100 mM NaCl. To demonstrate that sodium was driven force for choline uptake, we generated choline transport by diluted potassium acetate loaded cell with Tris-buffer or Tris-buffer containing 100 mM NaCl, the rate of choline uptake was increased when NaCl was presented at all pH (Figure 35A). To determine that choline was transported into the cell under potassium phosphate buffer at varied pH. The choline uptake was increased when pH was increased (Figure 35B). Cells (K loaded) diluted into Tris-buffer or Tris-buffer containing 100 mM NaCl transported choline at a higher pH showed more uptake than lower pH.

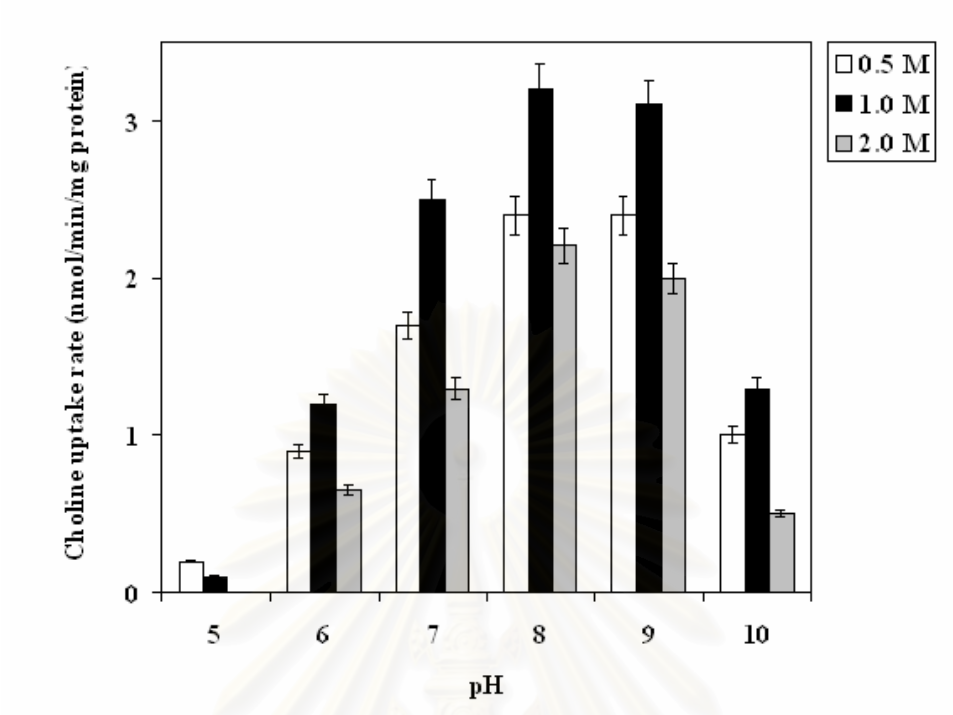


Figure 30 Effects of pH on choline uptake into *A. halophytica*. *A. halophytica* was grown in BG_{11} medium containing various NaCl concentrations from 0.5 M, 1.0 M, and 2.0 M for 7 days. After that cells were centrifuged and resuspended in new BG11 medium containing various NaCl concentration and pH 5 – 10. Data are means from three independent experiments with vertical bars representing standard errors of the means.

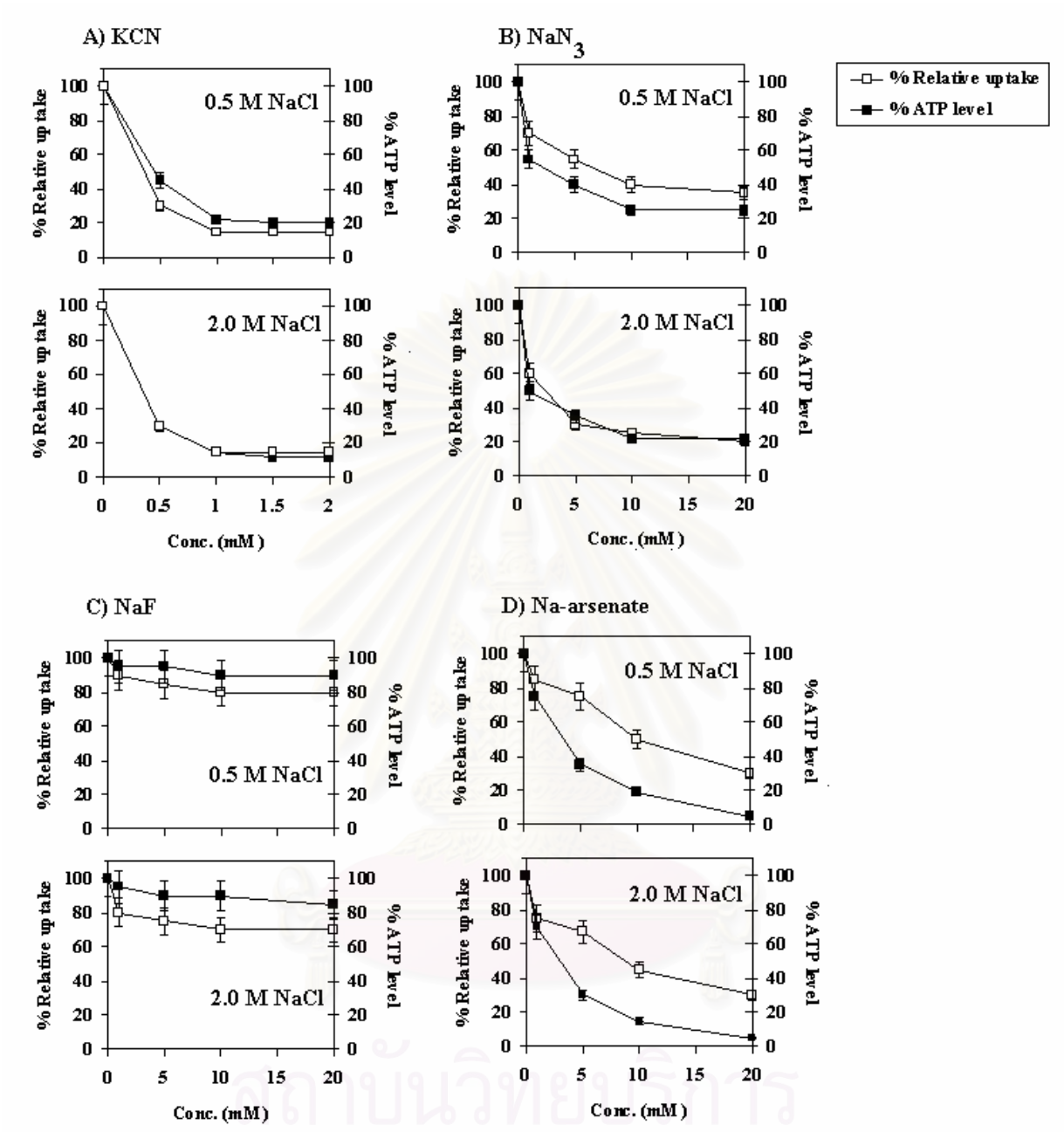


Figure 31 Effects of inhibitor on choline uptake into *A. halophytica*. A, B, C, and D showed affect of KCN, NaN_3, NaF, and Na-arsenate, respectively. Data are means from three independent experiments with vertical bars representing standard errors of the means.

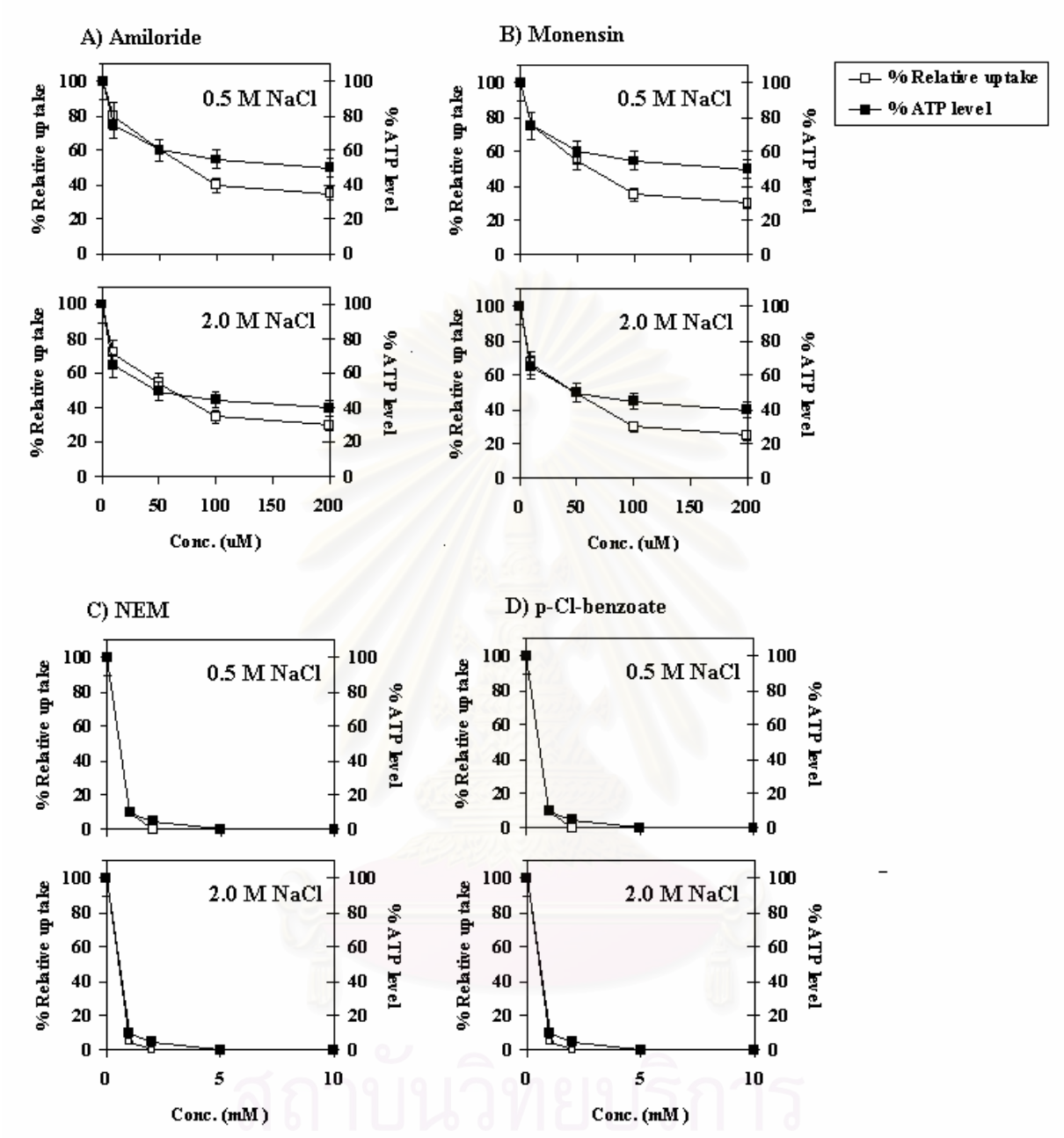


Figure 32 Effects of inhibitor on choline uptake into *A. halophytica*. A, B, C, and D showed affect of amiloride, monensin, NEM, and p-chloromercurobenzoate (p-Cl-benzoate), respectively. Data are means from three independent experiments with vertical bars representing standard errors of the means.

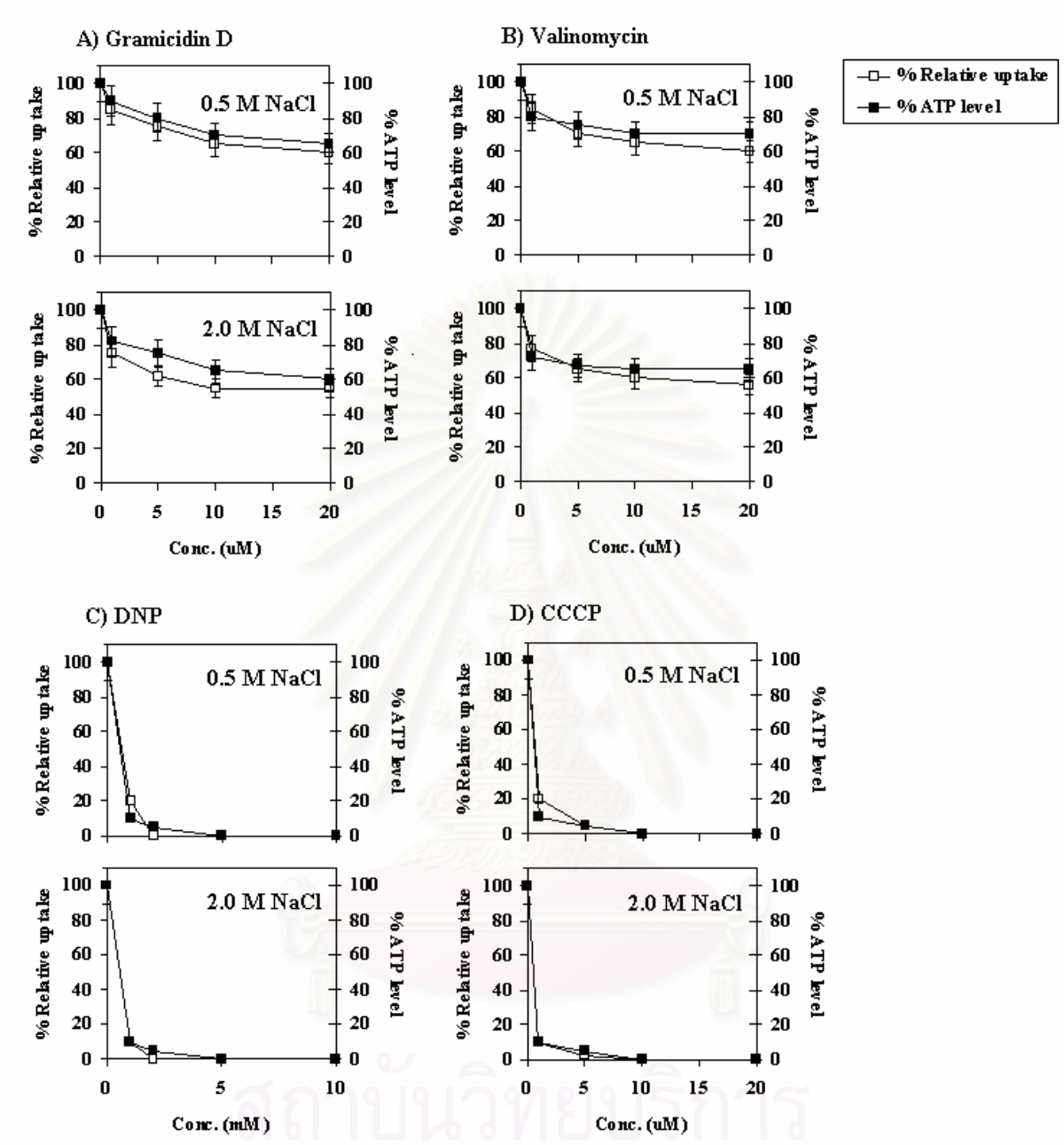


Figure 33 Effects of inhibitor on choline uptake into *A. halophytica*. A, B, C, and D showed affect of Gramicidin D, valinomycin, DNP, and CCCP, respectively. Data are means from three independent experiments with vertical bars representing standard errors of the means.

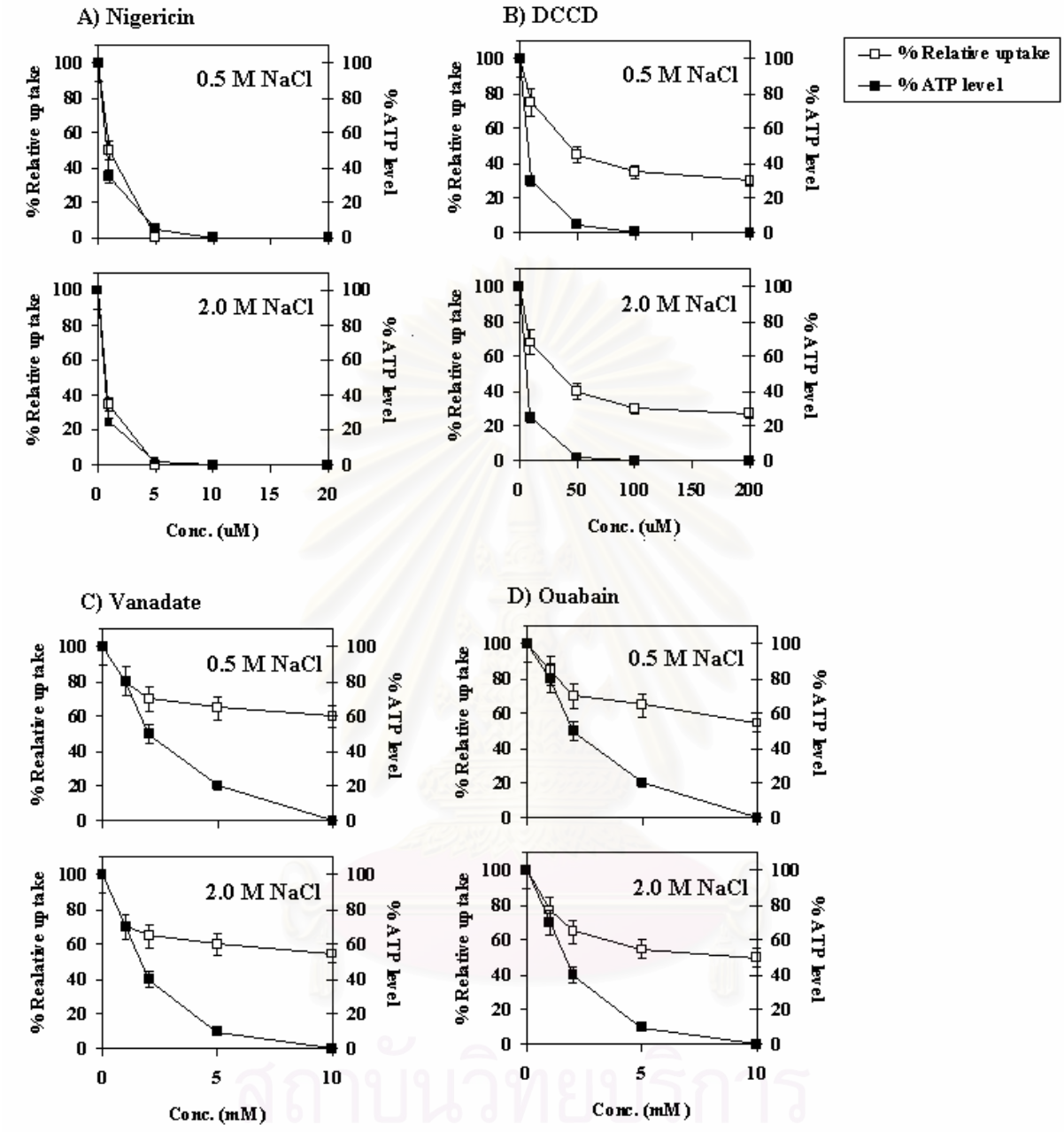


Figure 34 Effects of inhibitor on choline uptake into *A. halophytica*. A, B, C, and D showed affect of nigericin, DCCD, vanadate, and ouabain, respectively. Data are means from three independent experiments with vertical bars representing standard errors of the means.

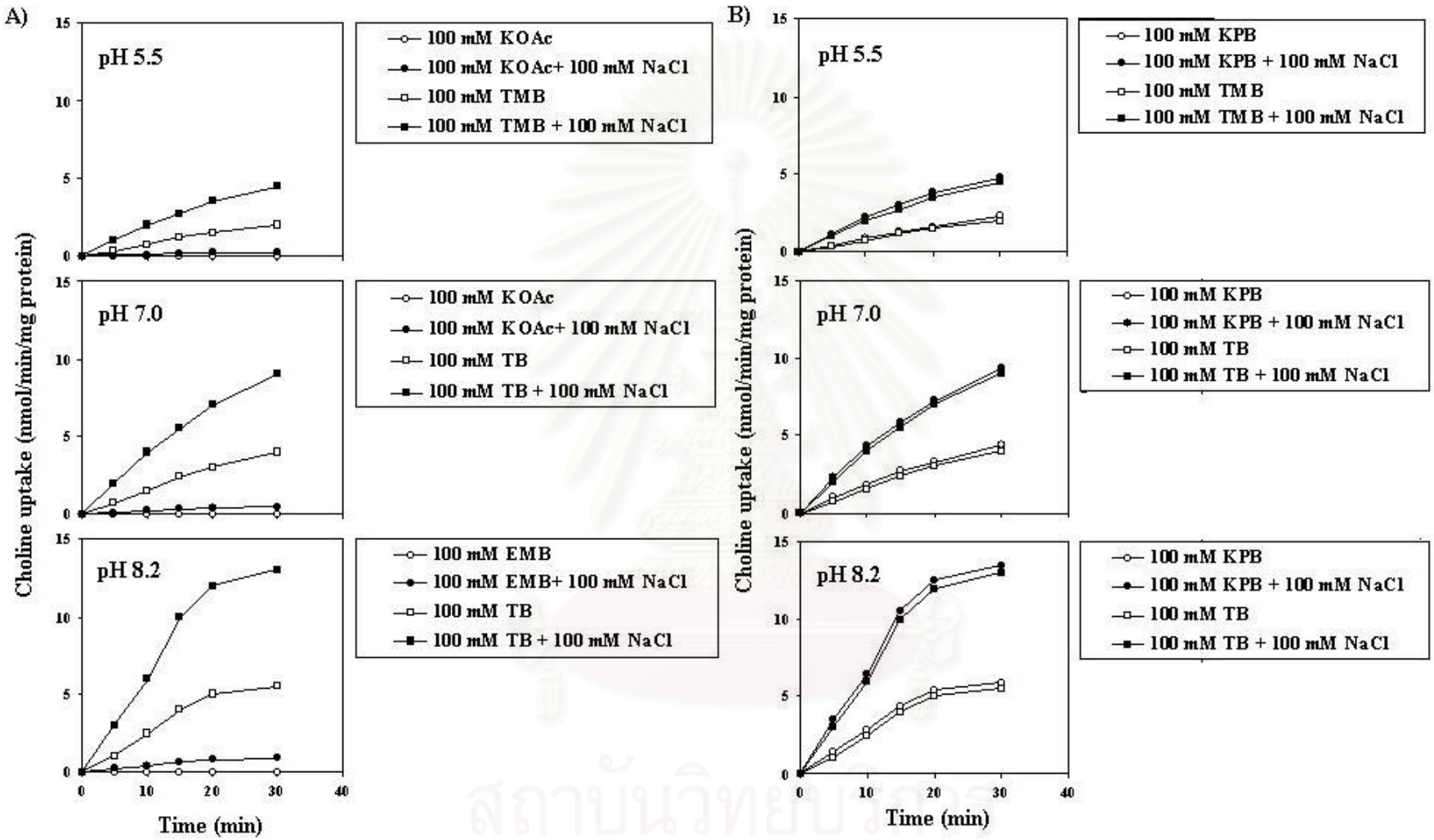


Figure 35 Chromatogram of artificial proton gradient and membrane potential. A) artificial proton gradient by cells were supposed with KOAc or EMB at pH 5.5, 7.0, and 8.2 and energized by Tris-Cl buffer or Tris containing 100 mM NaCl. B) artificial membrane potential by cell were loaded with KPB and generated choline uptake in Tris or Tris buffer containing 100 mM NaCl.

3.5.10 Role of Δψ on nitrate uptake at different pHs.

To investigate whether another component of the proton motive force, Δψ, contributed the driving force for choline uptake, we artificially generated Δψ either by supplementation of KSCN or by valinomycin in the absence of K^+ and then monitored the uptake of choline. Table 9 shows that at the three pHs tested, the rates of choline uptake did not increase under the influence of Δψ by the addition of 20 mM KSCN to the medium (cell inside is more negative than the control). Indeed, a slight decrease of choline uptake was observed. Likewise, the generation of Δψ by an imposition of an outwardly-directed, valinomycin-mediated (in the absence of K^+) potassium diffusion gradient caused no increase of the rates of choline uptake at the three pHs. On the other hand, the dissipation of Δψ by the addition of valinomycin in the presence of K^+ resulted in a modest stimulation of the rates of choline uptake at the three pHs assayed (Table 9). Taken together, these results indicated that Δψ constituted little or no contribution for the energization of nitrate uptake in *A. halophytica*.

Table 9 Effect of $\Delta\psi$ on choline uptake at different pHs[a]

pH	Choline uptake (μmol.min^{-1}.mg^{-1} protein)			Choline uptake (μmol.min^{-1}.mg^{-1} protein)	
	Control	$\Delta\psi$ generated by		Control	$\Delta\psi$ dissipated by
		KSCN	Valinomycin (- K^+)		Valinomycin (+ K^+)
5.5	0.12 ± 0.01	0.10 ± 0.03	0.12 ± 0.07	0.13 ± 0.04	0.14 ± 0.03
7.0	0.18 ± 0.03	0.15 ± 0.04	0.16 ± 0.06	0.20 ± 0.08	0.22 ± 0.07
8.2	0.25 ± 0.02	0.22 ± 0.06	0.24 ± 0.08	0.24 ± 0.05	0.29 ± 0.06

[a] For KSCN and valinomycin (-K^+) experiments, 100 mM Tris-maleate buffer was used at pH 5.5 and 100 mM Tris-HCl buffer was used at pH 7.0 and 8.2. For valinomycin (+K^+) experiments, 100 mM potassium phosphate buffer was used for the three pHs. Data are means ± standard errors of the means (n = 3).

3.5.11 Detection of products

Thin layer chromatography (TLC) was performed on siligar plate. The running solvent was chloroform: methanol: 0.1 M HCl (65:30:4 v/v) until solvent end at solvent front. The Dragendorff's reagent was used to detect the analytes. The results gave approximate Rf values and color spots for standard choline (red) and standard betaine (orange) which were 0.33 and 0.54, respectively (Figure 36A). At interval time of ^{14}C choline uptake, cells were extracted with boiling methanol and separated analytes by centrifugation. Supernatants was ran by TLC and exposed on X-ray film or detected beta activity by beta scanner, data as shown in Figure 36B and 36C. The results showed that choline as osmoprotectant because it was not changed to other products within 60 min (Figure 37) and could not detect [^{14}C] CO2 when the reaction was reacted with NaOH within 60 min (data not shown).

