การนำเข้าไกลซีนและลักษณะสมบัติของตัวขนส่งไกลซีนในไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica*

นางสาวอาภรณ์ บัวหลวง

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GLYCINE UPTAKE AND CHARACTERIZATION OF GLYCINE TRANSPORTER IN HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica*

Miss Aporn Bualuang

A Dissertation Submitted in Partial Fulfillment of the Requirements
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Thesis Title	GLYCINE UPTAKE AND CHARACTERIZATION OF GLYCINE TRANSPORTER IN HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica*
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Field of Study	Biochemistry
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อาภรณ์ บัวหลวง : การนำเข้าไกลซีนและลักษณะสมบัติของตัวขนส่งไกลซีนในไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica*. (GLYCINE UPTAKE AND CHARACTERIZATION OF GLYCINE TRANSPORTER IN HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร.อรัญ อินเจริญศักดิ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร.Teruhiro Takabe, 105 หน้า.

ในงานวิจัยครั้งนี้ศึกษาเกี่ยวกับการนำเข้ากรดอะมิโนไกลซีนในไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica* แบ่งออกเป็นสองส่วน โดยส่วนแรกศึกษาเกี่ยวกับการตรวจสอบคุณลักษณะทางชีวเคมีของการนำเข้าไกลซีน และส่วนที่สองเกี่ยวกับลักษณะสมบัติของยีนตัวขนส่งกรดอะมิโนไกลซีน เลี้ยงเซลล์ในภาวะที่มีความเข้มข้นของเกลือตั้งแต่ 0.25 ถึง 3.0 โมลาร์ ความเข้มข้นของเกลือมากขึ้นส่งผลให้การเจริญของเซลล์ลดลงเนื่องมาจากภาวะเครียดจากเกลือ การเติมกรดอะมิโนไกลซีนความเข้มข้นจนถึง 40 มิลลิโมลาร์ช่วยส่งเสริมการเจริญของเซลล์ในภาวะปกติที่มีความเข้มข้นของเกลือโซเดียมคลอไรด์ (0.5 โมลาร์) การเติมกรดอะมิโนไกลซีนความเข้มข้นจนถึง 20 มิลลิโมลาร์ ภายใต้ภาวะเครียดที่มีความเข้มข้นของเกลือโซเดียมคลอไรด์ (2.0 โมลาร์) ช่วยให้การเจริญกลับคืนมาเหมือนการเจริญในภาวะปกติ อย่างไรก็ตามการเติมไกลซีนที่ความเข้มข้นสูงมากเกินไปส่งผลให้เกิดการยับยั้งการเจริญของเซลล์โดยที่ความเข้มข้นของไกลซีน 100 และ 60 มิลลิโมลาร์ยับยั้งการเจริญในภาวะปกติและภาวะเครียดจากเกลือตามลำดับ การศึกษาการนำเข้ากรดอะมิโนไกลซีนในเซลล์ของ *A. halophytica* พบว่าอายุของเซลล์ที่เจริญมาแปดวันให้ผลการนำเข้าดีที่สุด โดยแสดงค่าอิ่มตัวคงที่ของ มิเคลลิส เมนเทนเท่ากับ 160.80 ไมโครโมลาร์ และมีค่าความเร็วสูงสุดเท่ากับ 3.85 นาโนโมลต่อนาทีต่อมิลลิกรัมโปรตีน ค่าความเป็นกรดด่างที่ให้ผลการนำเข้าไกลซีนสูงสุดคือที่ 8.0 การนำเข้าไกลซีนถูกยับยั้งจากทั้งตัวยับยั้งพลังงานและตัวยับยั้งที่ทำลายเกรเดียนต์ของไอออน ซึ่งจากผลของตัวยับยั้งดังกล่าวอาจจะกล่าวได้ว่าการนำเข้าไกลซีนมีด้วยกันสองระบบที่เกี่ยวข้องกับการใช้พลังงาน จากข้อมูลการทำ shot gun sequence พบว่าใน *A. halophytica* มียีนตัวขนส่งอะลานีนหรือไกลซีนร่วมกับแคทไอออน (*ApagcS1*) ซึ่งยีนนี้มีขนาด 1443 คู่เบส ประกอบด้วยกรดอะมิโน 480 เรซิดิว จากการนำลำดับกรดอะมิโนไปวิเคราะห์ด้วย TMHTMM Server v.2 พบว่าประกอบด้วย 10 ทรานสเมมเบรน โดยยีน *ApagcS1* ถูกนำมาโคลนและศึกษาลักษณะสมบัติในเซลล์ *E. coli* JW4166 สายพันธุ์กลายที่ถูกทำลายยีนการขนส่งตัวไกลซีน พบว่า ApAgcS1 ต้องการ โซเดียมไอออนในการขนส่งไกลซีน โดยมีค่าอิ่มตัวคงที่ของ มิเคลลิส เมนเทนเท่ากับ 12.53 ไมโครโมลาร์ และมีค่าความเร็วสูงสุดเท่ากับ 35.74 นาโนโมลต่อนาทีต่อมิลลิกรัมโปรตีน การนำเข้าไกลซีนถูกยับยั้งด้วยกรดอะมิโนหลายชนิดซึ่งอาจบอกได้ว่ามีความจำเพาะต่อการนำเข้ากรดอะมิโนได้หลายชนิด การนำเข้าไกลซีนโดย ApAgcS1 เกิดได้ดีที่ภาวะเป็นกรดด่างเท่ากับ 9.0 เมื่อนำยีน *ApagcS1* เข้าไปในไซยาโนแบคทีเรีย *Synechococcus* sp. PCC 7942 Δ*natG* (สายพันธุ์กลายที่ถูกทำลายระบบขนส่งอะมิโนที่ไม่มีประจุ) โดยศึกษาผลการสะสมกรดอะมิโนในอาหาร พบว่ามีการสะสมของกรดอะมิโนซีรีน แอสปาราจีน กลูตามีน และไอโซลิวซีน ลดลงอย่างมีนัยสำคัญ ($p<0.05$)เมื่อเปรียบเทียบกับ *Synechococcus* sp. PCC 7942 Δ*natG* การแสดงออกของยีน *ApagcS1* ใน *A. halophytica* พบว่ามีการแสดงออกเพิ่มขึ้น 2 และ 3 เท่า หลังจากการทำให้อยู่ในภาวะเครียดจากเกลือและขาดไนโตรเจน 3 และ 1 ชั่วโมงตามลำดับ

ภาควิชา ชีวเคมี	ลายมือชื่อนิสิต .....
สาขาวิชา ชีวเคมี	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....
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APORN BUALUANG: GLYCINE UPTAKE AND CHARACTERIZATION OF GLYCINE TRANSPORTER IN HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica*. ADVISOR: PROF. ARAN INCHAROENSAKDI, Ph.D., CO-ADVISOR: PROF. TERUHIRO TAKABE, Ph.D., 105 pp.

This study on glycine uptake by alkaliphilic halotolerant cyanobacterium *Aphanothece halophytica* comprised two parts. The first part dealt with biochemical characterization of the uptake whereas the second part involved molecular characterization. Cells were grown under various concentrations of NaCl from 2.5-3.0 M. Increasing NaCl concentration reduced cell growth due to salt stress effect. Supplementation of glycine up to 40 mM enhanced the growth of *A. halophytica* under normal condition (0.5 M NaCl). Similarly, under salt stress condition (2.0 M NaCl), the addition of up to 20 mM glycine could reverse the effect of growth inhibition by salt stress. However, too high concentration of glycine (at 100 mM under normal condition, and 60 mM under salt stress condition) led to the complete inhibition of cell growth. The uptake of glycine in *A. halophytica* was monitored. *A. halophytica* grown for 8 days showed the highest glycine uptake rate. The uptake rate of *A. halophytica* exhibited saturation kinetics according to Michaelis-Menten kinetic parameters with an apparent K_m of 160.80 μM and V_{max} of 3.85 nmol/min/mg protein. The optimal pH for glycine transport was at pH 8.0. Both of dissipating ion inhibitors and metabolic inhibitors inhibited glycine uptake in *A. halophytica*. The results of uptake experiment suggested that there might be at least two systems of glycine transporter with regard to energy supply. Based on shot gun sequence, *A. halophytica* contained a gene homolog of alanine glycine cation symporter (*ApagcS1)*. The *ApagcS1* gene contains 1443 bp, encoding 480 amino acid residues. The prediction of deduced amino acid residue by transmembrane prediction program TMHTMM Server v.2 showed ApAgcS1 consisting of 10 transmembrane segments. *ApagcS1* was cloned and characterized in *E. coli* JW4166 (deficient in glycine transport) cells. The JW4166 transformant cell harboring an pApagcS1 required Na^+ for glycine uptake. ApAgcS1 showed kinetic parameters with an apparent K_m for glycine of 12.53 μM and the V_{max} value was 35.74 nmol/min/mg protein. Substrate specificity of ApAgcS1 showed broad specificity for amino acid transport with optimum activity at pH 9.0. Cells of *Synechococcus* sp. PCC 7942 Δ*natG* mutant harboring pSyn1_*ApagcS1* vector showed a reduction in the accumulation of amino acid in the medium with serine, asparagine, glutamine and isoleucine showing a significant reduction ($p<0.05$) when compared to *Synechococcus* sp. PCC 7942 Δ*natG* mutant harboring an empty vector. The level of mRNA for *ApagcS1* in *A. halophytica* cell was induced by NaCl and nitrogen deficiency stresses with up to 2-fold and 3-fold respectively after 3 h NaCl stress and 1 h without nitrate.

Department: Biochemistry Student's Signature .....

Field of Study: Biochemistry Advisor's Signature .....

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LIST OF ABBEVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Chl	Chlorophyll
$CaCl_2$	Calcium chloride
CCCP	carbonyl cyanide-m-chlorophenylhydrazone
EDTA	Ethylenediamine tetraacetic acid
h	Hour
°C	Degree Celsius
DCCD	N,N'-dicyclohexylcarbodiimide
DNP	2,4-dinitrophenol
g	Gram
HCl	Hydrochloride
LiCl	Lithium chloride
min	Minute
µCi	Microcurie
µM	Micromolar
µl	Microliter
$MgSO_4$	Magnesium sulfate
nmol	Nanomole
NEM	N-ethylmaleimide
PPO	2, 5-diphenyloxazole
POPOP	1, 4-bis (5-phenyloxazole-2-yl benzene
KCl	Potassium chloride

NaN_3	sodium azide
OD	Optical Density
KCN	Potassium cyanide
NaCl	Sodium chloride

CHAPTER I

INTRODUCTION

Amino acid transport systems are ubiquitously found in eukaryotic and prokaryotic cells. They belong to two superfamilies, the ATP-binding cassette (ABC) superfamily and the amino acid/polyamine/organo cation (APC) superfamily.

1.1 The ATP-binding cassette (ABC) superfamily

The ATP-binding cassette (ABC) superfamily comprises many systems sharing common characteristics. Normally, an ABC transporter is composed of 3 parts: membrane-integral domain, ATP-hydrolyzing domains and substrate binding domain. The model of the ATP-binding cassette (ABC) transport systems is shown in Figure 1.1 (Schneider and Hunke 1998) ABC systems are present in both prokaryotes and eukaryotes. They constitute probably the largest superfamily of proteins ever detected in living organisms (Saurin *et al.* 1999). Some families of this superfamily that are involved in amino acid and their derivatives transport is shown in Table 1.1 (Saier 2000). The classification system for membrane transport proteins is known as the Transporter Classification (TC) system. (Saier *et al.* 2006) THE TC 3.A.1.3 and TC 3.A.1.4 are primarily concerned with polar and non-polar amino acid transport, respectively.

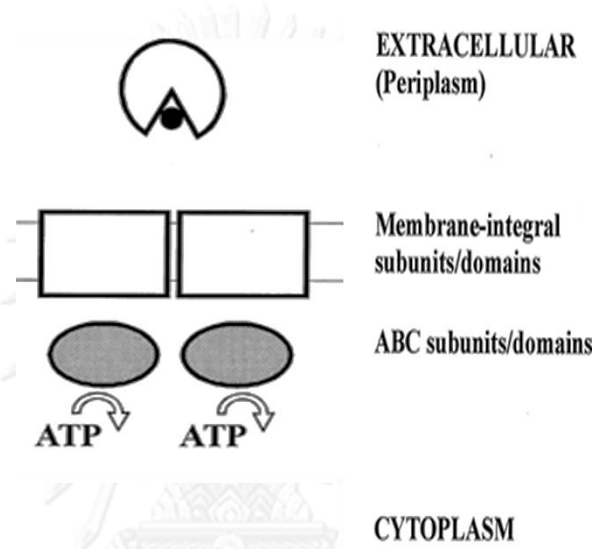


Figure 1.1 Model of the ATP-binding cassette (ABC) transport systems mainly represented by binding protein-dependent bacterial import systems. (Schneider and Hunke 1998)

Table1.1 The ATP-binding cassette (ABC) superfamily (Saier 2000).

TC no.	Name
ABC-type uptake permeases (prokaryotes)	
3.A.1.1	Carbohydrate uptake transporter 1 (CUT1) family
3.A.1.2	Carbohydrate uptake transporter 2 (CUT2) family
3.A.1.3	Polar amino acid uptake transporter (PAAT) family
3.A.1.4	Hydrophobic amino acid uptake transporter (HAAT) family
3.A.1.5	Peptide/opine/nickel uptake transporter (PepT) family
3.A.1.6	Sulfate uptake transporter (SulT) family
3.A.1.7	Phosphate uptake transporter (PhoT) family
3.A.1.8	Molybdate uptake transporter (MolT) family
3.A.1.9	Phosphonate uptake transporter (PhnT) family
3.A.1.10	Ferric iron uptake transporter (FeT) family
3.A.1.11	Polyamine/opine/phosphonate uptake transporter (POPT) family
3.A.1.12	Quaternary amine uptake transporter (QAT) family
3.A.1.13	Vitamin B_{12} uptake transporter ($VB_{12}T$) family
3.A.1.14	Iron chelate uptake transporter (FeCT) family
3.A.1.15	Manganese/zinc/iron chelate uptake transporter (MZT) family
3.A.1.16	Nitrate/nitrite/cyanate uptake transporter (NitT) family
3.A.1.17	Taurine uptake transporter (TauT) family
3.A.1.18	Putative cobalt uptake transporter (CoT) family
3.A.1.19	Thiamin uptake transporter (ThiT) family
ABC-type efflux permeases (bacterial)	
3.A.1.101	Capsular polysaccharide exporter (CPSE) family
3.A.1.102	Lipooligosaccharide exporter (LOSE) family
3.A.1.103	Lipopolysaccharide exporter (LPSE) family
3.A.1.104	Teichoic acid exporter (TAE) family
3.A.1.105	Drug exporter (DrugE1) family
3.A.1.106	Putative lipid A exporter (LipidE) family
3.A.1.107	Putative haem exporter (HemeE) family
3.A.1.108	β-Glucan exporter (GlucanE) family
3.A.1.109	Protein-1 exporter (Prot1E) family
3.A.1.110	Protein-2 exporter (Prot2E) family
3.A.1.111	Peptide-1 exporter (Pep1E) family
3.A.1.112	Peptide-2 exporter (Pep2E) family
3.A.1.113	Peptide-3 exporter (Pep3E) family
3.A.1.114	Probable glycolipid exporter (DevE) family
3.A.1.115	Na^+ exporter (NatE) family
3.A.1.116	Microcin B17 exporter (McbE) family
3.A.1.117	Multidrug exporter (DrugE2) family
3.A.1.118	Microcin J25 exporter (McjD) family
3.A.1.119	Drug/siderophore exporter 3 (DrugE3) family
ABC-type efflux permeases (mostly eukaryotic)	
3.A.1.201	Multidrug-resistance exporter (MDR) family
3.A.1.202	Cystic fibrosis transmembrane conductance exporter (CFTR) family
3.A.1.203	Peroxysomal fatty acyl CoA transporter (FAT) family
3.A.1.204	Eye pigment precursor transporter (EPP) family
3.A.1.205	Pleiotropic drug resistance (PDR) family
3.A.1.206	a-Factor sex pheromone exporter (STE) family
3.A.1.207	Conjugate transporter 1 (CT1) family
3.A.1.208	Conjugate transporter 2 (CT2) family
3.A.1.209	MHC peptide transporter (TAP) family
3.A.1.210	Heavy metal transporter (HMT) family

1.2 The amino acid/polyamine/organocation (APC) superfamily

The member of this superfamily possess the function as solute: cation symporter and solute: solute antiporters (Jack *et al.* 2000) found in several organisms ; bacteria, yeast, archaea , fungi, slime molds, unicellular eukaryotic protists, plants and animals (Saier 2000). Proteins of this superfamily are varying in length from 350 residues to 850 residues. The families within the APC superfamily are shown in Table 1.2 (Wong *et al.* 2012)

In 2000, Saier reviewed about the family of amino acid and their derivative transporter that belong to ACP superfamily. The ACP families are found in prokaryotes (Table 1.3) and The ACP families are found in eukaryotes (Table 1.4). The model of membrane topology of the amino acid transporter in this superfamily, Na^+-leucine transporter LeuT of *Aquifex aeolicus*, which is the leucine transporter of the neurotransmitter sodium symporter (NSS) family showed in Figure 1.2. The LeuT transporter consists of 12 TMS. (Yamashita *et al.* 2005)

Table1.2 Families within the APC superfamily (Wong *et al.* 2012)

TC no.	Family
2.A.3	APC family
2.A.15	BCCT family
2.A.18	AAAP family
2.A.21	SSS family
2.A.22	NSS family
2.A.25	AGCS family
2.A.30	CCC family
2.A.39	NCS1 family
2.A.40	NCS2 family
2.A.42	HAAAP family
2.A.53	SulP family

Table1.3 The ACP families found in prokaryotes (Saier 2000)

TC no.	Family	Substrate	Size range (residues)	No. TMSs	Organism*	No. members	Example
2.A.13	C_4-dicarboxylate uptake (Dcu) family	C_4-dicarboxylates	440	12	B	>10	Dicarboxylate uptake porter A, DcuA of *Escherichia coli*
2.A.15	Betaine/carnitine/ choline transporter (BCCT) family	Glycine betaine, carnitine, choline, proline	480–680	12	B	>10	Carnitine transporter, CaiT of *Escherichia coli*
2.A.25	Alanine/glycine:cation symporter (AGCS) family	Alanine, glycine	440–540	8–12	B	>10	Alanine/glycine transporter, DagA of *Alteromonas haloplanktis*
2.A.26	Branched chain amino acid:cation symporter (LIVCS) family	Branched chain amino acids	~440	12	B	>10	Branched-chain-amino-acid transporter, BraB of *Pseudomonas aeruginosa*
2.A.27	Glutamate:Na^+ symporter (GltS) family	Glutamate	~400	12	B	>10	Glutamate:Na^+ symporter, GltS of *Escherichia coli*
2.A.42	Hydroxy/aromatic amino acid permease (HAAAP) family	Hydroxy- and aromatic amino acids	400–450	11	B	>20	Tyrosine permease, TyrP of *Escherichia coli*; serine permease, SdaC of *Escherichia coli*
2.A.56	Tripartite ATP-independent periplasmic transporter (TRAP-T) family	C_4-dicarboxylates, acidic amino acids, sugars (?)	~1000 (three components)	12+4	B, A	>20	Dicarboxylate transporter, DctPQM of *Rhodobacter capsulatus*
2.A.75	L-Lysine exporter (LysE) family	Basic amino acids	190–240	5 or 6	B, A	>10	Lysine/arginine exporter, LysE of *Corynebacterium glutamicum*
2.A.76	Resistance to homoserine and threonine B (RhtB) family	Neutral amino acids and their derivatives (export)	190–240	5 or 6	B, A	>20	Homoserine/ threonine exporter, RhtB of *Escherichia coli*
2.A.78	Carboxylate/amino acid/amine transporter (CAAT) family	Carboxylates, amino acids and amines	260–320	10	B, A	>100	Putative acetate exporter, MadN of *Malonomonas rubra*

*B, bacteria; A, archaea.

