# การขนส่งพอลิเอมีนเข้าสู่ไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเคมี ภาควิชาชีวเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2550

# POLYAMINE TRANSPORT INTO CYANOBACTERIUM *Synechocystis* sp. PCC 6803

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**A Dissertation Submitted in Partial Fulfillment of the Requirements**
**for the Degree of Doctor of Philosophy Program in Biochemistry**
**Department of Biochemistry**
**Faculty of Science**
**Chulalongkorn University**
**Academic Year 2007**

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|---|---|
| **Thesis Title** | POLYAMINE TRANSPORT INTO CYANOBACTERIUM *Synechocystis* sp. PCC 6803 |
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| **Field of study** | Biochemistry |
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นายวุฒินันท์ รักษาจิตร์: การขนส่งพอลิเอมีนเข้าสู่ไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803 (POLYAMINE TRANSPORT INTO CYANOBACTERIUM *Synechocystis* sp. PCC 6803) อ. ที่ปรึกษา: ศ.ดร. อรัญ อินเจริญศักดิ์, อ. ที่ปรึกษาร่วม: Assoc. Prof. Pirkko Mäenpää, Ph.D. 167 หน้า.

ศึกษาลักษณะสมบัติของการขนส่งพอลิเอมีนเข้าสู่เซลล์ไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803 โดยติดตามการนำพิวเทรสซีนและสเปอร์มีดีนซึ่งติดสลากด้วยสารรังสีเข้าสู่เซลล์ ผลการศึกษาจลนพลศาสตร์พบว่า การขนส่งพิวเทรสซีนและสเปอร์มีดีนอิ่มตัวด้วยค่าคงที่มิเคลลิส เมนเทน ($K_m$) เท่ากับ 98 และ 67 ไมโครโมลาร์ และมีความเร็วสูงสุด ($V_{max}$) เท่ากับ 0.33 และ 0.45 นาโนโมลต่อนาทีต่อมิลลิกรัมโปรตีน ตามลำดับ แอคติวิตีการขนส่งพอลิเอมีนเปลี่ยนแปลงตามค่าพีเอช พิวเทรสซีนมีแอคติวิตีสูงสุดที่พีเอช 7.0 ขณะที่การขนส่งสเปอร์มีดีนมีแอคติวิตีที่เหมาะสมที่พีเอช 8.0 การขนส่งสเปอร์มีดีนถูกยับยั้งด้วยพิวเทรสซีนและสเปอร์มีน การขนส่งพิวเทรสซีนถูกยับยั้งด้วยสเปอร์มีดีนและสเปอร์มีนเช่นกัน ขณะที่กรดอะมิโนแทบจะไม่มีผลกระทบต่อการขนส่งพอลิเอมีน อีกทั้งพบว่าผลของการยับยั้งทางจลนพลศาสตร์ของการขนส่งสเปอร์มีดีนด้วยพิวเทรสซีนและสเปอร์มีนเป็นแบบไม่แข่งขัน (non-competitive) ด้วยค่าคงที่ $K_i$ เท่ากับ 292 และ 432 ไมโครโมลาร์ ตามลำดับ แสดงให้เห็นว่าตัวขนส่งพอลิเอมีนเข้าสู่ *Synechocystis* sp. PCC 6803 มีความจำเพาะต่อพอลิเอมีน การขนส่งพิวเทรสซีนและสเปอร์มีดีนถูกยับยั้งด้วยตัวยับยั้งพลังงานและไอโอโนพอร์ (ionophores) แสดงว่าการขนส่งพอลิเอมีนต้องการพลังงานจากเมตาบอลิซึมภายในเซลล์ รวมทั้งพลังงานที่เกิดจากเกรเดียนท์ของโปรตอน ($\Delta pH$) และเมมเบรน โพเทนเชียล ($\Delta\psi$) การเจริญเติบโตของเซลล์ *Synechocystis* ถูกหน่วงเหนี่ยวให้ช้าลงในสภาวะที่อาหารมีความเข้มข้นเกลือ NaCl สูง แต่เมื่อเติมพิวเทรสซีนและสเปอร์มีดีน ความเข้มข้นต่ำลงไปในอาหารที่มีความเข้มข้นเกลือสูงสามารถชะลอผลกระทบที่เกิดจากเกลือได้ แสดงให้เห็นว่าพิวเทรสซีนและสเปอร์มีดีนถูกขนส่งเข้าสู่เซลล์ *Synechocystis* และมีบทบาทสำคัญต่อการเจริญเติบโตของเซลล์ในสภาวะที่มีเกลือสูง การเพิ่มขึ้นของความเข้มข้นออสโมลาลิตีภายนอกจากเกลือและซอบิทอลมีผลทำให้แอคติวิตีของการขนส่งพิวเทรสซีนและสเปอร์มีดีนเพิ่มขึ้น 2 เท่าที่ความเข้มข้น 20 มิลลิออสโมลต่อกิโลกรัมและ 1.5 เท่าที่ความเข้มข้น 10 มิลลิออสโมลต่อกิโลกรัม ตามลำดับ

ผลศึกษาการแสดงออกของยีน *potD* ที่ระดับการถอดรหัส ด้วยเทคนิค reverse transcription PCR พบว่า ระดับของ *potD* transcript มีการเปลี่ยนแปลงอย่างมีนัยสำคัญเพื่อตอบสนองต่อความเครียดจากภายนอก อาทิเช่น ความเข้มแสง เกลือออสโมติก อุณหภูมิ และความเพียงพอของสารอาหาร ในช่วงระยะเวลา 3 วัน ขณะที่ระดับของ *potD* transcript ไม่เปลี่ยนแปลงในช่วงระยะเวลา 18 ชั่วโมงภายใต้ความเครียดแบบเดียวกัน อีกทั้งพบว่าการลดลงของระดับ *potD* transcript ภายใต้ความเครียดจากความร้อนสูงไม่ได้เป็นผลมาจากการลดลงของความเสถียร (stability) ของ *potD* transcript แต่เกิดจากการเปลี่ยนแปลงระดับการถอดรหัสเพื่อตอบสนองต่อความร้อนสูงของเซลล์ ผลการศึกษาการแสดงออกที่ระดับโปรตีน ด้วยเทคนิค immunoblot analysis พบว่า recombinant *Synechocystis* His-PotD protein มีความจำเพาะกับโมโนโคลนอล Anti-His antibody ซึ่งปรากฏแถบโปรตีนบนเมมเบรนที่ขนาด 46 กิโลดาลตัน เปอร์เซนต์ความเหมือนของลำดับกรดอะมิโนของโปรตีน PotD จาก *S ynechocystis* และ *Escherichia coli* เท่ากับ 24% แต่โครงสร้างการม้วนตัวของโปรตีน PotD ทั้งหมดและบริเวณตำแหน่งเร่งปฏิกิริยา (active site) เหมือนกัน อีกทั้งพบว่า โปรตีน PotD ไม่มีลำดับกรดอะมิโนนำสัญญาณทางด้านปลายอะมิโน (amino terminal signal sequences)



ภาควิชา.....ชีวเคมี.....ลายมือชื่อนิสิต.....

สาขาวิชา.....ชีวเคมี.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2550.....ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

##4673828023: MAJOR BIOCHEMISTRY
KEY WORD: *Synechocystis* sp. PCC 6803/POLYAMINES/SALT STRESS/TRANSPORT/ CYANOBACTERIA

WUTTINUN RAKSAJIT: POLYAMINE TRANSPORT INTO CYANOBACTERIUM *Synechocystis* sp. PCC 6803, THESIS ADVISOR: PROF. ARAN INCHAROENSAKDI, Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. PIRKKO MÄENPÄÄ, Ph.D., 167 pp.

The transport of polyamines into a moderately salt tolerant cyanobacterium *Synechocystis* sp. PCC 6803 was characterized by measuring the uptake of radioactively-labeled polyamines namely putrescine and spermidine. The results showed that putrescine and spermidine transport exhibited saturation kinetics typical of Michaelis -Menten kinetics with an apparent $K_m$ of 98 and 67 μM, respectively, and $V_{max}$ of 0.33 and 0.45 nmol/min/mg protein, respectively. The transport of putrescine was pH-dependent with highest activity at pH 7.0, whilst spermidine transport was pH-dependent with pH optimum 8.0. The competition experiment showed strong inhibition of spermidine uptake by putrescine and spermine. Also strong inhibition of putrescine transport was caused by spermine and spermidine. Additionally, amino acids were hardly inhibitory to polyamine uptakes. The inhibition kinetics of spermidine transport by putrescine and spermine were found to be non-competitive with $K_i$ values of 292 and 432 μM, respectively. These results suggest that the transport system in *Synechocystis* sp. PCC 6803 is highly specific for polyamines. The inhibition of putrescine and spermidine transport by various metabolic inhibitors and ionophores suggests that polyamine uptake is energy-dependent and proton motive force-dependent with the contribution of both $\Delta pH$ and $\Delta\psi$. The diminution of cell growth was observed in cells grown at high concentration of NaCl. The addition of low concentration of putrescine and spermidine relieved growth inhibition by salt stress, suggesting that exogenous putrescine and spermidine could be transported into *Synechocystis* cells and could act as growth promoter in the presence of high salt concentration. Upshift of the external osmolality generated by either NaCl or sorbitol caused an increased uptake with an optimum 2-fold increase at 20 mosmol/kg and 1.5-fold increase at 10 mosmol/kg for putrescine and spermidine transport, respectively.

At the post-transcriptional level, reverse transcription PCR demonstrated that the steady-state transcript amounts of the *potD* gene, encoding polyamine-binding protein were under regulation of a wide spectrum of long-term environmental stresses (3 days), namely light intensity, salt, osmotic, temperature and nutrient availability even if no short-term regulation (18 h) occurred under the same conditions. The decreased accumulation of the *potD* transcripts was not a result of a decreased stability of the transcript, but merely reflected an altered transcription activity by high temperature stress. Furthermore, the immunoblot analysis revealed that the recombinant *Synechocystis* His-PotD protein showed high specificity to monoclonal Anti-His antibody with a single protein band of 46 kDa. The sequence identity between *Synechocystis* PotD and *Escherichia coli* PotD was only 24 %, but the overall fold and the active site are well conserved. The putative polyamine-binding protein, PotD, has no amino terminal signal sequences.

Department.....Biochemistry.....Student's signature.....Wuttinun Raksajit

Field of study.....Biochemistry.....Advisor's signature.....Aran Incharoensakdi

Academic year.....2007.....Co-advisor's signature.....

# ACKNOWLEDGEMENTS

This study was carried out during the past five years at the Department of Biochemistry, Faculty of Science, Chulalongkorn University and Department of Plant Physiology and Molecular Biology, University of Turku Finland. Financial support from The Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (PHD/0115/2546), the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) and the Academy of Finland are gratefully acknowledged.

I wish to express my deepest gratitude to my supervisors, Professor Dr. Aran Incharoensakdi and Associate Professor Dr. Pirkko Mäenpää, for their excellent guidance, enthusiasm, instruction and support from the very beginning to the very end of my Ph.D. thesis. Especially I thank Professor Dr. Aran for endless patience and advice, and Associate Professor Dr. Pirkko for introducing me to the fascinating world of molecular biology and for her warm hospitality.

My gratitude is also extended to Professor Dr. Anchalee Tassanakajon, Assistant Professor Dr. Teerapong Buaboocha, Assistant Professor Dr. Supachitra Chadchawan, and Associate Professor Dr. Jarunya Narangajavana for serving as thesis committee, for their available comments and useful suggestion.