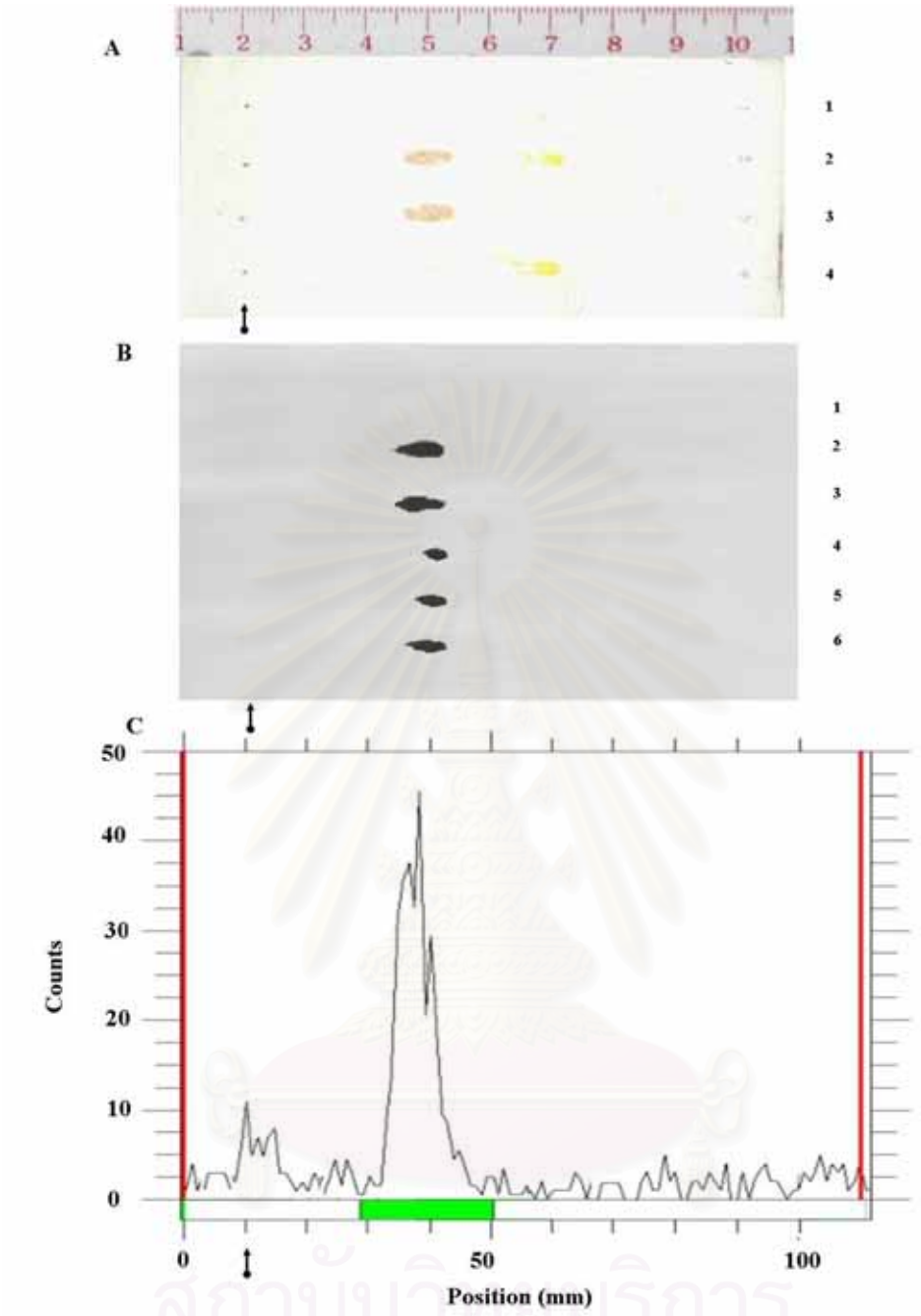


Figure 36 Thin layer chromatography (TLC), autoradiogram, and radiochromatogram of choline and choline product. A) TLC showed betaine and choline standard, lane 1 (no addition), lane 2 (mixture of choline and betaine), lane 3 (choline), and lane 4 (betaine); B) X-ray film of choline standard and uptake time, lane 1 (no addition), lane 2 and 3 (^{14}C-choline), and lane 4 – 6 (uptake at time 5, 10, 20 min, respectively). C) Radiochromatogram of ^{14}C choline was detected by beta scanner (arrow indicated origin position).

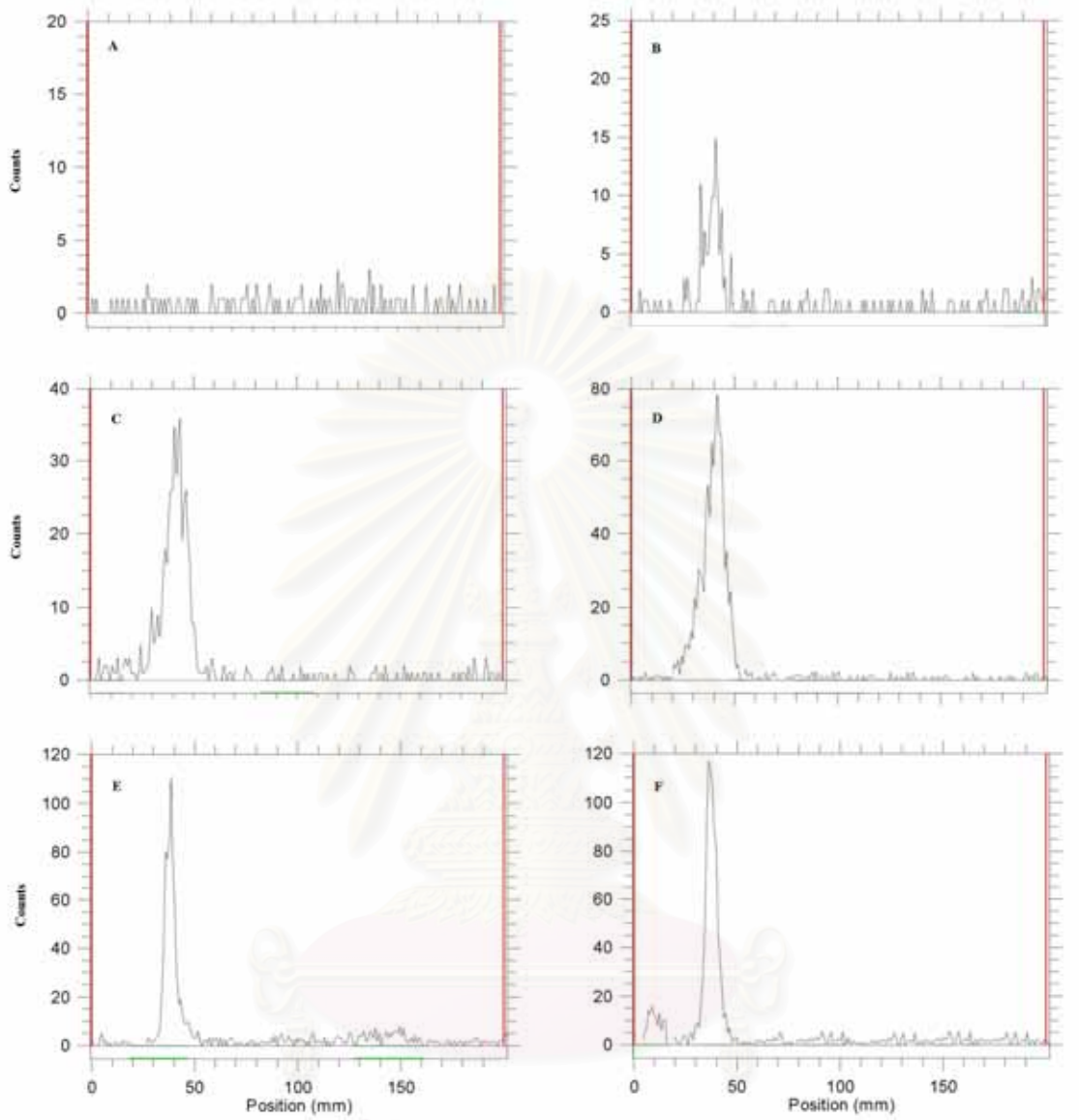


Figure 37 The radiochromatogram of choline after uptake into the cell at varied time. A, B, C, D, E, and F is uptake at 0, 5, 10, 20, 30, 60 min, respectively.

3.6 Uptake of choline uptake in membrane vesicles

3.6.1 Time course of choline uptake into membrane vesicles

Data above showed that choline transported in susceptible *A. halophytica* cells was energy dependent. Uptake was inhibited by the presence of CCCP or DNP, whereas significant uptake occurred when CCCP or DNP was presented in the medium. The clearly indicated that choline transport was driven by a proton motive force. The effects of ionophores on choline uptake in the presence of valinomycin were investigated. Valinomycin itself did not affect the uptake. Nigericin, which specifically cancels ΔpH, inhibited choline transport in a manner similar to that of CCCP or DNP. In contrast, valinomycin, which specifically cancels membrane potential, did not inhibit transport. These results indicated that choline transport depends on ΔpH but not membrane potential. The dependency of choline accumulation on ΔpH was confirmed by the results of an experiment with various medium pHs, i.e., the higher the pH of the medium, the higher the transport of choline. This result also suggests that the molecule taken up through the membrane is a protonated form of choline. We tested inhibition of transport by using inhibitors on sodium dissipation, amiloride, monensin, and gramicidin D showed that strongly inhibit choline uptake. The transport of choline depended on ΔpH and supported driven by sodium gradient.

Membrane vesicles were separated from *A. halophytica* and suspended in 100 mM potassium phosphate buffer, pH 7.0 containing 10 mM Mg_2SO_4 and 1 mM phenanzine methanosulfate (PMS). The initial rate of choline uptake was measured as shown in Figure 38. After that we tested choline uptake in membrane vesicles at various NaCl concentration (0 – 3,000 mM), using [methyl-^{14}C] choline (final concentration at 50 µM) as a substrate (Figure 39).

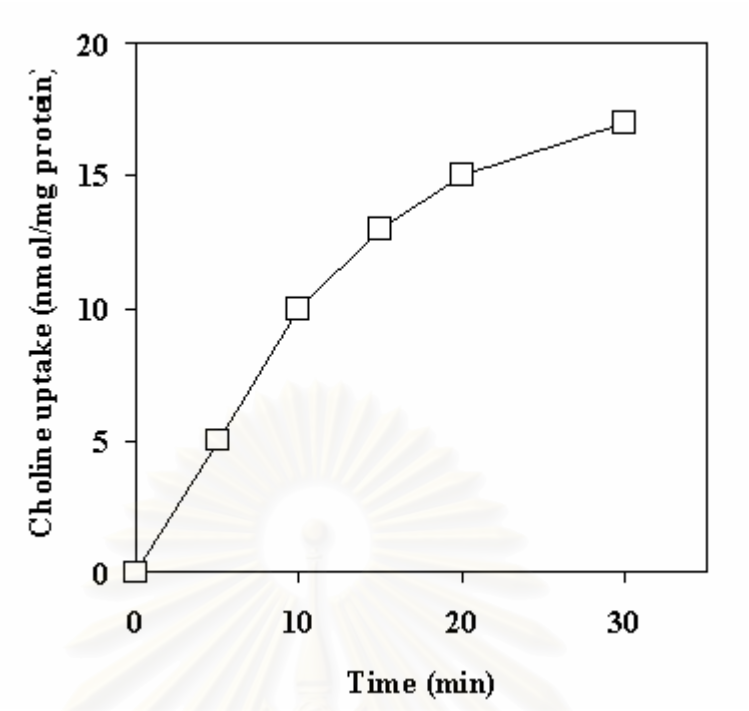


Figure 38 Time course of choline uptake into membrane vesicles at condition lacked NaCl.

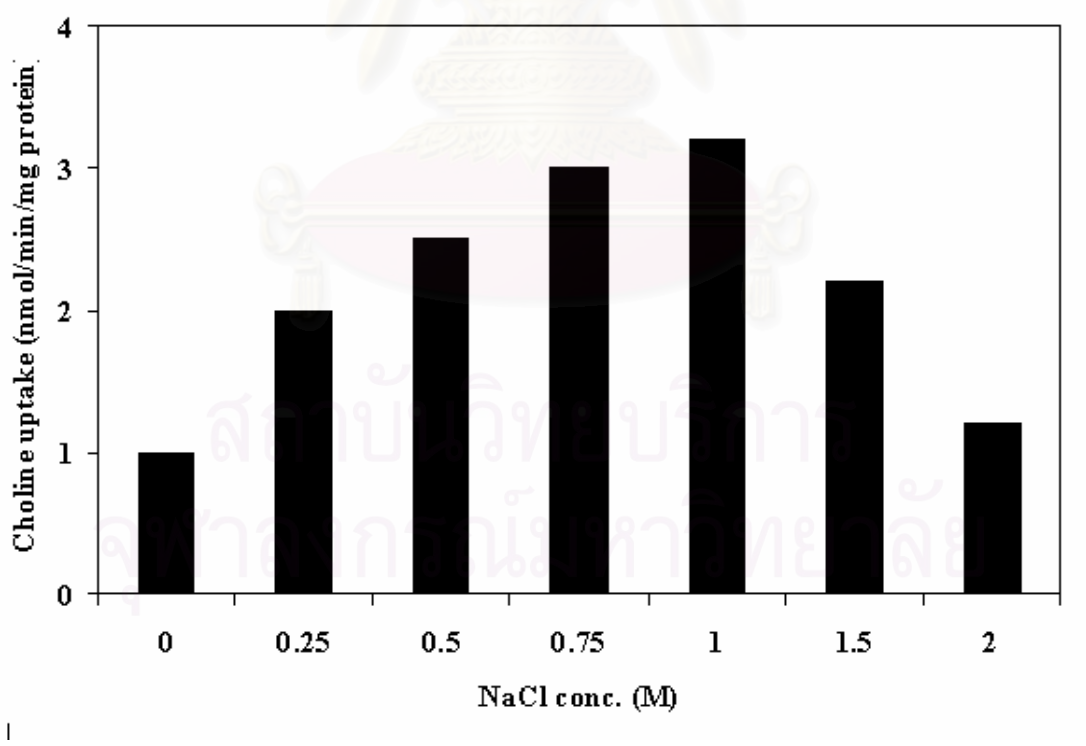


Figure 39 Effects of NaCl concentration on choline uptake into membrane vesicles.

3.6.2 Effect of various osmoticums and cations on choline uptake

A high rate of choline uptake was dependent on energization of cells with osmoticum, such as mannitol, sorbitol and sucrose. Choline uptake was also stimulated by various osmoticums with the same osmolarity of stress condition. There was hardly any difference in stimulation whether the osmolarity was increased with mannitol, sorbitol and sucrose, indicating that the osmolarity determined the uptake rate (Figure 40). Choline uptake was dependent on Na^+ and showed maximum uptake rate.

3.6.3 Kinetics of choline uptake

We also determined the concentration needed to saturate the uptake systems of osmotic stress condition on the rate of choline uptake by membrane vesicles. When membrane vesicles were assayed with 50 µM [methyl-^{14}C] choline uptake was linear for 10 min. Initial rates were determined over a wide range of choline concentrations (0 – 1 mM). The choline uptake system was saturable and displayed typical Michalis-Menten type kinetics (Figure 41). The Lineweaver-Burk transformation of the data under these conditions, the line of best fit was performed by using a least squares linear regression, the apparent Km and Vmax were shown in Table 10.

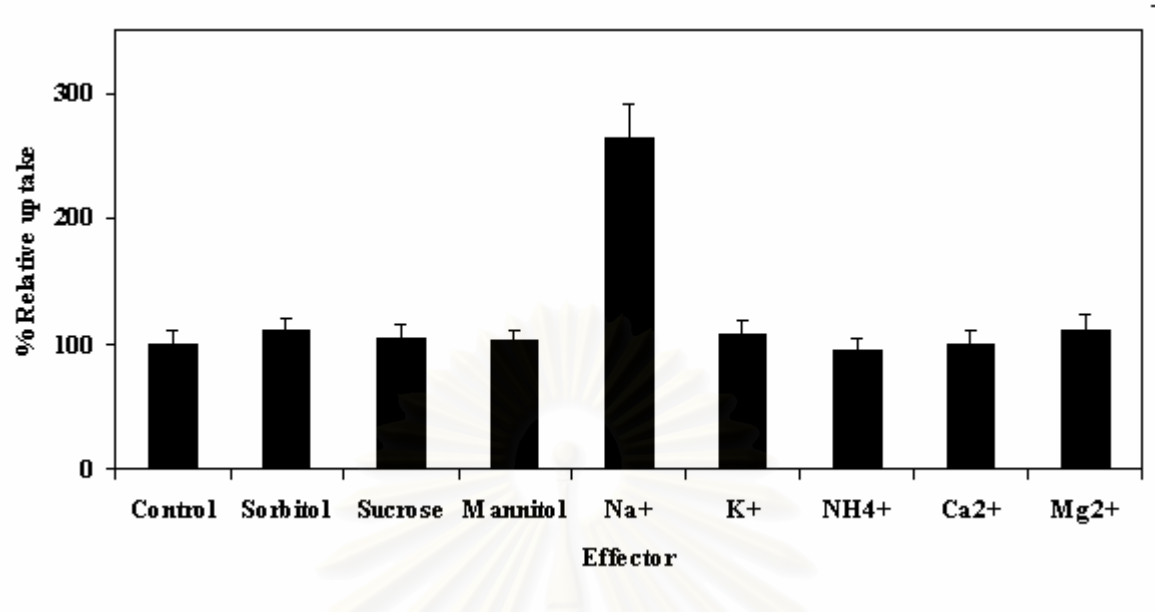


Figure 40 Effect of osmoticums and cations on choline uptake into membrane vesicles. The concentration of sucrose, sorbitol, and mannitol was 1.0 M and the concentration of cations was 0.5 M. Control is condition without effectors. Data are means from three independent experiments with vertical bars representing standard errors of the means.

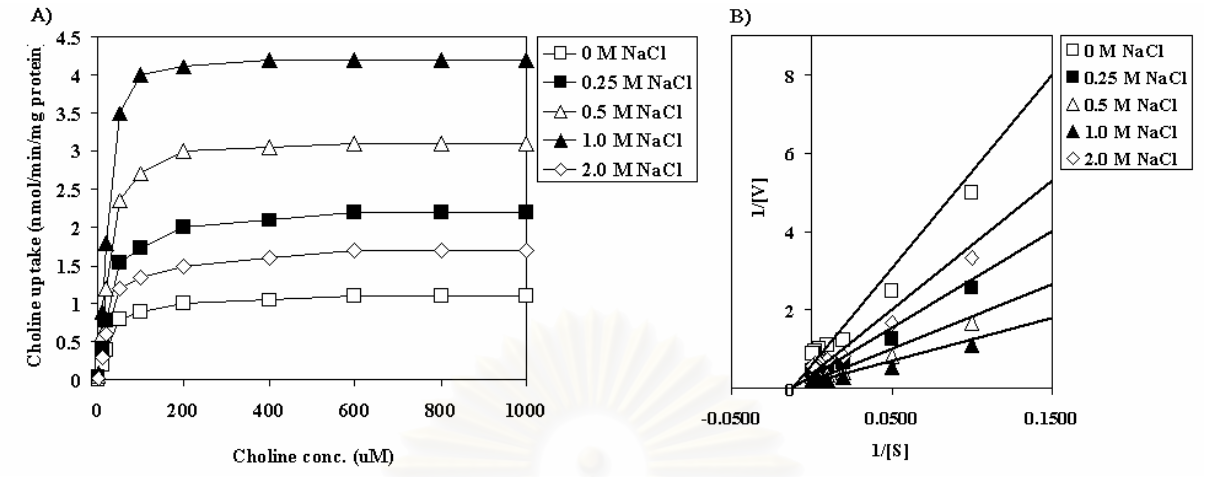


Figure 41 Kinetic of choline uptake at various NaCl concentrations. A) Saturation curve of choline uptake into membrane vesicles. B) Lineweaver-Burk plot of choline uptake into membrane vesicles. Data are means from three independent experiments with vertical bars representing standard errors of the means.

Table 10 Km and Vmax values of choline uptake into membrane vesicles at various NaCl concentrations.

NaCl conc. (M)	Km (μM)	Vmax (nmol/min/mg protein)
0	85.07	1.75
0.25	82.20	3.23
0.5	82.00	4.55
1.0	81.52	6.67
2.0	88.11	1.82

3.6.4 ΔpH dependent choline uptake and driven by Na^+ in membrane vesicles

Choline uptake in membrane vesicles is driven by ΔpH. In order to reveal whether ΔpH -dependent choline uptake requires energy, uptake of choline was investigated. Table 11 showed the experimental results obtained when Δψ and ΔpH were modified at three different extracellular pHs. In case of an extracellular pH of 5.5 and a control rate of uptake of 100, a complete inhibition in the uptake of labeled ^{14}C choline was obtained when ΔpH = 0 was imposed with potassium acetate buffer as well as when nigericin was presented in the assay medium. However, when a ΔpH was produced with potassium acetate buffer the rate of uptake was stimulated when compared to control at the same pH. When the medium at pH 5.5 was supplement with 20 mM KSCN, the Δψ increased and the uptake rate was increased to compare to control. A similar effect was obtained in membrane vesicles with EDTA and valinomycin in present or absence of extracellular potassium obtained when pH 7 and 8.2 were identical to those obtained when pH 5.5, excepting when an artificially ΔpH was imposed. There were some affect of sodium ion gradient when inhibitors (monensin) was presented at all pHs

Table 11 Effects of ΔpH, Δψ, and ΔpNa on uptake of choline at different pHs.

pH	Treatment	% uptake
5.5	Control	100
	ΔpH = 0 (KOAc)	0
	ΔpH (KOAc)	120
	Nigericin (1 μM)	25
	Nigericin (5 μM)	0
	KSCN (20 mM)	100
	Valinomycin (5 μM in TMB)	92
	Valinomycin (5 μM in KPB)	95
	Monensin (100 μM)	30
7.0	Control	100
	ΔpH = 0 (KOAc)	0
	ΔpH (KOAc)	115
	Nigericin (1 μM)	30
	Nigericin (5 μM)	0
	KSCN (20 mM)	98
	Valinomycin (5 μM in TB)	93
	Valinomycin (5 μM in KPB)	95
	Monensin (100 μM)	25
8.2	Control	100
	ΔpH = 0 (KOAc)	0
	ΔpH (KOAc)	105
	Nigericin (1 μM)	35
	Nigericin (5 μM)	0
	KSCN (20 mM)	100
	Valinomycin (5 μM in TB)	100
	Valinomycin (5 μM in KPB)	100
	Monensin (100 μM)	20

3.7 Cloning and characterization of betaine transporter gene from *A. halophytica.*

3.7.1 Cloning of betaine transporter gene from *A. halophytica*

Hitherto, a betaine transporter has not been reported in any cyanobacteria. Here one ORF homologous to the betaine transporter OpuD from *B. subtilis* was found from the shot gun clones of *A. halophytica*. The gene was isolated by PCR amplification and sequenced. The predicted gene product (Ap-BetT) consists of 565 amino acids with molecular mass of 64,655. The ClustalW analysis showed that Ap-BetT has 12 TM segments and could be classified as a member of the betaine choline carnitine transporter (BCCT) family of which common functional feature is that they transport the molecules with quaternary ammonium group. Ap-BetT exhibited highest level of identity (53 %) to ButA from a moderate halophilic lactic acid bacterium *Tetragenococcus halophila* (Baliarda *et al*., 2003), and exhibited some homology to BetP from *Corynebacterium glutamicum* (40 %) (Peter *et al*., 1996) and OpuD from *B. subtilis* (40 %) (Kappers *et al*., 1996), but very low level of identity to betaine/proline transporters ProP (29 %) from *E. coli* (Culham *et al*., 1993) and AmT1 (28 %) from mangrove (Waditee *et al.*, 2002). Alignment of eight transporters, Ap-BetT from *A. halophytica*, ProP from *E coli* (Culham *et al*., 1993), BetP (Peter *et al.*, 1996) and EctP (Peter *et al.*, 1998) from *C. glutamicum*, ButA from *T. halophila* (Baliarda *et al.*, 2003), OpuD from *B. subtilis* (Kappers *et al.*, 1996), BetS from *S. meliloti* (Boscari *et al*., 2002), and BetL in *L. monocytogenes* (Sleator *et al.*, 1999) showed highly conserved region in TM8, (347 WTVFYWG WWISWSPFVGMFIA367). Thirteen amino acid residues in this region are conserved among eight proteins. Interestingly, loop region connecting between TM VIII and IX is also highly conserved.

3.7.2 Expression of Ap-BetT in *E. coli* and its complementation of Na^+-sensitive phenotype

To examine the functional properties of Ap-BetT, its gene was expressed in the *E. coli* mutant MKH13 cells in which *betT, putPA, proP*, and *proU* genes were deleted. As shown in Figure 42A, Ap-BetT could be expressed in MKH13 cells. The expression level of Ap-BetT was almost independent on the concentrations of NaCl in growth medium. Addition of IPTG slightly increased the accumulation level of Ap-BetT. Figure 42B shows the photograph of *E. coli* MKH13 cells in the agar plate containing various concentrations of NaCl at pH 7.0. MKH13 cells transformed with the control plasmid could not grow in the medium containing 0.75 M or more of NaCl either with or without 1 mM betaine. However, the cells transformed with Ap-*betT* could grow in the medium containing 0.75 M NaCl when the growth medium contained 1 mM betaine (Figure 42B). Similar results were obtained in the agar plate at pH 9.0, but not at pH 5.0 (data not shown).

Since the MKH13 cells do not contain the proline transporters, ProP and ProU, we therefore tested whether Ap-BetT could allow the MKH13 cells to grow under high salinity in the presence of proline. The MKH13 cells transformed with Ap-*betT* could not grow in the MMA medium containing 0.75 M NaCl, 1 mM proline with- or without 1 mM IPTG (data not shown). These results suggest that Ap-BetT could take up betaine, but not of proline at neutral and alkaline pH .