Table1.4 The ACP families found in eukaryotes (Saier 2000)

TC no.	Family	Substrate	Size range (residues)	No. TMSs	Organism*	No. members	Example
2.A.3	Amino acid/polyamine/ organocation (APC) family	Amino acids, polyamines, organocations	440–630	12	B, A, E	>100	Lysine permease, LysP of *Escherichia coli*
2.A.18	Amino acid/auxin permease (AAAP) family	Amino acids, auxin and their derivatives	400–710	11	E (An, Pl, Y, F)	>30	Amino acid/auxin:H^+ symporter, Aux-1 of *Arabidopsis thaliana*
2.A.1	MHS family of the MFS	Various small molecules	400–600	12 or 14	B, A, E	>1000	Proline permease, PutP of *Escherichia coli*
2.A.17	Proton-dependent oligopeptide (POT) family	Peptides, nitrates, amino acids	450–600	12	B, E	>30	Dipeptide transporter, DtpT of *Lactococcus lactis*
2.A.21	Solute:sodium symporter (SSS) family	Sugars, amino acids, vitamins, nucleosides, inositols, iodide, urea	400–700	12–15	B, A, E	>30	Pantothenate:Na^+ symporter, PanF of *Escherichia coli*
2.A.22	Neurotransmitter: sodium symporter (NSS) family	Neurotransmitters, amino acids, osmolytes, taurine, creatine	600–700	12	B, A, E (An)	>50	Serotonin:Na^+ symporter of *Homo sapiens*
2.A.23	Dicarboxylate/ amino acid:cation (Na^+ or H^+) symporter (DAACS) family	C_4-dicarboxylates, acidic and neutral amino acids	420–580	10–12	B, A, E	>20	Glutamate/ aspartate permease, GltP of *Escherichia coli*
2.A.29	Mitochondrial carrier (MC) family	ATP/ADP, P_i, organic anions, H^+, carnitine/acyl carnitine, basic amino acids, FAD	300	6	E (mitochondria, peroxisomes)	>100	ATP/ADP exchanger of *Homo sapiens*
2.A.69	Auxin efflux carrier (AEC) family	Auxin (efflux)	600–700	8–12	B, A, E	~20	Auxin efflux carrier, PIN1

* B, bacteria; A, archaea; E, eukaryote; An, animal; Pl, plant; Y, yeast; F, fungi.

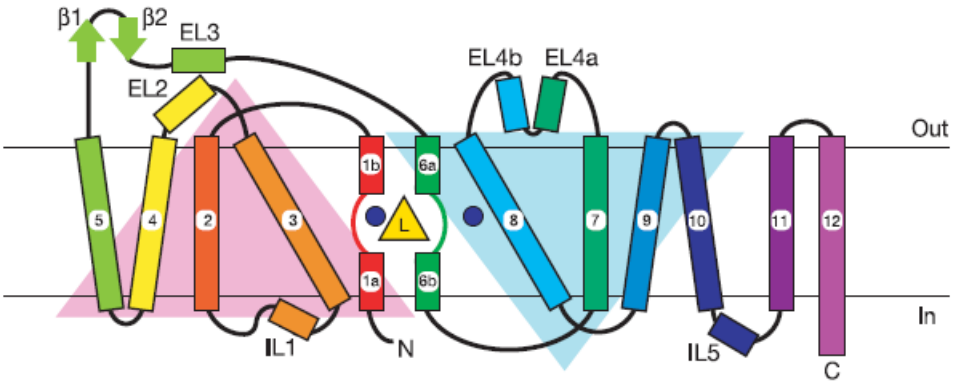


Figure 1.2 The $LeuT_{Aa}$ topology. The positions of leucine and the two sodium ions are depicted as a yellow triangle and blue circles, respectively.(Yamashita *et al*. 2005)

1.3 The alanine or glycine: cation symporter (AGCS) family

-The AGCS family consists of transporters that transport alanine and/or glycine in symport with Na^+ and/or H^+. The proteins have 8-12 putative transmembrane α-helical spanners with a length of 445-542 amino acyl residues. The members of this family that had been characterized are listed below:

-Gram negative marine bacterium which requires Na^+ for growth, *Altermonas haloplanktis* ATCC 1985. The Na^+-linked permease (DagA) which predicted amino acid residues consists of 542 ($M_{(r)}$ 58,955). Its profile of hydropathy suggests that it is composed of eight transmembrane segments with a long hydrophilic region between segments six and seven. DagA was found to significant similarity which the Na^+/proline permeases of *E. coli* and *Salmonella typhimurium* and the human and rabbit intestinal Na^+/glucose co transporters. (MacLeod and MacLeod 1992) . MacLeod and team (1992) reported the rate of glycine transport was 7.5 nmol/mg cell dry weights.

-Methanogenic archaeon; *Methanococcus maripaludis* which was isolated from salt marsh sediments, the *agcS* encodes alanine-sodium symport that transports either isomer of alanine. Moore and team used markerless mutagenesis technique to demonstrate roles for alanine dehydrogenase, alanine racemase and alanine permease (alanine transporter; a*gcS*), the result found that the permease is required for the transport of D and L isomer (Moore and Leigh 2005)

-*Thermophilic bacterium* PS3

An alanine carrier protein was isolated from membranes of the thermophilic bacterium PS3 using ion exchange column chromatography followed by high performance liquid chromatography with a hydroxylapatite column. The final

preparation consisted of a single polypeptide, with Mr = 42,500, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A polarity index of 33% was calculated from the amino acid analysis. Proteoliposomes reconstituted with the purified alanine carrier carried out an active alanine transport driven by either an electrochemical potential difference of protons or that of sodium ions.(Hirata *et al.* 1984)

Purified alanine carrier proteins were cleaved into peptides and amino acid sequence was analyses from these peptides, the encoding gene the alanine carrier protein (acp gene) was successful cloning from using synthesized a DNA probe. The 5'-flanking region was determined by an inverse polymerase chain reaction, and an open reading frame consisting of 1,335 nucleotides was found. The amino acid sequence deduced from the open reading frame consists of 445 amino acids, and all the partial amino acid sequences determined are included in the sequence. The calculated M(r) of 47,803 is significantly larger than the apparent M(r) of 42,500 as reported by Hirata, *et.al.* 1984. The in vitro translation experiment revealed that the product of the acp gene migrates at a position coinciding with that of the purified alanine carrier. Hydropathy analysis suggests that the protein contains at least 8 hydrophobic segments. A homology search on a database reveals relatively high scores of homology with either the Escherichia coli melibiose carrier or the human Na^+/glucose symporter and also similar to other Na^+-coupled symporters.(Kamata *et al.* 1992)

A novel insertion sequence (IS)-like element was found in the 5′-upstream region of the alanine carrier protein-encoding gene *(acp)* in the thermophilic

bacterium PS3 chromosomal DNA. The analysis of *acp* expression in *Escherichia coli* cells indicated that *IS1341* promotes the expression of *acp.* (Murai *et al*. 1995)

The alanine transporter (alanine carrier protein, ACP) gene of thermophilic bacterium PS3 was previously cloned and expressed in a functionally active form in Escherichia coli cells. To achieve controlled overproduction of the ACP protein, they designed a plasmid encoding a fusion protein comprising ACP joined to the carboxyl terminus of the maltose binding protein (MBP-ACP). Upon transduction of the plasmid into *E. coli* RM1 cells defective in alanine/glycine transport, the transport activity was expressed even before induction with 1-thio-beta-D-galacto-pyranoside (IPTG), and increased slightly on induction with IPTG at low concentrations. However, overexpression of the MBP-ACP gene, induced by higher concentrations of IPTG, resulted in death of the host cells. Hence they screened other host cells and found that the MBP-ACP fusion protein was produced in a large quantity in *E. coli* TB1 cells 3 h after IPTG induction. The MBP-ACP fusion protein was accumulated in cytoplasmic membranes in an amount reaching more than 20% of the total membrane protein. The affinity-purified MBP-ACP exhibited very low transport activity when reconstituted into proteoliposomes. (Kanamori *et al.* 1999)

The *acp* was cloned to *E. coli* AK340, which is detective in transporting glycine and D-alanine. The glycine transport assay was carrying out in *E. coli* transformant cell and found the K_t for glycine 4.0 μM.

The general transport reaction catalyzed by the AGCS family is: alanine or glycine (out) + Na^+ or H^+ (out) ⟶ alanine or glycine (in) + Na^+ or H^+ (in).

1.4 Amino acid transport in cyanobacteria

The cyanobacteria are a phototrophic group of eubacteria that carry out oxygenic photosynthesis. They mainly use inorganic sources of carbon and nitrogen for growth. Some cyanobacteria have been shown to be able to take up some amino acids mostly asparagine, glutamine and arginine for use as the nitrogen sources by a number of cyanobacteria, [i](Neilson and Larsson 1980, Vaishampayan 1982)

1.4.1 In freshwater cyanobacterium

Synechocystis sp PCC 6803, there were genes encoding elements for amino transport in four systems. The data were identified by insertion inactivation of ORFs from the genomic sequence. (Quintero *et al.* 2001).

1.4.1.1 Neutral amino acid transporter (Nat)

Nat belongs to the ATP binding cassette (ABC)-type transporter family (Quintero *et al.* 2001), (Picossi *et al.* 2005). The Nat-system consists of five subunits: two ATP-binding subunits (NatA, NatE), two transmembrane permease subunits (NatC, NatD) and the periplasmic substrate-binding protein (NatB). The Nat system plays a role in the diazotrophic growth of heterocystous strains (Picossi *et al.* 2005) and its action prevents the leakage of amino acids into the medium in unicellular strains (Labarre *et al.* 1987). Nat system transports neutral amino acid and histidine.

1.4.1.2 Basic amino acid transporter.

The transport of both L-(^{14}C)arginine and L-(^{14}C) glutamine was characterized and found that both of them was inhibited by L-arginine, L-glutamine, L-canavanine, L-citrulline, L-histidine, L-lysine, and L-ornithine, suggesting that common elements are involved in the transport of all of these amino acids in

Synechocystis sp.PCC 6803. (Flores and Muro-Pastor 1990). In 2001 Quintero and team constructed the mutation of putative gene of transport basic amino acid. The results imply that the products of the two ORFs encode elements of *slr1735* as *bgtA* and *sll1270* as *bgtB.* The Bgt system belongs to ABC transport family, *bgtA* encoding ATP-binding subunits, *bgtB* encoding periplasmic substrate-binding and integral membrane protein. Bgt stands for basic amino acid and glutamine transport.

1.4.1.3 Glutamate transport system

The glutamate transport activity of *Synechocystis* sp. strain PCC 6803 is Na^+-dependent and have identified two permeases involved in this activity: GltS, a monocomponent secondary permease, and GtrABC, a TRAP typetransporter systems (Quintero *et al.* 2001). GltS is a member of the APC superfamily but GltR is the member of ABC transport superfamily.

1.4.2 Filamentous cyanobacteria

Anabaena sp. strain PCC 7120 exhibits a measurable activity of transport (uptake from the external medium) of amino acids belonging to different chemical groups (basic, acidic, neutral polar, hydrophobic).

1.4.2.1 The neutral amino acid transporter I (N-I)

N-I is the product of the *natA*, *natB*, *natC*, *natD* and *natE* genes encoding ATPase subunits (NatA and NatE), transmembrane polypeptides (NatC and NatD) and a periplasmic substrate-binding protein (NatB) of an ABC-type transporter. It mainly transports proline and hydrophobic amino acids, but can also transport some neutral polar amino acids including glutamine. The genes encoding this transporter are expressed in vegetative cells but not in heterocysts, and the transporter is required for optimal diazotrophic growth (Picossi *et al.* 2005).

1.4.2.2 The neutral amino acid transporter II and basic amino acid transporter (N-II and Bgt)

The NatF, NatG, NatH and BgtA proteins constitute an ABC-type uptake transporter for acidic and neutral polar amino acids (N-II). The Bgt and N-II transport systems thus share the ATPase subunit, BgtA. These transporters together with the previously characterized ABC-type uptake transporter for proline and hydrophobic amino acids (N-I) account for more than 98% of the amino acid transport activity exhibited by *Anabaena* sp. strain PCC 7120. In contrast to N-I that is expressed only in vegetative cells, the Bgt and N-II systems are present in both vegetative cells and heterocysts, whereas Bgt is dispensable for diazotrophic growth, N-II appears to contribute together with N-I to the diazotrophic physiology of this cyanobacterium. (Pernil *et al.* 2008)

1.5 The halotolerant cyanobacterium *Aphanothece halophytica*

Aphanothece halophytica is an oxygen-evolving organism that can grow in a wide range of salinity conditions from 0.25 to 3.0 M NaCl concomitant with the accumulation of betaine (Incharoensakdi *et al.* 1986). *A. halophytica* synthesizes betaine from glycine by a three steps of methylation (Waditee *et al.* 2003). The previous report showed that an exogenous supply of serine or glycine to *Aphanothece halophytica* elevated intracellular accumulation of glycine betaine under salt stress. (Waditee *et al.,* 2007)

The sodium dependent glutamate transporter (ApGltS) from *A. halophytica* was characterized in *E. coli* ME9107 which is deficient in glutamate uptake. Kinetics studies revealed that ApGltS is a high affinity glutamate transporter with a $K_m \approx 5\mu M$

and competition experiments revealed that glutamate, glutamine, aspartate and asparagine inhibited glutamate uptake. (Boonburapong *et al.* 2012).

1.6 Glycine

Glycine represents the simplest amino acid commonly found in proteins. At neutral pH, glycine is zwitterion (Figure 13). Each amino acid has at least one amine and one acid functional group. Since an amino acid has both an amine and acid group which have been neutralized in the zwitterion, the amino acid is neutral unless there is an extra acid or base on the side chain. If neither is present then then the whole amino acid is neutral. The side chain (R-group) of glycine is H that it is a neutral amino acid.

Glycine biosynthesis can be synthesized by reactions of enzyme shown in Figure 1.4.

-The serine hydroxymethytrnsferase (SHMT) catalyzed the interconvention of serine and tetrahydrofilate(THF) to glycine and N^5,N^{10}-methylene-THF (CH_2-THF).

-The glycine cleavage system catalyzes the following reversible reaction:

Glycine + H_4folate + NAD^+ $\leftrightarrow$ 5,10-methylene-H_4folate + CO_2 + NH_3 +NADH + H^+

-Transamination from glyoxyrate to glycine by glyoxylate aminotransferase.

-Threonine aldolase catalyzed the pyidoxal phosphate-dependent, from threonine to glycine and acetaldehyde.

Furfure more in organism glycine can transport from the outside via the amino acid transporter across the membrane barrier. Glycine becomes degraded to CO_2, NH_3 and CH_2-THF by the reaction of glycine cleavage system or glycine decarboxylase complex (GDC) and also glycine plus CH_2-THF become to generate

serine by the reaction of SHMT. In addition glycine serves as the precursor of purine biosynthesis

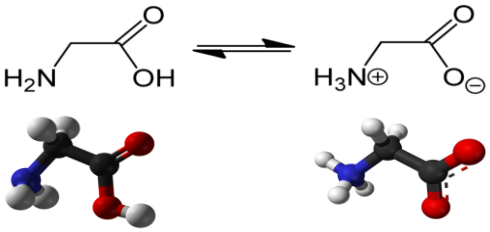


Figure 1.3 Structure of glycine

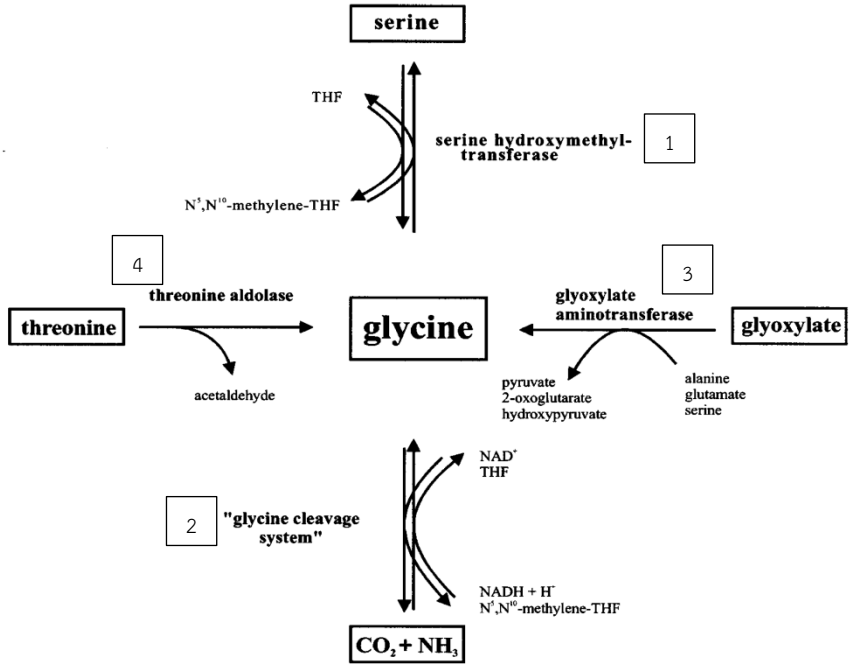


Figure 1.4 Scheme of enzymic reactions of glycine biosynthesis. Although glycine can be synthesized by the reactions of serine hydroxymethyltransferase(1), the glycine-cleavage system(2), glyoxylate aminotransferase(3), and threonine aldolase(4) Schlupen et al. 2003)

1.7 Salt stress and compatibles solutes in cyanobacteria

Under the osmotic stress organism have mechanism to adapt for survivals. High salt concentrations create two major harms for living systems. First, hypersaline condition can be deleterious to cells due to the concentration of inorganic ions outside is higher than inside since water is lost to the external medium and second the high ionic strength of the surrounding medium results in a continuous influx of inorganic ions (mainly Na^+ and Cl^-) which is rewarded by the accumulation of compatible solutes compounds. (Figure 1.5) The compatible solutes are low molecular weight organic compound range from sugars, ectoines, amino acids and their derivatives and polyols. The major compatible solutes found in cyanobacteria are sucrose, trehalose, glucosylglycerol and glycine betaine. Many strains of cyanobacteria accumulate mixtures of compatible solutes. Different salt tolerance groups of cyanobacteria used to classified group of accumulation.