My appreciation is also expressed to Dr. Paula Mulo, Dr. Saowarath Jantaro, Dr. Surasak Laloknam, for warm support, instruction, lending helping, and interesting discussion. I also thank all my co-authors for pleasant collabration; especially Anna- Maria Brandt, Dr. Tove Jansén and Dr. Tiina Salminen.

Sincere thanks are also extended to all members and friends, for lending a helping hand whenever needed, sharing the great time in laboratory, their warm assistance and friendship, at Department of Biochemistry Chulalongkorn University; Nok, Kook, Pond, Lah, Bong, Tent, Wansiri, Him, Ople, Nan, Jiab, Earn, Taum, Gift, Chao, Ae, Tik, Ake, Tipachai, P' Yui, P'Ann, P'Chai, P'Ohm, P'Cell, P'Kung, P'Tue, P'Neng, and at Biocity Turku Finland; Eevi, Ulla-Maija, Virpi, Kurt, Mika, Arto, Yagut, Anna, Esa, Taina, Natalia, Mirva, Marja, Marjaana, Mirja, Marjatta, Shahid, Mikko, Alexy, Minna, Julia, Pengpeng, Ilona, Sari, Tapani, Eira, Saijaliisa, Paivi, Outi and Colin.

Last, but not least, I wish to thank my family for keeping my feet tightly on the ground and my mind away from science when I was at home. It's always good to be the family.

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# LIST OF ABBEVIATIONS

| | |
|---|---|
| ATP | adenosine triphosphate |
| bp | Base pair |
| BCIP | 5-Bromo-4-chloro-3-indolyl phosphate |
| BSA | Bovine serum albumin |
| CCCP | Carbonyl cyanide *m*-chlorophenylhydrazone |
| Chl | Chlorophyll |
| Ci | Curie |
| cm | centimeter |
| CPM | Count Per Minute |
| DCCD | *N, N*$^{/}$ dicyclohexylcarbodiimide |
| DCMU | 3-(3,4-dichlorophenyl)-1,1-dimethylurea |
| ΔpH | pH gradient or proton gradient |
| Δψ | Membrane potential |
| g | gram |
| $^{o}$C | degree Celsius |
| kb | kilo base |
| kDa | kilo Dalton |
| h | hour |
| l | liter |
| mA | milliampere |
| min | minute |
| µl | microliter |
| ml | milliliter |
| mg | milligram |
| mM | millimolar |
| MW | Molecular Weight marker |
| µM | micromolar |
| M | Molar |
| NBT | *p*-Nitro blue tetrazolium chloride |
| nm | nanometer |
| OD | Optical Density |

| | |
|---|---|
| PotD | Periplasmic polyamine-binding protein D |
| PMF | Proton motive force |
| rpm | revolution per minute |
| UV | Ultraviolet |

# CHAPTER I

# INTRODUCTION

Natural occurring amines, derived from ammonia by substitution, can be divided into primary, secondary, tertiary and quaternary. A large number of these amines have been identified in higher plants. They could derive from amino acids decarboxylation or from aldehydes transmination that is an important source of simple aliphatic amines. Aliphatic polyamines (PAs), ubiquitous compounds classified as growth substances (Bagni, 1989), have been implicated in a large range of plant growth and developmental processes such as cell division (Bagni, 1989) and response to environmental stresses (Bouchereau *et al*., 1999), but their precise molecular mechanism of action still remains unclear (Bagni and Tassoni 2001). They play important roles in cellular processes such as regulating the structure and function of biological macromolecules, cell growth, cell death, embryonic development (Thomas and Thomas, 2001), cell cycle, proliferation, differentiation (Satriano *et al*., 2001; Bettuzzi *et al*., 1999), gene expression (Childs *et al*., 2003) and apoptosis (Seiler and Raul, 2005) in living organisms. PAs are poly cations at physiological pH, which have been shown to bind strongly *in vitro* to intracellular polyanions (Tabor and Tabor, 1985), including nucleic acids (Igarashi *et al*., 2000; Caldarera *et al*., 1975; Levy *et al*., 1974), acidic phospholipids (Tadolini *et al*., 1984) and many types of proteins, including enzymes whose activities are directly regulated by polyamines (Carley *et al*., 1983) and also ion channels (Williams, 1997). Other important compounds are aromatic amines and secondary amines, which react to nitrous acid resulting in

carcinogenic nitrosamines, such as dimethylnitrosamine which is present in *Solanum incanum* (Du Plessis *et al*., 1969).

**1.1 Polyamines**

PAs are present in all living cells, prokaryotes, eukaryotes, plants and animals (Tabor and Tabor, 1985). The most common ones are the diamine putrescine (Put; 1,4-diaminobutane), triamine spermidine (Spd; 1,8-diamino-4-azaoctane) and tetramine spermine (Spm; 1,12-diamino-4,9-diazododecane). Many other di- and polyamines are present in plant and microorganisms such as the diamines 1,3-diaminopropane and cadaverine (1,5-diaminopentene) (Table 1). Unusual polyamines have also been detected in bacteria, algae, fungi, animals and higher plants (Niitsu and Samejima, 1993). In the extreme thermophilic bacteria *Thermus thermophilus* at least 14 polyamines, among which some linear and branched pentamines, hexamines and heptamines, have been isolated (Table 1). Caldopentamine was present in considerable amount especially in bacterial cell grown at extremely high temperatures (Oshima, 1989). The control of polyamine levels is regulated in a very fast, sensitive and precise manner. To this control belong: *de novo* biosynthesis, degradation (oxidative deamination), conjugation and transport of PAs (Bouchereau *et al*., 1999; Urdiales *et al*., 2001).

**Table 1** Common and uncommon natural occurring aliphatic polyamines*

| Trivial name | Systematic name | Chemical structure |
|---|---|---|
| 1,3-Diaminopropane | 1,3-Diaminopropane | $NH_2(CH_2)_3NH_2$ |
| Putrescine | 1,4-Diaminobutane | $NH_2(CH_2)_4NH_2$ |
| Cadaverine | 1,5-Diaminopentene | $NH_2(CH_2)_4CHNH_2$ |
| Norspermidine (caldine) | 1,7-Diamino-4-azaheptane | $NH_2(CH_2)_3NH(CH_2)_3NH_2$ |
| Spermidine | 1,8-Diamino-4-azaoctane | $NH_2(CH_2)_3NH(CH_2)_4NH_2$ |
| sym-Homospermidine | 1,9-Diamino-5-azanonane | $NH_2(CH_2)_4NH(CH_2)_4NH_2$ |
| Thermine | 1,11-Diamino-4,8-diazaundecane | $NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$ |
| Spermine | 1,12-Diamino-4,9-diazadodecane | $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$ |
| Thermospermine | 1,12-Diamino-4,8-diazadodecane | $NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH_2$ |
| Homospermine | 1,13-Diamino-4,9-diazatridecane | $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_4NH_2$ |
| Caldopentamine | 1,15-Diamino-4,8,12-triazapentadecane | $NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$ |
| Homocaldopentamine | 1,16-Diamino-4,8,12-triazahexadecane | $NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH_2$ |
| Homopentamine | 1,19-Diamino-5,10,15-triazanonadecane | $NH_2(CH_2)_4NH(CH_2)_4NH(CH_2)_4NH(CH_2)_4NH_2$ |
| Caldohexamine | 1,19-Diamino-4,8,12,16-tetraazanonadecane | $NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$ |
| Homocaldohexamine | 1,20-Diamino-4,8,12,16-tetraazaeicosane | $NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH_2$ |
| N4-Aminopropyl-norspermidine | | $[NH_2(CH_2)_3]_3N$ |
| N4-Aminopropyl-spermidine | | $[NH_2(CH_2)_3]_2N[NH_2(CH_2)_4]$ |

* Bagni, N. and Tassoni, A. (2001)

### 1.1.1 Polyamine biosynthesis

The polyamine biosynthesis in higher plants and bacteria can be initiated directly by the decarboxylation of either ornithine or arginine, catalyzed by the enzymes ornithine decarboxylase (ODC; EC 4.1.1.17), which gives rise to putrescine directly and arginine decarboxyase (ADC; EC.4.1.1.19) to yield agmatine, which is subsequently converted to putrescine (Smith, 1985; Slocum, 1991). In mammals and fungi, ODC reaction only leads to putrescine formation. Spermidine and spermine are synthesized by the sequential addition of an aminopropyl group on to putrescine and spermidine, respectively. This step is catalyzed by the enzymes spermidine synthase (EC 2.5.1.16) and spermine synthase (EC 2.5.1.22), respectively. The aminopropyl group is taken from the decarboxylation of *S*-adenosylmethionine (SAM) by the enzyme *S*-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) (Kushad and Dumbroff, 1991; Bey *et al*., 1987) (Figure 1). A variety of other related compounds have been found in plants, including cadaverine (caddiamine), which is not as widely distributed as putrescine and is found in *Leguminoseae* and in the flower of *Arum lilies* (Slocum, 1991).

### 1.1.2 Polyamine degradation

Polyamines are oxidatively deaminated by the action of amine oxidases, including copper diamine oxidases (DAO; EC 1.4.3.6) and flavoprotein polyamine oxidases (PAO; EC 1.5.3.3), which oxidize either spermidine or spermine. In addition, DAO reaction catalyzed putrescine to pyrroline, hydrogen-peroxide and ammonia (Flores and Filner, 1985), while PAO yields pyrroline and 1,5-diabicyclononane, from spermidine and spermine, respectively, along with

diaminopropane and hydrogen-peroxide (Figure 2). Diaminopropane can be converted into β-alanine, whereas pyrroline can be further catabolized to γ-aminobutyric acid (GABA) (Hausman *et al*., 1997) in a reaction catalysed by pyrroline dehydrogenase (PDH) (Flores and Filner, 1985). The GABA is subsequently transaminated and oxidized to succinate, which is incorporated into the Krebs cycle. Thus, this pathway ensures the recycling of carbon and nitrogen from putrescine. On the other hand, the products of this catalytic process pathway including γ-aminobutyric acid (GABA), hydrogen-peroxide ammonia and aminoaldehydes produced during the degradation of polyamines also have biological significance as they trigger programmed cell death or apoptosis in certain cell types. Furthermore, decrease in polyamine levels due to catabolism may also be involved in regulating polyamines and driving cells to the apoptotic pathway (Thomas and Thomas, 2001; Seiler and Raul, 2005).