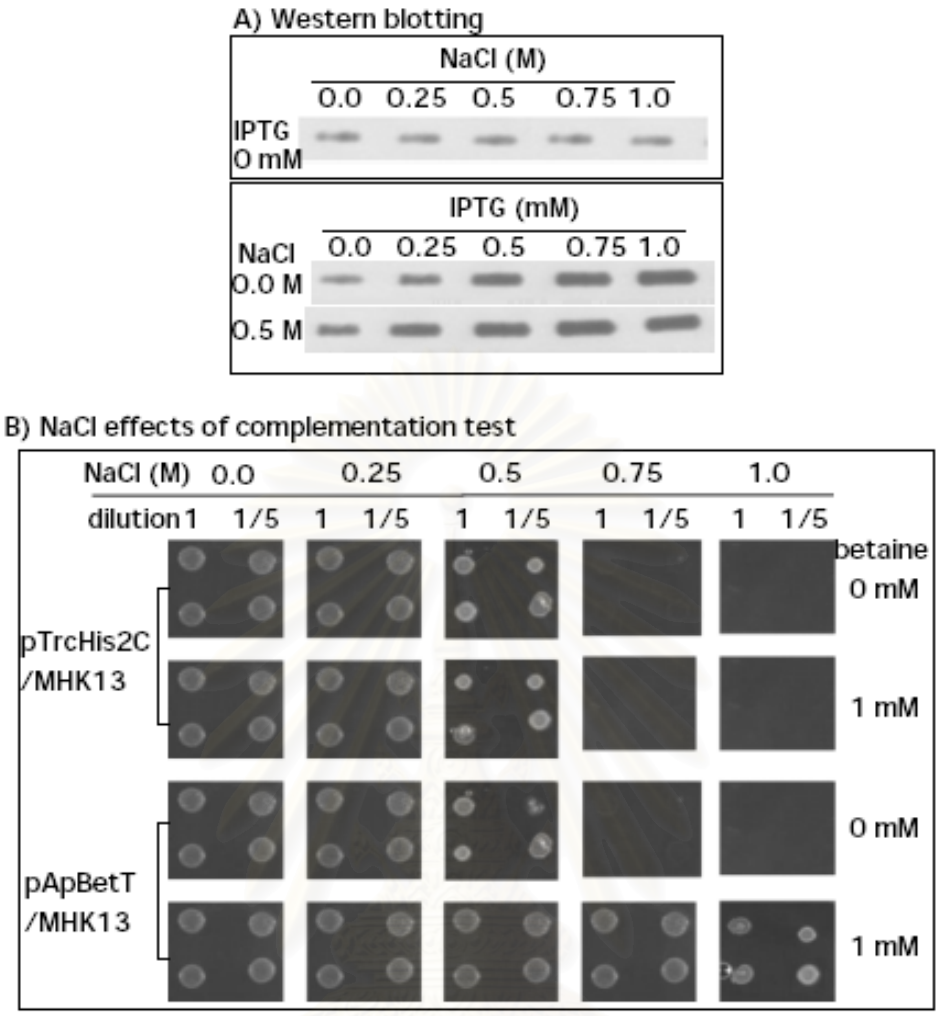


Figure 42 Expression and complementation of Ap-BetT in the salt sensitive *E. coli* MKH13 cells. A) Western blotting of Ap-BetT. The Ap-BetT expressing MKH13 cells were grown at 37°C in MMA (pH 7.0) containing 0.2 % glucose, ampicillin (50 µg/ml), and indicated concentrations of NaCl until optical density at 620 nm reached 0.3 and then the indicated concentrations of IPTG were added. After 5 h incubation, the cells were harvested, sonicated, and membrane fractions were used for Western blotting. B) Complementation test of betaine deficient MKH13 mutant cells by Ap-BetT. Ap-BetT expressing cells were grown for 5 days on the agar plates containing the indicated concentrations NaCl and betaine and then photographs were taken. The control represents MKH 13 cells transformed with pTrcHis2C.

3.7.3 Growth of MKH13 cells in the minimal medium A at various salinities and pHs.

The *E. coli* MKH13 cells are unable to grow in high osmolarity medium containing betaine due to the lack of betaine transport system as well as betaine synthesis genes. Figure 43A shows that without addition of NaCl, both control MKH13 cells and Ap-BetT expressing cells exhibited similar growth pattern. Upon the increase of NaCl concentrations, the growth of all cells became slower. However, the growth of Ap-BetT expressing cells supplemented with 1 mM betaine was always faster than that of other cells. The enhancement of growth by betaine was not observed when betaine was replaced by choline or proline. Addition of betaine to the control cells was also without effect for growth at high salinity conditions. These data indicate that Ap-BetT is involved in the uptake of betaine by the MKH13 cells, thus allowing their growth under high salinity.

Since the complementation of the salt-sensitive phenotype of MKH13 was observed at pHs 7.0 and 9.0, but not at pH 5.0, we examined the growth of MKH13 cells in the MMA medium containing 0.5 M NaCl at various pHs. The growth of control MKH13 cells was very slow at pH 5.0 (Figure 43B), increased upon the increase of pH up to pH 7.0, and decreased at pH 8.0 up to pH 10.0. All cells showed the similar pH dependent growth patterns. The growth of Ap-BetT expressing cells supplemented with 1 mM betaine was always faster than other cells. At extreme alkaline condition, pH 10, only Ap-BetT expressing cells supplemented with 1 mM betaine could grow (Figure 43B). These data suggest that Ap-BetT is active even at alkaline pH for the uptake of betaine by the MKH13 cells, thus allowing their growth at alkaline pH.

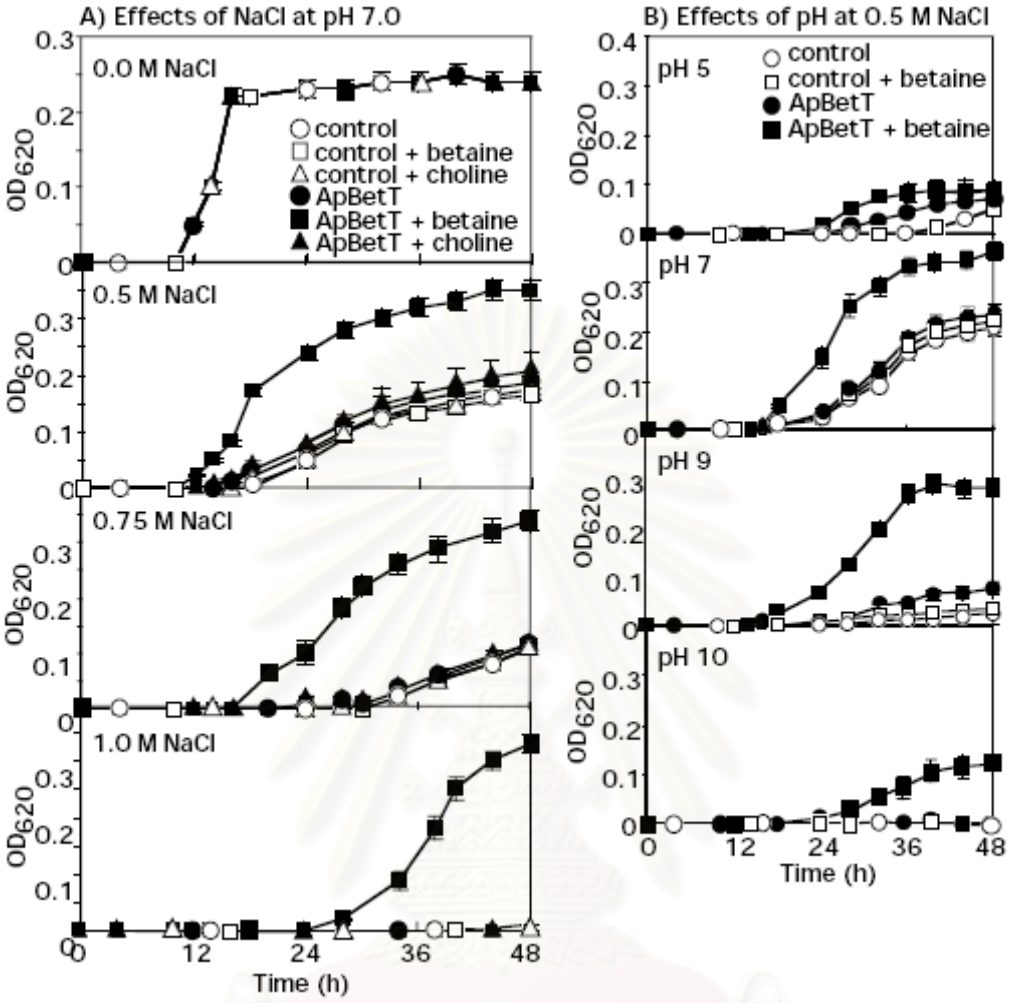
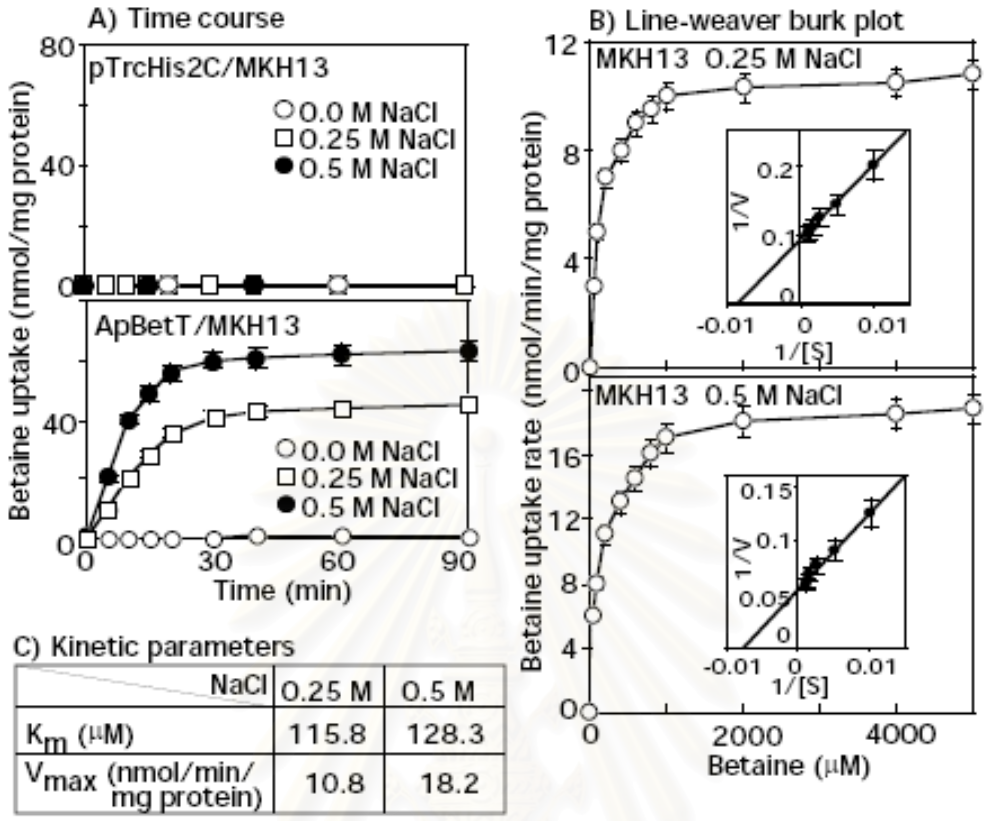


Figure 43 Effects of NaCl and pH on the growth of *E. coli* MKH13 cells expressing Ap-BetT. The MKH13 cells transformed by pApbetT or pTrcHis2C were grown on the MMA medium containing the indicated concentrations of NaCl. Betaine or choline (1 mM) was added when indicated. Growth was monitored by the absorbance at 620 nm. Each value shows the average of three independent measurements.

3.7.4 Kinetic properties of Ap-BetT in MKH13 cells.

To examine directly the transporter activity of Ap-BetT, we measured the kinetic parameters for this transporter. No measurable uptake of [1-^{14}C] betaine was observed for the MKH13 cells transformed with pTrcHis2C (Figure 44A). The MKH13 cells transformed with Ap-*betT* almost could not take up betaine in the absence of NaCl (Figure 44A). Addition of NaCl (0.25 M and 0.5 M) significantly increased the uptake of betaine (Figure 44A). The Vm values for betaine uptake by Ap-BetT at pH 7.0 and 0.25 M NaCl was 10.8 nmol betaine min^{-1}(mg protein) $^{-1}$, and increased about 2-fold when 0.5 M NaCl was added although the Km value for betaine uptake by Ap-BetT was almost independent on salinity (Figure 44B and 44C). No measurable uptake of [1-^{14}C] proline was observed for the MKH13 cells transformed with Ap-*betT* under any conditions (data not shown).



C) Kinetic parameters

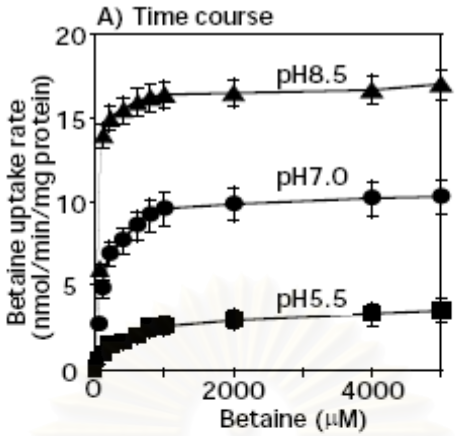
NaCl	0.25 M	0.5 M
K_m (μM)	115.8	128.3
V_{max} (nmol/min/mg protein)	10.8	18.2

Figure 44 Kinetics of betaine uptakes by Ap-BetT in MKH13 cells. A) Time courses of betaine uptakes by Ap-BetT expressing MKH13 cells at different salinity (pH 7.0). The transport was initiated by the addition of 0.1 mM [1-^{14}C] betaine. B) Double reciprocal plots of betaine transport kinetics by Ap-BetT expressing MKH13 cells. C) Kinetic parameters for betaine uptake by Ap-BetT in MKH13 cells at low (0.25 M) and high salinity (0.5 M) NaCl conditions. Data are means from three independent experiments with vertical bars representing standard errors of the means.

3.7.5 pH dependence of betaine uptake by Ap-BetT in MKH13 cells.

We examined pH dependence of betaine uptake by Ap-BetT in MKH13 cells. As shown in Figure 45A, MKH13 cells preferably uptaked [1-^{14}C] betaine at alkaline pH. The Vmax value at pH 8.5 was about 6-fold higher than that at pH 5.5, and the Km value at pH 8.5 was about 3-fold lower than that at pH 5.5 (Figure 45A and 45B). The optimum pH for the betaine uptake rate Vmax was about 9.0 (Figure 45C). In case of proline taken up by the expressing Ap-BetT cells, no measurable level was observed for the MKH13 cells at all pHs examined (data not shown).

These properties of Ap-BetT are quite different from those of mangrove betaine transporters, AmT1 and AmT2 (Waditee *et al.*, 2002). The mangrove AmT1 and AmT2 transport both betaine and proline whereas Ap-BetT could not transport proline (Figure 46A and 46B). Betaine uptake by AmT1 and AmT2 increased with the decrease of pH with the optimum pH was around 6.0 (Figure 46C). At pH 7.0, the betaine uptake rate by Ap-BetT was about 2-fold higher than that by AmT1 and AmT2. These results indicate that Ap-BetT is betaine transporter active at alkaline pH.



B) Kinetic parameters

pH	5.5	7.0	8.5
K_m (μM)	272	128	91.9
V_{max} (nmol/min/mg protein)	3.2	18.2	23.3

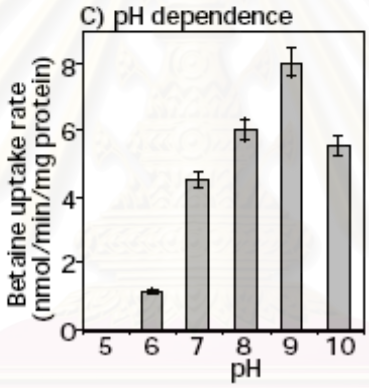


Figure 45 pH dependence of betaine uptake by Ap-BetT in MKH13 cells. A) Time courses of betaine uptake by Ap-BetT expressing MKH13 cells at different pH. The uptake medium contained 0.5 M NaCl. The values of pH were adjusted by KOH or MES. B) Kinetic parameters for betaine uptake by Ap-BetT in MKH13 cells at three different pHs. C) pH dependence of betaine uptake by Ap-BetT in MKH13 cells. The concentration of betaine was 0.1 mM. Each value shows the average of three independent measurements.

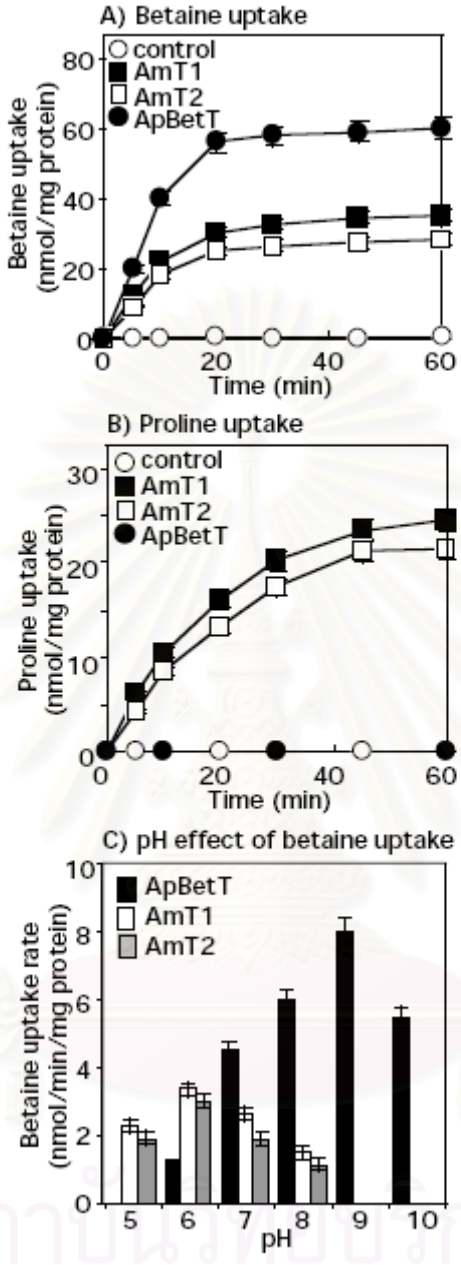


Figure 46 Comparison of betaine uptake between Ap-BetT, AmT1 and AmT2 in MKH13 cells. A) and B) Time courses of betaine (A) or proline (B) uptake by Ap-BetT expressing MKH13 cells. The uptake medium contained 0.5 M NaCl. C) pH dependence of betaine uptake by Ap-BetT, AmT1 and AmT2 in MKH13 cells. The concentration of betaine was 0.1 mM. Each value shows the average of three independent measurements.

3.7.6 Competitions for betaine uptake mediated by Ap-BetT.

To obtain the information on the substrate specificity, we performed the competition experiments. Consistent with the betaine uptake experiments (Figure 45A), the [1-^{14}C] betaine uptake by Ap-BetT in MKH13 cells was inhibited about 80 % and 90 % when 100-fold cold betaine was included in the uptake medium that contained 0.25 M NaCl and 0.5 M NaCl, respectively (data not shown). However, choline merely inhibited betaine uptake about 20 % and 10 % when the uptake medium contained 0.25 M NaCl and 0.5 M NaCl, respectively. Betaine aldehyde also exhibited similarly weak inhibition. By contrast, all other compounds such as GABA, proline, glutamate, aspartate, glutamine, asparagine, glycine, sarcosine, dimethylglycine, lysine, histidine, alanine, leucine, isoleucine, serine, cysteine, threonine, valine, phenylalanine, tryptophan, and methionine, did not inhibit the betaine uptake by Ap-BetT (data not shown). These results strongly suggest that the Ap-BetT is a transporter specific for betaine.

3.7.7 Sodium is required for betaine uptake by Ap-BetT.

To obtain the information on the co-substrate, we examined the uptake of Na^+, K^+, and betaine by MKH13 cells. Figure 47A shows that upon addition of NaCl, the Na^+ uptake occurs in all cells. However, the Ap-BetT expressing cells supplemented with betaine accumulated Na^+ more rapidly than other cells. The Ap-BetT expression was not enough for rapid accumulation of Na^+. Moreover, the lower panel of Figure 47A shows that only the cells expressing Ap-BetT and supplemented with betaine could accumulate betaine. Compared with the previous data, the lower rates of betaine uptake in Figure 47 were due to use of a lower concentration of betaine (0.025 mM). These results suggest that Na^+ was taken up together with betaine. Mole ratio of Na^+ and betaine uptake was almost equal to one (Figure 47A). Figure 47B shows that upon the addition of KCl, the intracellular K^+ increased with increasing of time. However, the accumulation levels of K^+ were almost the same in all cells, and no betaine uptake was observed in this case (lower panel of Figure 47B). Essentially similar pattern was observed upon the changes of concentrations of NaCl and betaine (Figure 47C). We also examined the effects of different cations and anions. Li^+, Cs^+, Rb^+, NH_4^+, Ca^{2+}, and Mg^{2+} could not replace Na^+ (data not shown). By contrast, NO_3^- and $H_2PO_4^-$ could replace Cl^- (data not shown). Sucrose and sorbitol were also ineffective for betaine uptake (data not shown). These results strongly suggest that the Ap-BetT is a Na^+-betaine symporter.

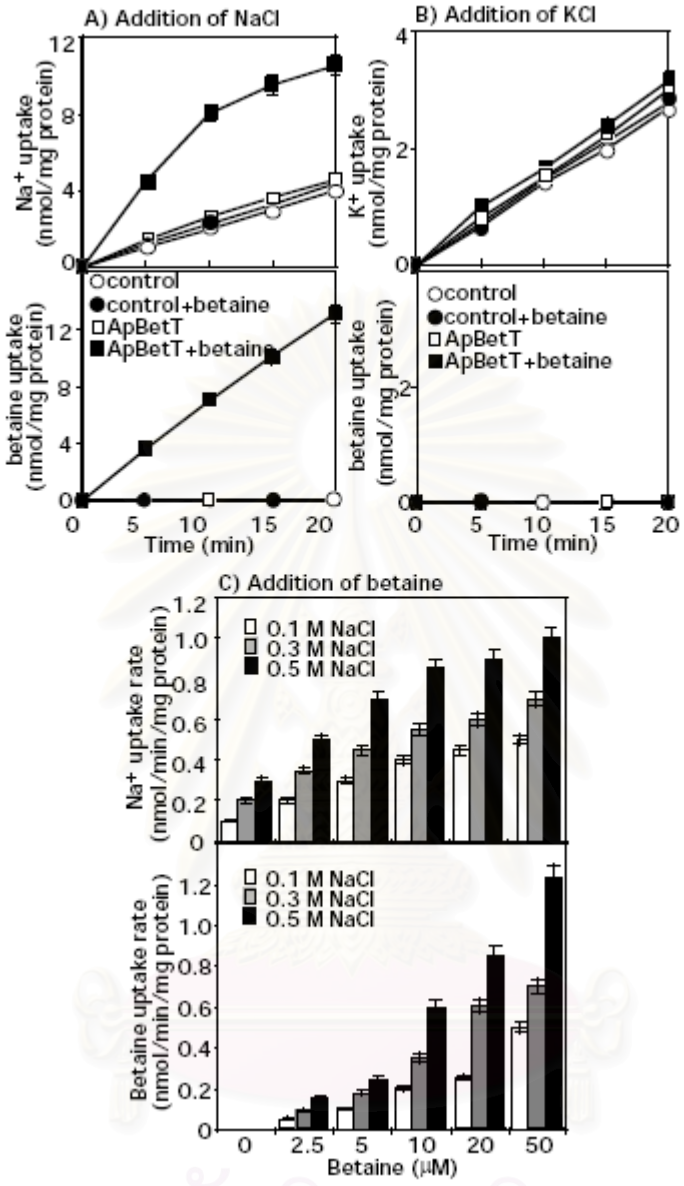
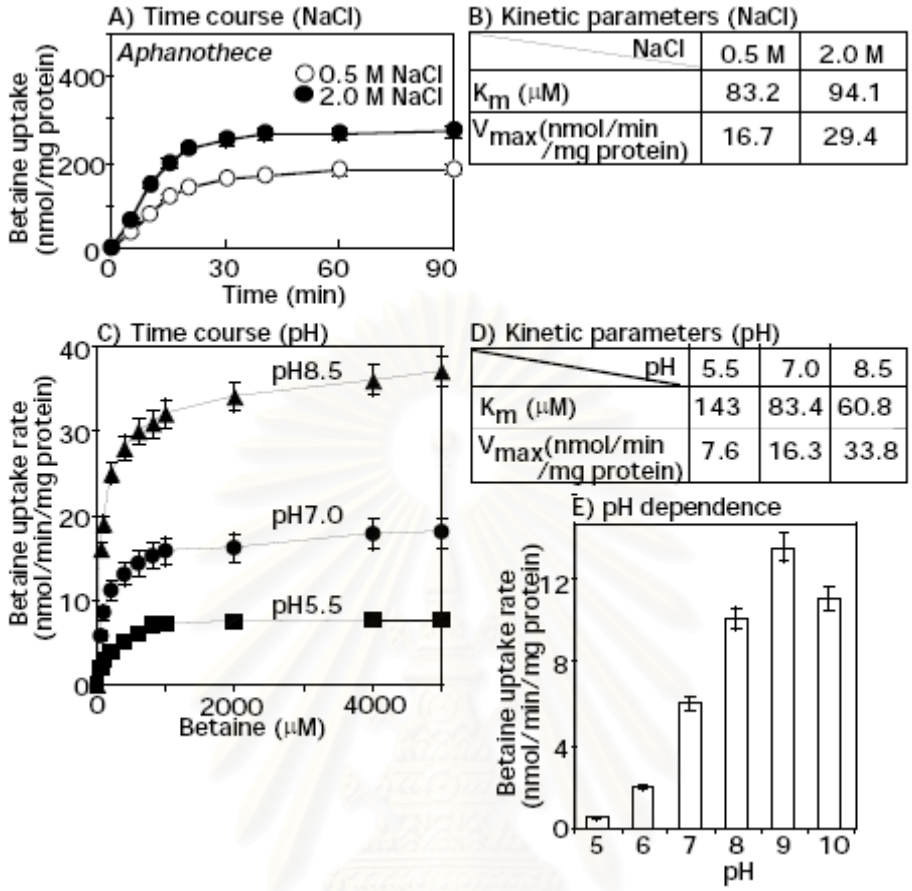


Figure 47 Uptakes of Na^+, K^+, and betaine by Ap-BetT in MKH13 cells. A) Time courses of Na^+ and betaine uptake upon addition of 0.5 M NaCl. NaCl was added to the assay medium. The concentration of betaine was 0.25 mM. B) Time courses of K^+ and betaine uptakes upon addition of 0.5 M KCl. KCl was added to the assay medium for MKH 13 cells transformed with pTrcHis2C or Ap-BetT. C) Uptakes of Na^+, K^+, and betaine by Ap-BetT in MKH13 cells under various concentrations of NaCl and betaine. Each value shows the average of three independent measurements.