Cyanobacteria inhabit environments which differ dramatically in their salinity Low salts tolerance can tolerate up to 0.7mol/L NaCl; Moderate salt tolerance upper tolerance limit is 1.8 mol!L NaCl and High salts tolerance strains use can tolerate salt concentrations up to 2.7 mol/L NaCl. (Mackey *et al.* 1984)

Cyanobacteria accumulate compatibles solute correlation with the habitats. (Figure 1.6) Low salt tolerance strains accumulated sucrose and trehalose as a compatible solutes, moderate salt tolerance accumulated of Glucosylglycerol as compatible and glycine betaine as compatible solute accumulated in strains of highest salt tolerance (Klähn and Hagemann 2011)

Glycine betaine (GB) is synthesized by a two-step oxidation from choline via the toxic intermediate betaine aldehyde, found in most bacteria and plants (Chen

and Murata 2002), whereas in the halophilic cyanobacterium *Aphanothece halophytica* GB has been demonstrated a three step methylation pathway that starts with glycine followed by sarcosine and dimethyglycine. (Waditee *et al.* 2003)

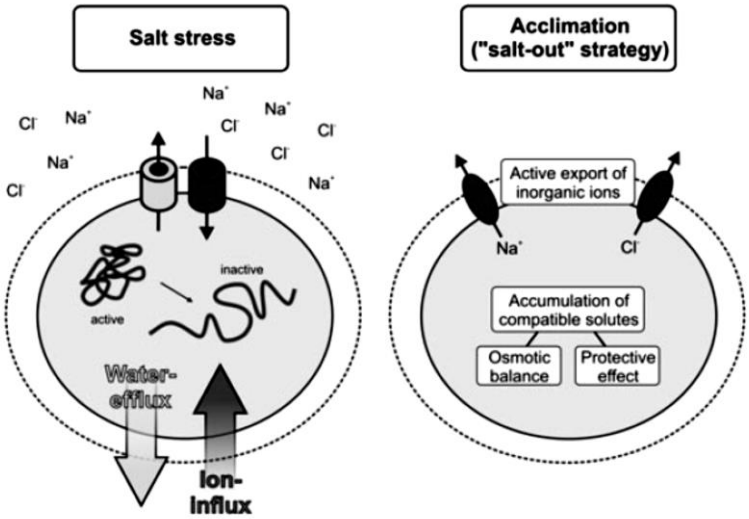


Figure 1.5 Schematic view on the principle of salt acclimation strategy of cyanobacteria.(Klähn and Hagemann 2011)

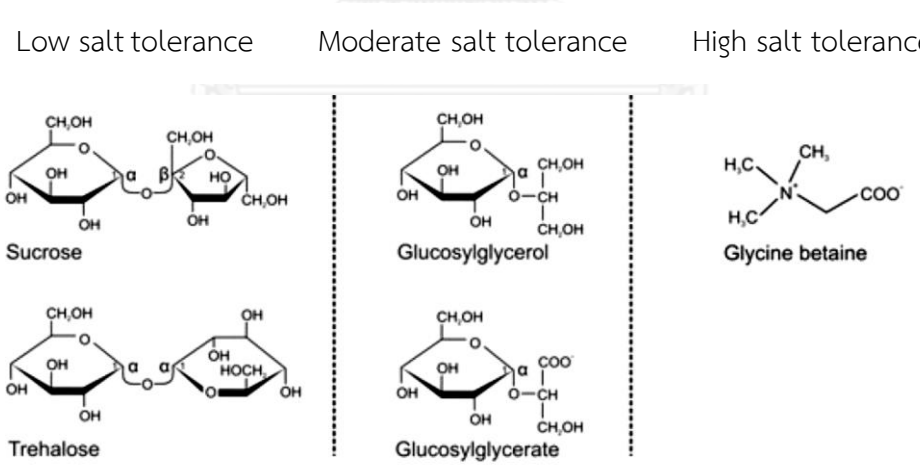


Figure 1.6 Structure of major compatible solutes of cyanobacteria in correlation with the preferred habitats

1.8 Objectives

1. To investigate the effect of glycine on growth of *Aphanothece halophytica*
2. To investigate glycine uptake of *A. halophytica*
3. To clone *A. halophytica* glycine transporter gene and express *A. halophytica* glycine transporter in *E. coli* mutant (deficient glycine transporter)
4. To characterize *A. halophytica* glycine transporter in transformant

CHAPTER II

MATERIALS AND METHODS

2.1 Instruments

Autoclave

: Model HA-30, Hirayama Manufacturing Cooperation, Japan

: Model MLS-3020, Sanyo Electric Co. Ltd., Japan

Auto pipette

: Pipetman, Gilson, France

Balances

: Model AB204-S, Mettler Toledo, Switzerland

: Model LC 620S, Sartorius, USA

DNA electrophoresis chamber

: Gelmate 2000, Toyobo, Japan

DNA Sequencer

: ABI310 genetics analyzer, Applied Biosystems, USA

Digital Illuminated Refrigerated Incubator Shaker

: Model Innova 4340, New Brunswick Scientific, Germany

Digital Lux meter FT710

: Taiwan

Electrophoresis unit

: Model Mini-protein II cell: Biorad, USA

Gel documentation system

: ImageMaster VDS, Pharmacia Biotech, USA

Genetic analyzer

: ABI PRISM 3100-Avant, Hitachi, Japan

High performance liquid chromatography

: Shimadzu class-vp, Japan

Laminar flow BVT-124

: International Scientific Supply, Thailand

Liquid Scintillation counter

: Model 3200C; Aloka Instruments Co., Tokyo, Japan

: LS 6500 Multi-Purpose Scintillation Counter, Beckman Coulter, USA

Microcentrifuge

: Kubota, Japan

Microscope

: Seek, Seek Inter Co. Ltd., Thailand

Microwave

: Model edition I, Daewoo Electronics America, USA

PCR Machine

: GeneAmp® PCR System 9700 Applied Biosystems, USA

pH meter

: PHM 83 Autocal pH meter, Radiometer, Denmark

Power supply

: Pharmacia, England

Refrigerated centrifuge

: Model Avanti J-30I, Beckman Coulter, USA

: Model Hettich MIKRO 22 R, UK

Spectrophotometer UV-VIS

: Model Biomate 3, Thermo Scientific,USA

Spectrophotometer UV-240

: Shimadzu, Japan

Trans-Blot Transfer Cell

: Bio-Craft, Japan

Vortex

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

Water bath

: Charles Hearson, England

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

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

Cobalt chloride : Fluka, Switzerland

Coomasie brilliant blue G-250 : Sigma, USA

Coomasie brilliant blue R-251 : 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

DL-lactate : 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

[U-^{14}C] glycine : Perkin Elmer, USA

HEPES : Sigma, USA

Isoamylalcohol : Katayama Chem, Japan

Lithium chloride : Katayama Chem, Japan

Magnesium chloride : Merck Ag Darmstadt, Germany

Magnesium sulfate : Merck Ag Darmstadt, Germany

Nigericin : Sigma, USA

Monensin : Sigma, USA

N-ethylmaleimide : Sigma, USA

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

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

Nitroblue tetrazolium (NTB) : Katayama Chem, Japan

Ouabain : Sigma, USA

p-chloromercurobenzoate: Sigma, USA

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

Spectinomycin : Sigma, USA

Sucrose : Katayama Chem, Japan

Trilithium cicrate tetrahydrate :Wako,Japan

Tris-hydrochloride : Katayama Chem, Japan

Triton X-100 : Packard, USA

Valinomycin : Sigma, USA

Vanadate : Sigma, USA

2.3 Supplies

Midisart 2000 filter : Sartorius, Germany

Polyvinylidine Fluoride membrane (PVDF) : Whatman International, England

Whatman No.1 filter paper : Whatman International, England

2.4 Kit

BigDye terminator v3.1 cycle sequencing kit : Applied Biosystems, USA

DNA cleanup kit :QIAquick Gel Extraction Kit, QIAGEN,Geranay

DNA ligation kit : TaKaRa, Japan

DNA marker : DynaMarker DNA High D #DM122 (300 to 10,000bp),
BioDynamics Labolatory Inc, Japan

GeneAmp dNTP mix : Roache, USA.

Genomic DNA extraction kit : DNeasy Plant Mini Kit, QIAGEN, Geramany

Plasmid extraction kit :QIAprep Spin Miniprep Kit, QIAGEN**,** Geramany

TA cloning kit : Invitrogen, USA

cDNA synthesis kit : PrimeScript First Strand cDNA Synthesis Kit TaKaRa, Japan

2.5 Enzymes and restriction enzymes

AmpliTaq Gold : Roache, USA

KOD plus : Toyobo, Japan

Lysozyme : Sigma, USA

Proteinase K : Katayama Chem, Japan

Restriction enzymes : New England Biolabs, UK

RNase A : US Biological, USA

2.6 Organisms

- *Aphanothece halophytica* was originally isolated from solar lake in Israel. The organism was kindly provided by Professor Dr. Teruhiro Takabe of the Research institute of Meijo University, Japan).

-*Escherichia coli* strains were obtained from Professor Dr. Teruhiro Takabe of the research institute of Meijo university, Japan.

Strain DH5α (Woodcock *et al.* 1989) genotype (F^-, ϕ80d*lacZ*ΔM15, Δ (*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17 (rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1) was used as a host for plasmid propagation.

Strain JW4166 (Baba *et al.* 2006) genotype F-, Δ *(araD-araB) 567*, Δ *lacZ4787* (::rrnB-3), λ^-, *rph-1*, Δ *(rhaDrhaB) 568*, Δ*cycA757::kan*, *hsdR514* was used as a host for biochemical studies of ApagcS1 expression.

-*Synechococcus* sp. PCC 7942 is a freshwater cyanobacterium, and was obtained from Professor Dr. Teruhiro Takabe of the Research Institute of Meijo University, Japan.

Synechococcus sp. PCC 7942 wild type strain

Synechococcus sp. PCC 7942 Δ*natG* mutant strain (constructed by Miss Bongkoj Boonburapong, unpublished)

2.7 Plasmids

The four plasmids, namely pCR 2.1, pMD20, pTrcHis2C and pSyn1 vector were used in this study. Circle maps are shown in APPENDIX 1, 2, 3 and 4 respectively.

- pCR 2.1 TA cloning (Invitrogen, USA): vector for cloning contains 3'-T overhangs for direct ligation of Taq-amplified PCR products.

-pTrcHis2C (Invitrogen, USA): vector for expression of recombinant proteins contains C-terminal 6xHis tags in *E. coli*. Moreover, this vector contains ampicillin resistance gene which allows selection of the plasmid in *E. coli*. (Invitrogen, USA)

-pMD20 (Takara,Japan): vector for cloning with a single 3'-terminal thymidine at both ends. The T-overhang ends at the cloning site improve the efficiency of ligation of PCR products which contain A-overhangs at 3'-ends.

-pSyn_1 (Invitrogen, USA): vector for expression of *Synechococcus* cells; this vector contains spectinomycin resistance gene which allows selection of the plasmid in *Synechococcus cells*.

2.8 Oligonucleotides

Table 2.1 PCR primers for PCR amplification glycine transporter gene from *A. halophytica* genomic DNA

Primer	Primer sequences	Amplified fragment length (bp)
BamHI_A5'_F_pTrc	5’GGATCCGGGAAAGTTAAACTCG TGAGCA 3’	1,948
SalI_A_R_pTrC	5’GTCGACTCGAAACATACCCGCC TCAA 3’	

Table 2.2 PCR primers for sequencing glycine transporter

Primer	Primer sequences
M13-F	5’ GTAAAACGACGGCCAGT 3’
M13-R	5’ CCTTTGTCGATACTGGTACT 3’
A_sym_mid_F	5’GAACCTGACGGCACTTATCGAG 3’

Table 2.3 PCR primers for PCR amplification glycine transporter gene from pApagcS1

Primer	Primer sequences	Amplified fragment length (bp)
*Bam*HI_A5'_F_pTrc	5’GGATCCGGGAAAGTTAAACTCGT GAGCA 3’	1,977
6xHisstop *Bam*HI-R	5'ACGGATCCTCAATGATGATGATG ATGATG 3'	

Table 2.4 PCR primers for PCR amplification glycine transporter gene from *A. halophytica* cDNA

Primer	Primer sequences	Amplified fragment length (bp)
A_sym_mid_F	5’GAACCTGACGGCACTTATCGAG 3’	398
Acp1RT_R	5’ATGCCACCGGTAATCGCTGAATC 3’	

2.9 Methods

2.9.1 The effect of glycine on growth of *A. halophytica*

A. halophytica, a short cylindrical shape cyanobacterium shown in Figure 2.1, was grown photoautotrophically in BG11 medium plus 18 mM $NaNO_3$ and Turk Island salt solution, pH of medium was adjusted to pH 7.6 as described in APPENDIX 5 .The NaCl concentration of the culture medium was adjusted to a range from 0.25 to 3.0 M as desired. Cotton-plugged 250-ml conical flasks containing 100 ml of medium were used and shaken on a reciprocal shaker. The culture flasks were incubated at 30°C under continuous fluorescent white light (30 μmolphoton/m^2/s). To study the effect of glycine, *A. halophytica* cells were transferred to new BG11 medium plus 18 mM $NaNO_3$ and Turk Island salt solution with various concentrations of NaCl and supplemented with 0–100 mM glycine. The growth was monitored by measuring an optical density at 730 nm (OD_{730}) with a spectrophotometer.

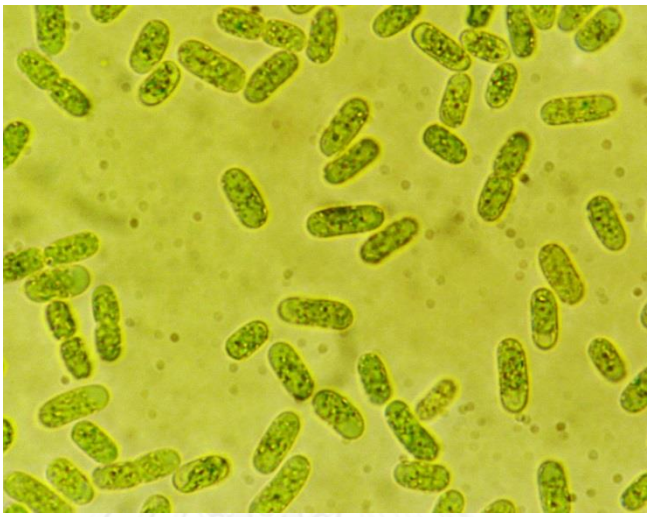


Figure 2.1 Microscopic picture of *A. halophytica* grown in BG11 medium plus 18 mM NaNO3 and Turk island salt solution at day 14th

.

2.9.2 Characterization of glycine transport in *A. halophytica*

2.9.2.1 Glycine uptake assay in *A. halophytica*

Cells at 10 day cultivation (mid log phase) were harvested by centrifugation (8,000 x g, 15 min, 4°C), washed twice with 25 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES)-KOH buffer pH 7.6 containing 0.5 M sorbitol. The uptake experiment was initiated by adding [U-^{14}C] -glycine with a specific activity of 8 µCi/µmol at a final concentration of 50 µM. The cells suspension was withdrawn rapidly and filtered through cellulose acetate filters (0.45 µm pore size; Sartorious). The filters were washed with 3 ml of buffer and the radioactivity trapped in the cells were added with scintillation fluid (APPENDIX 6) and determined with a liquid scintillation counter. Protein content was determined by a method of Bradford (Bradford 1976)using bovine serum albumin as a standard.

2.9.2.2 Kinetics of glycine uptake in *A. halophytica*

The glycine uptake was done as described in 2.9.2.1 by varying the concentrations of [U-^{14}C] -glycine concentration from 0 to 700 µM.

2.9.2.3 Effect of pH on glycine uptake in *A. halophytica*

The glycine uptake was done as described in 2.9.2.1 by varying the pH from 5.5 to 10.0 using 50 mM MES buffer pH 5.5-6.5, 50 mM HEPES pH 7.0-9.0 and 50 mM CAPS buffer pH 9.5-10.0.

2.9.2.4 Effect of cell age on glycine uptake in *A. halophytica*

The glycine uptake was done as modified in 2.9.2.1 by varying cultivation time of *A. halophytica* from day 2 to 30**.**

2.9.2.5 Effect of NaCl concentration on glycine uptake *in A. halophytica*

The glycine uptake was done as described in 2.9.2.1 by varying the concentrations of NaCl concentration from 0 to 1M in the assay medium.

2.9.2.6 Substrate specificity of glycine uptake in *A. halophytica*

The specificity of glycine uptake was determined by measuring the initial rate of [U-^{14}C] glycine uptake in the presence of 20 folds excess of unlabeled competitive substrate in the assay medium.

2.9.2.7 Effect of metabolic inhibitors and ion gradient dissipators on glycine uptake in *A. halophytica*

Cells at day 8th (mid log phase) .were pre-incubated with the tested compound(s) for 30 min. The cells were assayed for glycine uptake as described in 2.10.2.1 in the presence of different metabolic inhibitors.

2.9.3 Cloning of *A. halophytica* glycine transporter gene and expression in *E. coli* mutant (deficient in glycine transporter)

2.9.3.1 *A. halophytica* genomic *D*NA extraction

A. halophytica grown in BG11 medium plus 18 mM $NaNO_3$ and Turk Island salt solution with 0.5 M NaCl under continuous fluorescent white light was used for extraction of chromosomal DNA. Cells were harvested by centrifugation at 8,000 *g* for 15 min at 4 °C, and then Chromosomal DNA was extracted by using DNeasy Plant Mini Kit, (QIAGEN, Geramany). To determine concentration and purity of chromosomal DNA, THE sample was checked by measuring the ratio of OD_{260}/OD_{280}.

2.9.3.2 Cloning of *A. halophytica* glycine transporter gene

A. halophytica genomic DNA prepared from step 2.9.3.1 was used as a template DNA for cloning glycine transporter gene. The *ApagcS1* was amplified by PCR using the primer set *Bam*HI_A5'_F_pTrc / *Sal*I_A_R_pTrC (Table 2.1). The sequences of A5'_F_pTrc and *Sal*I_A_R_pTrC have *Bam*HI and *Sal*I restriction enzyme site, respectively. The amplified 1,948 bp DNA fragment was ligated to the multiple cloning site of pCR 2.1 (Invitrogen, USA). The resulting plasmid, pCR 2.1_*ApagcS1*, was transformed first into *E. coli* DH5α cells by heat shock method (APPENDIX 7). The positive clones were selected on LB agar (APPENDIX 8) containing 100 μg/ ml ampicillin and Xgal to a final concentration of 40 ug/ml, incubation at 37 °C for 16 hrs and the plasmids were extracted by QIAprep Spin Miniprep Kit, (QIAGEN, Geramany). Insert fragment of an expected size 1,948 kb was sequenced with 3 primers as shown in Table 2.1.