### 1.1.3 Polyamine conjugation

In nature, polyamines often occur as free molecular bases, but they can also be associated with small molecules like phenolic acids (conjugated forms) and also to various macromolecules like proteins (bound forms). The most common amine conjugates, i.e. polyamines and aromatic amine conjugates, covalently linked to hydroxycinnamic acids have also been shown to occur at high levels in plants (Martin-Tanguy, 1985) and are thought to be correlated with developmental phenomena. They occur as water-soluble or as water-insoluble forms. In the former, the single amine group of an aliphatic amine is linked with a phenolic cinnamic acid. The water-insoluble forms can be divided into two classes. In the first, each terminal amine group of an aliphatic amine is bound to cinnamic acid, while in the second

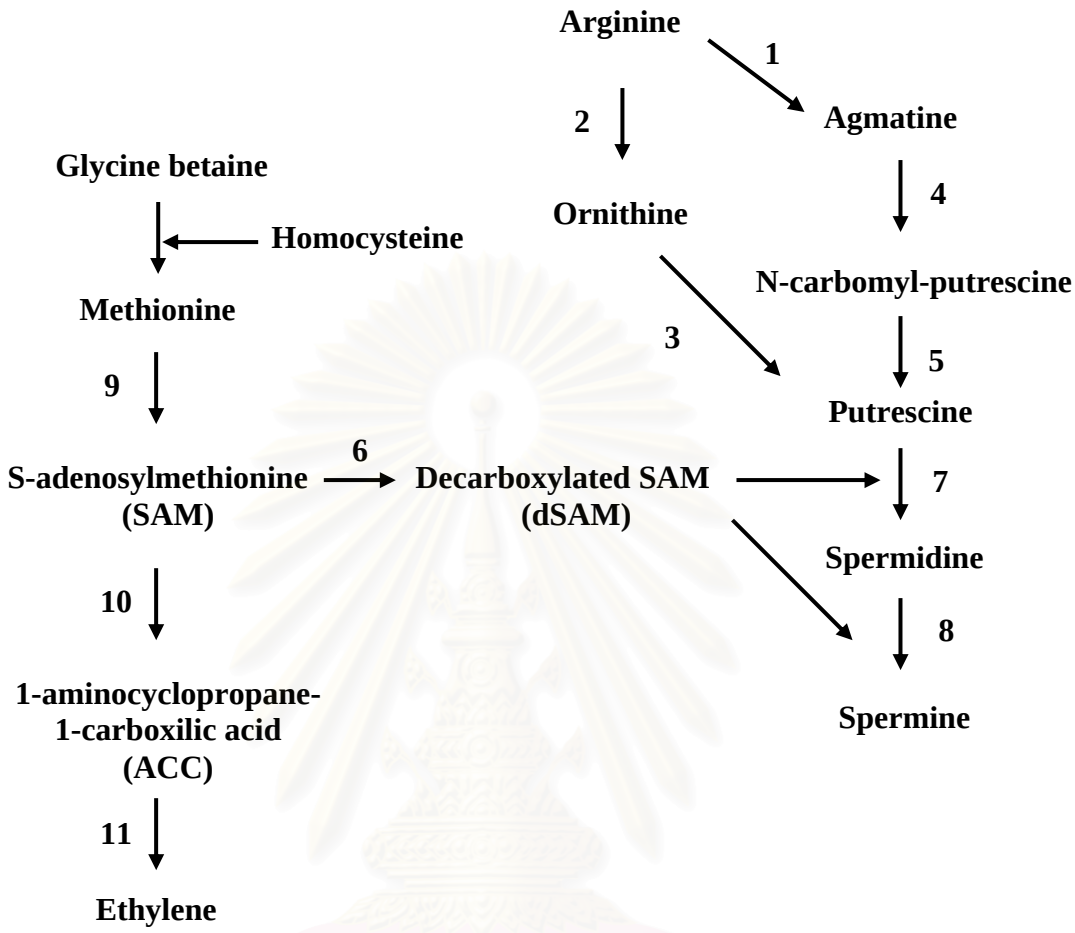


**Figure 1** The pathway of polyamine synthesis (modified from Bouchereau *et al*., 1999). 1. Arginine decarboxylase (ADC); 2. Argenase; 3. Ornithine-decarboxylase (ODC); 4. Agmatine iminohydrolase; 5. N-carbomoyl putrescine amidohydrolase; 6. S-adenosyl-methionine decarboxylase (SAMDC); 7. spermidine synthetase; 8. spermine synthetase; 9. SAM synthase; 10. ACC synthase; 11. ACC oxidase

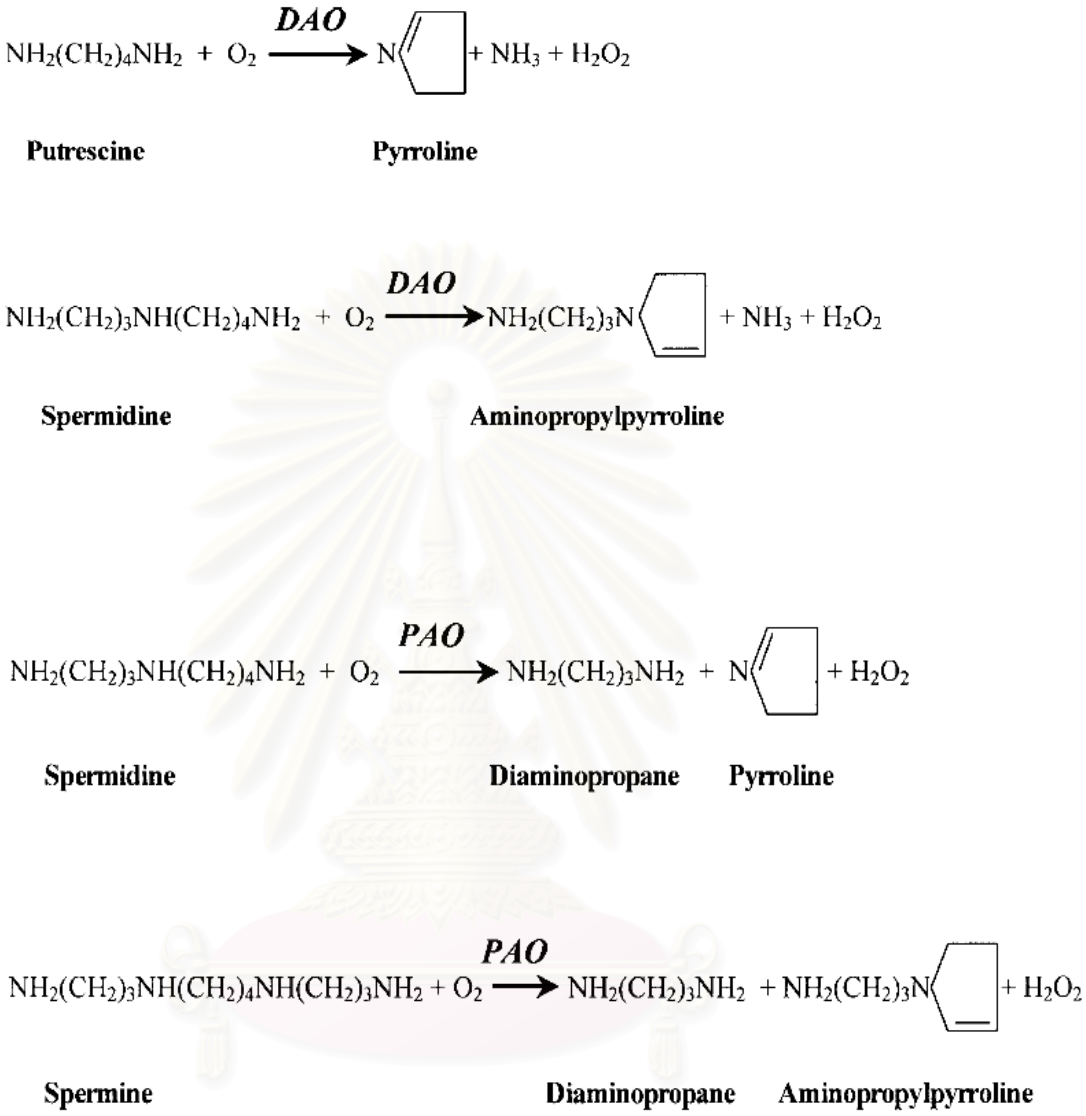




**Figure 2** Diamino oxidase (DAO) and polyamine oxidase (PAO) mechanisms of action in plants

(Bagni and Tassoni, 2001)

class the amine group of an aromatic amine is linked to cinnamic acid (Figure 3). These amine conjugates are fund in roots but they do not normally exist in shoots. In the root of tobacco, increases in water-soluble and water-insoluble conjugates have been shown before flowering (Martin-Tanguy *et al*., 1990) and occur in shoot apices upon floral initiation (Havelange *et al*., 1996). The synthesis of polyamine conjugates has been elucidated in tobacco callus (Nagrel, 1989). They are conjugated by the formation of an amide linkage, utilizing ester of Co-A for the provision of the activated carboxyl groups such as cinnamoylputrescine (Martin-Tanguy *et al*., 1988), which are formed by non-specific putrescine caffeyl-CoA transferase. Therefore, the synthesis of the water-insoluble polyamine such as di-p-coumaroylputrescine, di-p-coumaroylspermidine, di-feruloylputrescine and di-feruloylspermidine has not been elucidated (Martin-Tanguy, 1997). Furthermore, posttranslational covalent linkage of polyamines to protein is catalyzed by a class of enzymes known as transglutaminases (EC; 2.3.2.13) (Margosiak *et al*., 1990).

### 1.1.4 Polyamine transport

Polyamines are protonated molecules carrying positive charges on each nitrogen atom at physiological or cellular pH. Many authors have compared these molecules to $Ca^{2+}$ and $Mg^{2+}$, but they differ from such cations in the homogeneous distribution of the charge along the carbon chain (Seiler *et al*., 1996; Wallace *et al*., 2003). For that reason many researchers in the last decade have focused their efforts on the quest for polyamine carriers. Polyamine-specific carriers are widely distributed in prokaryotes and eukaryotes and can replenish polyamine pools upon inhibition of the biosynthetic enzymes (Seiler *et al*., 1996). The transport of polyamines classified as ABC [ATP binding cassette]-type transporters (Higgins, 1992) have been well

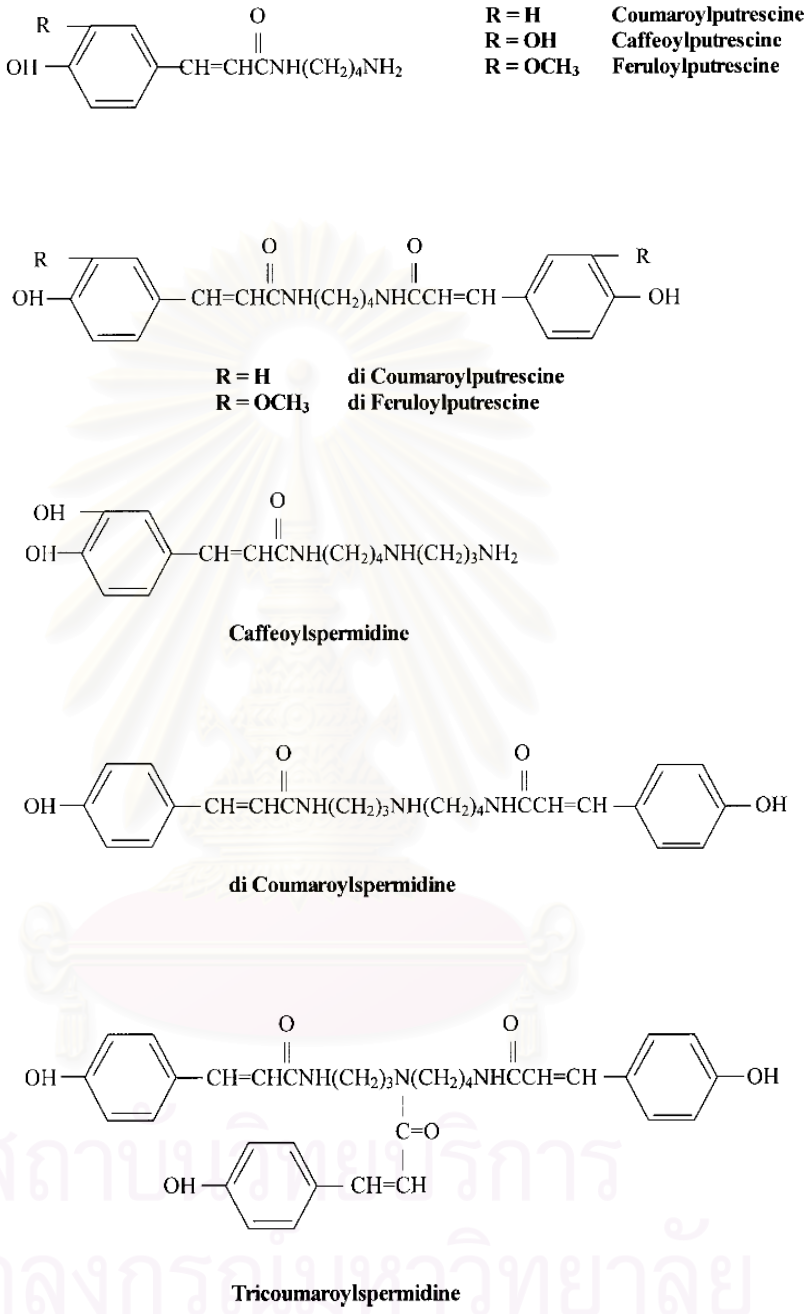


**Figure 3** Conjugates of hydroxycinnamic acids and polyamines (**Bagni and Tassoni, 2001**)

characterized in bacteria, yeast, parasites, and animals but not in cyanobacteria (Igarashi and Kashiwagi, 1999; Tassoni *et.al*., 2002; Rinehart and Chen, 1984).