3.7.8 Betaine uptake in *A. halophytica* cells.

Next, we examined the betaine uptake in *A. halophytica* cells. As shown in Figure 48A, *A. halophytica* cells could take up betaine very actively. *A. halophytica* cells were grown at 0.5 M, after washing cells, betaine uptake was measured at various salinity conditions. The Km values were not so much affected by NaCl. However, the Vmax values significantly increased at high salinity (Figure 48B). The pH dependence of betaine uptake was also similar to that by MKH13 cells. The Km values were decreased with increasing pH and the Vmax values were increased with increasing pH. (Figure 48C and 48D).The optimum pH was about pH 9.0 (Figure 48E). These facts suggest that either Ap-BetT is a major betaine transporter in *A. halophytica* cells or there might be other types of betaine transporter with similar pH- and salt-dependence.



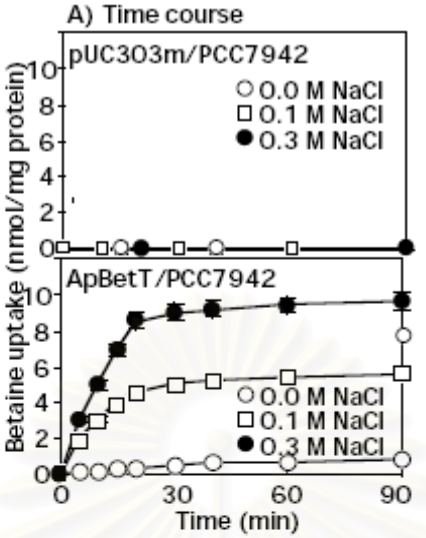
NaCl	0.5 M	2.0 M
K_m (μM)	83.2	94.1
V_{max} (nmol/min /mg protein)	16.7	29.4

pH	5.5	7.0	8.5
K_m (μM)	143	83.4	60.8
V_{max} (nmol/min /mg protein)	7.6	16.3	33.8

Figure 48 Kinetics of betaine uptakes by *A. halophytica* cells. A) and C) Time courses of betaine uptake, B) and D) Kinetic parameters for betaine uptake. E) pH dependence of betaine uptake. In A), the assay medium contains 0.5 or 2.0 M NaCl and 1 mM betaine at pH 7.0. In C), the assay medium contains 0.5 M NaCl at the indicated pH. Each value shows the average of three independent measurements.

3.7.9 Transformation *of Synechococcus* sp. PCC7942 with Ap-*betT*.

To examine functional properties of Ap-BetT, a fresh water cyanobacterium *Synechococcus* sp. PCC7942 was transformed with the Ap-*betT* gene. Figure 49A shows that the cells transformed with a vector pUC303-Bm did not uptake betaine with or without the addition of NaCl. This result suggests that *Synechococcus* cells could not uptake betaine. It should be mentioned that we previously observed that wild type *Synechococcus* cells could uptake choline (Nomura *et al.,* 1995). No measurable uptake of [1-^{14}C] betaine was observed for the *Synechococcus* cell transformed with ApBetT when the growth medium lacked NaCl (Figure 49A). The betaine uptake increased with increasing the concentrations of NaCl (Figure 49A). The Km values were almost independent on the concentrations of NaCl (Figure 49B and 49C). The optimum pH for betaine uptake was about 9.0. These properties are similar to those in MKH13 cells.



B) Kinetic parameters

NaCl	0.1 M	0.3 M
K_m (μM)	284	253
V_{max} (nmol/min /mg protein)	1.2	1.6

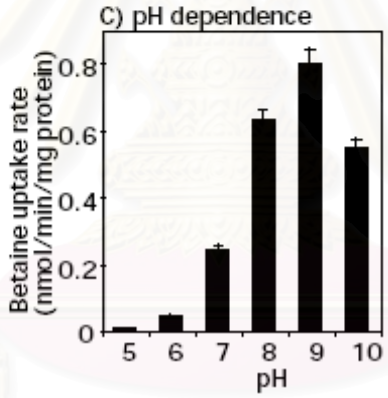


Figure 49 Betaine uptake by Ap-BetT in *Synechococcus* sp. PCC7942 cells. A) Time courses of betaine uptake in *Synechococcus* sp. PCC7942 cells transformed with pUC303m or pUC303-ApBetT at three different salinity conditions. B) Kinetic parameters for betaine uptake in *Synechococcus* sp. PCC7942 cells. C) pH dependence of uptake rate. Each value shows the average of three independent measurements.

3.7.10 Overexpression of Ap-BetT conferred the salt tolerance of a fresh water cyanobacterium *Synechococcus* sp. PCC 7942.

We examined the salt tolerance of *Synechococcus* sp. PCC 7942 cells at pH 7.0. As shown in Figure 50A, the growth of wild type and transformed cells was almost the same when the growth medium lacked NaCl. In BG_{11} medium containing 0.4 M NaCl, the control *Synechococcus* cells could not grow whereas the transformant cells with Ap-BetT supplemented with betaine could grow. The transformant cells expressing Ap-BetT supplemented with betaine could grow in the medium containing 0.5 M NaCl and also in sea water. Interestingly, the Western blotting of Ap-BetT showed that Ap-BetT protein was detected at the plasma membrane fractions and its level increased with increasing of salinity (Figure 50E). These results suggest an important role of Ap-BetT for salt tolerance.

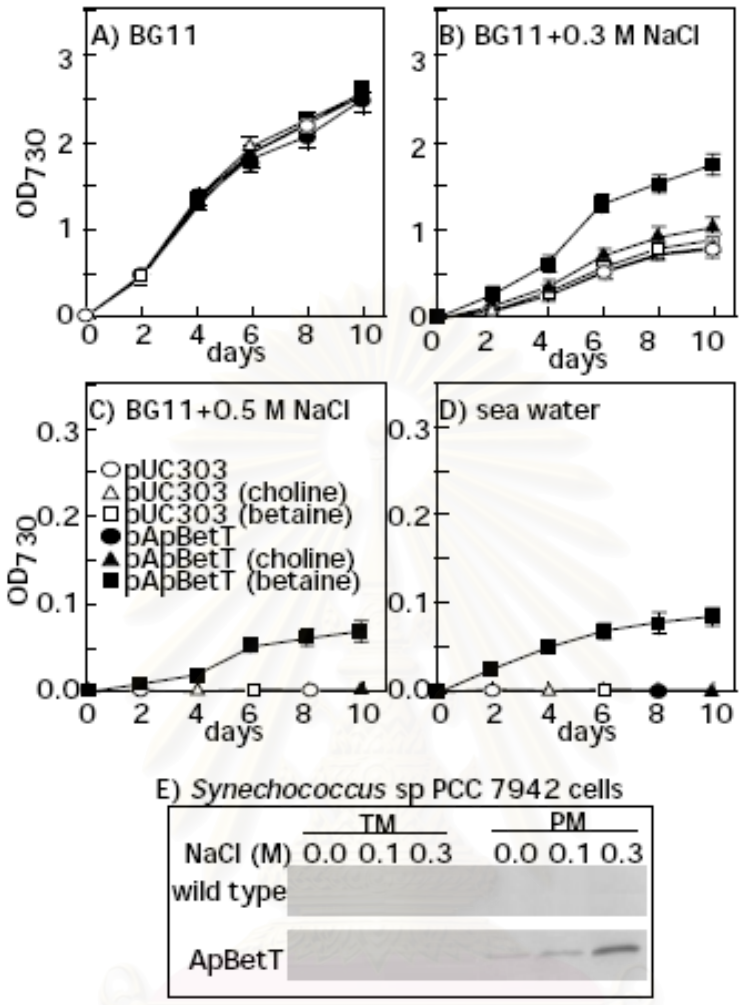


Figure 50 Salt tolerance of *Synechococcus* sp. PCC 7942 cells expressing Ap-BetT. The fresh water *Synechococcus* sp. PCC 7942 cells transformed with vector only or Ap-BetT were grown in BG_{11} medium containing 0.0 M NaCl (A), 0.4 M NaCl (B), 0.5 M NaCl (C), or in sea water (D) with the supplement of 1 mM choline or betaine. E) Immunoblotting analyses of Ap-BetT expressing cells. Thylakoid membrane (TM) and plasma membrane (PM) fractions were prepared. In each lane, 50 μg membrane proteins were loaded. Transporter proteins were detected using an antibody raised against His-tag. Each value shows the average of three independent measurements.

CHAPTER IV

DISCUSSION

The results shown in this study clearly indicated that there is energy-dependent nitrate uptake in *A. halophytica.* The energetic component for nitrate uptake was ascribed to the contribution by the proton motive force (Δp) which consists of two components, i. e. the pH gradient (ΔpH) and the membrane potential (Δψ) with the relationship, $\Delta p = \Delta\psi - 59\Delta pH$, where ΔpH equals the pHout minus pHin (The value 59 is a combination of constants for expression of ΔpH in millivolts at 25^{o}C). This conclusion is based on the results showing (i) inhibition of nitrate uptake by various protonophores (Table 3), (ii) an increased nitrate uptake when glucose or lactate was added as an energy source (Table 4).

The data from Figure 8 show an increase of nitrate uptake upon artificially generating ΔpH. This increase was observed independent of external pH. On the other hand, the suppression of ΔpH always resulted in a significant decrease of nitrate uptake, and again was independent of external pH. This behavior is expected when the ΔpH term of the proton motive force is the driving force for nitrate uptake. In contrast to the complete abolition of 4-hydroxybenzoate uptake in *Klebsiella planticola* strain DSZ1 when ΔpH was suppressed (Allende *et al*., 2002), our results showed some nitrate uptake activity remaining when ΔpH was suppressed (Figure 8). However, this nitrate uptake activity disappeared when ΔpH was suppressed in the presence of monensin (data not shown). Thus, it was suggested that Na^{+}-electrochemical gradient dissipated by monensin (Pressman, 1976) was involved in nitrate uptake. The dependence of nitrate uptake on Na^{+} was previously reported in A. halophytica (Incharoensakdi and Wangsupa, 2003). The fact that the protonophores, CCCP, 2, 4-DNP and

gramicidin D could not completely inhibit the uptake of nitrate (Table 3) also supports the notion that there exists another energetic component in addition to proton motive force, i. e. the so called sodium motive force which was previously suggested to play a role in nitrate uptake in *Anacystis nidulans* R2 (Rodriguez *et al*., 1992; Rodriguez *et al*., 1994). The contribution by either a proton or a sodium gradient to amino acid and glycine betaine transport has previously been shown in some bacteria (Ekiel *et al*., 1985; Proctor *et al.*, 1997).

The overall results in Figure 8 and Table 5 suggest that the uptake of nitrate by *A. halophytica* is driven by ΔpH. The contribution by Δψ was rather unlikely due to the findings that Δψ generated by either KSCN or valinomycin in the absence of K^+ had no stimulatory effect on the uptake of nitrate in acidic, neutral, and alkaline conditions (Table 5). Moreover, when Δψ was dissipated by valinomycin in the presence of K^+, no depression of nitrate uptake was observed (Table 5). The absence of an inhibitory effect by valinomycin suggests the absence of the contribution of Δψ to nitrate uptake. On the contrary, a slight stimulation of nitrate uptake occurred under the condition depleted of Δψ by valinomycin ($+K^+$) treatment (Table 5). This stimulation could occur due to (i) the action of Na+-motive force (Na^+-gradient driven) since valinomycin specifically abolishes Δψ contributed by K^+ transport (Reed, 1979) and/or (ii) an increase of ΔpH term of the proton motive force is generated by a compensatory mechanism, i. e. an increase in ΔpH simultaneous to a decrease in Δψ (Reed, 1979; Kroll and Booth, 1981). Similar observations were reported for 4-hydroxybenzoate uptake in Klebsiella planticola whereby ΔpH was also implicated as the driving force (Allende *et al.*, 2002). Previously, it was also shown in the cyanobacterium *Plectonema boryanum* that ΔpH is the major energetic component with no involvement of Δψ for energy coupling in photosynthesis (Padan and Schuldiner, 1978).

Extracellular pH also affected the uptake of nitrate in *A. halophytica.* It was shown that raising the extracellular pH from 5.5 to 8.2 resulted in the enhancement of the rate of

nitrate uptake (Figure 9). The dependence of betaine uptake on the extracellular pH was previously reported in *Lactococcus lactis* (Molenaar *et al.*, 1993). However, the increase of the extracellular pH resulted in the decrease of betaine uptake. This inverse relationship between extracellular pH and betaine uptake was proposed to be mediated by the intracellular K^+ concentration. It is intriguing to speculate that the intracellular Na^+ might mediate nitrate uptake in response to extracellular pH in *A. halophytica*. Previous work has shown that *A. halophytica* contains an Na^+/H^+ antiporter which can confer salt tolerance on the cells (Waditee *et al.*, 2001; Waditee *et al*., 2002). Apart from protecting cells against salt stress, Na^+/H^+ antiporter can also regulate intracellular H^+ levels (Padan and Schuldiner, 1996). During the generation of a pH gradient across the membrane, an increase in the extracellular pH would lead to a decrease in the pH gradient and consequently the intracellular Na^+ might also decrease. The observed increase of nitrate uptake at increasing extracellular pH (Figure 9) might be accounted for by an increase of Na^+-gradient mediated by Na^+/H^+ antiporter. Indeed, the activity of Na^+/H^+ antiporter in *A. halophytica* was shown to increase with increasing pH (Waditee *et al.*, 2001). Moreover, we also observed a reduction of nitrate uptake in *A. halophytica* in the presence of amiloride, an inhibitor of Na^+/H^+ antiporter (Mochizuki-Oda and Oosawa, 1985) (data not shown). Thus, the involvement of Na^+/H^+ antiporter in the uptake of nitrate cannot be precluded. That the role of Na^+/H^+ antiporter in the generation of sodium motive force to power Na^+/solute symport has previously been proposed for the transport of anions across the membranes (Krulwich and Guffanti, 1989; Espie and Kandasamy, 1994).

The fact that DCCD, the ATPase inhibitor, and the ionophores, CCCP, 2, 4-DNP, and gramicidin D strongly inhibited the uptake of nitrate (Table 3) highly suggests that the energy needed is provided by electron transport in the cytoplasmic membrane through a proton motive force, with the participation of ATP hydrolysis. Previous studies have shown that an

ATP-binding cassette (ABC)-type system with the two ATP-binding proteins is involved in nitrate uptake in the cyanobacterium *Synechococcus* sp. PCC7942 (Omata, 1995). Further work on the characterization of proteins responsible for nitrate uptake in *A. halophytica* is needed for a better understanding of nitrate uptake in cyanobacteria.

The living organisms; animals, plants, bacteria and others must be able to adapt to changes in the osmolarity of their environment. To adapt to these changes, they accumulate some compounds, named compatible solutes that confer protection against the deleterious effect of the low water activity (Canovas *et al,* 1998). The most bacteria and halotolerant cyanobacteria accumulate glycine betaine to protect cells against osmotic stress. The glycine betaine accumulation is an indication that the cells are adapting to the changes of external osmolarity.

The halotolerant cyanobacterium *A. halophytica* was studied by following the growth rate of cells in various NaCl concentration condition. The optimal growth rate was decreased when the cells were transferred to the medium with higher NaCl concentration. Our data showed *A. halophytica* could adapt to a broad range of salt concentrations from 0.5 – 3.0 M NaCl (Figure 10). We found that external provided choline and glycine betaine functioned as efficient osmoprotectants for *A. halophytica* (Figure 15 and 16). Choline and betaine activated the growth of *A. halophytica* over the whole range of salinities. The enhancement of growth by betaine was nearly identical at optimal salinity, but choline was less efficient than betaine. The fact that many bacteria prefer to transport external compatible solutes rather than to carry out the energetically more expensive de novo synthesis (Galinski, 1995) may account for the accumulation betaine in *A. halophytica* at 2.0 M NaCl.

The cessation of growth due to salt stress probably occurred as a result of energy being diverted to the initial adjustment of cellular volume by regulating the influx and efflux of certain ions. The influx of Na^+ and K^+ in *A. halophytica* in 100% sea water medium has

previously been shown to occur mostly in the first 24 hour (Reed *et al.*, 1984). Furthermore uptake and extrusion of Na^+ in *Synecocystis* PCC 6714 have also been shown to occur during the initial period of hypersaline treatment (Reed *et al*., 1985). It remains to be clarified how the movement of various solutes in and out of *A. halophytica* affects the osmoregulation of the cells. Results similar to those observed in *A. halophytica* have been recently reported for the effect of salt stress on growth of salinity-adapted cyanobacterium, *Spirulina platentis* (Vonshak *et al.*, 1996). Cells exhibited a lag period in growth when stress with 0.5 M or 0.75 M NaCl. However, normal growth resumed after the lag period. It is also noteworthy that the lag period after a salt-shock has also been observed in green algae, *Dunalilla salina* (Borowitzka *et al.*, 1990).

As a result, *A. halophytica* cells increased significantly in size. Thus, salt stress or osmotic stress had different effects on the proliferation of *A. halophytica*. To date, little attention has been paid to the morphological changes that occur in *A. halophytica* cell under stress conditions. Our results suggest that NaCl might arrest the formation of the shape of cells (Figure 11). The results demonstrated clearly that *A. halophytica* cells under salt stress were highly heterogeneous with respect to size and shape. The effects of salt stress on cell size of the halotolerant bacterium *S. aureus* was investigated in detail (Vijaranakul *et al*., 1995, 1997). Salt shock due to 2.5 m NaCl increased the cell size. The addition of betaine to the culture medium alleviated the adverse effects of salt stress by reducing cell size and accelerating cell division. These effects of betaine on cell size and cell division are similar to that of glucosylglycerol in *Synechocystis* sp. PCC 6803 cells (Ali *et al.*, 2003). Thus, glucosylglycerol and betaine might have similar effects on the regulation of cell size and might play similar roles in the protection of cell division. However, such does not seem to be the case for all compatible solutes.

The content of ions, betaine, and amino acid in *A. halophytica* under normal and stress condition was determined. Na^+, K^+, and NH_4^+ slight increased when cells were transferred and grown in stress condition but NO_3^- slight decreased if cells were transferred and grown in stress condition. High salt concentration imposes both a hyperosmotic and a hyperionic stress. *Halobacterium salinarum* was grown in the medium containing Na^+ (3.3 M) and K^+ (0.05 M) but Na^+ and K^+ inside the cells is 0.8 M and 5.3 M, respectively. *H. salinarum* uses K^+ as the compatible solute. High intracellular [K^+] required for ribosomal stability and for activity of various enzymes (Kushner, 1988). To resist the salt stress *A. halophytica* use ions for osmotic adjustment. Ion accumulation or ion exchanging in the cell keeps sodium away from the cytoplasm, while at the same time not so much different of ion inside the cell in both conditions. *A. halophytica* contains Na^+/H^+ antiporter and Ca^{2+}/H^+ antiporter to balance Na^+ or others ion to equibrium (Waditee *et al.*, 2002; Waditee *et al.*, 2004). Intracellular compartmentation of Na^+ within plants was found to have a decisive effect on plant salt tolerance (Apse *et al.*, 1999; Xiong *et al.*, 2002). To test directly whether the cellular compartmentalization of sodium in the cells resulted in a higher sodium uptake, we measured accumulation of sodium using an ion analyzer.

Of particular interest in this, amino acid contents under normal and stress conditions were determined. Glutamate and glutamine were major amino acid found at both conditions. A little further increases of glutamine at stress condition when compared with normal condition. Aspartate, proline, and glutamate were enhanced about two folds while glycine and arginine were decreased at stress condition. Glycine is a substrate for glycine betaine synthesis (Waditee *et al.*, 2003). Another factor may be accumulation of amino acids to concentrations which are in some way inhibitory. Exogenously supplied proline is osmoprotective for bacteria, facilitating growth in highly saline environments (Csonka, 1989; Strom *et al*, 1983; Csonka and Hanson, 1991; Yancey, 1994). Accumulation of proline in the

cytoplasm is accompanied by a reduction in the concentrations of less compatible solutes and an increase in cytosolic water volume (Cayley *et al*, 1991; 1992). Proline is an allosteric inhibitor of glutamate kinase and dehydrogenase (Umbarger *et al*., 1978). Inhibition of enzymes which catalyze the conversion of glutamic acid to glutamic acid semialdehyde could prevent proline accumulation beyond a certain concentration. It is also possible that osmoregulatory control via potassium accumulation requires less energy than de novo synthesis of free amino acids.

Potassium is generally less inhibitory to intracellular enzymes than is sodium (Aitken *et al.,* 1970; Anderson *et al*., 1982), but energy is required when K^+ accumulation must be maintained against a concentration gradient while other stress solutes are selectively excluded. Potassium concentrations in cells of *S. griseus* challenged by 1 M NaCl were about 6 times greater than the basal potassium concentration (35 mM) in the medium, whereas internal sodium concentrations were maintained at very low levels. However, the intracellular concentrations of free amino acids did not consistently vary between the two salts, indicating that the reduced cell yields did not result only from the energy cost of amino acid synthesis. Apparently some of the energy cost of response to NaCl stress was specific to the Na^+ ion. In *A. halophytica* indicates that tolerance to high salinity is associated with intracellular accumulation of free neutral amino acids and, under conditions of extreme salt stress, with selective internal concentration of potassium.

Exception of slight increasing of Na^+, K^+, NH_4^+, greatest increasing of some amino acid (asparagine, proline, and valine; there are many report which they are act as compatible solutes (Kalinski and Truper, 1994; Ko *et al*., 1994)), Betaine was found three-fold increasing under salt stress when compared with normal condition. This result showed that *A. halophytica* accumulation of betaine at high salinity. Previous research detailed that *A. halophytica* synthesized betaine by 2 steps of enzyme GSMT and DMT at high salinity

(Waditee *et al.*, 2003). Strategy was used by most extremely halophilic Bacteria is synthesis or uptake of organic osmolytes called compatible solutes which are highly soluble and compatible with cellular metabolism and the most important compatible solute is glycine betaine (Mackey *et al.*, 1984).

Halotolerant cyanobacterium *A. halophytica* was studied for growth at pH 6.5 – 10.5 in BG_{11} medium at normal and stress conditions. The patterns of growth curve showed the same pattern at all pH and the optimal pH for growth was 9.5. The growth of cells was very slow at pH 6.5 (Figure 12), increased upon the increase of pH up to pH 8.5, and decreased at pH 9.5 up to pH 10.5. All cells showed the similar pH dependent growth patterns. These data suggest that A. halophytica could grow well at alkaline pH. The interesting finding in this study is high betaine transport activity at alkaline pH. The optimal pH of some extremely halophilic Archaea, *Halobacterium salinarium*, *Natronobacterium gregoryi*, and *Natronococcus occultus* is 7.2, 9.5, and 9.5, respectively (Jone *et al*., 1987).

The study on the protein profiles from three fractions (cytoplasmic, periplasmic, and membrane fraction) in normal and salt treated condition (1.0 M and 2.0 M NaCl) using SDS-PAGE revealed that the NaCl treatment did induce significant changes in the pattern of proteins. It was found that the intensity of the 21, 29, 32, 36.5, 38.5, 41.5, 47, and 77.5 kDa protein bands in cytoplasmic fraction (Figure 17), called CP7 – CP1. The highest intensity band is CP6 (32 kDa). Previous report about salt stress induce the production of two enzymes (GSMT and DMT) for betaine synthesis in *A. halophytica* and these two enzymes, GSMT and DMT showed molecular mass as 31 kDa and 32 kDa, respectively (Waditee *et al*., 2003). For periplasmic fraction showed that 30.5, 33.0, 35.5, and 53.5 kDa, called PP4 – PP1, respectively (Figure 18). Thartdee (2001) reported that PP3 containing molecular mass as 34.6 kDa was highest band density after treated with 2.0 M NaCl. Eight protein bands were observed in membrane fraction 16.4, 18.7, 23.5, 26, 27.5, 38, 41.5, and 61 kDa, called MB8 –

MB1, respectively. The highest band density was MB2 (41.5). This result was similar to a previous work which showed *A. halophytica* Na^+/H^+ antiporter and betaine transporter containing molecular mass about 56 and 60 kDa, respectably (Waditee *et al.*, 2002).

Since it is well known that in different gram-negative bacteria choline uptake activity may occur through protein dependent transport system, all fractions at low or high osmolarity were subjected to non-denaturing PAGE in the presence of ^{14}C choline as described in materials and methods. All fractions showed higher intensity band when cells were treated with higher concentration of NaCl (Figure 20A, 20C, 20E). Figure 20B, 20D, and 20F showed X-ray films of cytoplasmic, periplasmic, membrane fraction, respectively. The cytoplasmic fraction could not found after it was exposure (Figure 20B). The weak band was observed at both conditions, normal and stress condition (Figure 20D). Membrane fraction was found strongly band at all conditions and enhanced band was observed when cells were transferred at stress condition and showed similar intensity band at 0.5 M and 2.0 M NaCl (Figure 20F).

The report for salt-adapted cells of *Synechosistis* sp. PCC 6803 showing accumulation of proteins on salt stress proteins has been performed using total cell extracts (Fulda and Hagemann, 1995). However, the cyanobacterial cell is composed of different compartments: periplasm, cytoplasm and lumen of thylakoids. The periplasm is particularly responsive to changes in salinity. Some of these proteins may be anchored to the cytoplasmic membrane or to the outer membrane; most are believed to be water-soluble and can be osmotic shock (Neu and Happel, 1965). Among the periplasmic proteins that are members of the osmotic stimulation in *E. coli*, the glycine betaine-binding protein of the ProU transport system (OsmY) and a periplasmic trehalose (TreA) have been identified (Gutierrez *et al*., 1989; Yim and Villarejo, 1992).

We had confirmed the choline binding protein activity by using radio active on all fractions. After cells were incubated with ^{14}C choline for 30 and 60 min and precipitated protein by adding of 10%TCA, results showed that could not found radio activity on cytoplasmic fraction. The membrane fraction indicated higher radio activity than periplasmic fraction at 30 and 60 min and not so much different of radio activity between at 30 and 60 min (Figure 21). These results showed that there is major choline binding protein in membrane fraction. To test the substrate specific for choline binding activity by using radioactivity showed that glycine betaine aldehyde, betaine, choline, and carnitine were strong inhibition and some effect inhibition by acetylcholine, phosphorylcholine, ethanolamine, and tri-methylamine (as shown in Table 6). At salt stress conditions, glycine betaine aldehyde, choline, and carnitine were strong inhibition and betaine was completely inhibition at 0.5 M and 2.0 M NaCl. From the results of the substrate binding activity studies, it appears that N-methyl group on one end of the molecule and the alcohol or aldehyde group (or at least a neutral group) on the other and is important in the recognition of choline by the uptake system. It appears that the change of the molecule is important in that most effective competitors are positively charge (acetylcholine and betaine aldehyde), whereas zwitterionic compounds closely related to choline (phosphorylcholine and betaine) are less effective.