2. 9.3.3 Construction of expression plasmid (pApagcS1)

The pCR 2.1_*ApagcS1* was double digested with *Bam*HI and *Sal*I. The cohesive end fragment was ligated into *Bam*HI and *Sal*I site of the digested pTrcHis2C expression vector. The resulting plasmid, pApagcS1 harboring *ApagcS1*, encoding glycine transporter gene fused in frame to six histidines at the C terminus, was transformed first into *E. coli* DH5α cells by heat shock method. The positive clones were selected on LB agar containing ampicillin, and Xgal and grown at 37 °C for 16 hrs before extracting the plasmids by QIAprep Spin Miniprep Kit, (QIAGEN, Geramany).

pApagcS1, which was double digested with *Bam*HI and *Sal*I, was confirmed from the mobility of agarose electrophoresis of DNA fragments (APPENDIX 9). The recombinant plasmid was extracted and then transformed to *E. coli* strain JW4166 cell, which was deficient for glycine uptake and was obtained from National Institute of Genetics (Mishima, Shizuoka, Japan). *E. coli* JW4166 was grown at 37 °C in LB medium containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml).

2.9.3.4 Transport assays

The *E. coli* JW4166 cells transformed with pTrcHis2C or pApagcS1 were grown overnight at 37 °C in LB medium containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml) and were inoculated into the same fresh medium with an OD_{620} of 0.4-0.6 . Isopropyl β-D-1thiogalactopyranoside (IPTG) (1 mM) was added. After 1 h of incubation, cells were harvested, washed twice, and suspended to an OD_{620} of 2.0 in 100 mM Tris-HCl buffer pH 7.4 containing 4 mM $MgSO_4$ and 300 mM NaCl. Subsequently, the cell suspension was added with Tris-lactate pH 7.4 to a final

concentration at 2 mM and incubated for 10 min at 37 °C. The uptake was initiated by the addition of 1 μM [U-^{14}C] glycine. For K_m and V_{max} determinations, the concentrations of glycine were varied from 0 to 100 μM. Cells were collected on 0.2 μm cellulose nitrate filters (Advantec MFS, Chiba, Japan). Filters were washed with 3 ml of buffer, and the radioactivity trapped in the cells was measured with a liquid scintillation counter (Model 3200C; Aloka Instruments Co., Tokyo, Japan). Competitions for glycine uptake were performed in the presence of 100-fold molar excess competitors. Radiolabeled [U-^{14}C] glycine (111.7 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, USA).

2.9.4 Cloning of *ApagcS1* and expression in *Synechococcus* sp. PCC 7942 Δ*natG* mutant strain

The pApagcS1from step 2.9.3.3 was used as a template DNA for cloning ApagcS1 by PCR using the primer set *Bam*HI_A5'_F_pTrc/6xHisstop *Bam*HI-R (Table 2.3). The amplified 1,977 bp DNA fragment was ligated to the multiple cloning site of pMD20 (Takara,Japan). The resulting plasmid, pMD20_*ApagcS1*, was transformed first into *E. coli* DH5α cells by heat shock method. The positive clones were selected on LB agar containing 100 μg /ml ampicillin and and Xgal to a final concentration of 40 ug/ml and incubated at 37 °C for 16 hrs before extracting the plasmids by QIAprep Spin Miniprep Kit, (QIAGEN, Geramany).

The pMD20_*ApagcS1* was digested with *Bam*HI. The cohesive end fragment was ligated into *Bam*HI site of the digested pSyn_1 expression vector. The resulting plasmid, pSyn_1 harboring *ApagcS1* was transformed first into *E. coli* DH5α cells by

heat shock method. The positive clone was selected on LB agar with 25 µg/ml spectinomycin.

pSyn_1 *ApagcS1*, which was double digested with *Bam*HI and *Nco*I, was confirmed from the mobility of agarose electrophoresis of DNA fragments The recombinant plasmid was extracted and then transformed to *Synechococcus* sp. PCC 7942 Δ*natG* mutant strain in which insertion of chloramphenicol resistance gene was inserted into the middle of the *natG* coding region, by natural tramsformation (APPENDIX 10). The transformants were selected on BG11 agar plate supplemented with 25 µg/ml spectinomycin and 25 µg/ml chloramphenicol.

2.9.4.1 Determination of glycine and glutamine uptake in *Synechococcus* sp. PCC 7942 Δ*natG* mutant strain transformed with pSyn_1 or pSyn_1 ApagcS1

Cells were grown in BG11 medium containing antibiotics as desired (OD_{730} =0.5). Cells were collected by centrifugation, washed twice and resuspended to a concentration of 0.1 mg cell protein in 1ml assay medium (100 mM HEPES-KOH, pH 7.5 containing 300 mM NaCl). The uptake was initiated by the addition of 10 µM [U-^{14}C] glycine or 5 µM [U-^{14}C] glutamine. The uptake of cells was determined at interval time for 30 min.

2.9.4.2 Determination of amino acid in culture medium

Synechococcus sp. PCC 7942 Δ*natG* mutant cells transformed with pSyn_1 or pSyn_1 *ApagcS1* were grown in BG11 medium containing antibiotic as desired (OD_{730} =0.5). Cells were collected by centrifugation and incubated in the

new medium (10 mM NH_4Cl) for 24 hours. The filtered culture medium was analyzed by high performance liquid chromatography (HPLC). The method used for the analysis of amino acids involves a derivatization of amino acids with o-phthalaldehyde (OPA), which reacts with primary amines of amino acids, peptide and proteins to enable fluorescent detection and quantitation.

2.9.5 Reverse transcriptase-PCR (RT-PCR) analysis of *ApagcS1* in *A. halophytica* cell.

Culture of *A. halophytica* (10 mL, OD_{730} = 0.5) was harvested. The cell pellet was used for RNA extraction by TRIzol method. The cDNA was synthesized from 1 µg of total RNA by using cDNA synthesis kit: PrimeScript First Strand cDNA Synthesis Kit (Takara, Otsu, Japan). Portions of 2µl synthesized cDNA were subjected to PCR in a reaction volume of 20 µl. Specific primer sets A_sym_mid_F and Acp1RT_R (Table 2.4) were used for PCR at a final concentration of 0.2 µM in the PCR mixture. The PCR reactions were performed using AmpliTaq Gold (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The PCR conditions employed were: denaturation at 95 °C for 5 min; 28 amplification cycles with denaturation for 30 sec at 95 °C, annealing for 30 sec at 55 °C, and extension for 30 sec at 72 °C. The resulting reaction mixtures were subjected to 1.5% agarose electrophoresis and visualized by staining with ethidium bromide. The intensities of band were quantified by GelQuant.NETsoftware and normalized by setting the signals from cells before treatment to 1.0.

CHAPTER III

RESULTS

3.1 The effect of glycine on growth of *A. halophytica*

A. halophytica cells were grown photoautotrophically in BG_{11} medium using 18 mM $NaNO_3$ as nitrogen source and Turk Island salt solution, pH of medium was adjusted to 7.6. Cells were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of mediumand shaken on a rotary shaker with 160 rpm at 30 ^{o}C under continuous illumination of 30 μmolphoton/m^2/s. The NaCl concentration of the culture medium was adjusted to 0.5 M NaCl for normal condition (Figure 3.1A) and 2.0 M NaCl (Figure 3.1B) for salt stress condition supplemented with glycine ranging from 0-100 mM. Cells were harvested every three days and the growth rate was monitored by measuring the optical density of the culture at 730 nm with a spectrophotometer. The result showed that low concentration of glycine at up to 20 mM enhanced growth in both normal and stress conditions. However concentration of glycine higher than 60 mM inhibited growth and complete inhibition was observed at 100 mM glycine.

Growth of *A. halophytica* was determined in various concentrations of NaCl (0-3 M) and supplemented with 10 mM glycine. The result in Figure 3.2 showed that glycine could enhance growth in all conditions of NaCl and the highest growth was observed at 3.0 M NaCl.

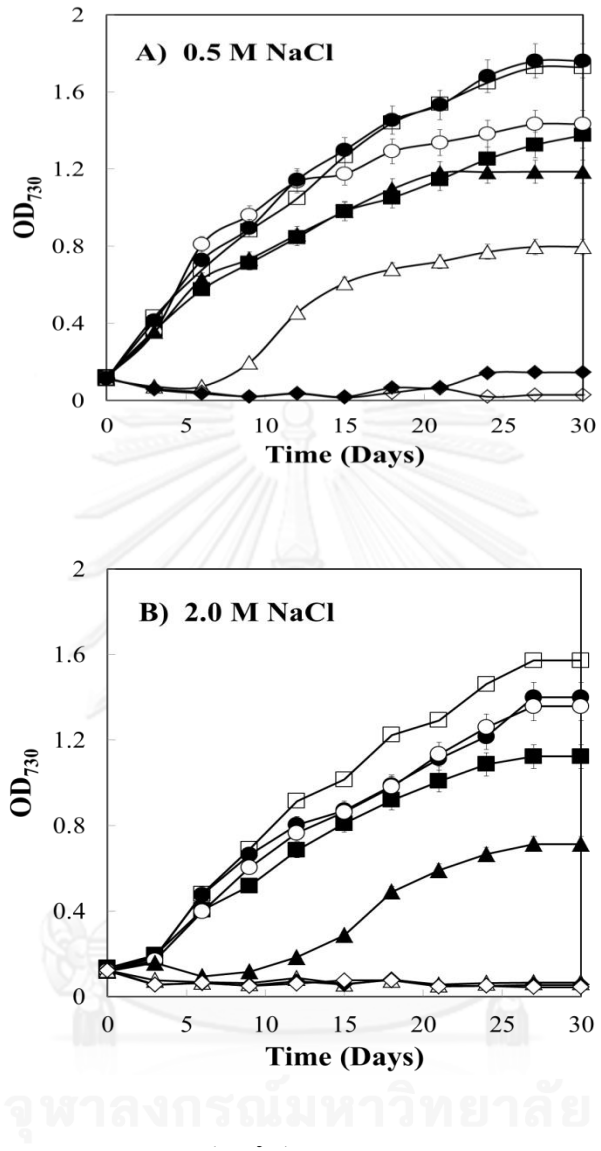


Figure 3.1 Growth of *A. halophytica* under 0.5 M NaCl normal conditions (A) 2.0 M NaCl stress conditions (B) supplemented with 0-100 mM glycine. The data are the average of 3 replicates ± S.E. (n=3)

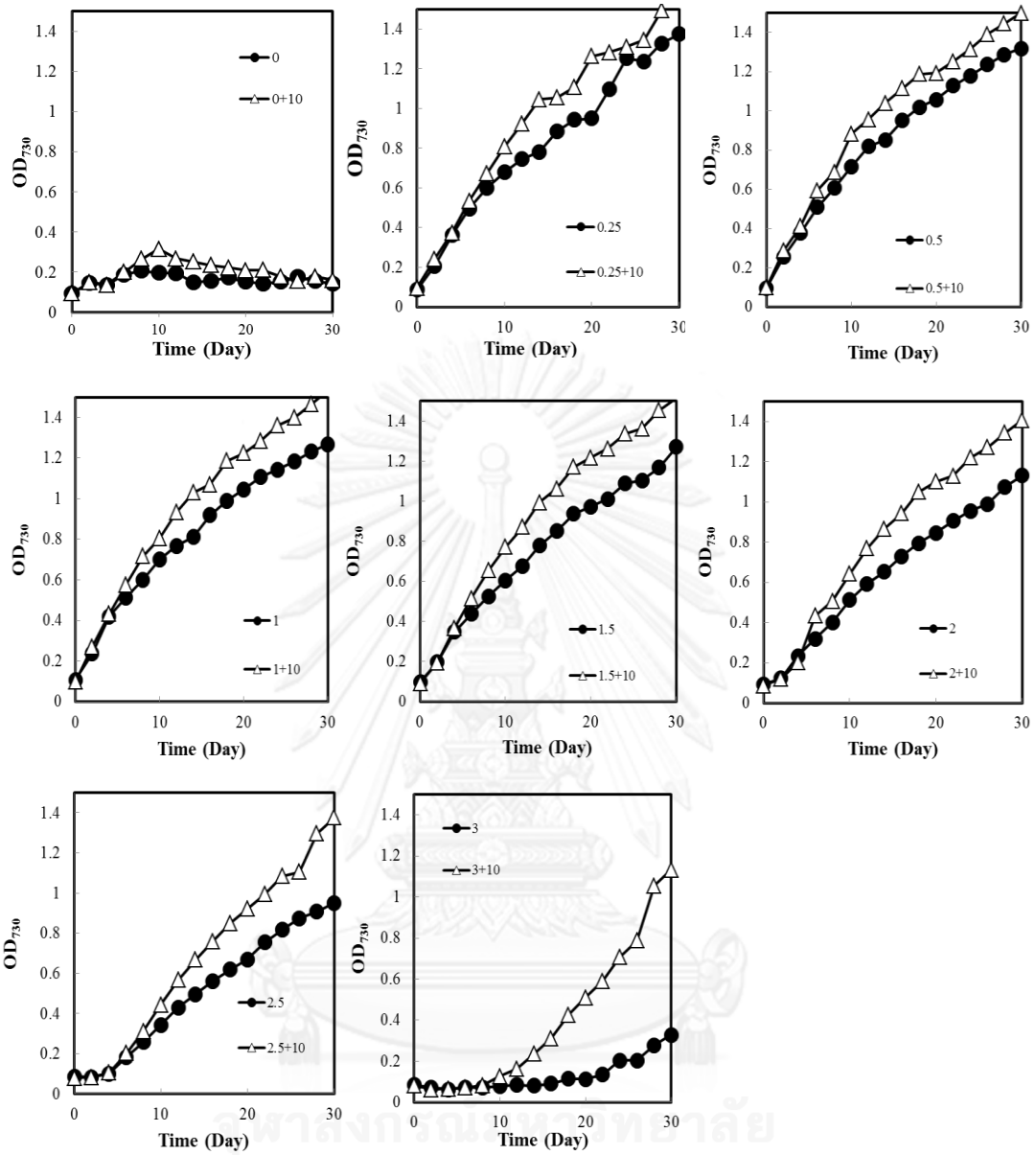


Figure 3.2 Growth of *A. haloophytica* under 0-3 M NaCl (●) and under 0-3 M NaCl supplement with 10 mM glycine (Δ)

3.2 Characterization of glycine uptake in *A. halophytica* cells

3.2.1 Time course of glycine uptake in *A. halophytica cells*

A. halophytica cells were grown in the growth medium containing 0.5 M NaCl. Cells at log phase (10 day culture) were harvested, washed twice with assay buffer pH 7.6 containing 0.5 M sorbitol. The uptake experiment was initiated by adding ^{14}C-glycine with a specific activity of 8 µCi/µmol at a final concentration of 50 µM. The glycine uptake of *A. halophytica* cells was determined at interval time for 30 minutes. The cells suspension was withdrawn rapidly and filtered through cellulose acetate filters. The filters were washed twice with 3 ml of buffer and the radioactivity trapped in the cells were added with scintillation fluid and determined with a liquid scintillation counter. The glycine can immediately be taken up by the cell with saturated glycine uptake after exposure to glycine about 10 minutes (Figure 3.3)

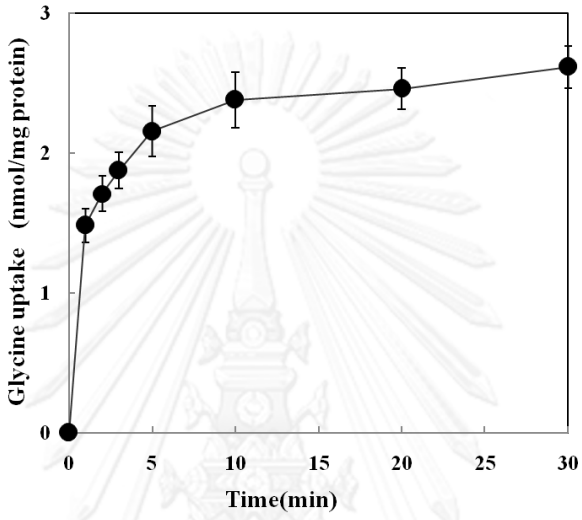


Figure 3.3 Time course of glycine uptake in *A. halophytica*. The data are the average of 3 replicates ± S.E. (n=3)

3.2.2 Effect of cell age of *A. halophytica* on glycine uptake

The effect of cell age was determined in culture from day 2 to day 30. The results in Figure 3.4 showed that the uptake rate slightly increased from day2 to day 8 and the highest uptake rate was observed at 8 days cultivation. Later, the uptake rate was slightly decreased when the cells were grown longer than 8 days.

3.2.3 Kinetics of glycine uptake in *A. halophytica*

The concentrations of glycine were varied from 0 to 700 μM. The results in Figure 3.5 showed saturable rate of uptake above 500 μM. The results exhibited saturation kinetics typical of Michaelis-Menten kinetics with an apparent K_m of 160.8 μM and the maximum velocity (V_{max}) of 3.85 nmol/min/mg protein

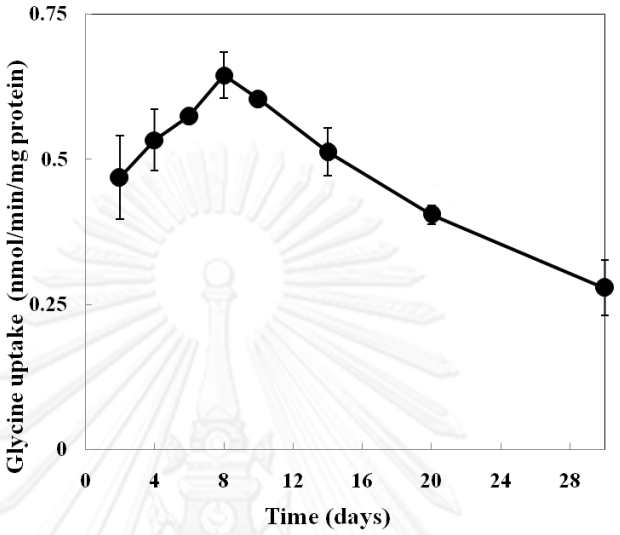


Figure 3.4 Effect of cell age on glycine uptake in *A. halophytica* cells. The data are the average of 3 replicates ± S.E. (n=3)

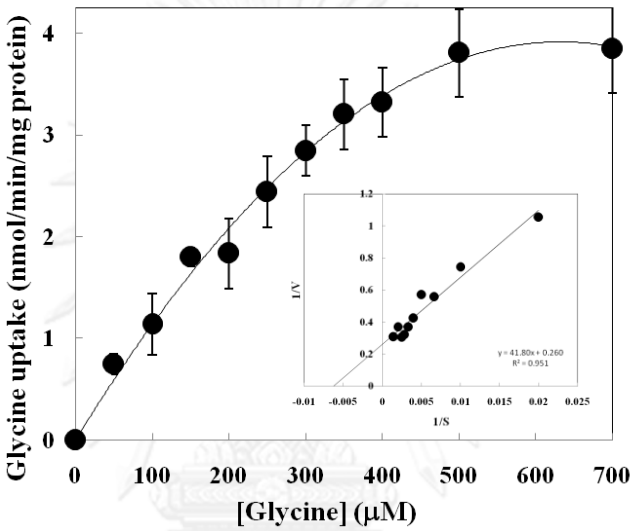


Figure 3.5 Effect of glycine concentration on glycine uptake in *A. halophytica* cells. The data are the average of 3 replicates ± S.E. (n=3)

3.2.4 Effect of external pH on glycine uptake in *A. halophytica*

From previous literature *A. hahophytica* can grow in the wide range of pH. To test the effect pH on glycine uptake the pH of assay buffer was varied from 5.5 to 10 by using 50 mM MES-KOH pH 5.5-6.5, 50 mM HEPES-KOH pH 7.0.-9.0 and 50 mM CAPS-KOH pH 9.5-10.0. The results from this experiment were represented as percentage of relative glycine uptake using data at pH 7.5 as 100 %. The highest activity of glycine uptake was at pH 8.0 (Figure 3.6).