#### 1.1.4.1 Polyamines transport in bacteria and yeast

The genes encoding polyamine transporter subunits have been identified in *Escherichia coli*. This transporter is a saturable, carrier-mediated and energy-dependent process. The genes of three transport systems were cloned (Igarashi and Kashiwaki, 1999; Igarashi *et al*., 2001). There are two polyamine uptake systems classified as ABC [ATP binding cassette]-type transporters (Tomii and Kanehisa, 1998, Linton and Higgins, 1998) consisting of a substrate-binding protein in the periplasm, integral channel-forming proteins (porins) and inner-plasma membrane side protein with ATPase activity to supply energy. First, the spermidine-preferential system consist of four proteins: a spermidine-binding protein (PotD), two porins (PotB, PotC) and ATPase (PotA) (Kashiwagi *et al*., 1995 and 1996) whereas the second system is putrescine-specific consisting of a substrate-binding protein (PotF), two porins (PotH and PotI) and ATPase (PotG) (Pistocchi *et al*., 1993). The third system is involved in both of uptake and excretion systems. Uptake system is dependent on membrane potential whereas the excretion system is an exchange reaction between putrescine and its amino acid precursor ornithine by activation of a putrescine-ornithine antiporter activity consisting of only PotE protein (Figure 4). The PotE protein encoded by the *potE* gene, together with *speF* (a gene encoding inducible ODC), constitute an operon in *E. coli* (Kashiwagi *et al*., 2000 and 1991). The role of *potE* has been described in *Escherichia coli* (Sciller *et al*., 2000).

In *Saccharomyces cerevisiae*, polyamine transporters which are encoded by set of *TPO* genes located on vacuolar membrane (Tomitori *et al*., 2001). This transporter was energy-dependent, proton gradient-dependent and saturation kinetics with $K_m$ values for spermidine and spermine were 0.2 and 0.7 mM, respectively (Kakinuma *et al*., 1992). $Mg^{2+}$ strongly inhibited polyamine transport (Maruyama *et al*., 1994) in this organism. The PotE protein has a long serine- and threonine-rich N-terminus domain, indicative of a post-translational regulatory mechanism mediated by serine or threonine kinases (Kakinuma *et al*., 1995). Four putative polyamine transporters, Tpo1p-Tpo4p, are found in the vacuolar membrane system to compartmentalize spermidine and spermine into vacuoles, in as much as free cytosolic polyamines are toxic for yeast (Tomitori *et al*., 1999). The carriers encoded by *TPO2* and *TPO3* are specific for spermine, whereas the transporters encoded by *TPO1* and *TPO4* recognize spermidine and spermine (Uemura *et al*., 2005) both: all are members of a family of multidrug-resistant carriers. Characterization studies of a *TPO1* deletion mutant have shown that Tpo1p is a spermidine exporter when cells are stressed with high spermidine concentrations (Albertsen *et al*., 2003). Another transporter, UGA4, which located on the vacuolar membrane, can catalyze the uptake of putrescine and 4-aminobutyric acid (Uemura *et al*., 2004). The uptake of putrescine and spermidine was dependent of extracellular $Na^+$ and pH (Basselin *et al*., 2000).

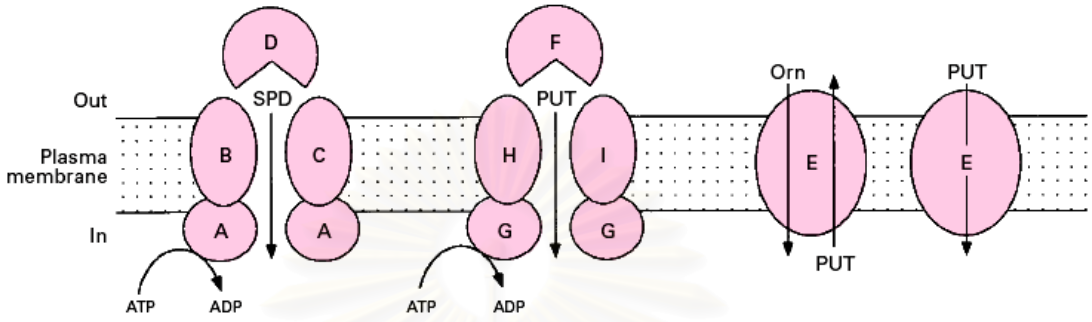


**Figure 4** Polyamine transport systems in *Escherichia coli*. ATPase (A, PotA and G, PotG**),** the substrate-binding proteins (D, PotD and F, PotF), channel-forming proteins (B, PotB; C, PotC, H, PotH and I, PotI) and the PotE protein (E) (Igarashi and Kashiwagi, 1999).

### 1.1.4.2 Polyamines transport in mammalian cells

Polyamine transport mechanisms have been recently studied in mammalian cells (Hoshino *et al*., 2005). Unlike their counterparts in yeast and *E. coli*, the transport is poorly characterized at the molecular level. Polyamine transport, uptake and efflux are net energy-consuming and saturable processes that can transport polyamines against significant concentration gradients, which suggests the existence of carrier-mediated mechanisms (Seiler and Dezeure, 1990; Khan *et al*., 1991; Grillo and Colombatto, 1994; Seiler *et al*., 1996). The mammalian polyamine transport activity is also acutely controlled by cell cycle events and hormonal stimulation (Lessard *et al*., 1995). The polyamine uptake into rat liver mitochondria is mediated by specific polyamine uniporter which is dependent on membrane potential and insensitive to medium pH (Tominello *et al*., 1992). Putrescine and spermidine transporters in mammalian cells have been identified (García-Fernández *et al*., 2005). Uptake and excretion are catalyzed by one and the same polyamine transporter (Sakata *et al*., 2000). A similar reversal of roles has also been observed in *E. coli* PotE, which catalyses both the uptake and excretion of putrescine (Kashiwagi *et al*., 1997). In animal cells, excess polyamines are metabolized to acetyl derivatives, which are better substrates for excretion than the polyamines themselves, although polyamines are better substrates for uptake than acetylpolyamines (Seiler, 1987). Polyamine transport is fully integrated into the regulatory system controlling polyamines concentration in mammalian cells.

As a general rule, factors increasing polyamine formation enhance their uptake from the extracellular environment, and conversely, situations where there is an excessive build-up of intracellular polyamines favor their removal. Several

proteins involved in polyamine transport regulation fine-tune polyamine levels in the cell. The ODC-antizyme (AZ) is a small polyamine-induced protein that plays a role in ODC inhibition by forming an AZ–ODC complex. Furthermore, AZ has been found to negatively regulate polyamine transport (Sakata *et al*., 2000). The AZ protein is fairly labile and the content of this molecule in the cell is subject to positive regulation by polyamines through a ribosomal frameshift mechanism (Ivanov *et al*., 2000), negative regulation by an antizyme inhibitor protein (an ODC-resembling protein which lacks ODC activity) and post-translational proteolytic modifications (Murakami *et al*., 1996). The excretion of polyamines is likewise a carrier-mediated process. Two systems seem to be involved in the diamine/polyamine efflux: a diamine exporter (AZ-independent process) which might be involved in the excretion of putrescine and *N*-acetylpolyamines (Xie *et al*., 1997); and the AZ-regulated polyamine transporter involved in the excretion of spermidine and spermine.

#### 1.1.4.3 Polyamine transport in parasites

The evidence of an influx system for polyamines in parasites was recently reported by Reguera *et al*., 2005. Previously, Kaur *et al.* (1986) revealed the effect of exogenous putrescine on leishmanial cells. Balaña-Fouce *et al.* (1989) provided the first description of an uptake system for protozoan parasites in *L. infantum* promastigotes. These authors showed that this organism is able to take up putrescine by a saturable, concentration- and energy-dependent, specific carrier not shared by higher polyamines, i.e. spermidine and spermine, other analogous polyamine such as MGBG, or basic amino acids. These results were conclusively confirmed in *Leishmania donovani*, the etiological agent of visceral leishmaniasis, in which a second polyamine carrier was described, in this case for spermidine (Kandpal

and Tekwani, 1997). The influx of this latter polyamine was found to be over seven times more effective than the uptake of putrescine, a diamine found not to share this mechanism, unlike spermine, which was observed to significantly inhibit spermidine uptake. Spermine, in turn, was not taken up at all by the putrescine transport system, and only moderately by the spermidine carrier. These findings were also observed in *Trypanosoma cruzi* (González *et al.*, 1992), *Leishmania mexicana* promastigotes (González and Algranati, 1994) and *Crithidia fasciculata* parasites (Calonge *et al.*, 1996a). In all these cases, putrescine uptake was specific and only scantly inhibited by spermidine, spermine and a number of variable length diamines.

A common feature in all the polyamine transporters described for parasites to date is their dependence on the proton motive force to take up the substrate. The fact that sodium ionophores, uncoupling agents and sulfhydryl group reagents were found to significantly inhibit polyamine influx in all the parasites studied suggests that the mechanism involved is coupled to a sodium electrochemical gradient (Balaña-Fouce *et al.*, 1989 and Kandpal and Tekwani, 1997). The molecular characterization of polyamine transporters in protozoa is a field open to research. No gene with this putative function has been identified in any of the major genome databases to date. When functionally expressed in *Xenopus laevis* oocytes, the polyamine transporter protein showed a high affinity for putrescine and spermidine (Enkvetchakul *et al*., 2003).

#### 1.1.4.4 Polyamines transport in cyanobacteria

In an initial survey of the polyamines of various cyanobacteria, spermine was not detected, although the organisms grew readily at a pH of 10 or

higher. Several strains and isolates of cyanobacteria [*Gloeocapsa uipicola* D, *Anacystis nidulans*, *Coccochloris peniocystis*, *Anabaena sp*., and *Plectonema boryanum*] were cultivated in the absence of an organic carbon source in the laboratory of Roger Stanier at the Pasteur Institute in Paris. The cells were centrifuged, lyophilized, and analyzed. All of the organisms examined contained high amount of spermidine, very low amount of putrescine, and were devoid of spermine. They differed widely in the base ratios of their DNA (Stanier *et al*., 1971). Putrescine, a diamine normally present at high (10 to 30 mM) concentrations in *E. coli*, was found at very low concentrations in the cells. Spermidine was clearly the major polyamine of these organisms. It was observed in deteriorating cultures in the stationary phase that spermidine was lost even before much chlorophyll had disappeared, and this led to a study of the metabolism of exogenous polyamines. Spermidine and spermine, although slightly toxic, were actively metabolized. Surprisingly, the natural diamines putrescine and cadaverine were quite toxic. The fact that exogenous putrescine was toxic at the concentration (0.15 mM) at which this normal metabolite is present within the cyanobacterium during exponential growth has led to additional studies of the mechanism of this effect (Ramakrishna *et al*., 1978; Guarino and Cohen, 1979).