Surface Plasmon Resonance technique was used to confirm binding activity on membrane fraction without radio isotope. This technique showed similar results by using radioactivity which membrane fraction was specific wide range of analyte and high specific binding activity on betaine, betaine aldehyde, choline, and carnitine, respectively (Table 7).

Choline uptake by *A. halophytica* in response to osmotic stress occurred via an inducible transport system. To study the effect of salinity on the uptake of choline, cells were incubated with normal or stress condition and hot choline 50 μM. There is substantial choline uptake of *A. halophytica* in osmotic stress condition, and this choline uptake activity is

stimulated about three-fold when the osmolarity of the stress condition is raised by the addition of NaCl. Figure 25 shows that the rapid uptake of ^{14}C choline occurred during the first 20 min of incubation but the uptake increased slowly after 40 min. The pattern of ^{14}C choline uptake was similar for control and stress condition. After uptake, choline might be used as a source of cell carbon and a precursor of the osmoprotectant glycine betaine. Salt stress was identified as one variable which favored the conversion of choline to glycine betaine and retention of glycine betaine in many microorganisms, presumably for its compatible solute functions. This stimulation is a true osmotic effect, since it can be triggered by increase with sorbitol, mannitol, and sucrose (Incharoensakdi and Kanchanathat, 2002).

Choline could be taken up without osmoticum (Figure 26) and the uptake was stimulated by Na^+. The optimal Na^+ for choline uptake was 1.0 M NaCl when NaCl was higher than 1.0 M NaCl showed reduction of choline uptake. At 2.0 M NaCl showed choline uptake activity not so much different at 0.5 M NaCl but showed lower activity than optimal about 2 folds. When the substrate concentration was varied the choline uptake system followed typical Michaelis-Menten kinetics. Using Lineweaver-Burk transformation of the data, the apparent Km's of control and stress condition (1.0 M and 2.0 M NaCl) were 246.78, 262.19, and 299.51 μM, respectively. Choline transport systems have been described in a variety of microorganisms, including a marine Pseudomonad (Snipes *et al.,* 1974) *Pseudomonas aeruginosa* (Salvano *et al*., 1989), *E. coli* (Styrovold *et al*., 1986), *R. meliloti* (Pocard *et al.,* 1983) and *S. cerevisiae* (Hosaka *et al.,* 1980). Choline transport systems may function in the transport of choline for use as a carbon, energy, and nitrogen source or use in osmoregulation (Abee *et al.*, 1990; Pocard *et al*., 1983).

From the results of the substrate specific studies, the results showed similar for substrate specific for choline binding protein activity. Betaine and betaine aldehyde indicated complete inhibition choline uptake at salt stress conditions. Indeed, a detailed genetic and

biochemical analysis of choline glycine betaine pathway in *E. coli* has proven that choline has no osmoprotective properties (Styrvold *et al.*, 1986). *B. subtilis* thus share ability to oxidize choline to glycine betaine for osmoprotective purpose with a number of gram-negative and gram-positive bacteria (Abee *et al.*, 1990; Bernard *et al.*, 1986; D'Souza-Ault *et al.*, 1993; Kaenjak *et al.*, 1993; Lanfald and Strom, 1986; Le Rudier *et al.*, 1984; Rozwadowski *et al*., 1991). This oxidation is a two step process with glycine betaine aldehyde as an intermediate. In microorganisms, it can involve either a soluble choline oxidase that is proficient for both enzymatic reactions and a combination of a membrane-bound choline dehydrogenase (which also can oxidase glycine betaine aldehyde to glycine betaine) and soluble glycine betaine aldehyde dehydrogenase with a high degree of substrate specificity (Rozwadowski *et al.*, 1991).

A sudden osmotic upshock stimulates immediately the activity of preexisting choline transporters, and subsequent de novo synthesis is required to achieve maximal choline uptake activity. Thus, the expression of structural gene is essential for the response to changes in medium osmolarity. Both a modulation of the activity of the choline transporter and stimulation at the level of gene expression contribute also to the increase in choline transport observed in *E. coli* under high osmolarity growth conditions (Echoo, 1988; May *et al.*, 1986). A number of environmental factors (osmolarity, availability of oxygen and temperature) and the presence of choline in the growth medium all influence the transcription of the betT gene, which encodes the *E. coli* choline transport protein (Echoo, 1988). High osmolarity, low phosphate concentrations and the availability of choline are also known to stimulate the expression of the gene(s) for an effective choline transport system in *Staphylococcus aureus* that serves to scavenge choline from environment for synthesis of osmoprotectant glycine betaine (Kaenjak *et al.*, 1993). The requirement of Na^+, but not other cations such as K^+, Li^+, Rb^+, Ca^{2+}, and Mg^{2+}, for the uptake of choline by *A. halophytica* (Figure 18A) suggest that

choline transport need Na^+ for couple activity. HCO_3^- and CO_3^{2-} showed some effect for choline uptake activity.

The interesting finding in this study is high choline uptake activity at alkaline pH at all NaCl concentration testing. The optimal pH for choline uptake was 8 and at pH 9 the activity was decreased till pH 10. At pH 5 showed little choline uptake activity and could not uptake at pH 5 on 2.0 M NaCl. The dependence of betaine uptake on the extracellular pH was previously reported in *Lactococcus lactis* (Molenaar *et al.*, 1993). However, the increase of the extracellular pH resulted in the decrease of betaine uptake. This inverse relationship between extracellular pH and betaine uptake was proposed to be mediated by the intracellular K^+ concentration. It is intriguing to speculate that the intracellular Na^+ might mediate nitrate uptake in response to extracellular pH in *A. halophytica.*

The results shown in this study clearly showed that there is energy-dependent choline uptake in *A. halophytica*. The energetic component for choline uptake was ascribed to the contribution by the proton motive force (Δp) which consists of two components, i. e. the pH gradient (ΔpH) and the membrane potential (Δψ). This conclusion is based on the results showing (i) inhibition of choline uptake by various protonophores (Figure 31 – 34), (ii) an increased choline uptake when glucose or lactate was added as an energy source (Table 8).

The data from Figure 35 show an increase of choline uptake upon artificially generating ΔpH. This increase was observed independent of external pH. On the other hand, the suppression of ΔpH always resulted in a significant decrease of choline uptake, and again was independent of external pH. This behavior is expected when the ΔpH term of the proton motive force is the driving force for choline uptake. In contrast to the complete abolition of 4-hydroxybenzoate uptake in *Klebsiella planticola* strain DSZ1 when ΔpH was suppressed (Allende *et al.*, 2002), our results showed choline could not uptake when ΔpH was suppressed. However, this choline uptake activity disappeared when ΔpH was suppressed in

the presence of monensin. Thus, it was suggested that Na^+-electrochemical gradient dissipated by monensin (Pressman, 1976) was involved in choline uptake. The dependence of nitrate uptake on Na^+ was previously reported in *A. halophytica* and nitrate uptake into *A. halophytica* depends on ΔpH (Incharoensakdi and Wangsupa, 2003; Incharoensakdi and Laloknam, 2005). The inhibitors sodium p-chloromercurobenzoate (SH reagent), dinitrophenol (proton motive force), with N-ethylmaleimide and sodium p-chloromercurobenzoate the affect protein structure (complete inhibit choline uptake activity) (Figure 32C and 32D). The fact that the protonophores, CCCP, 2, 4-DNP and nigericin could completely inhibit the uptake of choline (Figure 33C, 33D, and 34A) also supports that choline uptake depend on proton motive force. There exists another energetic component in addition to proton motive force, i. e. the so called sodium motive force because there are some inhibitors for dissipate Na^+ gradient for example monensin (Figure 32B), Amiloride (Figure 32A), and gramicidin D (Figure 33A) which affect on choline uptake activity. Previously work suggested playing a role in nitrate uptake in *Anacystis nidulans* R2 depend on proton motive force and sodium motive force (Rodriguez *et al.*, 1992; Rodriguez *et al.*, 1994). The contribution by either a proton or a sodium gradient to amino acid and glycine betaine transport has previously been shown in some bacteria (Ekiel *et al*., 1985; Proctor *et al.*, 1997).

The overall results in Figure 31 – 35 suggest that the uptake of nitrate by A. halophytica is driven by energy and depend on ΔpH. The fact that DCCD, ouabain, and vanadate the ATPase inhibitor, and the ionophores, CCCP, 2, 4-DNP, and gramicidin D strongly inhibited the uptake of nitrate (Figure 31 – 34) highly suggests that the energy needed is provided by electron transport in the cytoplasmic membrane through a proton motive force, with the participation of ATP hydrolysis. Previous studies have shown that an ATP-binding cassette (ABC)-type system with the two ATP-binding proteins is involved in

nitrate uptake in the cyanobacterium *Synechococcus* sp. PCC7942 (Omata, 1995). Further work on the characterization of proteins responsible for nitrate uptake in *A. halophytica* is needed for a better understanding of nitrate uptake in cyanobacteria. The uptake choline by *A. halophytica* was susceptible to a variety of inhibitory agent. Effect of inhibitors for ATPase, proton motive force and Na^+ electrochemical gradients on the initial rate of ^{14}C choline uptake via osmotic stress condition were studied. Choline uptake into the cells is believed to be dependent on the proton motive force; therefore, it was not surprising that dinitrophenol, which is known to disrupt the proton motive force, was inhibitory.

Recently the uptake of choline with subsequent conversion to glycine betaine has been reported for *Synechococcus* PCC 7942 transformed with *E. coli* bet genes (Nomura *et al*., 1995). Choline uptake by *Synechococcus* PCC 7942 was also strongly inhibited by the uncouplers suggesting the presence of an energy-dependent transport system in *Synechococcus*. Future day concerning choline uptake in A. halophytica may be important in understanding process. Previously a glycine betaine uptake system has been reported in *A. halophytica* (Moor *et al*., 1987).

We tested product of choline after transported in to the *A. halophytica* at various time by $^{14}CO_2$ testing (data not shown), thin layer chromatography (TLC) and autoradiography (Figure 36 and 37). 14CO2 could not detect and choline did not change after incubation time was started until 60 min. This results summarized that choline acts an osmoprotectant. *E. coli* K-12 strains C600, C3000, and *Listeria monocytogenes* could use betaine and choline as osmoprotectant and thermoprotectant (Teresa *et al*., 1999; Beumer *et al*., 1994).

To characterize specific choline uptake activity by membrane vesicles were studied. The choline uptake into intact *A. halophytica* cells and membrane vesicles showed similar physiological properties. When the substrate concentration was varied the choline uptake system followed typical Michaelis-Menten kinetics. Using Lineweaver-Burk transformation

of the data, the apparent Km's of all concentration of NaCl (0 – 2 M) were nearly identical value about 82 - 88 μM. Vmax showed increasing choline uptake when NaCl concentration was increased and limited at 1.0 M NaCl. The overall results and Table 9 suggest that the uptake of choline *A. halophytica* membrane vesicles is driven by ΔpH. The contribution by Δψ was rather unlikely due to the findings that Δψ generated by either KSCN or valinomycin in the absence of K^+ had no stimulatory effect on the uptake of nitrate in acidic, neutral, and alkaline conditions (Table 9). Moreover, when Δψ was dissipated by valinomycin in the presence of K^+, no depression of choline uptake was observed (Table 9). The absence of an inhibitory effect by valinomycin suggests the absence of the contribution of Δψ to choline uptake. On the contrary, a slight stimulation of nitrate uptake occurred under the condition depleted of Δψ by valinomycin ($+K^+$) treatment (Table 9). This stimulation could occur due to (i) the action of Na^+-motive force (Na^+-gradient driven) since valinomycin specifically abolishes Δψ contributed by K^+ transport (Reed, 1979) and/or (ii) an increase of ΔpH term of the proton motive force is generated by a compensatory mechanism, i. e. an increase in ΔpH simultaneous to a decrease in Δψ (Reed, 1979; Kroll and Booth, 1981). Similar observations were reported for 4-hydroxybenzoate uptake in *Klebsiella planticola* whereby ΔpH was also implicated as the driving force (Allende *et al.*, 2002). Previously, it was also shown in the cyanobacterium *Plectonema boryanum* that ΔpH is the major energetic component with no involvement of Δψ for energy coupling in photosynthesis (Padan and Schuldiner, 1978).

All of data above showed that choline as an osmoprotectant and energy dependent by part of ΔpH. We would like to characterize choline uptake gene system by using molecular biology. Whole genome project of *A. halophytica* was started to study by Professor Dr. Teruhiro Takabe at Meijo University, Nagoya, Japan and detailed that no choline transporter gene containing in *A. halophytica*. *A. halophytica* contains betaine transporter gene while we try to isolate and characterize this gene.

In this work, we show that a halotolerant cyanobacterium *A. halophytica* has at least one BCCT-type betaine transporter. Based on the data that the choline, betaine, and proline transport-deficient *E.coli* MKH13 mutant cells became Na^+-tolerant by transformation with the Ap-*betT1* (Figure 42 and 43), and the membrane vesicles of these transformants exhibited the uptake of betaine (Figure 44 – 47), Ap-BetT could be assigned as betaine specific transporter. The requirement of Na^+, but not other cations such as K^+, Li^+, Rb^+, Ca^{2+}, and Mg^{2+}, for the uptake of betaine by Ap-BetT (Figure 44A) suggest that Ap-BetT is a Na^+-betaine symporter. This is also supported by the results showing that addition of NaCl increased Na^+ uptake concomitantly with the increase of betaine uptake with similar mole ratio (Figure 47A).

The interesting finding in this study is high betaine transport activity at alkaline pH. To our knowledge, the alkaline optimum pH such as 9.0 has not been reported previously in any transporters. For alkaliphile, active uptake of betaine at alkaline pH would be beneficial for cells to survive under these conditions. The H^+ uptake by Na^+/H^+antiporter is important to keep the cytoplasmic pH neutral. Na^+/H^+antiporter could extrude Na^+ into the extracellular space. To maintain H^+ homeostasis at alkaline pH, re-entry of Na^+ is required for optimal pH homeostasis (Padan *et al*., 2005). The Na^+-betaine symporter could be a re-entry route. Co-operation of Na^+-betaine symporter and Na^+/H^+ antiporter would lead to the adjustment of pH homeostasis as well as the accumulation of betaine essential for osmotic balance. This strategy is particularly important for *A. halophytica* to survive under salt stress and alkaline pH.

The pH dependence of betaine transporter from *A. halophytica* is quiet different from that of mangrove betaine transporters AmT1 and AmT2 (Waditee *et al.*, 2002). The mangrove AmT1 and AmT2 transport both betaine and proline as shown in Figure 46A and 46B. Betaine uptake by AmT1 and AmT2 increased with the decrease of pH, and the optimum pH was around 6.0 (Figure 46C). Although the importance of charged C-terminal domain of BetP (Rubenhagen *et al.*, 2000) and ProP (Wood *et al.*, 2005) for osmosensing have been demonstrated, no

structural information is available for the pH sensing of betaine transporter. Therefore, it would be interesting to identify the amino acid residues involved for pH sensing in Ap-BetT and AmT.

Figure 49A showed that the cells transformed with a vector pUC303m did not uptake betaine even in the presence of NaCl. Since *Synechococcus* sp. PCC7942 cells could not synthesize betaine (Nomura *et al.*, 1995). Transformation of *Synechococcus* sp. PCC7942 cells is easy. Therefore, these facts suggest that *Synechococcus* cells might be used as a model system to study the effects of betaine synthesis and betaine transport for the stress tolerance of cyanobacterium.

It is well demonstrated that betaine transporter is essentially regulated by the activation of the transporter (Poolman *et al.*, 2004). However, Figure 50E shows that the level of Ap-BetT protein increased with increasing salinity. Molecular mechanisms of this remain to clarify.

Figures 50C and 50D show that overexpression of Ap-BetT could confer the salt tolerance of *Synechococcus* cells in such a way that it is capable of growth in the medium containing 0.5 M NaCl and even in sea water. Hitherto, the attempt to improve the salt tolerance by the genetic transformation of betaine transporter has not been reported except for one recent study (Boscari *et al.*, 2004). The *Bradyrhizobium japonicum* strain USDA110, *Rhizobiaceae*, was transformed with the *betS* gene of *Sinorhizobium meliloti* that is a major BCCT type betaine transporter. Whereas betaine transport was absent in the USDA110 strain, salt-treated transformed cells accumulated large amounts of betaine (Boscari *et al.*, 2004). The transformant could grow in the medium containing 80 mM NaCl, but not 100 mM NaCl whereas the wild type USDA110 strain could not grow at 80 mM NaCl. Since the wild type *Synechococcus* PCC 7942 cells could not grow in the medium containing 380 mM NaCl (Nomura *et al.*, 1995; Waditee *et al.*, 2002), the present study showed the significant

improvement of salt tolerance mediated by Ap-BetT. The overexpression of ApNhaP antiporter from *A. halophytica* (Waditee *et al.*, 2002) and overexpression of glycine methylation genes from *A. halophytica* (Waditee *et al*., 2005) significantly improve the salt tolerance of the fresh water cyanobacterium *Synechococcus*, making it capable of growth in seawater. Therefore, overexpression of Ap-BetT, methylation genes, and ApNhaP together in cyanobacteria is an interesting and suitable model to understand the limiting factors hindering the improvement of salt tolerance in any organisms. Recently, we showed that the overexpression of glycine methylation genes significantly improve the stress tolerance of model plant *Arabidosis* (Waditee *et al.*, 2005). Therefore, the introduction of a set of genes with multiple functions to important crop plants such as rice is also an interesting challenge for the development of salt-tolerant crop plants.

CHAPTER V

CONCLUSIONS

The results of the present investigation can be summarized as follows;

1. Nitrate uptake was inhibited by various protonophores suggesting the coupling of nitrate uptake to the proton motive force.

2. An artificially-generated pH gradient across the membrane (ΔpH) caused an increase of nitrate uptake.

3. The suppression of ΔpH resulted in a decrease of nitrate uptake.

4. The increase of external pH also resulted in an enhancement of nitrate uptake.

5. The generation of the electrical potential across the membrane (Δψ) resulted in no elevation of the rate of nitrate uptake.

6. The valinomycin-mediated dissipation of Δψ caused no depression of the rate of nitrate uptake. Thus, it is unlikely that Δψ participated in the energization of the uptake of nitrate.

7. Na^+-gradient across the membrane was suggested to play a role in nitrate uptake since monensin which collapses Na^+-gradient strongly inhibited nitrate uptake.

8. Exogenously added glucose and lactate stimulated nitrate uptake in the starved cells.

9. *N*, *N*′- dicyclohexylcarbodiimide, an inhibitor of ATPase, could also inhibit nitrate uptake suggesting that ATP hydrolysis was required for nitrate uptake. All these results indicate that nitrate uptake in *A. halophytica* is ATP-dependent, driven by ΔpH and Na^+-gradient.

10. Choline and glycine betaine stimulated the growth of *A. halophytica* over the whole range of salinities.

11. Cell morphology of *A. halophytica* was change when transferred to osmolarity medium.

12. Glycine betaine was enhanced when stimulated by salt stress condition.

13. Ion contents showed that not so much different change under normal and stress condition.

14. Aspartate, serine, glutamine, proline, valine, lysine was observed to increase when cells was treated by salt stress.

15. All of fractions, cytoplasmic, periplasmic, and membrane fraction were induced by salt stress to produce some protein bands.

16. Membrane fraction contains choline binding protein activity when confirmed tested with radio-protein precipitation, autoradiography, and surface plasmon resonance technique.

17. Betaine, choline, betaine aldehyde, and carnitine showed strong inhibition of choline binding protein activity (radio-protein precipitation technique) and indicated high substrate specific for binding protein (surface plasmon resonance).

18. Choline uptake activity is stimulated by addition of NaCl and optimal choline uptake at 1.0 M NaCl.

19. Choline uptake can be triggered by increase with osmoticum (sorbitol, mannitol, and sucrose).

20. Lineweave-Burk transformation of the data, the apparent Km under non stress and salt stress condition at 1.0 M and 2.0 M were 246.78, 262.19 and 299.51 μM, respectively, the Vmax were 12.12, 24.10 and 10.71 nmol/min/mg protein, respectively.

21. Betaine and betaine aldehyde were strong inhibition for choline uptake at normal condition and completely inhibited at stress condition.

22. Na^+ was specific for choline uptake activity.

23. The optimal pH for choline uptake activity was pH 9.

24. The uptake of choline by *A. halophytica* was susceptible to a variety of inhibitory agent and CCCP, DNP, and nigericin showed complete inhibition choline uptake activity at both conditions.

25. Choline uptake was driven by ΔpH.

26. *A. halophytica* used choline as osmoprotectant because could not detect other products from reaction.

27. The *A. halophytica* membrane vesicles indicated similar physiological properties for choline uptake like intact cells.

28. The Km value for choline uptake into membrane vesicles at various salinity showed nearly value of 81 – 88 μM and Vmax was increased when medium was increased of NaCl concentration.

29. *A. halophytica* contains a betaine transporter gene (Ap-*betT*) which exhibits high homology to betaine transporters from *Bacillus subtilis* OpuD.

30. Ap-*bet*T in *E. coli* mutant strain MKH13, which lacks choline, betaine, and proline transport systems, only betaine uptake was restored.

31. Competition experiments demonstrated that choline, GABA, betaine aldehyde, sarcosine, dimethylglycine, and amino acids such as proline were not effective competitors for ApBetT-mediated betaine transport.

32. Ap-BetT could complement the Na^+-sensitive phenotype of the *E. coli* mutant MKH13.

33. Ap-BetT showed efficient uptake of betaine, but not proline.

34. Sodium, but not potassium markedly enhanced betaine uptake rates.

35. The mole ratio of Na^+ and betaine taken up was almost equal to one, thus suggesting Na^+-betaine symporter.

36. Ap-BetT exhibited strongly pH dependent betaine uptake activities, high at alkaline pH, with the optimum pH around 9.0.

37. Similar kinetic properties were observed in *A. halophytica* cells. Overexpression of Ap-*betT* in the freshwater cyanobacterium *Synechococcus* sp. PCC 7942 enhanced the salt tolerance of cells to the extent that the freshwater cyanobacterium could grow in sea water.

38. *A. halophytica* contains Na^+-betaine symporter which contributes to the salt stress tolerance at alkaline pH.

REFFERENCES

Abee, T., Palmen, R., Hellingwerf, K. J., and Konings, W. N. 1990. Osmoregulation in *Rhodobacter sphaeroides*. **J. Bacteriol**. 172: 149 – 154.

Aitken, D. M., A. J. Wicken, and A. D. Brown. 1970. Properties of a halophil nicotinamide-adenine dinucleotide phosphate-specific isocitrate dehydrogenase. **Biochem. J**. 116:125 – 134.

Albright, C. D., Lui, R., and Bethea, T. C. 1996. Choline deficiency induces apoptosis in SV40-immortalized CWSV-1 rat hepatocytes in culture. **FASEB**. J. 10: 510.

Ali, F., Laszlo, M., Ronan, S., Kay, M., Iwane, S., Martin, H., and Norio, M. 2003. Glucosylglycerol, a Compatible Solute, Sustains Cell Division under Salt Stress. **Plant Physiology**. 131: 1628 – 1637.

Allende, J. L., Gibello, A., Fortun, A., Mengs, G., Ferrer, E., and Martin, M. 2000. 4-Hydroxybenzoate uptake in an isolated soil *Acinetobacter* sp. **Curr. Microbiol**. 40: 34 – 39.

Allende, J. L., Gibello, A., Fortun, A., Sanchez, M., and Martin, M. 2002. 4-Hydroxybenzoate uptake in *Klebsiella planticola* strain DSZ1 is driven by ΔpH. **Curr. Microbiol.** 44: 31 – 37.

Anderson, C. B., and L. D. Witter. 1982. Glutamine and proline accumulation by *Staphylococcus aureus* with reduction in water activity. **Appl. Environ. Microbiol**. 43:1501 – 1503.

Anderson, P. A., Kaasen, I., Styrvold, O. B., Boulnois, G., and Storm, A. R. 1988. Molecular cloning, physical mapping and expression of the bet genes governing the

osmoregulatory choline-glycine betaine pathway of *Escherichia coli*. **J. Gen. Microbiol**. 134: 1737 – 1746.

Bagnasco, S., Balaban, R., Fales, H. M., Yang, Y. M., and Burg, M. 1986. Predominant osmotically active organic solutes in rat and rabbit renal medullas. **J. Biol. Chem**. 261: 5872 – 5877.

Beumer, R. R., Te Giffel, Cox, C. L. J., Rombouts, F. M., and Abee, T. 1994. Effect of Exogenous Proline, Betaine, and Carnitine on Growth of *Listeria monocytogenes* in a Minimal Medium. **App. Envi. Microbiol.** 2: 1359 – 1363.

Bird C, Wyman M. 2003. Nitrate/Nitrite assimilation system of the marine picoplanktonic cyanobacterium *Synechococcus* sp. Strain WH 8103: effect of nitrogen source and availibity on gene expression. **Appl Environ Microbiol** 69: 7009 – 7018.

Boch, J., Kempf, B., and Bremer, E. 1994. Osmoregulation in *Bacillus subtilis*: synthesis of the osmoprotectant glycine betaine from exogenously provide choline. **J. Bacteriol**. 176: 5364 – 5371.

Boch, J., Kempf, B., Schmid, R., and Bremes, E. 1996. Synthesis of the osmoprotectant glycine betaine in *Bacillus subtilis*: characterization of the gbsAB genes. **J. Bacteriol**. 178: 5121 – 5159.

Booth, I. R. 1985. Regulation of cytoplasmic pH in bacteria. Microbiol. Rev. 49: 359 – 378.

Booth, I. R., and Louise, P. 1999. Managing hypoosmotic stress: aquaporins and mechanosensitive channels in *Escherichia coli*. **Curr. Opin. Microbiol.** 2: 166 – 169.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microorganism quantities of protein utilizing the principle of protein-dye binding. **Anal. Biochem**. 72: 248-254.

Brouquisses, R., Wiegel, P., Rhodes, D., Yocum, C. F., and Hanson, A. D. 1989. Evidence for a ferridoxin-dependent choline monooxygenase from Spinach chloroplast stroma. **Plant Phisiol.** 90: 322 – 329.

Brown, A. D. 1976. Microbial water stress. **Bacteriol Rev**. 40: 803 – 846.

Byerrum, R. U., Sato, C. S., and Ball, C. D. 1956. Utilization of betaine as a methyl group donor in tobacco. **Plant Physiol.** 31: 374 – 377.