3.2.5 Substrate specificity of glycine uptake in *A. halophytica*

To determine the specificity of the uptake, we performed the competition experiments. The [U-^{14}C] glycine uptake was inhibited by about 70% when 20-fold "non label" glycine was included in the assay buffer (Figure 3.7B). Neutral amino acid; alanine, serine and cysteine strongly inhibited glycine uptake by50- 60%. Glycine uptake was slightly inhibited (20-40%) by asparagine, valine, glutamine, methionine, threonine, leucine, proline and isoleucine. Acidic amino acids (aspartate and glutamate shown in Figure 3.7C) and basic amino acids (lysine arginine and histidine shown in Figure 3.7A) did not inhibit glycine uptakes.

3.2.6 Effect of metabolic inhibitors and ion dissipaters on glycine uptake in *A. halophytica*

The effects of various agents were tested and the results are shown in Table 3.1. To determine the requirement of energy for glycine uptake, uncoupling agents; carbonyl cyanide-m-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol (DNP), ATP synthase inhibitor; N,N'-dicyclohexylcarbodiimide (DCCD) and N-ethylmaleimide (NEM)

and respiratory chain inhibitors; sodium azide (NaN_3) and potassium cyanide (KCN) were tested. The result showed that these inhibitors could inhibit glycine uptake about 50% when compared with control. The results of ion dissipater (amiloride; Na^+/H^+ antiporter inhibitor, monensin; Na^+ ionophore, valinomycin and gramicidin D; K^+ ionophore and nigericin; ionophore) on glycine uptake corresponded with results of metabolic inhibitors .

3.2.7 Effect of NaCl on glycine uptake in *A. halophytica*

The effect of NaCl on gycine uptake was determined and the result is shown in Figure 3.8. Concentrations of NaCl were varied from 0 to 1 M NaCl. The glycine uptake was decreased with increasing concentration of NaCl.

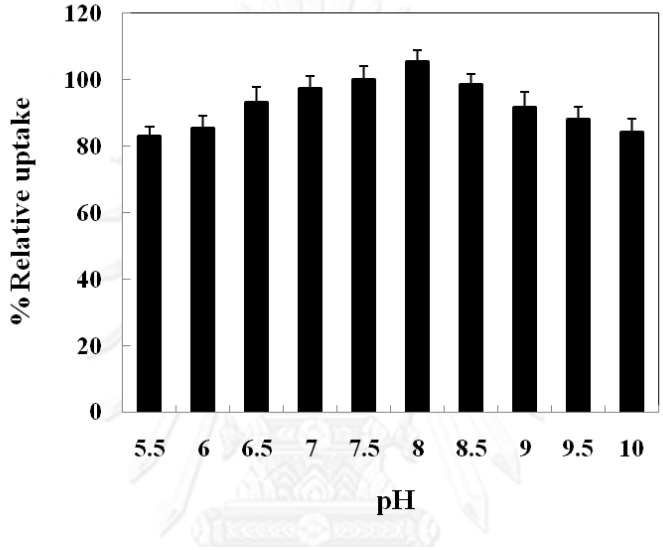


Figure 3. 6 Effect of external pH on glycine uptake in *A. halophytica*. The data are the average of 3 replicates ± S.E. (n=3)

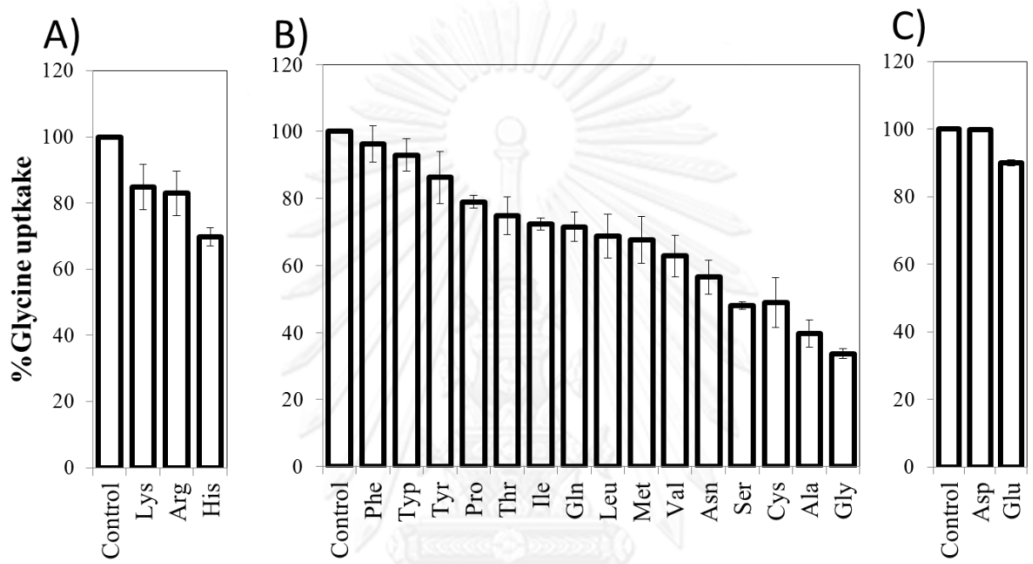


Figure 3.7 Effect of various amino acids on glycine uptake in *A. halophytica*, A) basic amino acid, B) neutral amino acid and C) acidic amino acid. The data are the average of 3 replicates ± S.E. (n=3)

Table 3.1 Effect of metabolic inhibitors and ion dissipaters on glycine uptake in *A. halophytica*

(A) Metabolic inhibitors		Conc.	Glycine uptake (%)
	None		100
Uncoupling agent	CCCP	0.1mM	49.52
	DNP	1 mM	48.67
ATP Synthase inhibitor	DCCD	1 mM	55.24
	NEM	0.1mM	57.18
Respiratory chain inhibitor	NaN_3	1 mM	76.98
	KCN	1 mM	100
(B) Ion gradient dissipaters			
Na^+/H^+ antiporter inhibitor	Amiloride	0.1mM	44.68
Na^+ ionophore	Monensin	1 mM	54.96
K^+ ionophore	Valinomycin	0.1mM	39.99
	Gramicidin D	0.1mM	59.87
Ionophore	Nigericin	0.1mM	52.39

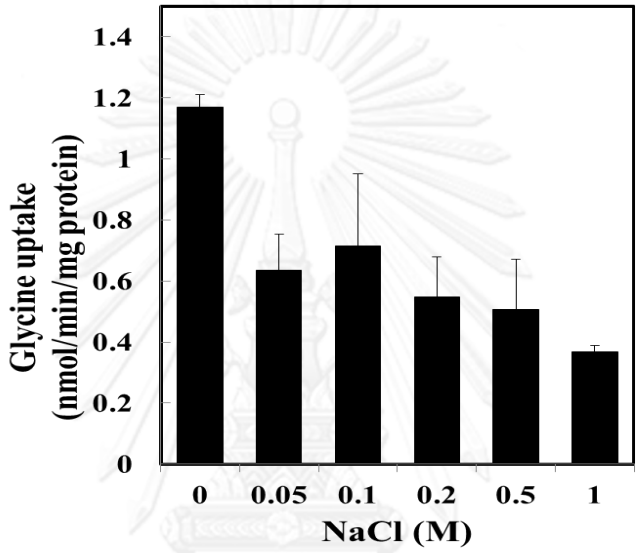


Figure 3. 8 Effect of NaCl on glycine uptake in *A. halophytica*. The data are the average of 3 replicates ± S.E. (n=3)

3.3 Cloning of *A. halophytica* glycine transporter gene and expression in *E. coli* mutant (deficient with glycine transporter)

3.3.1 Identification of *A. halophytica* glycine transporter gene

We searched the homologous gene in *A. halophytica* based on the shot gun sequencing of *A. halophytica*, and found that *A. halophytica* contains a gene (alanine/glycine cation symorter, *ApagcS1*). The deduced amino acid sequence (APPENDIX 11) was used as a query for domain search. The result of domain search using interproscan showed that *ApagcS1* contains sodium: alanine symporter (IPR001463). The prediction of transmembrane helices in proteins was analyzed by TMHTMM Server v.2 (http://www.cbs.dtu.dk/services/TMHMM/). The result of transmembrane prediction showed that ApacgS1 consists of 10 transmembrane segments (TMS) (Figure 3.9).

3.3.2 Cloning of *A. halophytica* glycine transporter gene

A. halophytica genomic DNA was used as template for PCR amplification as described in materials and methods 2.9.3. The PCR product is shown by agarose gel electrophoresis in Figure 3.10. The amplified fragment contained 500' upstream region (UTR) before coding sequence and the amplified fragment of *ApagcS1* is 1,948 bp (APPENDIX 12). DNA fragment was ligated to the multiple cloning site of pCR 2.1 (Invitrogen, USA). The resulting plasmid, pCR 2.1_*ApagcS1* was transformed into *E. coli* DH5α cells by heat shock method. The positive clones were selected on LB agar and the plasmids were extracted. Insert fragment of an expected size was sequenced with 3 primers as shown in Table 2.2.

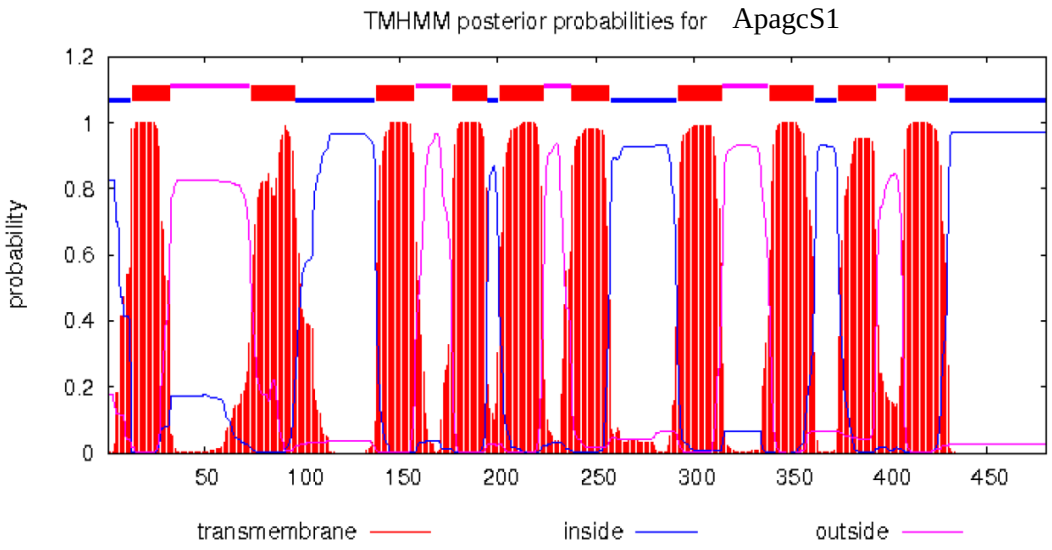


Figure 3.9 Transmembrane segments prediction of ApagcS1. The Transmembrane segments were represented with red boxes.

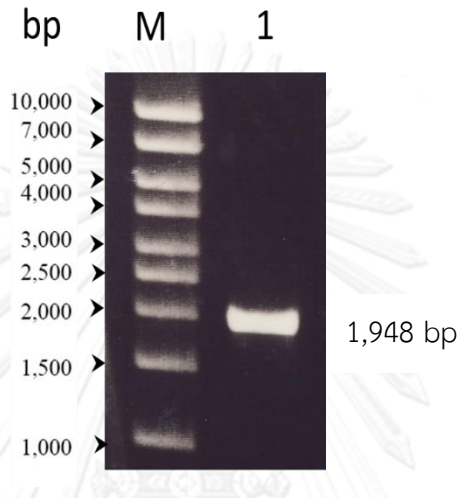


Figure 3.10 Agarose gel electrophoresis of the amplified fragment. The PCR products were separated on 0.8% agarose gels and visualized by ethidium bromide staining.

Lane M DynaMarker DNA High D #DM122

Lane 1 PCR product of *ApagcS1*

3.3.3 Construction of expression vector *E. coli* mutant (deficient in glycine transporter)

The pCR 2.1_*ApagcS1* was double digested with *Bam*HI and *Sal*I. The cohesive end fragment was ligated into *Bam*HI and *Sal*I site of the digested pTrcHis2C expression vector. The resulting plasmid, pApagcS1 harboring *ApagcS1* fused in frame to six histidines at the C terminus was transformed first into *E. coli* DH5α cells by heat shock method. The positive clones were selected and the plasmids were extracted.

The recombinant plasmid was extracted and before transforming to expressing host cell the recombinant plasmid was confirmed by digestion with restriction enzyme (Figure 3.11). The expected size of fragment that digested with restriction enzyme showed in Table 3.2. Then recombinants plasmid was transformed to *E. coli* strain, JW4166 cell, deficient in glycine uptake which was obtained from the Keio collection of single-gene knockouts constructed through a collaboration of the Inst. of Adv. Biosci. at Keio Univ., the Nara Inst. of Sci. and Technol, Japan and Purdue Univ., USA. The *E. coli* JW4166 transformant cell was grown at 37 °C in LB medium containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml).

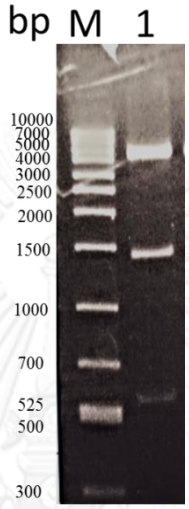


Figure 3.11 Agarose gel electrophoresis of the pApagcS1 digested with restriction enzyme

Lane 1 pApagcS1 digested with *Bam*HI/*Nco*I/*Sal*I

Table 3.2 Restriction enzymes used for the digestion of pApagcS1 and expected size of DNA fragments

	Restriction Enzymes	Expected DNA Fragments (bp)
pApagcS1	*Bam*HI/*Nco*I/*Sal*I	4310, 1394, 548, 4

3.3.4 Transport assays

The *E. coli* JW4166 cells transformed with pTrcHis2C or pApagcS1 were grown overnight at 37 °C in LB medium containing antibiotic as desired and were inoculated into the same fresh medium with an OD_{620} of 0.4-0.6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (1 mM) was added. After 1 h of incubation, cells were harvested, washed twice, and suspended to an OD_{620} of 2.0 in 100 mM Tris-HCl buffer pH 7.4 containing 4 mM $MgSO_4$ and 300 mM NaCl. Subsequently, the cell suspension was added with Tris-lactate pH 7.4 to a final concentration at 2 mM for 10 min at 37 °C, and the uptake was initiated by the addition of 1 μM [U-^{14}C] glycine. After incubation at indicated time cells were filtered through membrane, washed twice with buffer and determined by liquid scintillation counter No measurable uptake of [U-^{14}C] glycine was observed for *E. coli* JW4166 cells transformed with pTrcHis2C (empty vector) as shown in Figure 3.12. The initial rate of [U-^{14}C] glycine uptake was occurred in the first 1 min and the uptake was saturated above 5 minutes.

3.3.4.1 Effect of ion on glycine uptake in *E. coli* JW4166 cells transformed with pApagcS1

The effect of ion on glycine uptake such as Na^+, Li^+ and K^+ is shown in Figure 3.13. The concentrations were varying from 0 to 1000 mM. The significantly increased uptake of glycine occurred with the maximum uptake at 200 and 300 mM NaCl. However KCl and LiCl were ineffective, indicating that ApagcS1 is a Na^+-dependent glycine transporter.

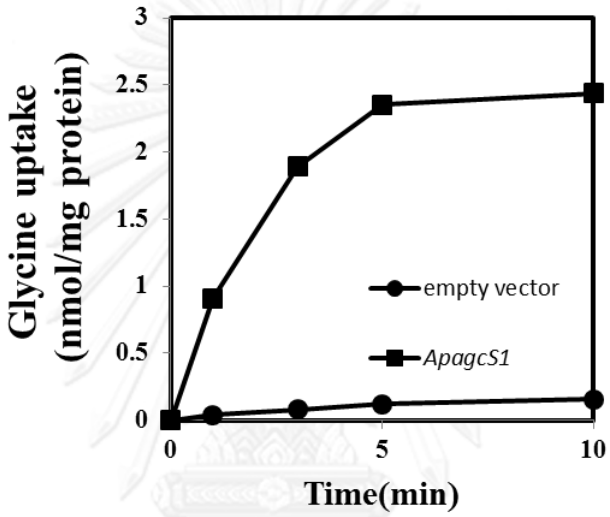


Figure 3.12 Time course of glycine uptake in *E. coli* JW4166 transformant cells. The data are the average of 3 replicates ± S.E. (n=3)

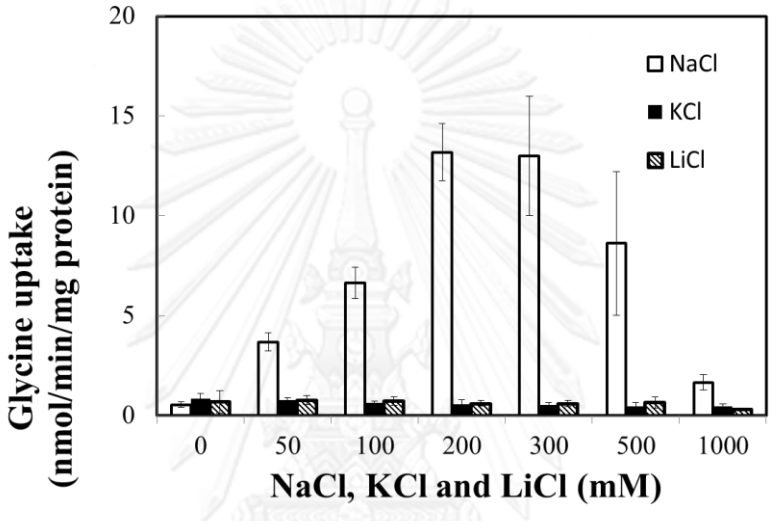


Figure 3.13 Ion dependency of glycine uptake in *E. coli* JW4166 transformant cells. The data are the average of 3 replicates ± S.E. (n=3)

3.3.4.2 Kinetic properties of ApagcS1 in *E. coli* JW4166 transformed cells

The saturation curve of glycine uptake is shown in Figure 3.14. The concentrations of glycine were from 0 to 100 μM. The glycine uptake rates were increased with the increase of glycine. The K_m and V_{max} values were determined by Nonlinear regression (curve fit). Michaelis-Menten enzyme kinetics was performed using GraphPad Prism version 6.00 for Windows, GraphPad Software. The K_m value was 12.53 μM and the V_{max} value was 35.74 nmol/min/mg protein.