Because this pattern is so distinctive, the cyanobacteria may become organisms of choice for the study of the metabolism and function of polyamine. In cyanobacterium *Synechocystis* PCC6803, only a PotD-like protein is detectable (Kaneko *et al*., 1996; Igarashi and Kashiwaki, 1999). The PotD-like protein may share channel-forming proteins and membrane-associated ATPase with the other uptake system in *Synechocystis* PCC 6803. Another possibility is that the similarity between

*E. coli* and *Synechocystis* genes may be too low to easily detect equivalents of the *pot* genes (Kaneko *et al*., 1996). However, the importance of polyamine uptake by living cells is far from clear, since all organisms have enzymes able to synthesize them and maintain optimum levels for their metabolism. Indeed, most of studies for the uptake of polyamine were carried out in *E. coli* and yeast (Igarashi and Kashiwaki, 1999). Genes for several kinds of putative polyamine transport systems have been described in living organism in which the whole genome has been sequenced (Table 2).

**1.2 Environmental factors have an effect on *Synechocystis***

All organisms have some capacity to adapt to environmental stress, but the extent of this adaptive capacity varies widely. Heat, cold, high pressure, and acid or alkaline conditions can all produce stress. Bacteria adapt easily to environmental stress, usually through changes in the enzymes and other proteins they produce. These adaptations enable bacteria to grow in a variety of conditions. Gradual exposure to the stress, for example, may enable bacteria to synthesize new enzymes that allow them to continue functioning under the stressing conditions or that enhance their capacity to deal with the stressing agent. Or they may resist environmental stress in other ways. Some bacteria that live in extremely acidic conditions can pump out acid from their cell. Some kinds of bacteria thrive in hydrothermal vents on the ocean floor or in oil reservoirs within Earth, at high pressures and temperatures as high as 120$^{o}$C. Other kinds can live at temperatures as low as –12$^{o}$C in Antarctic brine pools. Other bacteria have adapted to grow in extremely acid conditions, where mines drain or minerals are leached from ores and sulfuric acid is produced. Others grow at extremely alkaline or extremely salty conditions. Still others can grow in total absence of oxygen. Bacteria able to function in these extreme conditions generally cannot function under

conditions we consider normal. Most of earlier studies relating to stress responses have been done as the external symptoms of abiotic challenge become acute (Bouchereau *et al*., 1999) but their precise molecular mechanism of action still remains unclear (Bagni and Tassoni, 2001). Photosynthetic bacteria must acclimate to response in external stimuli by strictly regulating the expression of distinct sets of genes, as shown in *Synechocystis* (Murata and Suzuki, 2005).

Cyanobacteria must acclimate to changing light intensity, osmolarity, temperature and nutrient availability in their environments by strictly regulating the expression of distinct sets of genes, as shown in *Synechocystis* (Murata and Suzuki, 2005). Light is not only energy source, but also a signal in regulation of cyanobacterium gene expression (Hübschmann *et al*., 2005; Gill *et al*., 2002; Hihara *et al*., 2001). The responsiveness of photosynthesis genes to white light has recently become obvious in studies of *Synechocystis* global gene expression profile (Huang *et al*., 2002). Transcriptional levels of a polyamine biosynthesis gene (ADC) were observed to remain unchanged under light-to-dark transition in *Synechocystis* (Jantaro *et al*., 2005). The genome-wide responses of gene expression of *Synechocystis* under high osmolarlity stresses strongly induced the essential genes for salt acclimation in *Synechocystis* (Kanesaki *et al*., 2002; Marin *et al*., 2004). These regulations were perceived as distinct signals through the Hik-Rre systems (Shoumskaya *et al*., 2005) which may respond by direct osmolarity-induced activation of the transport or by enhanced expression of the corresponding gene. Correspondingly the accumulation of cellular polyamine levels was increased after exposure long-term salt and osmotic stress in *Synechocystis* (Jantaro *et al*., 2003). Non-optimal temperature, either low or high temperature, is an effective abiotic factor, which is known to influence

polyamine metabolism in plants (Bouchereau *et al*., 1999). The expression of certain cold-inducible genes in *Synechocystis* was perceived as distinct signals through the histidine kinase system (Suzuki *et al*., 2001).

Chilling stress (22$^{o}$C) was observed to enhance the transcriptional levels of a gene encoding sulfate transport ATP-binding system in *Synechocystis* (Inaba *et al*., 2003). Additionally, heat stress (42$^{o}$C) was noticed to markedly increase the specific activity of polyamine biosynthetic enzyme (ADC) even though did not affect on the steady-state transcriptional levels of ADC gene in *Synechocystis* (Jantaro *et al*., 2005). In addition to nutrient availability, effects of deficiency of nourishing have been studied in cyanobacteria (Schwarz *et al*., 2005). Characterization of photosynthetic gene expression under in iron deficiency was investigated in *Synchocystis* (Odom *et al*., 1993). Recently, iron deficiency was observed to markedly increase the accumulation of sets of genes for iron transporter after 12-h limitation in *Synechocystis* (Singh *et al*., 2003, Katoh *et al*., 2001). The effects of nitrogen starvation on the abundance of pigment molecules in *Synechocystis* have been well documented (Richaud *et al*., 2001). The decrease in chlorophyll and phycobilisome content leads to dramatic change in cell color. Furthermore, nitrogen deficiency was noticed to significantly enhance the transcriptional levels of *glnA* and *glnN* genes after 12-h limitation in *Synechocystis* (Reyes *et al*., 1997).

**Table 2** Genes for polyamine transport*****

| Bacterium or archaeon | Size of chromosome (kbp) | Number of genes annotated | Genes suggested to be linked to polyamine transport |
|---|---|---|---|
| Archaea | | | |
| *Archaeoglobus fulgidus* | 2178 | 2407 | *potABC* |
| Bacteria | | | |
| *Bacillus subtilis* | 4215 | 4100 | *blt* |
| *Borrelia burgdorferi* | 911 | 850 | *potABCD* |
| *Escherichia coli* | 4639 | 4289 | *potABCD, potFGHI, potE* |
| *Haemophilus influenza* | 1830 | 1709 | *potABCD, potD, potE* |
| *Mycoplasma genitalium* | 580 | 467 | *potABC* |
| *Mycoplasma pneumoniae* | 816 | 677 | *potABC* |
| *Rickettsia prowazekii* | 1112 | 834 | *potE* |
| *Synechocystis sp. PCC 6803* | 3573 | 3169 | *potD* |
| *Treponema pallidum* | 1138 | 1031 | *potABCD* |

***** Igarashi and Kashiwagi, 1999

### 1.3 Lineage of cyanobacterium *Synechocystis* sp. PCC 6803

The algae which are the simplest members of the plant kingdom have a considerable and increasing economic importance; they have both beneficial and harmful effects on human life. Blue-greens are not true algae. They have no nucleus, the structure that encloses the DNA, and no chloroplast, the structure that encloses the photosynthetic membranes, the structures that are evident in photosynthetic true algae. In fact, blue-greens are more like to bacteria which have similar biochemical and structural characteristics. The process of nitrogen fixation and the occurrence of gas vesicles are especially important to the success of nuisance species of blue-greens. The blue-greens are widely distributed over land and water. Their fossils have been identified as over three billion years old. They were probably the chief primary producers of organic matter and the first organisms to release elemental oxygen, $O_2$, into the primitive atmosphere, which was until then free from $O_2$. Thus blue-greens were most probably responsible for a major evolutionary transformation leading to the development of aerobic metabolism and to the subsequent rise of higher plant and animal forms. They are referred to in literature by various names, chief among which are Cyanophyta, Myxophyta, Cyanochloronta, Cyanobacteria, blue-green algae, blue-green bacteria.

The majority of blue-greens are aerobic photoautotrophs: their life processes require only oxygen, light and inorganic substances. A species of Oscillatoria that is found in mud at the bottom of the Thames, are able to live anaerobically. They can live in extremes of temperatures -60°C to 85°C, and a few species are halophilic or salt tolerant (as high as 27%, for comparison, percentage of salt in seawater is 3%). Blue-greens can grow in full sunlight and in almost complete darkness. They are often

the first phototrophic organism to colonize bare areas of rock and soil, as an example subsequent to cataclysmic volcanic explosion (at Krakatoa, Indonesia in 1883). At the onset of nitrogen limitation during bloom conditions, certain cells in *Anabaena* and *Aphanizomenon* evolve into heterocysts, which convert nitrogen gas into ammonium, which is then distributed to the neighboring cells of a filament. In addition, blue-greens that form symbiotic (mutually beneficial) relationships with a wide range of other life forms, can convert nitrogen gas into ammonium.

Finally, at the onset of adverse environmental conditions, some blue-greens can develop a modified cell, called an akinete. Akinetes contain large reserves of carbohydrates, and owing to their density and lack of gas vesicles, eventually settle to the lake bottom. They can tolerate adverse conditions such as the complete drying of a pond or the cold winter temperatures, and, as a consequence, akinetes serve as "seeds" for the growth of juvenile filaments when favorable conditions return. Heterocysts and akinetes are unique to the blue-greens. Unicellular and filamentous blue-greens are almost invariably present in freshwater lakes frequently forming dense planktonic populations or water blooms in eutrophic (nutrient rich) waters. In temperate lakes there is a characteristic seasonal succession of the bloom-forming species, due apparently to their differing responses to the physicol- chemical conditions resulting from thermal stratification. Usually the filamentous forms (*Anabaena* species, *Aphanizomenon flos-aquae* and *Gloeotrichia echinulata*) develop first soon after the onset of stratification in late spring or early summer, while the unicellular-colonial forms (like *Microcystis* species) typically bloom in mid-summer or in autumn. The main factors which appear to determine the development of planktonic populations are light, temperature, pH, nutrient concentrations and the presence of organic solutes.

Among prokaryotes, cyanobacteria are the only organisms capable of oxygenic photosynthesis, and there is evidence to suggest that they are the progenitor(s) of plant plastids. Their phylogenetic position in the bacterial kingdom is still obscure, although recent analysis of ancient genes has indicated a genetic relationship with Gram-positive bacteria (Hansmann and Martin, 2000; Xiong *et al*., 2000). *Synechocystis* sp. PCC 6803 is unicellular non-nitrogen ($N_2$)-fixing cyanobacterium and a ubiquitous inhabitant of fresh water. It has been one of the most popular organisms for genetic and physiological studies of photosynthesis for two major reasons; it is naturally transformable by exogenous DNA (Grigorieva and Shestakov, 1982) and grows heterotrophically at the expense of glucose (Rippka e*t al*., 1979; Williams, 1988). The entire genome of *Synechocystis* sp. PCC 6803 was sequenced in 1996 (Kaneko *et al*., 1996). It was also the first phototrophic organism to be fully sequenced. The availability of the entire genome sequence has allowed closer studies on the structure of each gene and the organization of the entire genome, resulting in a better understanding of the unique genetic characteristic of phototrophic organisms. Since then, a tremendous amount of information has been generated through functional genomics, and proteome and transcriptome studies. '*Synechocystis*' has been defined as a unicellular coccoid, or spherical cyanobacterium lacking gas vesicles or a sheath. They divide by binary fission at two 74 or three successive planes.