Cayley, S., Lewis, B.A., and Record, M. T. Jr 1992. Origins of the osmoprotective properties of betaine and proline in *Escherichia coli* K-12. **J. Bacteriol**. 174: 1586 – 1595.

Cayley, S., Lewis, B.A., Guttman, H.J., Record, M. T. Jr. 1991. Characterization of the cytoplasm of *Escherichia coli* K-12 as a function of external osmolarity. Implications for protein-DNA interactions *in vivo*. **J. Mol. Biol**. 222: 281 – 300.

Choquet, C. G., Ahonkhai, I., Klein, M., and Kushner, D. J. 1991. Formation and role of glycine betaine in themodulately halophile *Vibrio costicola*. **Arch. Microbiol**. 155: 153 – 158.

Crawford, N. M. and Glass, A. D. M. 1998. Molecular and physiological aspects of nitrate uptake in plants. **Trends Plant Sci.** 3: 389 – 395.

Csonka, L. N. 1981. Proline over-production results in enhanced osmotolerance in *Salmonella typhimurium*. **Mol. Gen. Genet.** 182: 82 – 86.

Csonka, L. N. 1989. Physiological and genetic responses of bacteria to osmotic stress. **Microbiol. Rev.** 53: 121 – 147.

Csonka, L. N., and Hanson, A. D. 1991. Prokaryotic osmoregulation: Genetics and Physiology. **Annu. Rev. Microbiol**. 45: 569 – 606.

Ekiel, I., Jarrel, K. F., and Sprot, G. D. 1985. Amino acid biosynthesis and sodium-dependent transport in *Methanococcus voltae*, as revealed by 13C-NMR. **Eur. J. Biochem**. 149: 437 – 444.

Espie, G. S. and Kandasamy, R. A. 1994. Monensin inhibition of Na^+-dependent HCO3 -transport distinguishes it from Na^+-independent transport and provides evidence for Na+/HCO3 -symport in the cyanobacterium *Synechococcus* UTEX 625. **Plant Physiol**. 104: 1419 – 1428.

Flores, E. and Herrero, A. 1994. Assimilatory nitrogen metabolism and its regulation; in **The Molecular Biology of Cyanobacteria**, Bryant, D. A. (ed.), Kluwer Academic Publishers, Dordrecht, the Netherlands. PP: 487 – 517.

Fulda, S. and Hageman, M. 1995. Salt treatment induces accumulation of flavodoxin in the cyanobacterium *Synechocystis* sp. PCC 6803. **J. Plant Physiol**. 146: 520 – 526.

Fougere, F. and Le Rudulier, D. 1990. Glycine betaine biosynthesis and catabolism in bacteroids of Rhizobium meliloti; effect of salt stress. **J. Gen. Microbiol**. 136: 2503 – 2510.

Galinski, E. A. 1994. Osmoleculation in bacteria. **Adv. Microbiol. Physiol**. 37: 273-328.

Galinski, E. A. and Truper, H. G. 1982. Betaine, a compatible solute in the extremely halophilic phototrophic bacterium *Ectothiorhodospira halochlochlosis*. **FEMS**. Microbiol. Lett. 13: 357 – 360.

Galinski, E. A., and Herzog, R. M. 1990. The role of treharose as a substitute for nitrogen-containing compatible solutes (*Ectothiorhorospira halochloris*). **Arch. Microbiol.** 153: 607 – 613.

Garcia-Sanchez, M. J., Jaime, M. P., Ramos, A., Sanders, D., and Fernandez, J. A. 2000. Sodium-dependent nitrate transport at the plasma membrane of leaf cells of the marine higher plant *Zostera marina* L. **Plant Physiol.** 122, 879 – 885.

Garlick, S., Oven, A., and Padan, A. 1977. Occurrence of facultative an oxygenic photosynthesis among filamentous and unicellular cyanobacteria. **J. Bacteriol.** 129: 623 – 629.

Gilson, E., Alloing, G., Schmitdt, T., Claverys, J. P., Dudler, R., and Hofnung, M. 1988. Evidence for high affinity binding-protein dependent transport systems in gram-positive bacteria and in *Mycoplasma*. **EMBO**. J. 7: 3971 – 3974.

Guerrero, M.G., Romero, J.M., Rodriguez, R., and Lara, C. 1990. Nitrate transport in cyanobacteria. In: Ullrich, W.R., Rigano, O., Fuffi, A., Aparicio, P.J. (eds) **Inorganic nitrogen in plants and microorganisms**. Berlin: Springer Verlag. P: 79 – 85.

Gunnewijk, M. G. W., and Poolman, B. 2000. HPr (His~P)-mediated phosphorylation differently affects counterflow and proton motive force-driven uptake via the lactose transport protein of *Streptococcus thermophilus*. **J. Biol.Chem**. 275: 34080 – 34085.

Gutierrez, C., Ardourel, M., Bremer, E., Middendort, A., Boos, W., and Ehmann, U., 1989. Analysis and DNA sequence of the osmoregulated treA gene encoding yhe periplasmic treharase of *Escherichia coli* K-12. **Mol. Gen. Genet**. 217: 347 – 354.

Hanson, A. D., May, A. M., Grumet, R., Bode, J., Jamieson, G. C., and Rhodes, D. 1985. Betaine synthesis in chenopods: Localization in chloroplasts. **Proc. Natl. Acad. Sci. USA**. 82: 3678 – 3682.

Hanson, A. D., Rathinasabapathi, B., Chamberlain, B., and Gage, D. A. 1991. Comparative physiological evidence that beta-alanine betaine and choline-o-sulfate act as compatible osmolytes in halophytic *Limonium* species. **Plant Physiol**. 97: 1199 – 1205.

Haubrich, D. R., and Gerber, N. H. 1981. Choline dehydrogenase assay, properties and inhibitors. **Biochem. Pharmacol.** 30: 2993 – 3000.

Huchins, R. W., Ellefson, W. L., and Kashket, E. R. 1987. Betaine transport imparts osmotolerance on a strain of *Lactobacillus acidophilus*. **Appl. Environ. Microbiol**. 53: 2275 – 2281.

Ikuta, S. D., Imamura, S., Misaki, H., and Horiuti, Y. 1997. Purification and characterization of choline oxidase from *Arthrobacter globioformis*. **J. Biochem**. 82: 1741 – 1749.

Incharoensakdi, A. and Karnchanatat, A. 2003. Salt stress enhances choline uptake in the halotolerant cyanobacterium *Aphanothece halophytica*. **Biochim. Biophys. Acta**. 1621: 102-109.

Incharoensakdi, A. and Kum-arb, U. 1998. Betaine aldehyde dehydrogenase from a halotolerant cyanobacterium *Aphanothece halophytica*: purification, properties, and regulation by salinity. **J. Sci. Soc. Thailand.** 24: 231-240.

Incharoensakdi, A., and Laloknam, S. 2005. Nitrate uptake in the halotolerant cyanobacterium *Aphanothece halophytica* is energy-dependent driven by ΔpH. **J. Biochem. Mol. Biol.** 38: 468 – 473.

Incharoensakdi, A. and Waditee, R. 2000. Degradation of glycine betaine by betaine-homocysteine methyltransferase in *Aphanothece halophytica*: effect of salt downshock and starvation. **Curr. Microbiol**. 41: 227 – 231.

Incharoensakdi, A. and Wangsupa, J. 2003. Nitrate uptake by the halotolerant cyanobacterium *Aphanothece halophytica* grown under non-stress and salt-stress conditions. **Curr. Microbiol**. 47: 255 – 259.

Incharoensakdi, A. and Wutipraditkul, N. 1999. Accumulation of glycine betaine and its synthesis from radioactive precursors under salt-stress in the cyanobacterium *Aphanothece halophytica.* **J. Appl. Phycol**. 11: 515-523.

Incharoensakdi, A., Takabe, T., and Akazawa, T. 1986. Effect of betaine on enzyme activity and subunit interaction of ribulose 1, 5-biphosphate carboxylase/oxygenase from *Aphanothec halophytica.* **Plant. Physiol.** 81: 1044-1049.

Ishitani, M., Nakamura, T., Han, S. Y., and Takabe, T. 1995. Expression of the betaine aldehyde dehydrogenase gene in barley in response to osmotic stress and abcisic acid. **Plant Mol. Biol**. 27: 307 – 315.

Jebbar, M., von Blohn, C., and Bremer, E. 1997. Ectoine functions as an osmoprotectant in *Bacillus subtilis* and is accumulated via the ABC-transport system OpuC. **FEMS. Microbiol Lett**. 154: 325 – 330.

Joshi, A. K., Ahmed, S. and Ferro-Luzzi, G. 1989. Energy coupling in bacterial periplasmic transport systems. **J. Biol. Chem**. 264: 2126 – 2133.

Kapper, R. M., Kempf, B., and Bremer, F. 1996. Three transport systems for the osmoprotectant glycine betaine operate in *Bacillus subtilis*: characterization of OpuD. **J. Bacteriol**. 178: 5071 – 5079.

Kempf, B., and Bremer, E. 1995. OpuA, an osmotically regulated binding protein-dependent transport system for the osmoprotectant glycine betaine in Bacillus subtilis. **J. Biol. Chem**. 270: 16701 – 16713.

Kiene, R. P. 1998. Uptake of choline and its conversion to glycine betaine by bacteria in esturine waters. **Appl. Environ. Microbiol**. 64: 1045-1051.

Knipper, M., Kahle, C., and Breer, H. 1991. Purification and reconstitution of the high affinity choline transporter. **Biochim. Biophys. Acta**. 1065: 107 – 113.

Ko, R., Smith, L. T., and Smith, G. M. 1994. Glycine betaine confers enhanced osmotolerance and crotolerance on *Listeria monocytogenes*. **J. Bacteriol.** 176: 426 – 431.

Kroll, R. G. and Booth, I. R. 1981. The role of potassium transport in the generation of a pH gradient in *Escherichia coli*. **Biochem. J.** 198: 691 – 698.

Kruip, J. Karapetyan, N. V., Terkhovas, I. V., and Rogner, M. 1999. In vitro oligomerization of membrane protein complex. **J. Biol. Chem**. 274: 18181 – 18188.

Krulwich, T. A. and Guffanti, A. A. 1989. The Na^+cycle of extreme alkalophiles: a secondary Na^+/H^+ antiporter and Na^+/solute symporters. **J. Bioenerg. Biomembr**. 21: 663 – 677.

Kushner, D.J. 1988. "What is the 'true' internal environment of halophilic and other bacteria?" **Can. J. Microbiol**. 34:482 – 6.

Laemmli, U.K. 1970. Cleavage of Structural Proteins during the assembly of head of bacteriophage T4. **Nature**. 227: 680 – 685.

Lamark, T., Rokenes, T. P., McDougall, J., and Strom, A. 1996. The complex bet promotors of *Escherichia coli* regulation on oxygen (ArcA), choline (BetI), and osmotic stress. **J. Bacteriol**. 178: 1655-1662.

Lanfald, B. and Strom, A. R. 1986. Choline-glycine betaine pathway confers a high level of osmotic tolerance in Escherichia coli. **J. Bacteriol**. 165: 849-855.

Lanyi, J. K. 1974. Salt-dependent properties of proteins from extremely halophilic bacteria. **Bacteriol. Rev.** 38: 272 – 290

Lara, C., Romero, J. M. and Guerrero, M. G. 1987. Regulated nitrate transport in the cyanobacterium *Anacystis nidulans.* **J. Bacteriol**. 169: 4376 – 4378.

Le Rudulier, D. and Bouillard, L. 1983. Glycine betaine, an osmotic effectorin Klebsiella pneumoniae and other members of the *Enterobacteriaceae*. **Appl. Environ. Microbiol.** 46: 152 – 159.

Le Rudulier, D., Storm, A. R., Dandekar, A. M., Smith, L. T., and Valentine, R. C. 1984. Molecular biology of osmoregulation. **Science**. 224: 1064 – 1068.

Lin, C. S. and Wu, R. D. 1986. Choline oxidase and choline dehydrogenase. **J. Prot. Chem**. 5: 193 – 200.

Mackey, M. A., Norton, R. S., and Borowitzka, L. J. 1984. Organic osmoregulatory solutes in cyanobacteria. **J. Gen. Microbiol**. 130: 2177 – 2191.

Mackinney, G. 1941. Absorption of light by chlorophyll solutions. **J. Biol. Chem**. 140: 314 – 322.

Marcin, P.M., Hanna, C., Jacek, S., Bozena, S., Grzegorz, K., Lukasz, K., Artur, O., Celia, F.B., and Krzysztof, W.S. 2004. Inducible heat shock protein 70 promotes myelin acute antigen presentation by HLA classII. **J. Immunol.** 172: 202 – 213.

Martin, D. D., Ciulla, R. A., and Roberts, M. F. 1999. Osmoadaptation in archaea. **Appl Environ Microbiol.** 65: 1815 – 1825.

Matoh, T., Watanabe, J., and Takahashi, E. 1987. Sodium, potassium, chloride and betaine concentrations in isolated vacuoles from salt-grown *Atriplex gmelini* leaves. **Plant Physiol.** 84: 173 – 177.

Meharg, A. A. and Blatt, M. R. 1995. NO3- transport across the plasma membrane of *Arabidopsis thaliana* root hairs: kinetic control by pH and membrane voltage. **J. Membr. Biol.** 145: 49 – 66.

Miller, A. J. and Smith, S. J. 1996. Nitrate transport and compartmentation in cereal root cells. **J. Exp. Bot**. 47: 843-854.

Mochizuki-Oda, N. and Oosawa, F. 1985. Amiloride-sensitive Na^+-H^+ antiporter in *Escherichia coli*. **J. Bacteriol.** 163: 395 – 397.

Mohammad, F. A. A., Reed, R. H., and Stewart, W. D. P. 1983. The halophilic cyanobacterium *Synechocystis* DUN 52 and its osmotic response. **FEMS. Microbiol. Lettl**. 16: 287 – 290.

Molenaar, D., Hagting, A., Alkema, H., Driessen, A. J. M., and Konings, W. N. 1993. Characteristics and osmoregulatory roles of uptake systems for proline and glycine betaine in *Lactococcus lactis*. **J. Bacteriol**. 175: 5438 – 5444.

Moore, D. J., Reed, R. H., and Stewart, W. D. P. 1987. A glycine betaine transport system in *Aphanothece halophytica* and other glycine betaine-synthesising cyanobacteria. **Arch. Microbiol.** 147: 399 – 405.

Murakami, H., Sawada, N., Koyabu, N., Ohtami, H., and Sawada, Y. 2000. Characteristics of choline transport across the blood-brain barrier in mice: correlation with in vitro data. **Pharm. Res.** 17: 1526 – 1530.

Nagasawa, T., Mori, N., Tani, Y., and Ogata, K. 1976. Characterization of choline dehydrogenase from *Pseudomonas aerugenosa* A-16. **Agric. Biol. Chem**. 40: 2077 – 2084.

Neu, H. C. and Heppel, L. A. 1965. The release of enzyme from *Escherichia coli* by osmotic shock and during the formation of spheroplast. **J. Biol. Chem**. 240: 3685-3692.

Nomura, M., Ishitani, M., Takabe, T., Rai, A. K., and Takabe, T. 1995. *Synechococcus* sp. PCC 7491 transformed with *Escherichia coli* bet genes produces glycine betaine from choline and acquires resistance to salt stress. **Plant Physiol**. 107: 703 – 708.

Ohta-Fukuyama, M., Miyake, T., Emi, S., and Yamano, T. 1980. Identification and properties of prosthetic group of choline oxidase from *Alcaligenes* sp. **J. Biochem.** 88: 197 – 203.

Omata, T. 1995. Structure, function and regulation of the nitrate transport system of the cyanobacterium *Synechococcus* sp. PCC 7942. **Plant Cell Physiol**. 36: 207 – 213.

Oren, A. 1986. Intracellular salt concentrations of the anaerobic halophilic eubacteria *Haloanaerobium praevalens* and *Halobacteroides halobius.* **Can. J. Microbiol.** 32: 4 – 9.

Oren, A. 1990. Formation and breakdown of glycine betaine and trimethylamine in hypersaline environments. **Antonie v. Leeuwenhoek**. 58: 291 – 298.

Oren, A. 1999. Bioenergetic aspect of Halophilism. Microbiol. **Mol. Biol. Rev**. 63: 334 – 338.

Oren, A., Heldal, M., and Norland, S. 1997. X-ray microanalysis of intracellular ions in the anaerobic halophilic eubacterium *Haloanaerobium praevalens.* **Can. J.Microbiol.** 43: 588 – 592.

Oren, A., Simon, G., and Galinski, E. A. 1991. Intracellular salt and solute concentrations in *Ectothiorhodospira marismortui*: glycine betaine and N'-carbamoyl glutamineamide as osmotic solutes. **Arch. Microbiol.** 156: 350 – 355.

Otto, R., Lageveen, R.G., Veldkamp, H., Konings, W. 1982. Lactate efflux-induced electrical potential in membrane vesicles of *Streptococcus cremoris*. **J. Bacteriol**.49: 733 – 738.

Padan, E. and Schuldiner, S. 1978. Energy transduction in the photosynthetic membranes of the cyanobacterium *Plectonema boryanum*. **J. Biol. Chem**. 253: 3281 – 3286.

Padan, E. and Schuldiner, S. 1996. Bacterial Na+/H+ antiporters: molecular biology, biochemistry, and physiology; in Handbook of **Biological Physics**, Konings, W. N., Kaback, H. R. and Lolkema, J. S. (eds.), Elsevier Science, Amsterdam, the Netherlands. PP: 501 – 531.

Panfili, G., Manzi, P., Compagnon, D., Scaciglia, L., and Palleschi, G. 2000. Rapidly assay of choline in food using microwave hydrolysis and choline biosensor. **J. Agric. Food Chem.** 48: 3430 – 3407.

Perroud, B. and Le Rudulier, D. 1985. Glycine betaine transport in *Escherichia coli*: osmotic modulation. **J. Bacteriol**. 161: 393 – 401.

Pocard, J. A., Benard, T., Smith, L. T., and Le Rudulier, D. 1989. Characterization of three choline transport activities in *Rhizobium meliloti*: modulation by choline and osmotic stress. **J. Bacteriol.** 171: 531 – 537.

Pourkomailian, B. and Booth, I. R. 1994. Glycine betaine transport by *Staphylococcus aureus*: evidence for feedback regulation of the activity of the two transport systems. **Microbiol.** 140: 3131 – 3138.

Pressman, B. C. 1976. Biological application of ionophores. **Annu. Rev. Biochem**. 45: 501 – 530.

Proctor, L. M., Lai, R., and Gunsalus, R. P. 1997. The methanogenic archaeon *Methanosarcina thermophila* TM-1 possesses a high affinity glycine betaine transporter involved in osmotic adaptation. **Appl. Environ. Microbiol.** 63: 2252 – 2257.

Reed, P. W. 1979. Ionophores. Meth, **Enzymol**. 55: 435 – 454.

Reed, R. H., Chudek, J. A., Foster, R., and Stewart, W. D. P. 1984. Osmotic adjustment in cyanobacteria from hypersaline environments. **Arch. Microbiol**. 138: 333 – 337.

Reed, R. H., Richardson, D. L., and Stewart, W. D. P. 1985. Na+ uptake and extrusionin the cyanobacterium *Synechocystis* PCC 6714 in response to hypersaline treatment. Evidence for transient changes in plasmalemma Na^+ permeability. **Biochim. Biophysic. Acta.** 814: 347 – 355.

Reed. R. H. and Stewart, W. D. P. 1985. Evidence for turgor-sensitive K+ influx in the cyanobacterium *Anabaena variabilis* ATCC 29413 and Synechocystis PCC 6714. **Biochim Biophysic Acta.** 812: 155 – 162.

Rengpipat, S., Lowe, S. E., and Zeikus, J. G. 1988. Effect of extreme salt concentrations on the physiology and biochemistry of *Halobacteroides acetoethylicus.* **J. Bacteriol**.170: 3065 – 3071.

Roberts, M. F. 2004. Osmoadaptation and osmoregulation in archaea: update 2004. **Front Biosci**. 9: 1999 – 2019.

Robertson, D. E., Noll, D., Roberts, J. A., Menaia, G. F., and Boone, D. R. 1990. Detection of osmoregulator betaine in methanogens. **Appl. Environ. Microbiol**. 56: 563 – 565.

Robinson, S. P. and Jones G.P. 1986. Accumulation of glycine betaine in chloroplast provides osmotic adjustment during salt stress. **Aust. J. Plant Physiol**. 13: 659 – 668.

Rodriguez, R., Guerrero, M. G. and Lara, C. 1994. Mechanism of sodium/ nitrate symport in *Anacystis nidulans* R2. **Biochim. Biophys. Acta.** 1187: 250 – 254.

Rodriguez, R., Lara, C. and Guerrero, M. G. 1992. Nitrate transport in the cyanobacterium *Anacystis nidulans* R2: kinetic and energetic aspects. **Biochem. J**. 282: 639 – 643.

Rosentein, R., Futter-Bryniok, D., and Gotz, F. 1999. The choline-converting pathway in *Staphylococcus xylosus* C2A: genetic and physiological characterization. **J. Bacteriol**. 181: 2273 – 2278.

Rothschild, H. A. and Barron, E. S. G. 1954. The oxidation of betaine aldehyde by betaine aldehyde dehydrogenase. **J. Biol. Chem**. 209: 511 – 523.

Sakaguchi, K. 1960. Betaine as a growth factor for *Pediococcus soyae* VIII studies on the activities of bacteria in soy sauce brewing. **Bull. Agri. Chem. Soc. Jpn**. 24: 489 – 496.

Sandeep, P., John, E., Kaplan, M., and Fred, L. M. 1997. Protein, not adenosine or adenine nucleotides, mediates platelt decrease in endothelial permeability. **The Am. Physiol. Soc**., 12: 199 – 207.

Shkedy-Vinkler, C. and Avi-Dor, Y. 1975. Betaine-induced stimulation of respiration of high osmolarities in halotolerant bacterium. **Biochem. J.** 150: 219 – 226.

Skiba, W. E., Taylor, M. P., Wells, M. S., Mangum, J. H., and Awad, W. M. J. 1982. Human hepatic methionine biosynthesis purification and characterization of betaine: homocysteine S-methyltransferase. **J. Biol. Chem**. 257: 14944 – 14948.

Smith, L. T., Pocrad, J. A., Berward, T., and Le Rudulier, D. 1988. Osmotic control of glycine betaine biosynthesis and degradation in *Rhizobium meliloti*. **J. Bacteriol**. 143: 176 – 181.

Storm, A. R. and Kaasen, I. 1993. Treharose metabolism in Escherichia coli: stress protection and stress regulation of gene expression. **Mol. Microbiol.** 29: 285 – 296.

Styvold, O. B., Falkenberg, P., Lanfald, B., Eshoo, M. W., Bjornsen, T., and Storm, A. R. 1986. Selection, mapping and characterization of osmoregulatory mutants of

Escherichia coli blocked in the choline-glycine betaine pathway. **J. Bacteriol**. 165: 856 – 863.

Summers, P. S. and Weretilnyk, E. A., 1993. Choline synthesis in spinach in relation to salt stress. **Plant Physiol**. 103: 1269 – 1276.

Takabe, T., Incharoensakdi, A., Arakawa, K. and Yokota, S. 1988. CO2 fixation and RuBisCO content increase in a highly halotolerant cyanobacterium *Aphanothece halophytica*, grown in high salinity. **Plant Physiol.** 88: 1120 – 1124.

Teresa, C., Nathalie, D. C., and Alexandre, G. 1999. Thermoprotection by glycine betaine and choline. **Microbiol**. 51: 135 – 177.

Umbarger, H. E. 1978. Amino acid biosynthesis and its regulation. **Annu. Rev. Biochem**. 64:171 – 185.

Ventosa, A., Nicto, J. J., and Oren, A. 1998. Biology of moderately halophilic aerobic bacteria. **Microbiol. Mol. Biol**. **Rev**. 62: 504 – 544.

Vijaranakul, U., Nadakavukaren, M. J., de Jonge, B. L. M., Wilkinson, B. J., and Jayaswal, R. K. 1995. Increased cell size and shortened peptidoglycan interpeptide bridge of NaCl-stressed *Staphylococcus aureus* and their reversal by glycine betaine. **J Bacteriol** 177: 5116 – 5121.

Vijaranakul, U., Nadakavukaren, M. J., Bayles, D. O., Wilkinson, B. J., and Jayaswal, R.K. 1997. Characterization of an NaCl-sensitive *Staphylococcus aureus* mutant and rescue of the NaCl-sensitive phenotype by glycine betaine but not by other compatible solutes. **Appl Environ Microbiol** 63: 1889 – 1897.

Vyas, S. and O'Regan, S. 1985. Reconstitution of carrier-mediated choline transport in proteoliposome prepare from presynaptic membranes of Torpedo electric organ, and its internal and external ionic requirements. **J. Membr. Biol**. 85: 111 – 119.

Waditee, R., Hibino, T., Nakamura, T., Incharoensakdi, A., and Takabe, T. 2002. Overexpression of Na+/H+ antiporter confers salt tolerance on a fresh water cyanobacterium, making it capable of growth in sea water. **Proc. Natl. Acad. Sci. USA** 99: 4109 – 4114.

Waditee, R., Hibino, T., Tanaka, Y., Nakamura, T., Incharoensakdi, A., and Takabe, T. 2001. Halotolerant cyanobacterium *Aphanothece halophytica* contains an Na+/H+ antiporter, homologous to eukaryotic ones, with novel ion specificity affected by C-terminal tail. **J. Biol. Chem.** 276: 36931 – 36938.

White, R. F. and Demain, A. L. 1971. Catabolism of betaine and its relationship to cobalmin overproduction. **Biochim. Biophysic. Acta.** 237: 112 – 119..

Williams, J. G. K. 1988. Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803. **Meth. Enzymol** 167: 766-778.

Wood, J. M. 1999. Osmosensing by bacteria: signal and membrane based sensors. **Microbiol. Mol. Biol. Rev**. 63: 230-262.

Wood, J. M., Erhard, M., Laszlo, N. C., Reinhard, K., Bert, P., Tiemen, vander. H., and Linda, T. S. 2001. Osmosensing and osmoregulatory compatible solute accumulation by bacteria. **Compare. Biochem. Physiol. Part A**. 130: 437-460.

Wyn Jones, R. G., Storey, R., Leigh, R. A., Ahmad, N., and Pollard, A. 1977. A hypothesis on cytoplasmic osmoregulation. **In Regulation of cell membrane activities in higher plants**, edited by E. Marre and C. Ciferri. Elsevier, North Holland, Amsterdam. P: 121 – 136.

Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D., and Somero, G. N. 1982. Living with water stress: evolution of osmolyte systems. **Science**. 217: 1214 – 1222.

Yancey, P. H. 1994. Compatible and counteracting solutes. In **"Cellular and Molecular Physiology of Cell Volume Regulation"** (K Strange ed), CRC Press, Boca Raton, pp. 81 – 109.

Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D., and Somero, G. N. 1982. Living with water stress: evolution of osmolyte systems. **Science**. 217: 1214 – 1222.

Yim, H. H. and Villarejo, M. 1992. A new hyperosmotically inducible gene, encodes a periplasmic protein in *Escherichia coli*. **J. Bacteriol.** 174: 3637 – 3644.

Zahran, H. H. 1997. Diversity, adaptation and activity of the bacterial flora in saline environment. **Biol. Fertil. Soils.** 25: 211 – 223.

Zeisel, S. H. 2000. Choline: an essential nutrient for humans. **Nutrition**. 16: 669 – 671.

Zeisel, S. H. and Blusztajn, J. K. 1994. Choline and human nutrition. **Annu. Rev. Nutr**. 14: 269.

Zhang, J., Blusztaln, J. K., and Zeisel, S. H. 1992. Measurement of the formation of betaine aldehyde and betaine in rat liver mitochondria by high pressure liquid chromatography-radioenzymatic assay. **Biochem. Biophysic. Acta.** 1117: 333 – 339.

APPENDICES

APPENDIX 1

BG_{11} medium

Turk Island Salt Solution + modified BG_{11} medium contained the following component:

1. Preparation of Turk Island Salt Solution

Stock solution A:	KCl	33.3	g
	$MgCl_2.6H_2O$	275.0	g
	$CaCl_2.2H_2O$	73.3	g
	And made up to 5 litres with distilled water		
Stock solution B:	MgSO4.7H2O	347.0	g
	And then made up to 5 litres with distilled water		

To make Turk Island Salt Solution, 500 ml of Stock solution A was added to 500 ml of Stock solution B. To this mixture 140.8 g of NaCl was added and the final volume was made to 5 litres distilled water.

2. Composition of modified BG_{11} medium (BG_{11} medium + $NaNO_3$ solution)

$NaNO_3$ (75 g/500 ml)	50	ml
KH_2PO_4 (8 g/200 ml)	05	ml
$MgSO_4$.7H2O (15 g/200 ml)	05	ml
$CaCl_2.2H_2O$ (7.2 g/200 ml)	05	ml
Na_2CO_3 (4 g/200 ml)	05	ml
Citric acid (1.2 g/200 ml)	05	ml
EDTA.Na_2 (0.2 g/200 ml)	05	ml
*Trace element A_5 Solution + Co	05	ml

*Trace element A_5 Solution + Co cont A_5 Solution contained the following component in gram per litre H_3PO_4 [2.86], $ZnSO_4.7H_2O$ [0.2], $CuSyptO_4.5H_2O$ [0.08], $MnCl_2.4H2O$ [1.81], $Na_2MnO_4.2H_2O$ [0.39], and $Co(NO_3)_2.6H_2O$ [0.049].

Culture medium of *Aphanothece halophytica* was prepare by adding all solution of item 2 at indicated volume to 5 litres of Turk Island Salt solution and the pH was adjusted to 7.6 by slowly adding 2 M NaOH. The medium was sterilized by autoclaving at 15 lb/inch^2 for 15 minutes.

APPENDIX 2

Preparation of 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-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	18.2	g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100ml with distilled water.

2 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	24.2	g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100ml with distilled water.

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	6.1	g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100ml with distilled water.

1 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	12.1	g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100ml with distilled water.

10% SDS

SDS	10	g
Distilled water	100	ml

10% ammoniumpersulfate

Ammoniumpersulfate	1	g
Distilled water	10	ml

0.5% bromophenol blue

bromophenol blue	0.05	g
distilled water	10	ml

Solution B (SDS-PAGE)

2 M Tris-HCl, pH 8.8	75	ml
10% SDS	4	g
Distilled waster	21	ml

Solution C (SDS-PAGE)

1 M Tris-HCl, pH 6.8	50	ml
10% SDS	4	ml

2. Non-denaturing PAGE

12.0% separating gel

30% acrylamide solution	4.17	ml
1.5 M Tris-HCl, pH 8.8	2.50	ml
Distilled water	3.33	ml
10% ammoniumpersulfate	50	μl
TEMED	5	μl

5.0% stacking gel

30% acrylamide solution	1.67	ml
0.5 M Tris-HCl, pH 8.8	2.50	ml
Distilled water	5.8	ml
10% ammoniumpersulfate	50	μl
TEMED	5	μl

Sample buffer

0.5 M Tris-HCl, pH 6.8	1	ml
Glycerol	0.8	ml
0.5% bromophenol blue	0.5	ml
Distilled water	5.8	ml

Electrophoresis buffer (1,000 ml)

(25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-amenomethane	3.0	g
Glycine	14.4	g

Dissolved in distilled water to 1000 ml. Do not adjust pH with acid or base (final pH should be 8.3).

3. SDS-PAGE

12.0% separating gel

30% acrylamide solution	4.17	ml
Solution B	2.50	ml
Distilled water	3.33	ml
10% ammoniumpersulfate	50	µl
TEMED	5	µl

5.0% stacking gel

30% acrylamide solution	1.67	ml
Solution C	2.50	ml
Distilled water	5.8	ml
10% ammoniumpersulfate	50	µl
TEMED	5	µl

Sample buffer

0.5 M Tris-HCl, pH 6.8	0.6	ml
Glycerol	0.8	ml
0.5% bromophenol blue	0.5	ml
10% SDS	2.0	ml
2-mercaptoethanol	0.5	ml
Distilled water	5.8	ml

One part of sample buffer was added to fourth part of sample. The mixture was heated 5 minutes in boiling water before loading to the gel.

Electrophoresis buffer (1,000 ml)

(25 mM Tris, 192 mM glycine)

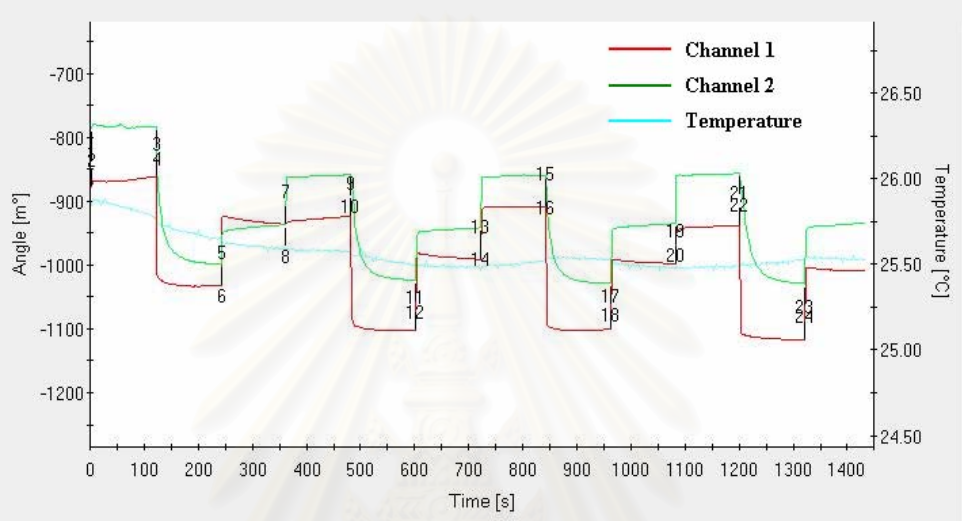
Tris (hydroxymethyl)-amenomethane	3.0	g
Glycine	14.4	g
SDS	1.0	g

Dissolve in distilled water to 1000 ml. Do not adjust pH with acid or base (final pH should be 8.3).

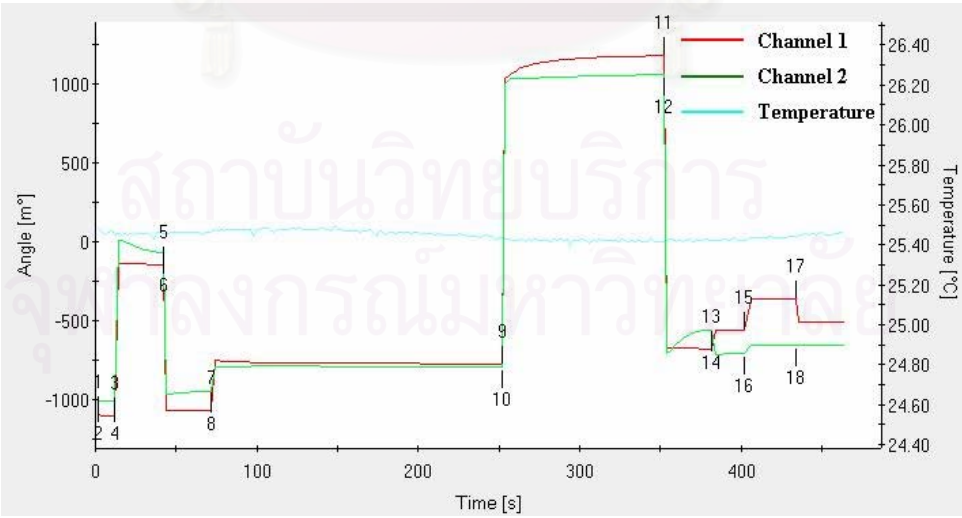
APPENDIX 3

Sensorgram of SPR

A) Sensorgram shows steps of gold disk activation

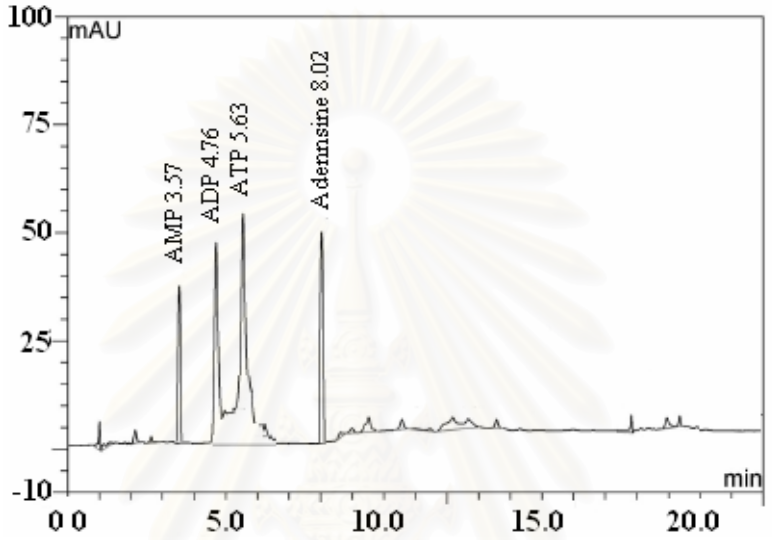


B) Sensorgram shows steps of immobilized membrane

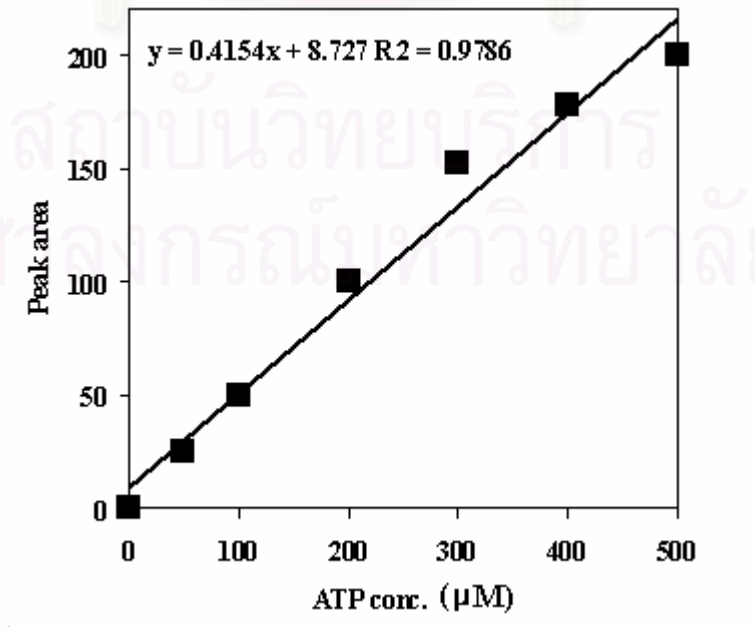


APPENDIX 4

HPLC chromatogram showing standard of AMP, ADP, ATP, and adenosine.



Standard curve of ATP standard



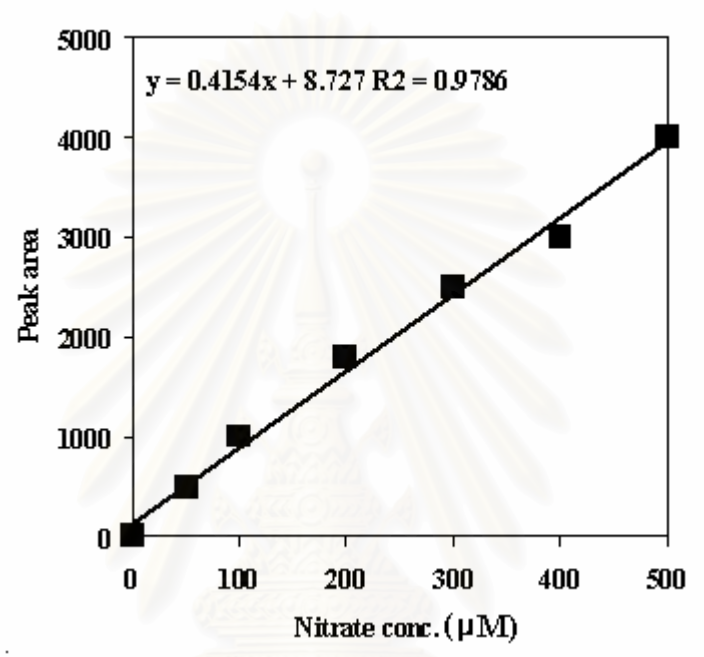
APPENDIX 5

Optical and Electron Microscopy

Optical microscopy was performed with a microscope (Axioskop FL, Carl Zeiss, Gottingen, Germany) that was equipped with a high-definition image-capture camera (model HC-1000, Fujix, Tokyo). For electron microscopy, cells were pelleted by centrifugation at 3,000*g* for 5 min and then immediately fixed for 1 h with 2% (v/v) glutaraldehyde in 100 mm sodium phosphate (pH 7.2). After rinsing overnight in sodium phosphate buffer, samples were post-fixed in 1% (v/v) osmium tetraoxide for 1 h before dehydration by passage through a graded ethanol series (50%–100%, v/v). Then samples were infiltrated with and embedded in resin (Araldite CY-212, Ouken, Tokyo). Thin sections were mounted on copper grids, stained with uranyl acetate, and examined under an electron microscope (model 1200EX, JEOL, Tokyo).

APPENDIX 6

Standard curve of nitrate



APPENDIX 7

Chlorophyll content determination

1) The 795 µl of 80% acetone was added into the Microcentrifuge tube.
2) Added 5 µl cell suspensions.
3) Vortex for 2 – 3 min.
4) Centrifuged at 10,000xg for 5 min at 4 ^{o}C.
5) Measured the OD at 663 nm.
6) Calculated the chlorophyll content;

   [OD_{663} x 12.7 x (800/5)]/1000 = ……. µg ChlA/ µl

   Coefficient constant = 12.7

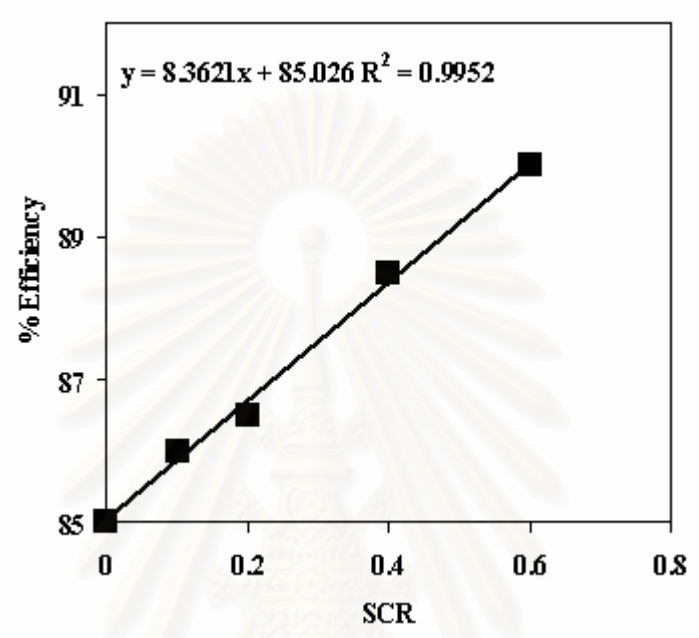
APPENDIX 8

Scintillation fluid (1,000 ml) as follows;

Dissolve 5.5 g PPO (2, 5-diphenyloxazole) and 0.1 g POPOP [1, 4-bis (5-phenyloxazole-2-yl) benzene] in 1,000 ml of a solution composed of 667 ml Toluene and 333 ml of Triton X-100. Make certain that the contents are completely dissolved before the solution is used. The solution should be stored in a brown bottle in a cool dark place.

APPENDIX 9

Standard curve of ^{14}C



APPENDIX 10

LB medium

Composition per 1 litre:

Bacto tryptone	10	g
Yeast extract	05	g
NaCl	10	g

Dissolve all composition with 800 ml of distilled water; adjust the pH to 7.0 with 6 M NaOH. Adjust volume of solution to 1 litre with deionized water. The medium was sterilized by autoclaving at 15 lb/in^2 for 15 minutes.

APPENDIX 11

Minimal Medium A (MMA)

5 x Stock MMA solution (1 litre)

K_2HPO_4	10.5	g
KH_2PO_4	04.5	g
$(NH_4)_2SO_4$	01.0	g
Sodium citrate.$2H_2O$	00.5	g
$MgSO_4.7H_2O$	00.1	g

Dissolve all composition with 800 ml of distilled water; adjust the pH to 6.7 with 6 M NaOH. Adjust volume of solution to 1 litre with deionized water. The medium was sterilized by autoclaving at 15 lb/in^2 for 15 minutes.

5 M NaCl (100)

NaCl	29.2	g

Dissolve with 100 ml of distilled water was sterilized by autoclaving at 15 lb/in^2 for 15 minutes.

20% glucose (100 ml)

Glucose	20.0	g

Dissolve with 100 ml of distilled water was sterilized by filtered through 0.45 μm nitrocellulose membrane.

100 mg/ml ampicillin (1 ml)

Ampicillin	100	mg

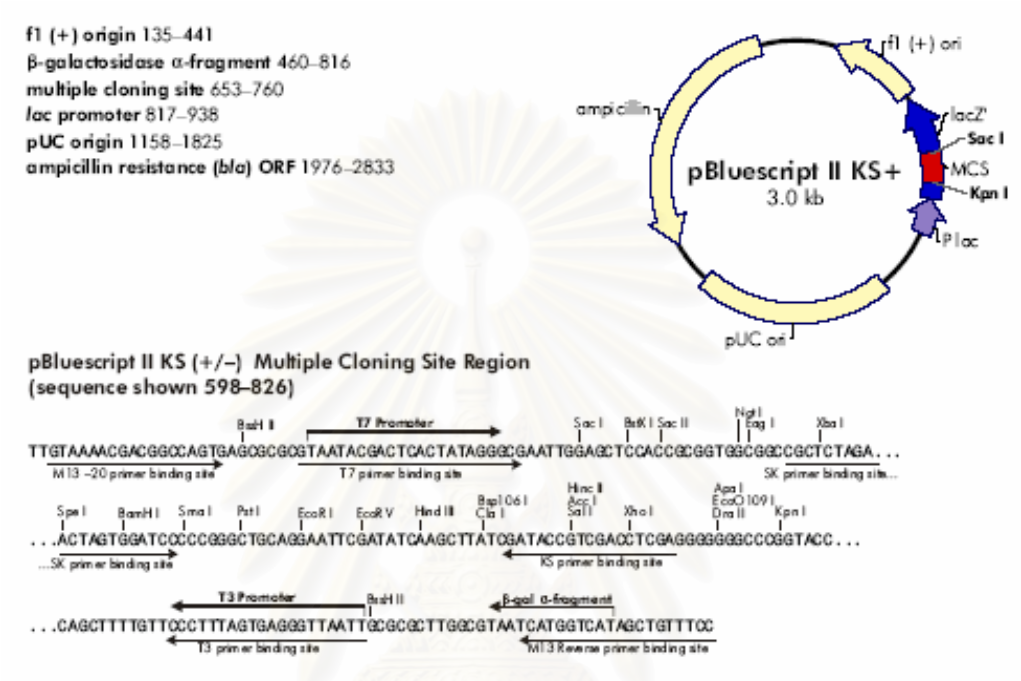
Dissolve with 1 ml of distilled water was sterilized by filtered through 0.45 μm nitrocellulose membrane.

1 M IPTG (1 ml)

Sterile distilled water

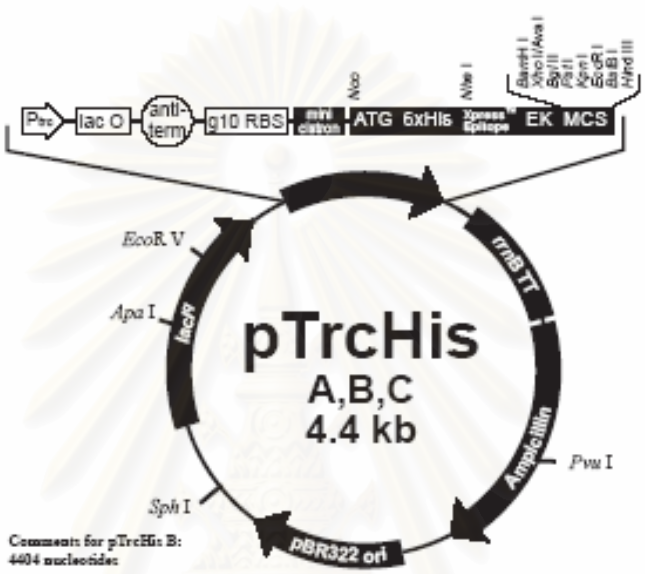
APPENDIX 12

The gene map of pBluescript II KS^{+}



APPENDIX 13

The gene map of pTrcHis2C

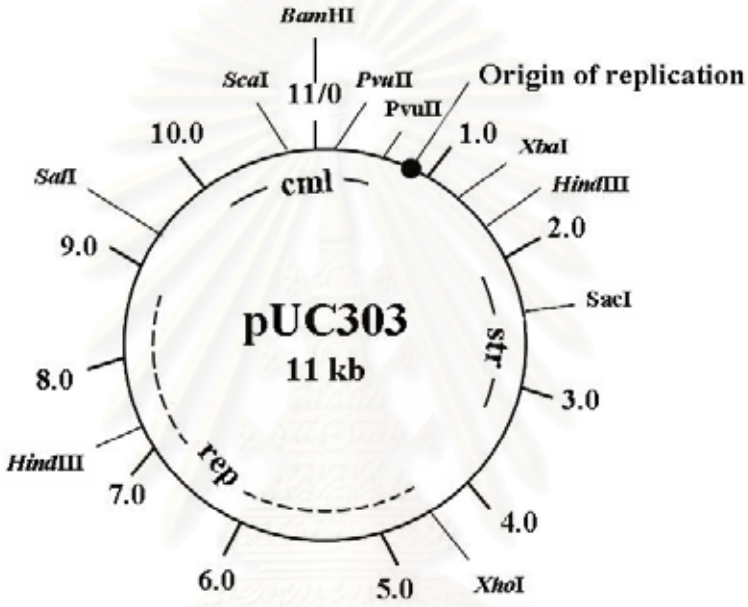


Comments for pTrcHis B:
4404 nucleotides

trc promoter: bases 191-221
lac operator: bases 228-248
rrnB anti-termination sequences: bases 264-333
T7 gene 10 translational enhancer: bases 346-354
Ribosome binding site: bases 370-374
Mini-cistron: bases 383-409
Polyhistidine and enterokinase cleavage site: bases 425-504
Xpress™ epitope: bases 482-505
Multiple cloning site: bases 515-554
rrnB transcriptional termination sequence: bases 637-794
Ampicillin resistance ORF: bases 1074-1934
pBR322 origin: bases 2079-2752
lac I^{q} ORF: bases 3281-4365

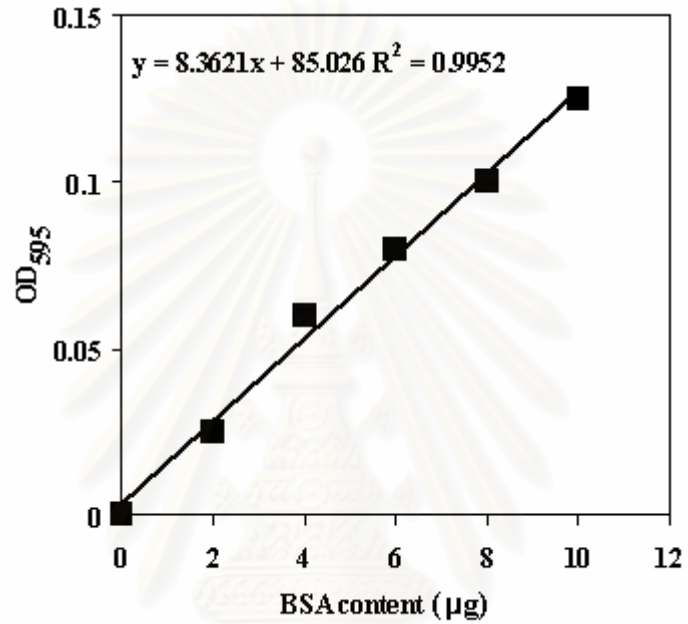
APPENDIX 14

The gene map of pUC303



APPENDIX 15

Chromatogram of BSA standard



APPENDIX 16

Agarose gel electrophoresis

To measure the size and amount of DNA in the sample, 0.8 – 1.5% agarose gel (consist of 0.5 mg/ml ethidium bromide) in 1xTBE buffer (89 mM Tris-Cl, 89 mM boric acid and 2.5 mM EDTA pH 8.3) consisting 0.5 mg/ml ethidium bromide was used. The DNA sample was mixed with 1/5 volume of loading dye (0.25% bromophenol blue, 0.25% xylene cyanolFF and 30% glycerol in water) before loading into the well of gel which submerged in the 1xTBE buffer in an electrophoresis chamber. An appropriate amount of λ/*Hin*dIII or λ/*Eco*RV was also load to the gel to serve DNA marker. Generally, the gel was run at 100 volts until bromophenol blue migrated to the other edge. The DNA band was visualized under UV light and photograph. The concentration and molecular weight of DNAs sample were estimated by comparing with the intensity and relative mobility of λ/*Hin*dIII or λ/*Eco*RV. The standard DNA bands λ/*Hin*dIII are 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.5 kb, respectively. For the standard DNA bands λ/*Eco*RV are 5.7, 5.3, 4.6, 3.8, 2.8, 2.6, 1.9, 1.6, 1.4, 0.7, 0.5, and 0.2 kb, respectively.