3.3.4.3 Effect external of pH on glycine uptake in *E. coli* JW4166 transformed with pApagcS1

The effect of pH on glycine uptake by ApagcS1 in JW4166 cells were tested with the pH ranging from 5.5-10.0 as shown in Figure3.15. The optimum pH was around 9.0. However at the acid pH 5.5-6.5, the glycine uptake activities were low.

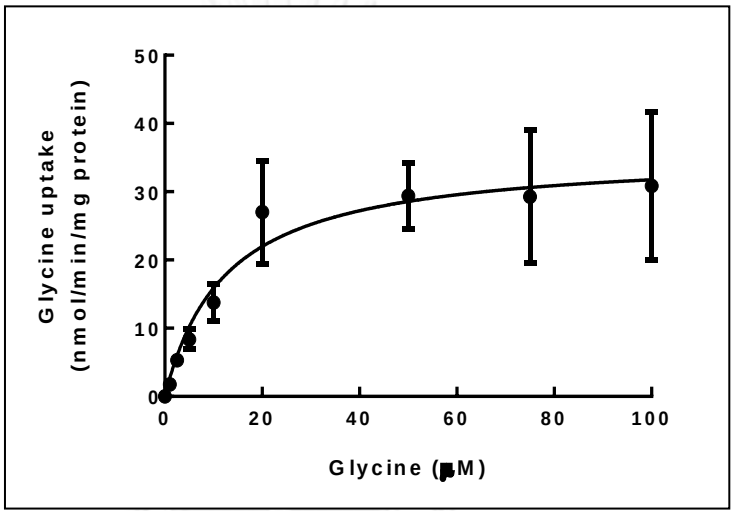


Figure 3.14 Saturation curve of glycine uptake in *E. coli* JW4166 transformed with pApagcS1. The data are the average of 3 replicates ±S.E. (n=3)

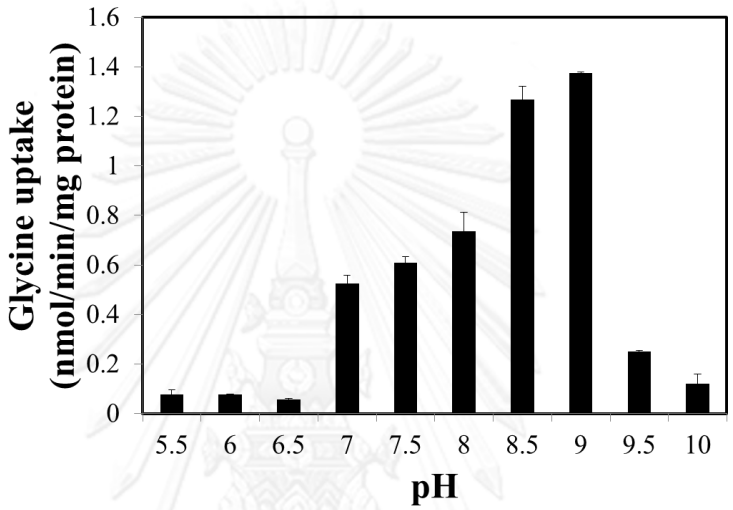


Figure 3.15 Effect of pH on glycine uptake in *E. coli* JW4166 transformed with pApagcS1. The data are the average of 3 replicates ±S.E. (n=3)

3.3.4.4 Substrate specificity of glycine uptake in *E. coli* JW4166 transformed with pApagcS1

To determine the specificity of the glycine uptake by ApagcS1, the competition experiments were determined with 100 fold of competitor. The [U-^{14}C] glycine uptake by ApagcS1 in JW4166 cells was inhibited by 100-fold nonlabel glycine about 85% (Figure 3.16). Glutamine and asparagine strongly inhibited glycine uptake by 95%. Methionine, alanine cysteine and serine moderately inhibited glycine uptake about 40-60%. Slightly inhibition about 30% was observed by isoleucine, tryptophan leucine histidine tyrosine lysine and phenylalanine. These results show that the ApagcS1 transporter were broad specific for amino acid.

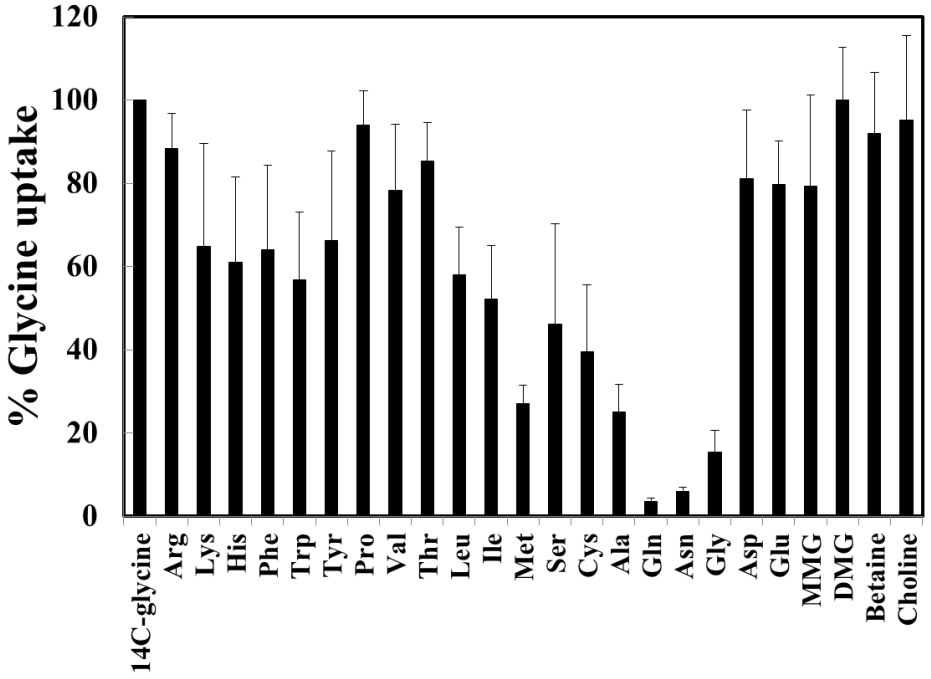


Figure 3.16 Substrate specificity of glycine uptake in *E. coli* JW4166 transformed with pApaGcS1.The data are the average of 3 replicates ±S.E. (n=5)

3.4 Cloning of *ApagcS1* and expression in *Synechococcus* sp. PCC 7942 Δ*natG* mutant strain

The pApagcS1 from step 2.9.3.3 was used as a template DNA for cloning ApagcS1 by PCR using the primer set *Bam*HI_A5'_F_pTrc/6xHisstop *Bam*HI-R (Table 2.3). The amplified 1,977 bp DNA fragment (Figure 3.17) was ligated to the multiple cloning site of pMD20 (Takara, Japan). The resulting plasmid, pMD20_*ApagcS1*, was transformed first into *E. coli* DH5α cells by heat shock method. The positive clones were selected on LB agar containing 100 μg/ml ampicillin and and Xgal to a final concentration of 40 μg/ml followed by incubation at 37 °C for 16 hrs and the plasmids were extracted by QIAprep Spin Miniprep Kit, (QIAGEN, Germany).

The pMD20_*ApagcS1* was digested with *Bam*HI. The cohesive end fragment was ligated into *Bam*HI site of the digested pSyn_1 expression vector. The resulting plasmid, pSyn_1 harboring *ApagcS1* was transformed first into *E. coli* DH5α cells by heat shock method. The positive clone was selected on LB agar with 25 μg/ml spectinomycin.

pSyn_1 *ApagcS1*, which was double digestion with *Bam*HI and *Nco*I, was confirmed from the mobility of agarose electrophoresis of DNA fragments (Figure 3.18) and the expected size is shown in Table 3.3. The recombinant plasmid was extracted and then transformed to *Synechococcus* sp. PCC 7942 Δ*natG* mutant strain, with insertion of chloramphenicol resistance gene inserted into the middle of the *natG* coding region, by natural tramsformation. The transformants were selected on BG11 agar plate supplemented with 25 μg/ml spectinomycin and 25 μg/ml chloramphenicol.

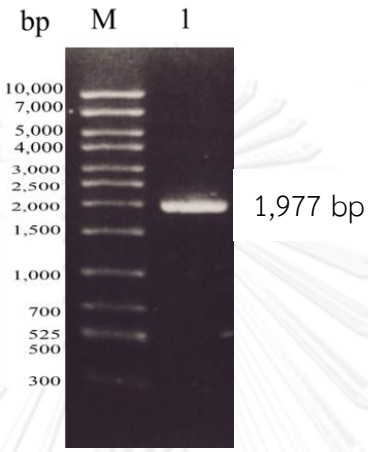


Figure 3.17 Agarose gel electrophoresis of the amplified fragment. The PCR products were separated on 0.8% agarose gels and visualized by ethidium bromide staining.

Lane M DynaMarker DNA High D #DM122

Lane 1 PCR product of *ApagcS1*

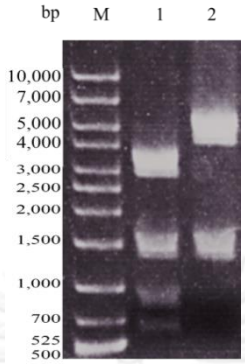


Figure 3.18 Agarose gel electrophoresis of the pSyn_1 ApagcS1 digested with restriction enzyme

Lane MDynaMarker DNA High D #DM122

Lane 1 pSyn_1 *ApagcS1* digested with *Bam*HI/*Nco*I

Lane 2 pSyn_1 *ApagcS1* digested with *Nco*I

Table 3.3 Restriction enzymes used for the digestion of pSyn_1 ApagcS1 and expected size of DNA fragments

	Restriction Enzymes	Expected DNA Fragments (bp)
pSyn_1 *ApagcS1*	*Bam*HI/*Nco*I	2958, 1394, 824, 525
pSyn_1 *ApagcS1*	*Nco*I	4532,1399

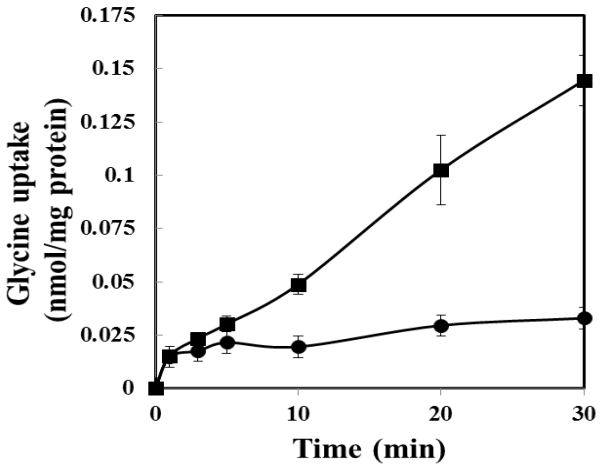
3.4.1 Determinations of glycine and glutamine uptake in *Synechococcus* sp. PCC 7942 Δ*natG* mutant strain transformed with pSyn_1 or pSyn_1 *ApagcS1*

Synechococcus sp. PCC 7942 Δ*natG* mutant strain transformed with pSyn_1 or pSyn_1 *ApagcS1* was grown in BG11 medium containing antibiotics as desired. Cells at mid log phase (OD_{730}=0.5) were harvested by centrifugation, washed twice and re-suspended to a concentration of 0.1 mg cell protein in 1ml assay medium (100 mM Hepes-KOH, pH 7.5 containing 300 mM NaCl). The uptake was initiated by the addition of 10 μM [U-^{14}C] glycine or 5 μM [U-^{14}C] glutamine. The uptake of cells was determined at interval time for 30 min. *Synechococcus* sp. PCC 7942 Δ*natG* mutant strain transformed with pSyn_1 *ApagcS1* could take up both glycine and glutamine (Figure 3.19) compared with no uptake in *Synechococcus* sp. PCC 7942 Δ*natG* mutant strain transformed with pSyn_1.

3.4.2 Determination of amino acid in culture medium

Extracellular amino acids were determined by high performance liquid chromatography (HPLC) in samples of filtered culture medium after inoculation of cell in the new medium (10 mM NH_4Cl) at 24 hours. Accumulation of amino acid in the extracellular medium of *Synechococcus* sp. PCC 7942 Δ*natG* mutant cell harboring pSyn1 vector showed the accumulation of amino acid in medium higher than that of wild type about 1 to 4 fold especially serine, glutamine, alanine and tyrosine (Figure 3.20). Interestingly, cell of *Synechococcus* sp. PCC 7942 Δ*natG* mutant harboring pSyn1_*ApagcS1* vector showed a reduction in the accumulation of amino acid in medium. Serine, asparagine, glutamine and isoleucine were significantly decreased ($p<0.05$) when compared to *Synechococcus* sp. PCC 7942 Δ*natG* mutant harboring an empty vector.

A)



B)

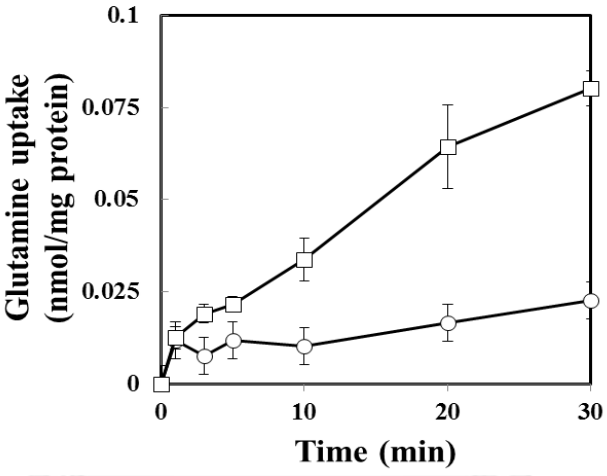


Figure 3.19 Time course of A) glycine uptake in *Synechococcus* sp. PCC 7942 Δ*natG* mutant strain transformed with pSyn_1 (●) and in *Synechococcus* sp. PCC 7942 Δ*natG* mutant strain transformed with pSyn_1 ApagcS1 (■) B) glutamine uptake in *Synechococcus* sp. PCC 7942 Δ*natG* mutant strain transformed with pSyn_1 (○) and in *Synechococcus* sp. PCC 7942 Δ*natG* mutant strain transformed with pSyn_1 ApagcS1 (□).

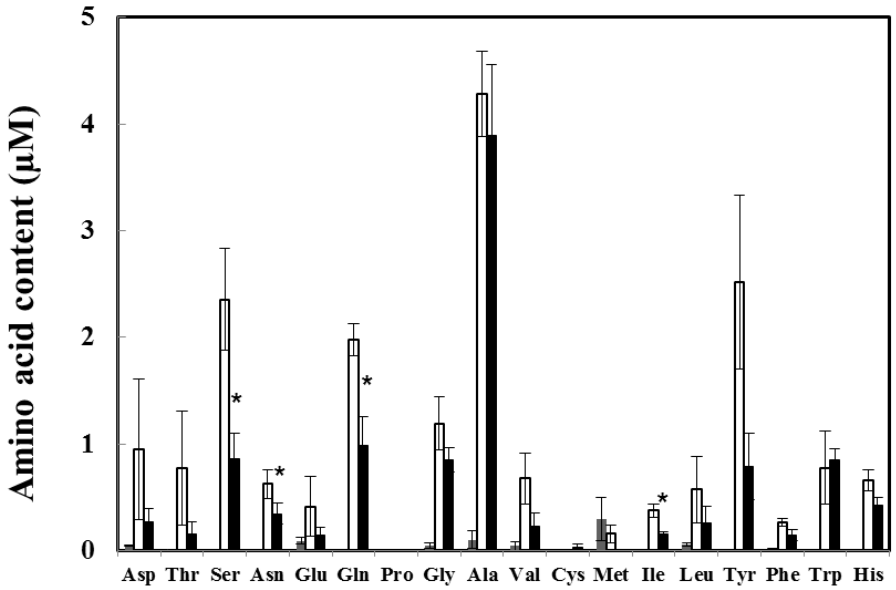


Figure 3.20 Amino acid in culture medium of *Synechococcus* sp PCC 7942 wild type (gray bar) *Synechococcus* sp. PCC 7942 Δ*natG* mutant harboring pSyn_1 (white bar) and pSyn_1 ApagcS1 (black bar) after 24 hour. The data are the average of 3 replicates ± S.E. (n=3)

3.5 Reverse transcriptase-PCR (RT-PCR) analysis of *ApagcS1* in *A. halophytica* cell.

Expression of *A. halophytica ApagcS1* was examined under 2 conditions, the effect of NaCl stress (0.5 M to 2.5 M NaCl) (Figure 3.21) and nitrogen deficiency condition (Figure 3.22). The relative expression of gene was set to 1 at time zero. Expression of the *ApagcS1* was found to be induced by salt stress with up to 2 fold after 3 h of stress. The effect of nitrogen on gene expression of *ApagcS1* revealed that the expression of *ApagcS1* was induced by 3 fold after 1 hour without nitrate. These results indicate that the expression of *ApagcS1* is induced upon salt stress and nitrogen deficiency.