Based on their GC contents, many cultured strains of *Synechocystis* can be classified into three groups; the marine group, the low GC group and the high GC group (Holt *et al*., 1994). Strain PCC 6803 belongs to the latter group, whose members, including PCC 6714, have been mostly isolated from freshwater. They also

have the propensity to utilize glucose and grow heterotrophically (Rippka *et al*., 1979). There are four culture substrains of *Synechocystis* ('PCC', 'ATCC', 'GT' (glucose-tolerant) and 'Kazusa'), all of which were derived from the Berkeley strain 6803, which was isolated from freshwater in California by R. Kunisawa (Stanier *et al*., 1971) (Figure 6). It was originally believed that these subcultures were the same. For this reason, they were grouped together under the name of *Synechocystis* sp. PCC strain number 6803 (Rippka and Herdman, 1992). However, the four substrains show certain differences in phenotype. A single representative clone of the GT strain was established for complete genome sequencing (Kazusa strain), while the other strains have been maintained and transferred without sequencing the genome. For example, the Berkeley and PCC strains are motile, while the others are apparently non-motile. Both PCC and ATCC strains are sensitive to glucose, whereas the glucose tolerant (GT) strain was intentionally generated under defined conditions (Williams, 1988; Bartesevich and Pakrasi, 1995). The complete nucleotide sequence of *Synechocystis* sp. PCC 6803 was determined in 1996. This was the first genome of a photoautotrophic organism to be fully sequenced. Sequencing was carried out using a clone-by-clone strategy based on the physical map of the genome, resulting in a highly accurate sequence. The circular genome encoding 3167 genes namely amino acid biosynthesis (84 genes), biosynthesis of cofactors, prosthetic group and carriers (108 genes), cell envelope (64 genes), cellular process (62 genes), central intermediary metabolism (31 genes), energy metabolism (86 genes), fatty acid, phospholipid and sterol metabolism (35 genes), photosynthesis and respiration (131 genes), nucleic acid and metabolism (38 genes), general regulatory functions (147 genes), DNA replication, recombination and repair (49 genes), transcription (24 genes), translation (144 genes), transport and binding proteins (158 genes), other

categories (255 genes), and function unknown (1,751 genes) was originally deduced to be 3,573,470 bp long (Figure 7). Cyanobacteria owe the ubiquitous distribution to remarkable capacities to adapt to varying environmental conditions. A few data are available regarding adaptation of natural populations, most studies having been performed on a few model strains selected for specific adaptive capacities to a chosen environmental or stress factors as well as for solving agricultural problems (Ikeuchi, and Tabata, 2001). Therefore, *Synechocystis* is a useful tool in the study of the biochemistry and genetics of cyanobacteria (Joset *et al*., 1996).

**OBJECTIVES OF THIS RESEARCH**

1. To study polyamine transport with respect to energetics and kinetics to *Synechocystis* sp. PCC 6803
2. To reveal if transcription of the *potD* gene (*slr0401*) of *Synechocystis* sp. PCC 6803 is under long or short term environmental regulation
3. To characterize the expression of PotD protein of *Synechocystis* sp. PCC 6803 and the structurally modeling it

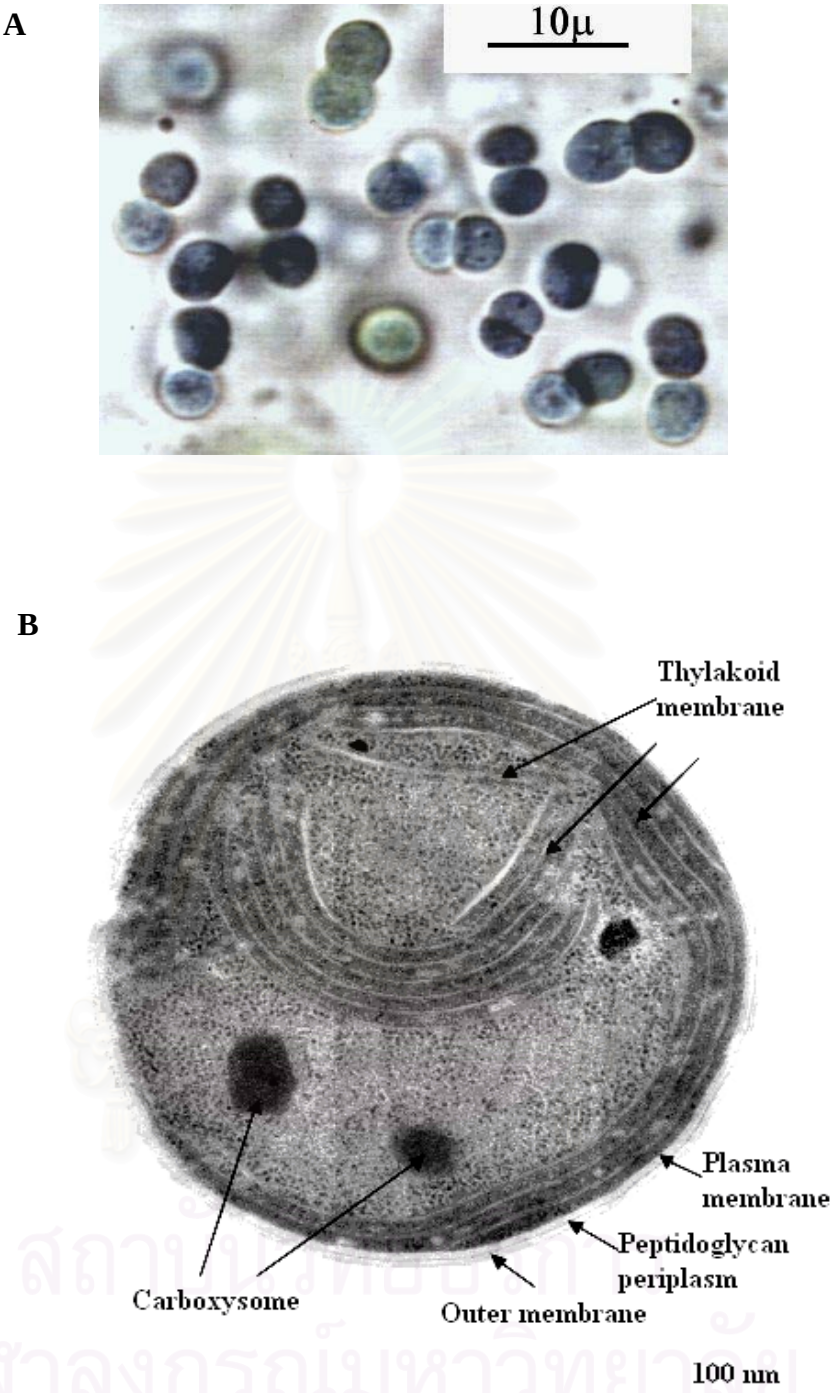


**Figure 5** (**A**) *Synechocystis* sp. PCC 6803 cells (**B**) Schematic representation of a thin section of a cyanobacteria cell (www-cyanosite.bio.purdue.edu/images/images.html)

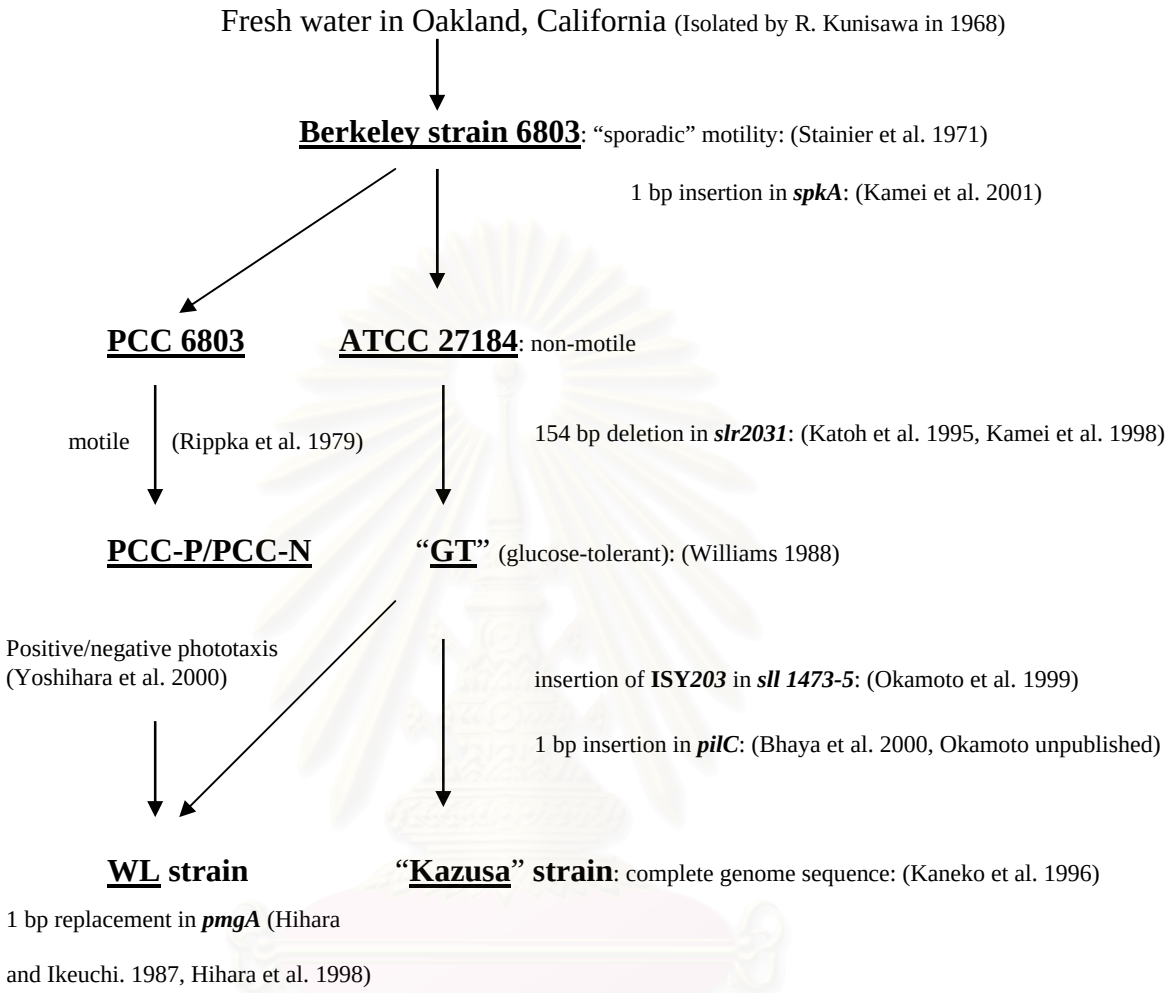


**Figure 6** Strain history of *Synechocystis* sp. PCC 6803 (Ikeuchi and Tabata, 2001)

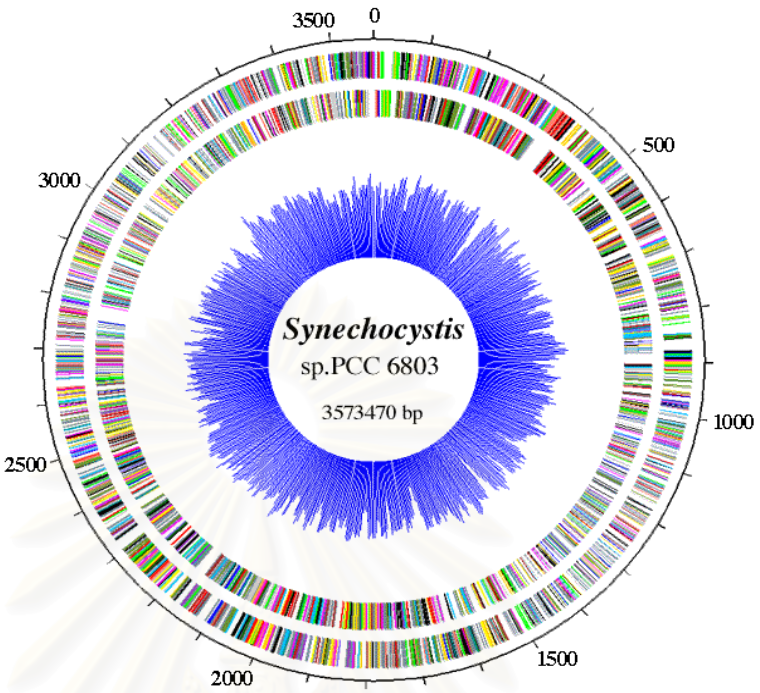


**Figure 7** The circular genome of *Synechocystis* sp. PCC 6803 according to Cyanobase (www.kazusa.or.jp/cyano/Synechocystis).