APPENDIX 17

^{14}C-glycine betaine preparation

^{14}C-glycine betaine was prepared by the reaction of choline oxidase from *Alcaligenase* sp. (Ikuta, *et al.,* 1977). The radioactive substrate ^{14}C-choline (55 µCi/ µmol) was converted to ^{14}C-glycine betaine aldehyde by adding choline oxidase and incubated 25 ^{0}C for 4 h. The intermediate product, ^{14}C-glycine betaine aldehyde was converted to ^{14}C-glycine betaine by adding NaOH (final concentration 0.17 M) and H_2O_2 (final concentration 10 %) and incubated overnight. The product, ^{14}C-glycine betaine, was separated by ion exchange chromatography (Dowex 50W, 50x4-200, hydroxyl form) and eluted by 2 M NH_3. The solution was lyophilized and checked for the purity by autoradiography.

APPENDIX 18

Immunoblotting of membrane protein of ApBetT expressing cells

Protein content of everted membrane vesicles of *E. coli* prepared from French Press was determined according to Bradford's method. The same everted membrane vesicles were used for immunoblotting experiment. Fifty-microgram of membrane protein was separated by 12.5% sodium dodecyl polyacrlamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane using blotting transfer buffer (Appendix 19). Blotting was done at 150 mA/inch^2 for 1 hr followed by blocking in blocking solution (Appendix 19) for 2 hr. The nitrocellulose membrane was incubated with primary antibody (an antibody raised against 6-histidine, 6X-His tag) for 1 hr 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 second antibody (an antibody raised against mouse) for 1 hr and washed 3 times with 100 ml PBS plus 5% skim milk buffer for 15 min. The nitrocellulose membrane was visualized after incubation with the detection reagent for 30 min (Appendix 20).

APPENDIX 19

Buffer for western blotting

PBS buffer (Phosphate buffer saline) (1,000 ml)

10 mM sodium phosphate pH 7.4

150 mM NaCl

Blocking buffer

10% (w/v) skim milk and 0.01% Tween20 in PBS buffer

Blotting transfer buffer (1,000 ml)

39 mM glycine

48 mM Tris-base

0.037% SDS

20% methanol

APPENDIX 20

Detection reagent for western blotting

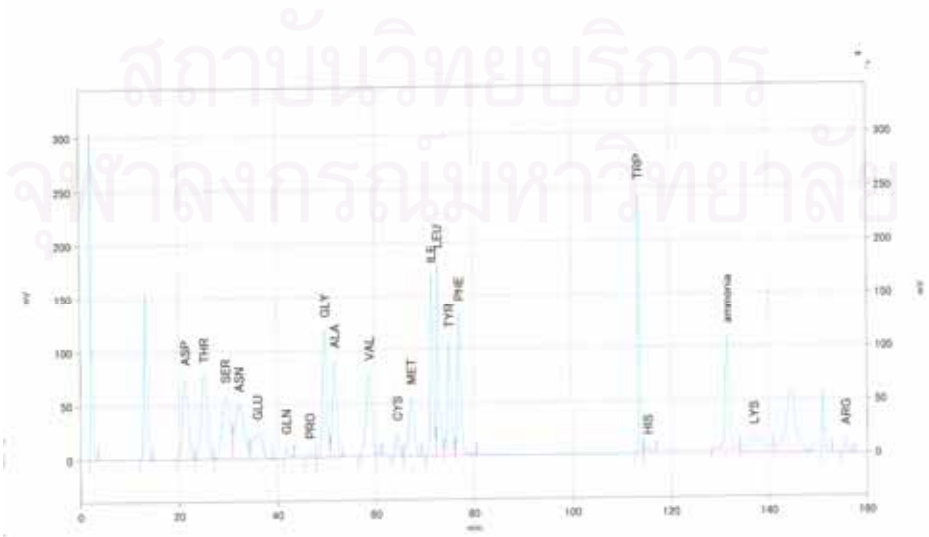
150 mM Barbital pH 9.6	18	ml
0.1% NTB (Nitro Blue Tetrazolium)	2	ml
1 M $MgCl_2$	80	μl
0.5% BCIP (5-bromo-4-chloro-3-indoyl phosphate)	200	μl

Detection reagent for western blotting should be freshly prepared and used within 30 minutes. When the bands are of the desired intensity, wash the membrane with deionized water 2 – 3 times and take photograph.

APPENDIX 21

Amino acid analysis

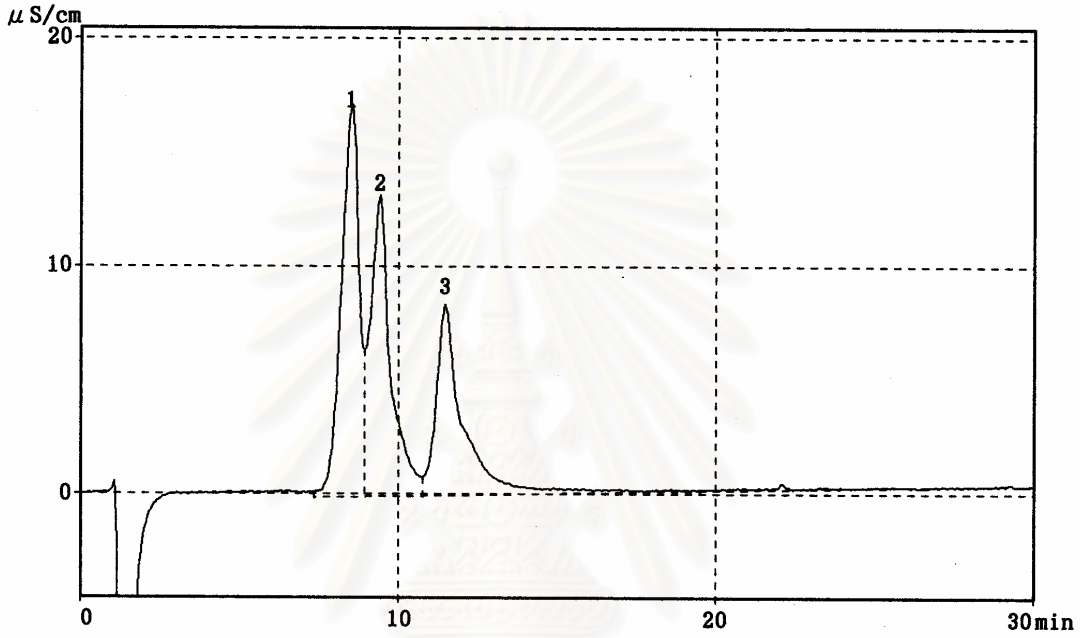
Cells were homogenized with 9 volume of 100% methanol and centrifuged for 5 minutes at 4oC with 1500xg. After centrifugation supernatant were transferred in another tube and remaining pellet were re-extracted with 90% methanol and centrifuged as above conditions. 90% methanol extract were pooled with previous 100% methanol extract and this pooled extract were dried in vacuum rotary evaporator at 43oC. After dry up, dried pellet were re-dissolved in MQ water (500ul) and same volume of chloroform were added and mixed on vortex. Again centrifuge for 5 minutes at 1500xg at 4oC. Upper aqueous phase were separated and filtered with 0.22-um membrane filter. Filtrate was again dried in vacuum rotary evaporator at 43oC. Dried samples can be stored at -20 or -80oC till analysis. Before analysis samples were dissolved in MA solution (pH 2.6, lithium citrate tetra hydrate, methoxy ethanol and perchloric acid) and start injection with amino acid analyzer with a shim pack Li column (Shimadzu). The chromatogram of amino acid analyzer for amino acid standard was shown below.



APPENDIX 22

Chromatograms of standard Na^+, NH_4^+, and K^+ were analyzed by Ion Analyzer

Peak 1 = Na^+, Peak 2 = NH_4^+, and Peak 3 = K^+



APPENDIX 23

Chromosomal DNA extraction

A. halophytica grown in modified BG_{11} plus 0.5 M NaCl under continuous fluorescent white light was used for extraction chromosomal DNA. 14-day-old culture (mid-exponential growth) was harvested by centrifugation at 4,000 rpm for 15 min at 4 ^{0}C, washed twice with SET buffer (20% sucrose, 50 mM EDTA, and 50 mM Tris-Cl buffer, pH 7.6). Pellet was frozen at – 20 ^{0}C for 2 h, thawed at 65 ^{0}C for 10 min and resuspended in SET buffer. Cells were lyzed by using lysozyme (final concentration 0.5 mg/ml), incubated at 37 ^{0}C for 30 min under gentle shaking. Subsequently, SDS and RNase were added at final concentration 0.5% and 0.25 mg/ml, respectively. After an incubation at 37 ^{0}C for 3 h, proteinase K 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 ^{0}C. The aqueous layer^{0}C was collected and reextracted at least three times with equal volume of phenol/chloroform/isoamylalcohol. High molecular weight DNA was precipitated by adding 2 volume of absolute ethanol and chilled at – 20 ^{0}C for 1 – 2 h. Chromosomal DNA was collected by centrifugation at 15,000 rpm for 5 min at 4 ^{0}C 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 buffer, 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 A_{260}/A_{280}.

APPENDIX 24

Preparation of plasmid by alkaline lysis method

A single colony of *E. coli* harboring recombinant plasmid was grown in 1.5 l of LB solution containing 50 μg/ml ampicillin at 37 ^{o}C for overnight with shaking (Appendix 2). The cells were harvested by centrifugation at 4,000 rpm for 10 min at 4 ^{o}C and suspended in 100 μl of solution I (50 mM glucose, 25 mM Tris-Cl buffer, pH 8.0 and 10 mM EDTA) by vigorous vortexing. After 5 minutes incubation at room temperature, the cells were lyzed by adding of 200 μl of freshly prepared solution II (0.2 N NaOH and 1% SDS), mixed by gently inversion and incubated on ice for 5 min. The cell lysate was neutralized by gently mixing with 150 μl of 3 M sodium acetate buffer, pH 4.8 followed by 5 min incubation on ice. The mixture was centrifuged at 15,000 rpm for 5 min at 4 ^{o}C. The clear lysate was collected, extracted once with phenol/chloroform/isoamylalcohol (25: 24: 1). Subsequently, the plasmid was precipitated by adding 2 volumes of ice-cold absolute ethanol, mixed by inversion several times before incubated at – 20 ^{o}C for 10 min and then centrifuged for 10 min at 15,000 rpm at 4 ^{o}C. The plasmid was washed with 70% ethanol and recollected by centrifugation for 3 min. Finally, the air-dried pellet was dissolved in 20 μl TE buffer and stored at – 20 ^{o}C.

APPENDIX 25

Preparation of competent cells

A single colony of *E. coli* DH5α was incubated to 2 ml of LB medium and inoculation at 37 ^{o}C with vigorous shaking. This culture was reinoculated to fresh LB medium and incubated 37 ^{o}C with vigorous shaking for 3 – 4 hours until the OD620 reach 0.4 – 0.6. The culture was standed on ice for 10 min and centrifuged at 4,000 rpm for 10 min at 4 ^{o}C. Cell pellet was resuspended in 0.05 volumes of TSB-DMSO free medium and stand on ice for 10 min. This cell suspension was dispensed in 100 μl aliquot into 1.5ml Microcentrifuge tubes and stored at –70 ^{o}C.

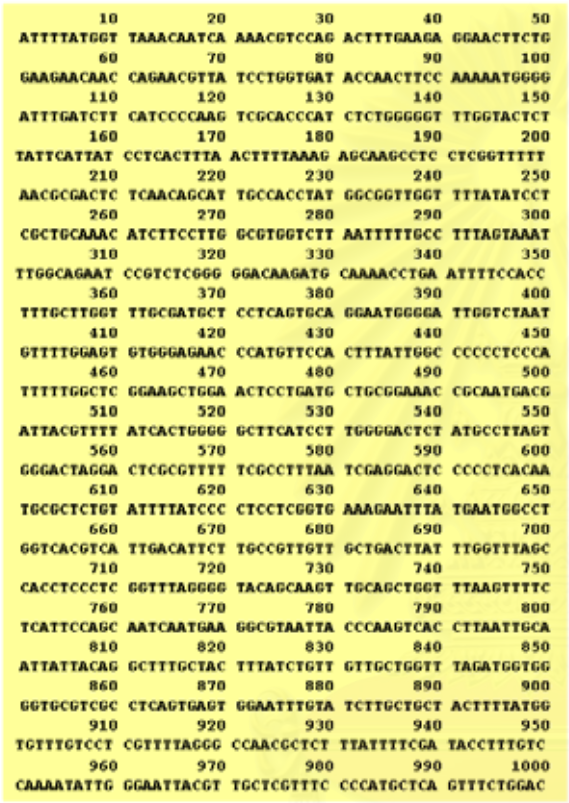
APPENDIX 26

Transformation

One hundred microlitres of competent cells was thawed on prior to the adding of 1 – 3 µl of plasmid DNA or ligation mixture. The transformation mixtures were flicked 2 – 3 times and stand on ice 10 min. Subsequently, the mixture was heated to 42 ^{o}C for 90 second further on ice 5 min. The mixture was diluted with 918 µl of 1xTSB-DMSO-Glucose medium (855 µl 1xTSB, 45 µl DMSO, and 18 µl 1 M Glucose) and gently shaking at 37 ^{o}C for 60 min. Cell suspension was spread on selection medium as desired.

APPENDIX 27

Nucleotide sequence of ApBetT



```
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        60         70         80         90        100
GAAGAACAAC CAGAACGTTA TCCTGGTGAT ACCAACTTCC AAAAATGGGG
       110        120        130        140        150
ATTTGATCTT CATCCCCAAG TCGCACCCAT CTCTGGGGGT TTGGTACTCT
       160        170        180        190        200
TATTCATTAT CCTCACTTTA ACTTTTAAAG AGCAAGCCTC CTCGGTTTTT
       210        220        230        240        250
AACGCGACTC TCAACAGCAT TGCCACCTAT GGCGGTTGGT TTTATATCCT
       260        270        280        290        300
CGCTGCAAAC ATCTTCCTTG GCGTGGTCTT AATTTTTGCC TTTAGTAAAT
       310        320        330        340        350
TTGGCAGAAT CCGTCTCGGG GGACAAGATG CAAAACCTGA ATTTTCCACC
       360        370        380        390        400
TTTGCTTGGT TTGCGATGCT CCTCAGTGCA GGAATGGGGA TTGGTCTAAT
       410        420        430        440        450
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       460        470        480        490        500
TTTTTGGCTC GGAAGCTGGA ACTCCTGATG CTGCGGAAAC CGCAATGACG
       510        520        530        540        550
ATTACGTTTT ATCACTGGGG GCTTCATCCT TGGGGACTCT ATGCCTTAGT
       560        570        580        590        600
GGGACTAGGA CTCGCGTTTT TCGCCTTTAA TCGAGGACTC CCCCTCACAA
       610        620        630        640        650
TGCGCTCTGT ATTTTATCCC CTCCTCGGTG AAAGAATTTA TGAATGGCCT
       660        670        680        690        700
GGTCACGTCA TTGACATTCT TGCCGTTGTT GCTGACTTAT TTGGTTTAGC
       710        720        730        740        750
CACCTCCCTC GGTTTAGGGG TACAGCAAGT TGCAGCTGGT TTAAGTTTTC
       760        770        780        790        800
TCATTCCAGC AATCAATGAA GGCGTAATTA CCCAAGTCAC CTTAATTGCA
       810        820        830        840        850
ATTATTACAG GCTTTGCTAC TTTATCTGTT GTTGCTGGTT TAGATGGTGG
       860        870        880        890        900
GGTGCGTCGC CTCAGTGAGT GGAATTTGTA TCTTGCTGCT ACTTTTATGG
       910        920        930        940        950
TGTTTGTCCT CGTTTTAGGG CCAACGCTCT TTATTTTCGA TACCTTTGTC
       960        970        980        990       1000
CAAAATATTG GGAATTACGT TGCTCGTTTC CCCATGCTCA GTTTCTGGAC
```

```
      1010       1020       1030       1040       1050
TGAATCCTTT GGATCAGGAG AAGAAAGCAC CTGGCAAAAT AGTTGGACGG
      1060       1070       1080       1090       1100
TCTTTTATTG GGGTTGGTGG ATTTCCTGGT CTCCGTTTGT GGGAATGTTT
      1110       1120       1130       1140       1150
ATCGCCCGTG TTTCCAGAGG TCGTACCATT CGTGAATTTG TGATGGGCGT
      1160       1170       1180       1190       1200
TCTGATTGCA CCCACGCTTC TGTCTTTTCT CTGGCTGTCA ACAATGGGCG
      1210       1220       1230       1240       1250
GAGCAGCCTT AAATCTTCAG TTAACCAATG CAGGAGATAT TGCTGCTGCG
      1260       1270       1280       1290       1300
GTGCAAGAAA ACGTTGCCAC AGCTATGTTT GTGATGTTGG ATAACTTCCC
      1310       1320       1330       1340       1350
CTTAACGGCA CTAAGCTCCA TTGTGGGAAT TTTACTGGTG ACCATCTTCT
      1360       1370       1380       1390       1400
TTGTCACTTC CTCTGACTCA GGCTCGCTGG TTGTTGATAA TTTGACTTCA
      1410       1420       1430       1440       1450
GGGGGAAAAC TGGATTCTCC TGTACCACAG CGAGTGTTTT GGGCTGTGAT
      1460       1470       1480       1490       1500
GGAAGGTGTG GTTGCTGCGG TTTTACTCCT TGGCGGTGGC TTAACGGCTT
      1510       1520       1530       1540       1550
TACAGACGGC TGCGATTACA ACAGGTTTAC CTTTTGCAAT TGTATTGCTC
      1560       1570       1580       1590       1600
ATCATGTGTT ACAGCTTGAA TCAAGGGCTC TCGCAAGAAC TACAAGAACT
      1610       1620       1630       1640       1650
AGAAATGGCA GAACTGCGAG ATGCTGAAGC CAAACGAGAA GAAAAAATTG
      1660       1670       1680       1690       1700
CTGCAATTAT GGCTTCTCGT CAAGAAGCGC GATTTGCCCA AGATGAATAA
      1710       1720       1730       1740       1750
TGAT
```

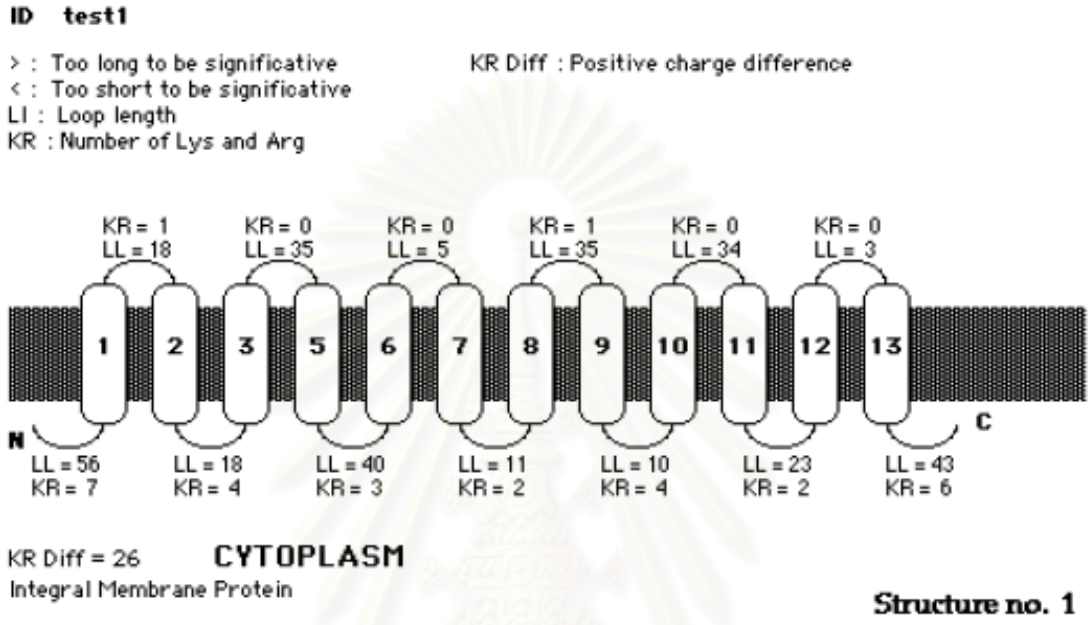
APPENDIX 28

Alignment of the deduced amino acid sequence of betaine transporters from four organisms.

ApBetT = *A. halophytica* betaine transporter**,** BacOpuD **=** *B. subtilis* betaine transporter**,** TetRa = *T. halophila* betaine transporter, and CoRBetP = *C. glutamicum* betaine transporter.

APPENDIX 29

Topology model of ApBetT



APPENDIX 30

Nucleotide sequence of ApBetT promotor

```
-420              360/CONTIG1215
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ATGCGC TTTATG GAACGA TATGCA GAAGAA A
ATGGCT TAGAAT TTAGTA AATCTT AACTTA ATTTGA GTTCAC TAACAT CTCGGT
AAATGA TTACAG TGGCGA GGGGGA TTTTTC C
TCGCCT TTTTTG CGAGAT TCTTCT CCAGAG GCTTTC CCAGGC GAAATC CTCGCC
TTCTGT TACGGT CAGCGT CGGGAA TAGCTC A
CAAGAG GCTGGT ATTAAA AACAGT TTTTCG CTATAA CTGACT AAAATG GCAAGT
TTTAAA ATGGAT CTGTCC TGCTCT TAAAAA A
ATTCAA CTCTTC CCATTA GGGGAG TCGATT AGGACG ATCTTT GAATCT CTTGTA
AAAAAC TCTTTT CAAAGA AAATAT TTTATG GTTAAA
                          +1M  V  K
```
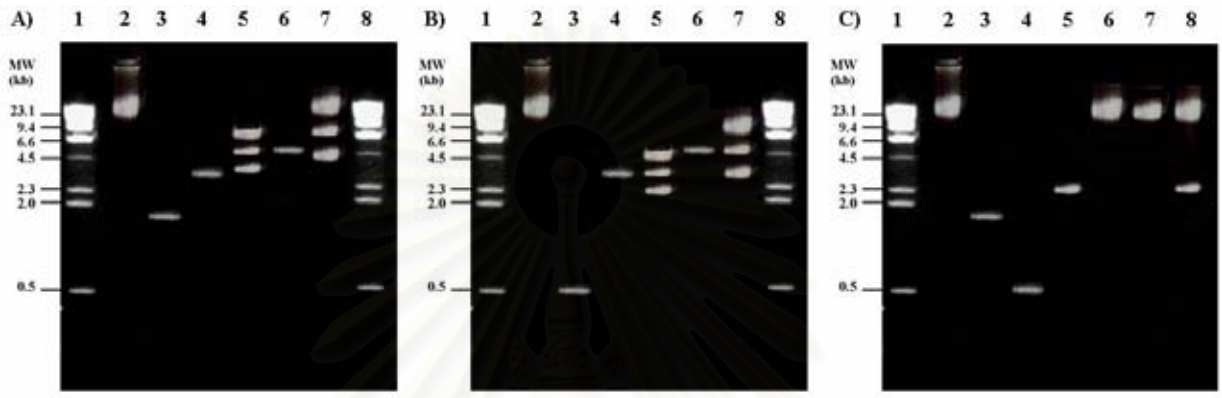

APPENDIX 31

Analysis of ApBetT gene by agarose gel electrophoresis



A) Analysis of ApBetT gene on 1% agarose gel electrophoresis

Lane 1, 8: λ/*Hind*III Lane 2: *A. halophytica* chromosomal DNA

Lane 3: PCR product corresponding to 1.7 kb (*ApBetT* gene)

Lane 4: pBluescript II KS^+/*Eco*RV Lane 5: *ApBetT*/ pBluescript II KS^+

Lane 6: pTrcHis2C/*Nco*I+*Sal*I Lane 7: *ApBetT*/ pTrcHis2C

B) Analysis of ApBetT promoter gene on 1% agarose gel electrophoresis

Lane 1, 8: λ/*Hind*III Lane 2: *A. halophytica* chromosomal DNA

Lane 3: PCR product corresponding to 0.5 kb (*ApBetT promotor* gene)

Lane 4: pBluescript II KS^+/*Eco*RV Lane 5: *ApBetT promotor*/ pBluescript II KS^+

Lane 6: pTrcHis2C/*Nco*I Lane 7: *ApBetT promotor*/ pTrcHis2C

C) Analysis of ApBetT with own promoter gene on 1% agarose gel electrophoresis

Lane 1: λ/*Hind*III Lane 2: *A. halophytica* chromosomal DNA

Lane 3: PCR product corresponding to 1.7 kb (*ApBetT* gene)

Lane 4: PCR product corresponding to 0.5 kb (*ApBetT promotor* gene)

Lane 5: PCR product corresponding to 2.2 kb (*ApBetT with own promotor* gene)

Lane 6: pUC303/*Bam*HI Lane 7: *ApBetT with own promotor*/ pUC303

Lane 8: *ApBetT with own promotor*/ pUC303/*Nco*I

APPENDIX 32

Dragendorff's reagent

Solution A

Bismuth nitrate	17	g
Tartaric acid	200	g

Adjust volume to 800 ml with distilled water

Solution B

Potassium iodide	160	g

Adjust volume to 400 ml with distilled water

Mix solution A and B

For use, 100 g tartaric acid is dissolved in 50 ml of the mixture A and B and 250 ml water.

BIOGRAPHY

Mr. Surasak Laloknam was born on May 23, 1972 in Bangkok, Thailand. He graduated with a Bachelor of Science degree in Biology from Srinakarinwirot University at Bangkhen in 1993 and Master of Science in Biochemistry from Chulalongkorn University in 1997, respectively. He followed work at the Office of Atomic for Peace, Ministry of Science and Technology. He has further studied for the Doctor of Philosophy (Ph.D.) degree in Program of Biotechnology, Chulalongkorn University since 2002.