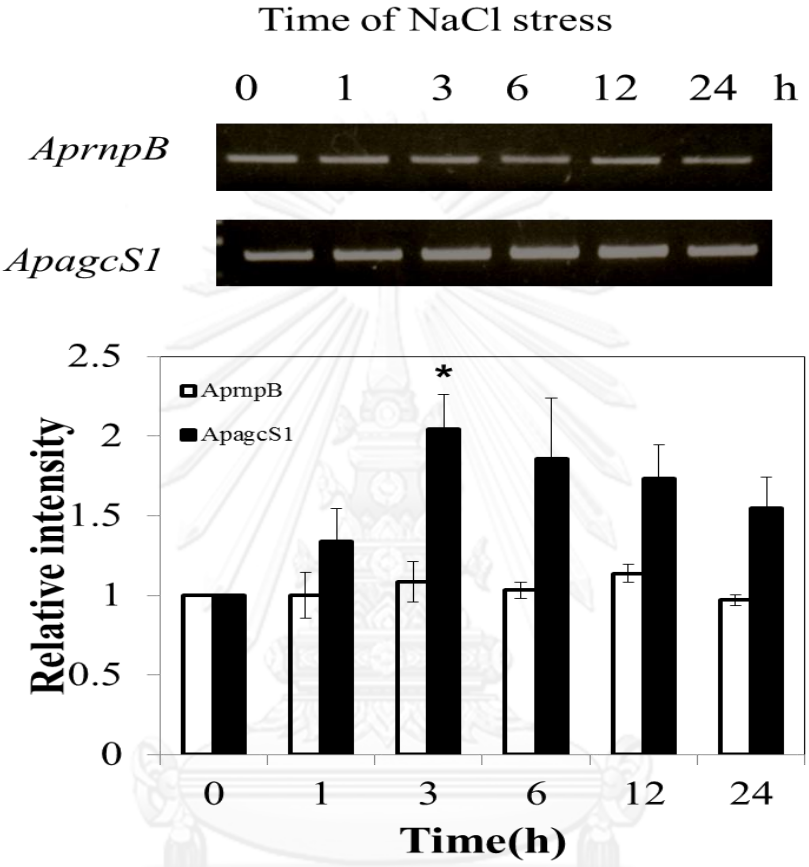


Figure 3.21 Expression of ApagcS1 in A. halophytica cells under the effect of NaCl stress. Cells were collected at 0, 1, 3, 6, 12 and 24 h of exposure to NaCl stress. PCR products were subjected to electrophoresis (A), followed by calculation of relative values of the amount of DNA fragments (B). RNase P (rnpB) gene was used as control. The values at time zero of each gene were set to 1. The data are the average of 3 replicates ± S.E. (n=3). Asterisks indicate significant difference ($P < 0.05$)

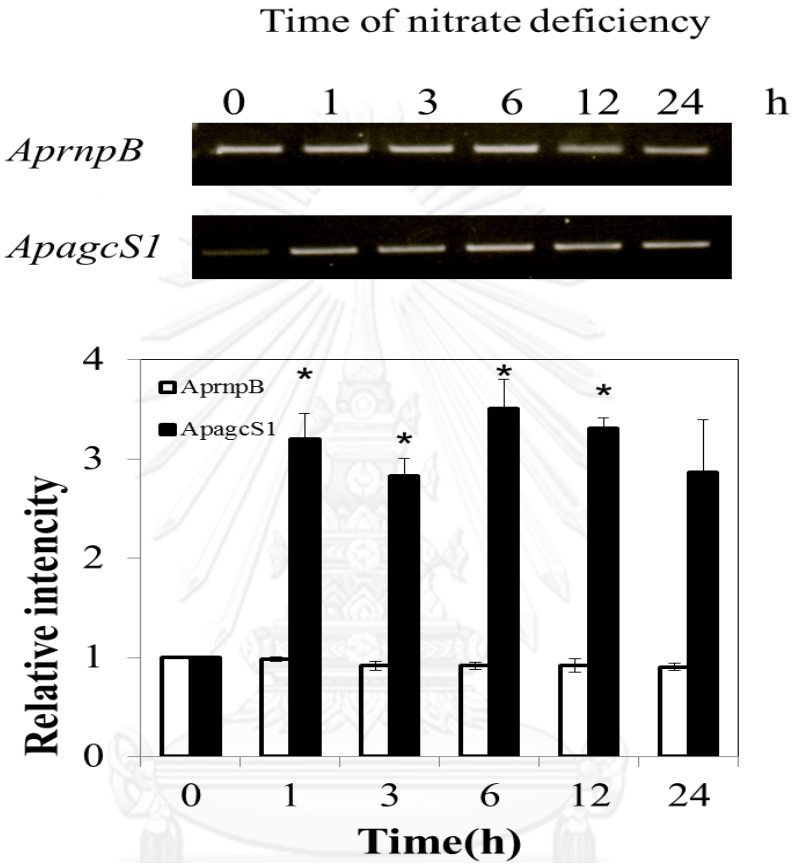


Figure 3.22 Expression of ApagcS1 in *A. halophytica* cells under nitrogen deficiency condition. Cells were collected at 0, 1, 3, 6, 12 and 24 h of exposure to nitrogen deficency. PCR products were subjected to electrophoresis (A), followed by calculation of relative values of the amount of DNA fragments (B). RNase P (rnpB) gene was used as control. The values at time zero of each gene were set to 1.The data are the average of 3 replicates ± S.E. (n=3). Asterisks indicate significant difference ($P < 0.05$).

CHAPTER IV

DISCUSSION

From the results of supplementation of glycine in normal medium (in BG11 medium plus 18 mM $NaNO_3$ and Turk Island salt solution, 0.5 M NaCl), it is clear that low concentration of glycine up to 20 mM promoted growth of *A. halophytica.* Previously *Synechocysis* sp. PCC 6803 showed a gradual decline in growth in the presence of increasing glycine levels (Eisenhut *et al.* 2007). However, high concentration of 100 mM glycine completely inhibited growth of *A. halophytica* whereas 40 mM glycine inhibited growth completely of *Synechocysis* sp. PCC 6803. Growth of *A. halophytica* was inhibited by NaCl, but supplementation of suitable concentration of glycine reduced the effect of NaCl. (Figure 3.2)

Glycine uptake was characterized in *A. hahophytica* cells. The uptake of glycine occurred at all cultivation time test and showed highest activity at mid log phase. The K_m for glycine uptake was 160.8 μM which is a high affinity uptake system when compared to other cyanobacteria. In filamentous cyanobacteria *Anabaena* sp. strain PCC 7120 the amino acid uptake was characterized for and it was found that a rather low affinity system with a K_m value for glycine about 2 μM (Montesinos *et al.* 1995). The maximum velocity (V_{max}) of glycine up take in *A. halophytica* is 3.85 nmol/min/mg protein or 13.9 nmol/min/mg Chl (APPENDIX 13). The result of *Anabaena* sp. strain PCC 7120 showed the maximum velocity (V_{max}) of 31nmol/min/mg Chl (Montesinos *et al.* 1995). For fresh unicellular cyanobacterium *Synnechocystis* sp. PCC 6003, it was reported that the initial rate of glycine transport was 15.0 nmol/min/10^{10} cell (Labarre *et al.* 1987), 102.8 nmol/ mg Chl /10 min (Montesinos *et al.* 1997) and 9.4 nmol /min/ mg Chl (Quintero *et al.* 2001). The

velocity of glycine uptake in *A. halophytica* was lower than *Anabaena* sp. strain PCC 7120 but slightly higher than *Synnechosystis* sp. PCC 6003.

The results in Table 3.1 showed that glycine uptake was sensitive to the uncoupling agent carbonyl cyanide m-chlorophenylhydrazone (CCCP) and N, N'-dicyclohexylcarbodiimide (DCCD), ATP synthase inhibitor dissipating both the chemical hydrogen ion concentration gradient (ΔpH) and the proton electrochemical gradient. The results of the effect of metabolic inhibitors suggest that the glycine uptake in *A. holophytica* is an energy dependent process which is in agreement with the results in *Anabaena* sp. Strain PCC 7120 where glycine uptake was inhibited by DCCD and CCCP (Montesinos *et al.* 1995). Interestingly glycine uptake in *A. halophytica* cells were inhibited by ion dissipaters such as monensin and amiloride (sodium ionophores), valinomycin and gramicidin D (potassium ionophores) and nigericin (ionophore). In cyanobacteria, there have been no data on the inhibition of glycine uptake by ion dissipaters. From the data of metabolic inhibitor and ion dissipaters found in *A. halophytica*, it might suggest that at least two systems of glycine uptake exist in *A. halophytica*, one system is energy dependent and the other one is ion dependent. This is different from filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 where glycine uptake occurred via Nat system (N-I and N-II; energy dependent TC 3.A.1.4 and TC 3.A.1.3 (Pernil *et al.* 2008), and from Nat system; energy dependent TC 3.A.1 reported in fresh water cyanobacterium *Synnechocystis* sp. PCC 6003 (Quintero *et al.* 2001).

Glycine uptake in *A. halophytica* was strongly inhibited by alanine serine and cysteine about 50-60 % and slightly inhibited (20-40%) by asparagine, valine, glutamine, methionine, threonine, leucine, proline and isoleucine. These amino acids

are neutral amino acids. The results are similar to those reported in other cyanobacteria where glycine was taken up via Nat system (neutral amino acid transpoter) in *Synnechocystis* sp. PCC 6003 (Quintero *et al.* 2001) and via Nat system (N-I and NII) in *Anabaena* sp. strain PCC 7120 (Pernil *et al.* 2008). However, it should be noted that neutral amino acids phenylalanine, tryptophan and tyrosine only slightly inhibited glycine uptake in *A. halophytica* suggesting that these amino acids might be transported by other transporters. For instance, in *E. coli* K12 separate transport systems were found for the following groups of amino acids: leucine, isoleucine, and valine; alanine, glycine, and serine; phenylalanine, tyrosine, and tryptophan; and methionine. (Piperno and Oxender 1968). The general aromatic amino acid permease, AroP, of *E. coli* is responsible for the active transport of phenylalanine, tyrosine, and tryptophan. PheP is a phenylalanine transporter that is normally responsible for phenylalanine transport (Pi *et al.* 1991).

Based on the shotgun sequence of *A.halophytica*, it was found that *A.halophytica* contains a alanine/glycine cation symporter. The transmembrane segment (TMS) prediction of ApagcS1 is 10 TMS which is different from the data of proteins in this family that have been reported, i.e. DagA of *Altermonas haloplanktis* ATCC 19855 and ACP of *Thermophilic bacterium* PS3 contain 8 TMS. (Kamata *et al.* 1992, MacLeod and MacLeod 1992) .

The protein in AGCS family is generalized as a cotransporter with cation Na^+ or H^+ which was drawn from the results employing NaCl, KCl and LiCl. The uptake of glycine in *E.coli* JW 4166 cells transformed with pAgcS1 responded to only NaCl which could suggest that ApagcS1 is sodium dependent. Previously, the alanine uptake in *Thermophilic bacterium* PS3 using reconstituted proteoloposomes was

shown to be driven by either an electrochemical potential difference of proton (addition of valinomycin) or that of sodium ions (100 mM Na phosphate pH7.0).(Kamata *et al.* 1992).

The kinetic parameters of glycine uptake of ApagcS1 in *E.coli* JW 4166 showed lower affinity (K_t for glycine 4.0 μM) than the K_t (apparent affinity constants of transport) of AK430 cell (*E.coli cycA* mutant transformed with acp gene). AK430 cell transformed with *cycA* gene (gene that encodes glycine transport in *E.coli*) showed K_t for glycine 2.0 μM (Akahane *et al.* 2003).

With respect to substrate specificity, asparagine leucine tyrosine lysine and isoleucine showed inhibition of glycine uptake by ApagcS1 but they did not inhibit glycine uptake by ACP. This was in contrast to aspartate, glutamate and threonine where inhibition of glycine uptake was observed by ACP but not by ApagcS1.

The results of amino acid excreted into culture medium of *Synechococcus* sp. PCC 7942 Δ*natG* mutant cell were similar to those reported for other cyanobacteria such as *Synechocystis* sp. PCC 6803 or *Anabaena* sp. PCC 7120 where mutation of neutral amino acid transport system (Nat) resulted in the leakage of hydrophobic amino acid especially alanine into the medium (Montesinos *et al.* 1995, Montesinos *et al.* 1997, Pernil *et al.* 2008). *Synechococcus* sp PCC 7942 Δ*natG* mutant harboring pSyn1_*ApagcS1* vector showed a reduction in the accumulation of some amino acids serine, asparagine, glutamine and isoleucine in the medium compared to *Synechococcus* sp PCC 7942 Δ*natG* mutant harboring an empty vector. The type of amino acids that were decreased in the medium corresponded with the amino acids that competed with glycine for the uptake by the *E. coli* JW4166 cells transformed with pApagcS1.

The uptake of glycine experiment was done at pH 7.6 in *A. halophytica* cells and pH 7.4 in *E. coli* transformant cells. Histidine is a basic amino acid having imidazole as a side chain with a pKa of. 6.1. Therefore at tested pH for glycine uptake higher than pKa of imidazole, most molecules of histidine exist in neutral form. As a consequence, some inhibition of glycine uptake by histidine was observed in *A. halophytica* cells and in *E. coli* transformant cells

CHAPTER V

CONCLUSION

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

1 Supplementation of low concentration of glycine in growth medium promoted growth of *Aphanothece halophytica* at all NaCl concentrations tested (0.25 – 3.0 M NaCl); however, high concentration of supplemented glycine inhibited growth.

2 The transport of glycine in *A. halophytica* was shown to exhibit saturation kinetics according to Michaelis-Menten kinetic parameters with an apparent K_m of 160.80 μM and V_{max} of 3.85 nmol/min/mg protein.

3 The inhibition of glycine uptake by metabolic and ion-dissipating inhibitors in *A. halophytica* suggested that, with regard to energy supply, there are at least two systems of glycine transporter, metabolic energy dependent and ion gradient dependent.

4 Based on shot gun sequence, *A. halophytica* consisted of gene homolog to alanine glycine cation symporter (*ApagcS1)* that exhibited high homology to alanine or glycine :cation symporter (AGCS) family. The *ApagcS1* gene contains1443 bp, encoding 480 amino acid residues.

5 The prediction of deduced amino acid residue by transmembrane prediction program TMHTMM Server v.2 showed ApAgcS1 consisting of 10 transmembrane segments.

6 The JW4166 transformant cell harboring pApagcS1 required Na^+ for glycine uptake. ApAgcS1 showed kinetic parameters with an apparent K_m for glycine of 12.53 μM and the V_{max} value was 35.74 nmol/min/mg protein. Substrate specificity of ApAgcS1 showed broad specificity for the transport of amino acids and optimum activity occurred at pH 9.0.

7 Based on the results of the reduced accumulation of amino acids in the medium, it was suggested that ApAgcS1 might be involved in the recapture of leaked amino acids, whereby serine, asparagine, glutamine, and isoleucine were the main target.

8 The level of mRNA for *ApagcS1* in *A. halophytica* cell was induced by NaCl and nitrogen deficiency stresses.

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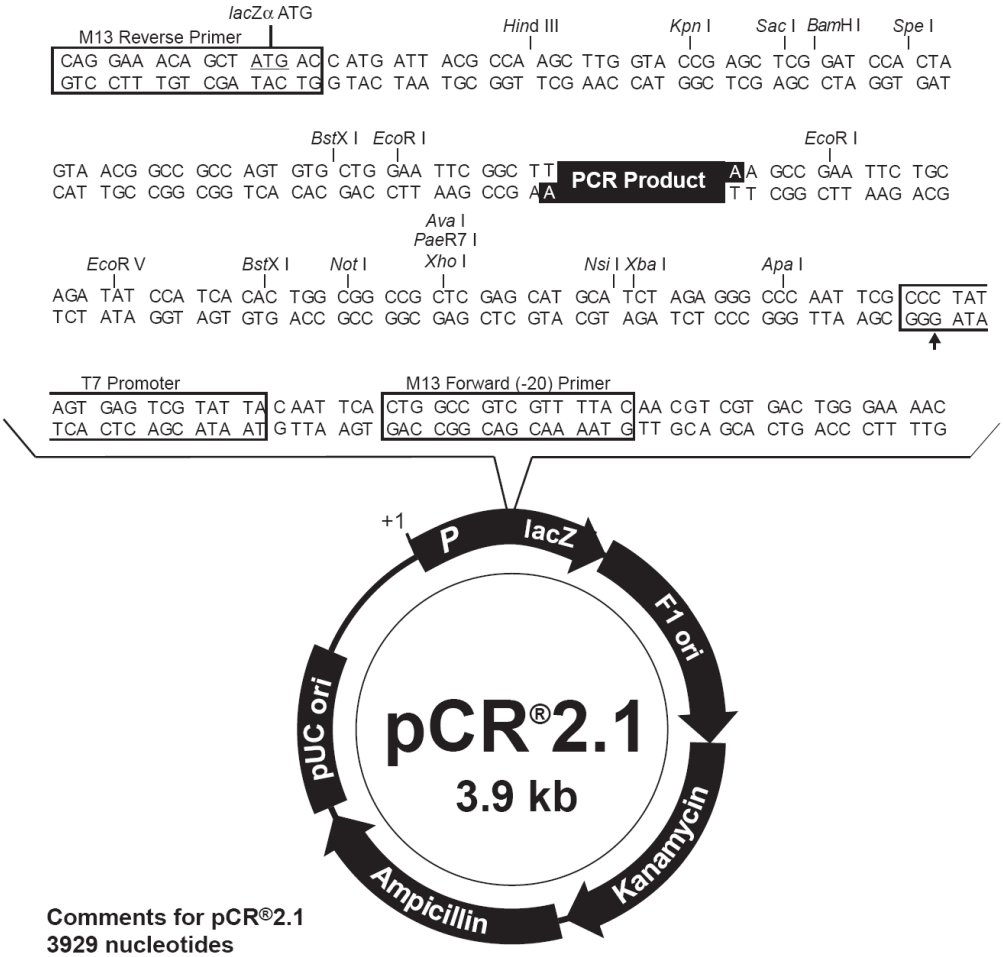
APPENDIX

APPENDIX 1

pCR2.1

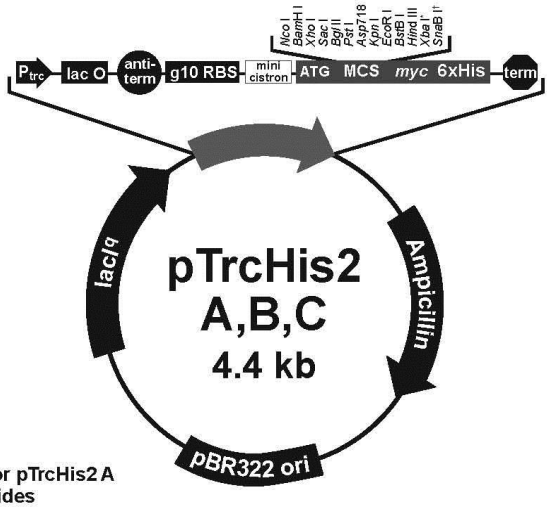


Comments for pCR®2.1
3929 nucleotides

*Lac*Zα gene: bases 1-545
M13 Reverse priming site: bases 205-221
T7 promoter: bases 362-381
M13 (-20) Forward priming site: bases 389-404
f1 origin: bases 546-983
Kanamycin resistance ORF: bases 1317-2111
Ampicillin resistance ORF: bases 2129-2989
pUC origin: bases 3134-3807

APPENDIX 2

pTrcHis2C



Comments for pTrcHis2 A
4406 nucleotides

trc promotor region: bases 190-382
-35 region: bases 193-198
-10 region: bases 216-221
lac operator (*lac*O): bases 228-248
*rrn*B antitermination signal: bases 264-333
gene 10 region: bases 346-354
Ribosome binding site: bases 369-373
pTrcHis forward priming site: bases 370-390
Minicistron ORF: bases 383-409
Reinitiation RBS: bases 398-403
Expression ATG: bases 413-415
Multiple cloning site: bases 411-464

* *Xba* I is only found in pTrcHis2 B

† *Sna*B I is only found in pTrcHis2 C

pTrcHis forward priming site
Mini cistron
RBS
RBS
Nco

```
361  AAAATTAAAG AGGTATATAT TA ATG TAT CGA TTA AAT AAG GAG GAA TAA ACC
                              Met Tyr Arg Leu Asn Lys Glu Glu *** 
```

*Bam*H I *Xho* I *Sac* I *Bgl* II *Pst* I *Asp*718 I *Kpn* I *Eco*R I *Bst*B I *Hin*d III *Sna*B I

```
413  ATG GATCCGAGCT CGAGATCTGC AGCTGGTACC ATATGGGAAT TCGAAGCTTA CGTA
     Met
```

myc epitope tag
Sal I

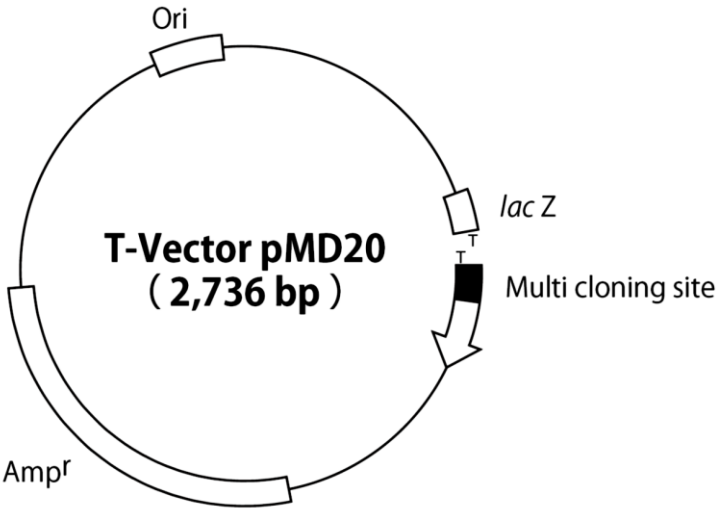
```
461  GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT AGC GCC GTC GAC CAT
     Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Ser Ala Val Asp His
```