# CHAPTER II

# MATERIALS AND METHODS

## 2.1 MATERIALS

### 2.1.1 Equipments

| | |
|---|---|
| Autoclave | Model HA-30, Hirayama Manufactering Cooperation, Japan |
| Balances | METTLER PJ360 DeltaRange® GWB, USA |
| | IKAMAG® REO DREHZAHL ELECTRONIC |
| | Precisa 800M SWISS QUALITY, USA |
| Centrifuge | Jouan MR 1812, USA |
| | SORVALL® MC 12V DUPONT, USA |
| | HERMLE Z233 MK, USA |
| Electrophoresis Unit | BIO-RAD PROTEIN® II xi Cell, USA |
| French press cell disrupter | THERMO ELECTRON COPORATION, USA |
| Geiger counter | RADOS RDS-120 Universal Survey Meter, USA |
| Geldoc® enabled | DNA visualisation UV light, USA |
| GENE pulser apparatus | BIO-RAD, USA |
| Laminar flow | BVT-124 International Scientific Supply, Thailand |
| Light source unit | Prekeo S250 Zeiss IKON, Japan |
| PCR apparatus | PERKIN ELMER DNA Thermal Cycler, Japan |
| pH meter | ORION model 420A, USA |

| | |
|---|---|
| Power supply | BIO-RAD POWER PAC 1000, USA |
| | BIO-RAD Model 1000/500 |
| Scintillation counters | LS6500 Multi-Purpose Scintillation Counter, BECKMAN COULTER, USA |
| Shaker | Innova™ 2100 PLATFORM SHAKER, USA |
| Spectrophotometer | SPECTRONIC® GENESYS™2, USA |
| | Jenway UV/VIS 6400, USA |
| Ultracentrifuge | BECKMAN COULTER OPTIMA™ L-100XP, USA |
| Vortex | Model K-550-GE, Scientific Industries, USA |
| Water bath | THERMOMIX® B B.BRAUN, USA |

**2.1.2 Chemicals**

| | |
|---|---|
| 2,4-Dinitrophenol(DNP) | Merck, Germany |
| 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) | Sigma, USA |
| 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) | Sigma, USA |
| Acetic acid | BDH, England |
| Acetone | Merck, Germany |
| Acrylamide | Merck, Germany |
| Agarose | Promega Corporation, USA |
| Agmatine | Sigma, USA |
| Amiloride | Sigma, USA |
| Ammonium persulfate | Merck, Germany |
| Brilliant blue | Sigma, USA |
| Bromophenol blue | Sigma, USA |

| | |
|---|---|
| BSA (Fraction V) | Sigma, USA |
| Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) | Sigma, USA |
| Chloramphenicol | Sigma, USA |
| Chloroform | Merck, Germany |
| Dithiothreitol | Sigma, USA |
| Dimethylformamide (DMF) | Sigma, USA |
| EDTA | Sigma, USA |
| Ethanol | Scharlau Chemie S.A., Spain |
| Ethidium bromide | Sigma, USA |
| Ferricyanide | Sigma, USA |
| Ficoll | Phamacia Biotech, USA |
| Formaldehyde 37% | Merck, Germany |
| Glucose | Sigma, USA |
| Glycerol | Scharlau Chemie S.A., Spain |
| Glycine | Sigma, USA |
| Glyoxal | Sigma, USA |
| Gramicidine D | Sigma, USA |
| Isoamylalcohol | Sigma, USA |
| Isopropanol | Sigma, USA |
| Mercaptoethanol | Sigma, USA |
| Methanol | Scharlau Chemie S.A., Spain |
| Metylene blue | Sigma, USA |
| *N, N*′ dicyclohexylcarbodiimide (DCCD) | Sigma, USA |
| *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) | Sigma, USA |

| | |
|---|---|
| *N*-Ethylmaleimide | Sigma, USA |
| Nigericin | Sigma, USA |
| Ouabain | Sigma, USA |
| *p*-Chloromercurisulfonic acid | Sigma, USA |
| *p*-Nitro blue tetrazolium chloride (NBT) | Sigma, USA |
| Phenol | Merck, Germany |
| Potassium cyanide | Sigma, USA |
| Putrescine dihydrochloride | Sigma, USA |
| Putrescine-1,4-$^{14}$C dihydrochloride | Sigma, USA |
| Pyridoxal-5-phosphate | Sigma, USA |
| Rifampicin | Sigma, USA |
| Silver nitrate | Sigma, USA |
| Sodium arsenate | Sigma, USA |
| Sodium bicarbonate | BDH, England |
| Sodium chloride | APS, Australia |
| Sodium citrate | Sigma, USA |
| Sodium dodecyl sulfate | Sigma, USA |
| Sodium fluoride | Sigma, USA |
| Sodium thiosulfate | Sigma, USA |
| Sorbitol | Sigma, USA |
| Spermidine trihydrochloride | Sigma, USA |
| Spermidine-8-$^{14}$C trihydrochloride | Sigma, USA |
| Spermine tetrahydrochloride | Sigma, USA |
| Sucrose | Sigma, USA |
| TEMED | BIO-RAD, USA |

| | |
|---|---|
| Toluene | BDH, England |
| Tris base | USB Corporation, USA |
| Triton X-100 | Packard, USA |
| Tween-20 | BIO-RAD, USA |
| Urea | Sigma, USA |
| Valinomycin | Sigma, USA |
| Xylene cyanol FF | Sigma, USA |

**2.1.3 Kits and suppliers**

| | |
|---|---|
| AIS™ The Analytical Imaging Station Operation program: | Imaging Research Inc. Ontario, Canada |
| 1 kb DNA Ladder | BioLabs, New England, England |
| -AffinPure Rabbit Anti-Mouse IgG Alkaline Phosphatase Conjugated | Machery-Nagel, USA |
| Anti-His Antibody | Amersham Biosciences, USA |
| DyNAzyme™ DNA Polymerase kit | FINNZYMES, Finland |
| Milipore Ultrafree-DA | Millipore Coorperation, USA |
| Ni Sepharose column kit | Amersham bioscience, USA |
| Nylon membrane filter | 0.45 and 0.22 μm., Sartorius, Germany |
| Parablot NCL membrane | Machery-Nagel, USA |
| PCR amplification kit | MBI Fermentas, Germany |
| Prestained Protein Ladder, 10-160 kDa | MBI Fermentas, Germany |
| Prime-a-Gene® Labeling System | Promega Corporation, USA |
| Ready-To-Go You-Prime First-Strand Beads kit | Amersham Biosciences, USA |
| RNA Ladder, High Range | MBI Fermentas, Germany |

| | |
|---|---|
| RQ1 Rnase-Free Dnase | Promega Corporation, USA |
| Whatman 3MM paper | Whatman International, England |

### 2.1.4 Organism

The two organisms, namely *Escherichia coli* and *Synechocystis* sp. PCC 6803 were used in this study.

**2.1.4.1 *Escherichia coli* strains** were obtained from the Laboratory of Biochemistry, Department of Biochemistry, Chulalongkorn University, Thailand.

Strain TOP10 ($F^-$ *mcr*A, Δ*(mrr-hsdRMS-mcrBC, ϕ80lacZΔM15 ΔlacX74 deoR rec1 araD139 Δ(ara-leu)7696galU galK rpsL* ($Str^R$) *endA1 nupG)* was used for DNA manipulation.

Strain BL21 (DE3) pLysS ($F^-$ *ompT hsdS*($r_Bm_B$) *gal dcm*(DE3) pLysS($Cm^R$)) was used for *PotD* expression.

**2.1.4.2 *Synechocystis* sp. PCC 6803** wild type strains (**Williams, 1988**), is glucose tolerant, and was obtained from the Laboratory of Plant Physiology and Molecular Biology, Department of Biology, University of Turku, Finland.


**Figure 8** *Synechocystis* sp. PCC 6803 in BG-11 medium

### 2.1.5 Plasmids

The two plasmids, namely pGEM®T Easy and pET-19b Vector were used in this study. Circle maps are shown in APPENDIX O and P

### 2.1.6 Oligonucleotides

#### 2.1.6.1 PCR primers for reverse transcription PCR

| Target gene | Name | Primers | Amplified fragment length (bp) |
|---|---|---|---|
| *16s* rRNA | forward-16s | 5′-AGTTCTGACGGTACCGTGATGA-3′ | 521 |
| | reverse-16s | 5′-GTCAAGCCTTGGTAAGGTTCT-3′ | |
| *potD* (*slr0401*) | forward-*potD* | 5′-CAGGGCAGTAGCAAAGAAGT-3′ | 530 |
| | reverse-*potD* | 5′-GGATCATCCACCAGAGCAAT-3′ | |

#### 2.1.6.2 PCR primers for *Synechocystis* chromosomal DNA

| Target gene | Name | Primers | Amplified fragment length (bp) |
|---|---|---|---|
| *potD* (*slr0401*) | forward-*potD* | 5’CCATATGAATTTACCCTGCTATTCCCGCCG 3’ | 1,150 |
| | reverse-*potD* | 5’CGGGATCCCTAAGCACTCCGCATGGTTT3’ | |

**2.1.6.3 PCR primers for sequencing**

| Recombinant gene | Name | Universal Primer |
|---|---|---|
| pGEMpotD gene | forward primer | T7 promoter |
| | reverse primer | SP6 promoter |
| pETpotD gene | forward primer | T7 promoter |
| | reverse primer | T7 terminator |

**2.2 Method for *Synechocystis* sp. PCC 6803**

**2.2.1 General methods**

**2.2.1.1 Culture conditions and experimental treatments of the cells**

The glucose-tolerant strain of *Synechocystis* sp. strain PCC 6803 (Williams, 1998) cells were grown photoautotrophically in BG-11 medium at 32$^{o}$C using a rotatory shaker at 110 rpm under continuous illumination of 50 μmol photons/m$^2$/s. The liquid cultures for the experiments were started at $OD_{730}$ being 0.25 and the cultures used for environmental conditions were in exponential growth phase (OD730 = 1.0~1.2). The experimental stress conditions used were: the control condition (32$^{o}$C, continuous light 50 μmol photons/m$^2$/s), darkness (18 hour and 3 day), followed by growth light for 5 min, salt stress (550 mM NaCl, 5 min and 3 day), hyperosmotic stress (300 mM D-sorbitol, 5 min and 3 day), low temperature (18$^{o}$C, 5 min and 3 day), high temperature (42$^{o}$C, 5 min and 3 day), ammonium chloride (18 h and 3 day) and iron deficiency (18 h and 3 day). Iron deficiency was induced by replacing ferric ammonium citrate in BG-11 with ammonium citrate. Ammonium chloride ($NH_4Cl$) was added to BG-11 medium instead of $NaNO_3$, the nitrogen source in BG-11 (Muro-

Poster *et al*., 2001). The BG-11 medium was always buffered with 20 mM TES buffer at pH 7.0.