ProBond™ binding domain

```
510  CAT CAT CAT CAT CAT TGA GTTTA
     His His His His His *** 
```

APPENDIX 3

pMD20



M13 primer RV

SP6 promoter

TTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCTATTTAGGTGACACTATA
AACACTCGCCTATTGTTAAAGTGTGTCCTTTGTCGATACTGGTACTAATGCGGTTCGATAAATCCACTGTGATAT

Sse8387 I

Hind III *Sph* I *Pst* I *Acc* I *Xba* I *Spe* I *Nde* I

GGGGAAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCTACTAGTCATATGGATT3' (cloned insert) ATC
CCCCTTTCGAACGTACGGACGTCCAGCTGAGATCTCCTAGATGATCAGTATACCTA 3' T TAG

Sma I
Ava I

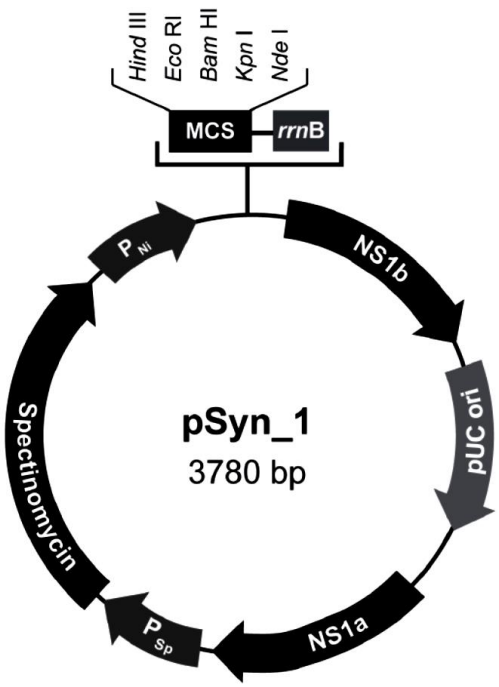
*Bam*H I *Kpn* I *Sac* I *Eco*R I

GGATCCCCGGGTACCGAGCTCGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCG
CCTAGGGGCCCATGGCTCGAGCTTAAGTGACCGGCAGCAAAATGTTGCAGCACTGACCCTTTTGGGACCGC

M13 primer M4

APPENDIX 4

pSyn_1



Features of pSyn_1

3780 nucleotides

pUC origin:	58–673
NS1a (neutral site 1a):	745–1543
Spectinomycin promoter (P_{Sp}):	1550–1683
Spectinomycin resistance gene:	1684–2694
Nickel promoter (P_{Ni}):	2699–2799
Multiple cloning site:	2800–2834
*rrn*B transcriptional termination region:	2841–2998
NS1b (neutral site 1b):	2999–3780

APPENDIX 5

BG-11 and BG-11 + Turk Island Salt Solution

Stock solution (100 ml)		Stock solution (500 ml)	
Solution I: K_2HPO_4	3.135 g	Solution VI:	
Solution II: $MgSO_4.7H_2O$	7.5 g	H_3BO_3	1.43 g
Solution III: $CaCl_2.2H_2O$	3.6 g	$ZnSO_4.7H_2O$	110 mg
Solution IV: Na_2CO_3	2.0	$Na_2MoO_4.2H_2O$	195 mg
Solution V:		$MnCl_2.4H_2O$	0.905 g
EDTA	0.1 g	$CuSO_4.5H_2O$	39.5 mg
Citric acid	0.1 g	$Co(NO_3)_2.6H_2O$	24.5 mg
Ferric ammonium citrate	0.6 g		
Sterile filtrate, store at 4 $^{\circ}C$		Sterile filtrate, store at 4 $^{\circ}C$	

BG11 medium (1,000 ml)

	Solid medium	Liquid medium
Bacto-agar	15 g	-
$NaNO_3$	1.5 g	1.5 g
Solution I	1 ml	1 ml
Solution II	1 ml	1 ml
Solution III	1 ml	1 ml
Solution IV	1 ml	1 ml
Solution V	1 ml	1 ml
Solution VI	1 ml	1 ml
H_2O added up to	1000 ml	1000 ml

BG11 medium + Turk island salt solution (1,000 ml)

	Solid medium	Liquid medium
Bacto-agar	15 g	-
$NaNO_3$	1.5 g	1.5 g
KCl	0.67 g	0.67 g
$MgSO_4.7H_2O$	6.92 g	6.92 g
$MgCl_2.6H_2O$	5.5 g	5.5 g
$CaCl_2.2H_2O$	1.47 g	1.47 g
Solution I	1 ml	1 ml
Solution II	1 ml	1 ml
Solution III	1 ml	1 ml
Solution IV	1 ml	1 ml
Solution V	1 ml	1 ml
Solution VI	1 ml	1 ml
NaCl (0.5M)	29.22 g	29.22 g
H_2O added up to	1000 ml	1000 ml

Remark: The culture medium was mixed, adjusted pH to 7.6 by adding 2 M NaOH, then added distrilled water to the final volume 1 litre. The medium was sterilized by autoclaving at 15 lb/in^2 for 15 minute.

APPENDIX 6

Scintillation fluid (1,000 ml) was prepared as follows:

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 were mixed. 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 7

E. coli transformation by heat shock method

1. Preparation of competent cells

200 μl overnight culture of *E. coli* DH5α or *E.coli* JW 4166was inoculated into 100 ml sterile LB medium, then grown at 37 °C, 250 rpm until OD_{620} reached 0.3-0.4. The culture was incubated on ice for 30 minutes. The cells were collected by centrifugation at 5,000 g, 4 °C for 10 minutes. The pellet was resuspended with 100 ml of cold 0.1 M $MgCl_2$ and centrifuged at 5,000 g, 4 °C for 10 minutes. A supernatant was discarded then the pellet was resuspended with 100 ml of cold 0.1 M $CaCl_2$ and incubated on ice for 30-60 minutes. After incubation, the cell suspension was centrifuge at 5,000 g, 4 °C, for 10 minutes. A supernatant was discarded then the pellet was resuspended with 3 ml of cold 0.1 M $CaCl_2$ containing 15% glycerol. The cell suspension was aliquot 100 μl/tube on ice prior to immediate drop into liquid nitrogen. The competent cells were stored at -80 °C until use.

2. Transformation

The plasmid was mixed with cold cell suspension in microcentifuge tube and place on ice for 30 min. The mixture was incubated at 42 °C for 1 min and placed on ice for 5 minutes. The cell suspension was transferred into a new sterile tube containing 1 ml of LB broth. The transformed cells were incubated at 37 °C, 200 rpm for 1 hr and spreaded onto the LB agar plate containing appropriate antibiotic. The plate was incubated at 37 °C overnight.

APPENDIX 8

LB medium

LB medium (1 liter)

	Liquid medium	Solid medium
Bacto tryptone	10 g	10 g
Yeast extract	5 g	5 g
NaCl	10 g	10 g
Agar	-	15 g

All compositions were dissolved together with 1liter of distilled water. The medium was sterilized by autoclaving at 15 lb/in^2 for 15 minute.

APPENDIX 9

Agarose gel electrophoresis for DNA

TBE buffer:

- Concentrated stock solution (per liter)

5X: 54 g Tris base

27.5 g Boric acid

20 ml 0.5 M EDTA (pH 8.0)

- Working solution

0.5X: 0.045 M Tris-borate

0.001 M EDTA

Preparation of agarose gel:

1. The edges of a clean, dry, glass was sealed plate and then molded on a horizontal section of the bench.

2. TBE electrophoresis buffer (100 ml) was prepared

3. 1 g of agarose was weighed and put into the TBE buffer.

4. The mixture was boiled for 2-3 minute with microwave.

5. Ethidium bromide (a stock solution of 1 mg/ml) was added into the gel solution by adjusting final concentration to 0.5 µg /mland then mixed.

6. The comb was placed in suitable position.

7. Agarose solution was poured onto the tray.

8. After gel was completely set (30-45 min at room temperature), the comb was removed carefully.

9. The gel was placed into the electrophoresis tank.

Method for running DNA on agarose gel:

To measure the size and the amount of DNA in the sample, 1.0% agarose gel (consist of 0.5 mg/ml ethidium bromide) in 1x TBE buffer (89 mM Tris-HCl, 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% bromphenol blue, 0.25% xylene cyanol FF and 30% glycerol in water) before loading into the well of gel with submerged in the 1x TBE buffer in an electrophoretic chamber. An appropriate amount of 100 bp or 1 kb GeneRuler™ (Fermentas, USA) was also load to the gel to serve as a DNA marker. Generally, the gel was run at 100 volts until bromphenol blue migrated to the other egde. 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 100 bp or 1 kb GeneRuler™ (Fermentas, USA).

APPENDIX 10

Plasmid transformation into *Synechococcus* sp. PCC 7942

1. Preparation of competent cells

The cells grown in 100 ml of BG-11 medium at 7th day were collected by centrifugation at 5,000 g, 4 ℃ for 10 minutes. The pellet was resuspended with 10 ml of new BG-11 medium and the cell suspension was aliquot 1 ml/tube for immediately transformation.

2. Transformation

The plasmid was mixed with 1 ml of the cell suspension in microtube and incubated on a rotary shaker at 30 °C, 160 rpm under dark overnight. The transformed cells were spreaded onto the BG-11 agar plate containing appropriate antibiotic. The plate was incubated at 30 ℃ until the green colonies appear and then subcultured into BG-11 liquid medium containing the same antibiotic as in the plate.

APPENDIX 11

Deduced amino acid sequence of ApagcS1 based on the shot sun sequencing of *ApagcS1* gene

>ApagcS1

```
  1 MDVNNWSKVG GTTRSRVRYG MSNKGNHGHS SAASVAGRVG AGNAGVAVAT GGGAWMWGGM
 61 ATSCTSVKRA DGTYRGGAYY GNRWAAVSVV TGGNASYTVA ASVDTGSRDM TAMMGMGAGG
121 VKRAHVAYVV MAGYMSAVTN DGSVATRNAG DSATGGGAAG VKRGSNAGGS ANVAAVAYVK
181 HVNGMSSVDT MVCTCTAAVS GVYGAVNGVT TNAMSGGAVT GAATSMYNYY GNSNSNHTNG
241 RVTAWGATDT TVGADTMGAV NAAVMGVGKV HDKRSGKTAA SDAKAWHSTV RVAGMR
```

APPENDIX 12

Nucleotide sequence of the *ApagcS1* gene congaing 500' UTR base on the shot sun sequencing of *A. halophytica,* the translation start and stop codon are indicated by bold and underline type, respectively.

>ApagcS1

```
   1 GGATCCGGAA AGTTAAACTC GTGAGCAGCA CAGTGGATGG GATCAATCTC AACTGAGGCA
  61 AGGACATCAA ACCCAGCTTG TTCAAAACCT AAAGTGATCC CACCAACTCC TGCAAATAAA
 121 TCAACTGCAA CTGCGCGTTT TTGTTGAAAT GTGTCCATCT GAAAACTACA ATCTCTCTTT
 181 TGATCTCGGA CTCATAGTTT AGCAGGTTCT TTGCCAATTC TGACTTTAGC TGCTATCGTT
 241 ACCCATTTTC CCCAGTCGAT CTCATCCTCA TATTTAATTG ATCTACAAAA TTTAATTTTC
 301 TCTTTATGCC GACATTACAA ATCTAGAACC AAGTTGGATA ACTCCTCTTC TCCAGAGAAG
 361 TGAGTGAGAA GTTTGATGAT TTCTACAGCG CGATCGCGCT CTTGGATAAA GATTATTTCG
 421 TATCTAAAGT TACAGTTCCA CAGGAAGTGA AATGTAAGAT GATGGAGTAT TTATTCGCAA
 481 TTTTTAGAGG AATGCTCACC CATGTTCG**AT G**TTCTCAACA ACCTCATTTG GAGTAAGCTC
 541 CTGATTGTCC TTTTAATTGG TATAGGACTC ACTTTTACAA TTCGCTCCCG TTTTGTTCAA
 601 TTCCGCTACT TGGGGCTAAT GTTGTCCAAC TTCAAGGAGG GGTTCAACCA TGAGGAAGGT
 661 CACCTCAGTT CCTTTCAGGC ACTTGCCCTA AGCGTTGCGG GTCGCGTGGG AGCGGGAAAT
 721 ATTGCGGGGG TCGCTGTCGC CATTACTTTA GGAGGACCAG GGGCAATCTT TTGGATGTGG
 781 TTAATTGGCT TAATCGGCAT GGCGACCAGT TTCTTCGAGT GTACTCTCAG CCAAGTCTTT
 841 AAGAGAGCCG AACCTGACGG CACTTATCGA GGGGGTCCCG CTTACTACAT TGAGCAAGGA
 901 TTGAATCAGC GCTGGTTAGC TGCCGTTTTC TCGGTTTTAC TGCTGGTGAC CTTTGGTTTG
 961 GGCTTTAATG CACTCCAATC TTATACAGTG GCTGCCTCGG TAGAGGATAC CTTTGGCTTA
1021 TCTCGTGACA TGACCGCCTT CATCATGATG GGGATGTTGG GCGCGATCAT TTTTGGCGGA
1081 GTCAAGCGTA TCGCTCACGT TGCCGAATAT ATCGTACCAG TCATGGCAAT TGGCTATTTC
1141 CTGATGTCCC TCGCAGTAAT TATCACCAAC CTAGACGGTA TTCCAAGCGT TTTTGCCACT
1201 ATTATTCGCA ATGCTTTTGG CTTTGATTCA GCGATTACCG GTGGCATTGG GGCTGCGATC
1261 ATTTTTGGCG TTAAGCGGGG GCTATTTTCT AACGAAGCGG GTTTAGGAAG TGCGCCTAAC
1321 GTTGCAGCAG TGGCTTATGT TAAACATCCT GTTAATCAGG GGATGCTTCA ATCTATAAGT
1381 GTCTTTATTG ACACCATGGT TCTGTGTACT TGTACCGCAG CCGTGATTTT ACTCTCTGGT
1441 GTTTATGAAC CCGGTGCAGA GGTCAACGGC GTGACGCTGA CCCAAAATGC CATGTCTGAA
1501 CAATTTGGTT TTTTAGGACA AGCCTTTGTC ACCTTCGGTT TAGCTCTGTT TGCTTTCACC
1561 TCGATGCTGT ATAACTATTA CTTGGGTGAA AATAGCCTCA ACTTCTTCAG CGAAGAAAAT
1621 CACACCCTCT TCAATGGCTT TCGTGTTTTA ACCCTTGCTT TGATTATCTG GGGCGCAACC
1681 CAAGATCTGA CAACAGTGTT TGGTTTTGCT GATCTTACTA TGGGGTTATT AGCCTTAGTG
1741 AACTTGGCAG CGCTGGTGAT GTTATTTGGG GTTGGATTAA AGGTATTGCA CGATTTCGAG
1801 AAACAGCTTC GCAGTGGCAA GCAACAACCC TTATTTACAG CAGCGAGCTT ACCTGAATTT
1861 GAAATTGATG CTAAGGCGTG GCCCCATGAA CCATCAGAGC CAGAACCAAC AGTTGAGCGG
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```
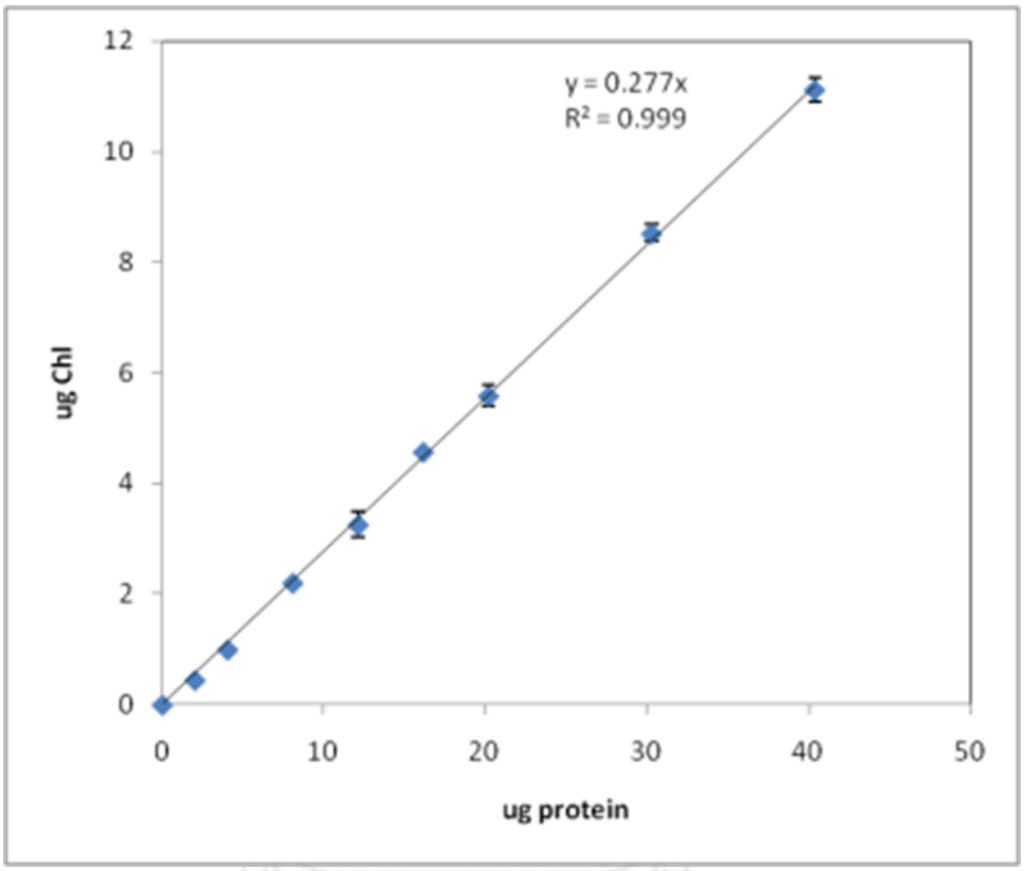

APPENDIX 13

Calculate mg protein to Chl



VITA

Miss Aporn Bualuang was born on January 16, 1979 in Nakornpathom, Thailand. She graduated with a Bachelor of Science degree in Biology from Faculty of Science, Silapakorn University, Nakornpathom, Thailand in2001 and Master of Science in Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok Thailand in2007. She has further studied for the Doctor of Philosophy (Ph.D) degree in Biochemistry Program, Faculty of Science, Chulalongkorn University since 2009. .