#### 2.2.1.2 Polyamine transport assays

Cells at late log phase were harvested by centrifugation (8,000xg, 10 min, 4$^{o}$C), washed twice with 50 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid(Hepes)-KOH buffer pH 7.6 containing 0.4% glucose and suspended in the same buffer to yield a protein concentration of 0.1 mg cell protein/ml. The cell suspension (95 µl) was preincubated at 37$^{o}$C for 5 min. The uptake experiment was initiated by the addition of 5 µl $^{14}$C-polyamine (Radiochemical Centre, Amersham, England) with a specific activity of 2 mCi/mmol at a final concentration of 50 µM. After incubation, the cells were rapidly collected on membrane filters (cellulose acetate, 0.45 µl pore size; Millipore Corp.). The filters were washed twice with 1 ml of cold buffer containing 1 mM specific polyamine to remove the adsorbed $^{14}$C-polyamine. The amount of amine adsorbed to the cell surface and the filter was less than 0.1% of the added amine under these experimental conditions. The radioactivity on the filter was determined with a liquid scintillation counter. Initial polyamine uptake rates were determined from the linear increase of uptake and are expressed as nanomoles of polyamine taken up per minute per milligram protein as determined by the method of Bradford (1976) using bovine serum albumin as a standard. The osmolality of the uptake assay medium was measured with a Wescor vapor pressure osmometer model 5520. For the assay of inhibition by substrate analogues, cells were added to a mixture of labelled substrate and 20-fold excess of unlabeled analogues. In inhibitory assays, cells were

preincubated with the inhibitor for 30 min at 37ºC before the addition of $^{14}$C-polyamine.

#### 2.2.1.3 Sequence alignment and structural modelling of *Synechocystis* PotD

In order to recognize a template for reliable modelling of *Synechocystis* PotD, a structure-based sequence alignment of spermidine/putrescine binding protein PotD in *E. coli* and the putrescine receptor in *E. coli* (PDB codes 1POT and 1A99, respectively; Sugiyama *et al.*, 1996b; Vassylyev *et al.*, 1998) was made. The sequences of spermidine/putrescine binding proteins of *Synechocystis* (*slr0401*), *Trichodesmium erythraeum* (Q3HGU2), *Nostoc* sp. PCC *7120* (alr0299), *Thermosynechococcus elongatus* (tll0716) and *Synechococcus* sp. PCC *7942* (syc1832_d) were added to the alignment by program MALIGN (Johnson and Overington, 1993) and modelling package. Gap positions were refined manually. Based on the structural information the N-terminal region (Met1-Gln31) and a loop region (Ala83-Thr102) were not modelled, because the known structures lack these regions.

The *Synechocystis* PotD was modelled based on the crystal structure of *E. coli* PotD (PDB code 1POT) according to the refined structure-based alignment using the program MODELLER (Sali and Blundell, 1993). The spermidine ligand and the conserved water molecule in the active site were included in the model. A set of ten models were created and after a visual examination the model with the lowest objective function derived by MODELLER was chosen for further examination. The conformation of the loop region (Ala83-Thr102) was decided to be excluded from the model. The final model of *Synechocystis* PotD was compared to the crystal structure

of *E. coli* PotD by superimposing them by using program VERTAA (Johnson and Lehtonen, 2000) within the Bodil visualization and modeling package (Lehtonen *et al.*, 2004).

In order to find signal sequences the N-terminal sequence was checked with SignalP 3.0 (Bendtsen *et al.*, 2004). The secondary structures were predicted using PredictProtein (Rost, 1996). The sequence alignment figure was generated using the program ESPript 2.2 implemented in ENDscript 1.1 (Gouet *et al.*, 1999; Gouet and Courcelle, 2002).

### 2.2.2 Molecular biology methods

#### 2.2.2.1 Extraction of total RNA (Hot phenol method)

The *Synechocystis* sp. strain PCC 6803 cells at the logarithmic phase were harvested from 50 ml of culture ($OD_{730}$ about 1.0) by centrifugation(4000xg, 10 min), then the cells pellet was immediately frozen in liquid nitrogen. Total RNA was extracted by the hot phenol method. Pellet was thawed and freezed, then resuspended in 250 µl of resuspension buffer (10 mM sodium acetate buffer pH 4.5, 0.3 M sucrose), and 75 µl of 250 mM EDTA buffer, pH 8.0, incubated at room temperature for 5 min. After incubation, 375 µl of lysis buffer (2% SDS, 10 mM sodium acetate buffer pH 4.5) was added and incubated at 65$^{o}$C for 3 min. The sample was mixed with 700 µl of hot phenol (pH 6.5, 65$^{o}$C) for 3 min. Cool down the solution on a freeze aluminium block for 10 sec and centrifuged at 12,000 rpm for 5 min at room temperature. The upper phase was taken and repeated by adding hot phenol. After centrifuged at 12,000 rpm for 5 min, the mixture was extracted once with equal volume of Phenol:Chloroform:Isoamyl alcohol (25:24:1), mixed gently and centrifuged at 12,000 rpm for 5 min. The RNA was precipitated by adding 1/10

volume of 3 M sodium acetate buffer, pH 6.0 and 2.5 volume of cold ethanol (-20$^{o}$C), incubated at -20$^{o}$C for 30 min. After incubation, mixture was centrifuged at 12,000 rpm for 10 min (4$^{o}$C). The pellet was washed with 70% ethanol, centrifuged at 12,000 rpm for 2 min. After the pellet was dried, resuspened in 50 µl of storage buffer (20 mM sodium phosphate buffer, pH 6.5). To determine concentration and purity of RNA, sample was diluted with storage buffer and checked by measuring the optical density at 260 nm, then checked quantity of RNA by running 0.8% agarose gel in TAE buffer, finally kept at freezer -80 $^{o}$C until used.

**2.2.2.2 Preparation of total RNA**

To remove chromosomal DNA contamination, RNA sample were treated with RQ1 RNase-Free DNase (Promega Corporation). The reaction mixtion contained estimately 50 µg of total RNA, 5 µl of 10x Buffer, 5 µl of RQ RNase-Free DNase and adjusted with RNA storage buffer to a total volume of 50 µl. After an incubation for 3 hours, the mixture was added 200 µl of 40 mM Tris-HCl, pH 8.0 and extracted with 250 µl of Phenol:Chloroform:Isoamyl alcohol (25:24:1), mixed gently and centrifuged at 12,000 rpm for 5 min. The RNA was precipitated by adding 1/10 volume of 3 M sodium acetate buffer, pH 5.2 and 0.6 volume of isopropanol (-20$^{o}$C), incubated at -20$^{o}$C for 30 min. After incubation, mixture was centrifuged at 12,000 rpm for 10 min (4$^{o}$C). The pellet was washed with 70% ethanol, centrifuged at 12,000 rpm for 2 min. After the pellet was dried, resuspened in 15 µl of storage buffer (20 mM sodium phosphate buffer, pH 6.5). To determine concentration and purity of RNA, sample was diluted with storage buffer and checked by measuring the optical density at 260 nm, then checked quantity of RNA by running 0.8% agarose gel in TAE buffer, finally kept at freezer -80 $^{o}$C until used.

#### 2.2.2.3 Reverse Transcription-Polymerase Chain Reactions (RT-PCR)

One step RT-PCR was performed using Ready-To-Go You-Prime First-Strand Beads Kit (Amersham Biosciences). Bring 5 μg total RNA to a volume of 23 μl using MQ-water. Heat at 65 $^{o}$C for 10 min, then chill on ice for 2 min. Transfer the RNA solution to the tube of first strand reaction mix beads. Add 50 pmol of each 3′-antisense specific primer, designed on the basis of sequence data from CyanoBase (http://bacteria.kazusa.or.jp/cyanobase/), to a final volume 33 μl. Let sit at room temperature for approximately 1 min. Mix the contents by gently votexing, or by repeatedly pipetting the mixture up and down. Mixture was centrifuged briefly to collect the contents at the bottom of the tube. Incubate at 37 $^{o}$C for 60 min. The completed first strand reaction is now ready for immediate PCR amplification. PCRs were performed using DNA polymerase (DyNAzyme$^{TM}$ DNA Polymerase kit). The initial pre-denaturation at 95$^{o}$C for 5 min was done, followed by 34 cycles of denaturation at 95$^{o}$C for 1 min, annealing at 55$^{o}$C for 1 min and extension at 72$^{o}$C for 1.30 min, followed by final extension at 72$^{o}$C for 2 min. The intensities of PCR products were aanalyzed by electrophoresis and autoradiography of a 0.8% agarose gel in 1x TAE buffer. Quantification was carried out using AIS$^{TM}$ program.

#### 2.2.2.4 Stability of *potD* transcript

*Synechocystis* cell culture (10 μg chl/ ml) was incubated under growth-light and high temperature (42$^{o}$C) conditions in the presence of rifampicin (500 μg/ml), an inhibitor of transcriptional initiation. Aliquots were taken after 0, 15, 30,

60 and 120 min of incubation, frozen immediately in liquid nitrogen and subjected to RNA extraction and RT-PCR analysis. The intensities of PCR products were aanalyzed by electrophoresis and autoradiography of a 0.8% agarose gel in 1x TAE buffer. Quantification was carried out using AIS$^{TM}$ program.

**2.2.2.5 Chromosomal DNA extraction**

Total DNA was extracted from exponentially growing cultures of *Synechocystis sp.* PCC 6803 ($OD_{730}$ > 0.8). The pellets were collected by centrifuging at 4000xg for 10 min and resuspended in 535 µl of TE-Buffer pH 8.0 containing10 mM EDTA. Then 60 µl of 20%SDS and 60 µl of proteinase K (1 mg/ml) or 3 µl of proteinase K (20 mg/ml) were added into the mixture and incubated at 37 $^{o}$C for 2 hr. After incubation, 100 µl of 5M NaCl and 80 µl of CTAB:NaCl (10%CTAB in 0.7M NaCl) were added and incubated at 65$^{o}$C for 10 min. The lysate was then centrifuged (10 min, 10,000xg) to remove major amount of polysaccharides, and the clear supernatants (containing total DNA) were transferred to a new microcentrifuge tube. After that, 800 µl (1:1 volume) of Chloroform:Isoamyl alcohol (24:1) was mixed and centrifuged at 12,000 rpm, room temperature for 5 min. Kept viscous supernatant in new microcentrifuge tube, added 1:1 volume of Phenol:Chloroform:Isoamyl alcohol (25:24:1) and then centrifuged at 12,000 rpm, room temperature for 5 min. Kept supernatant in new microcentrifuge tube, added 0.6 volume of isopropanol then incubated on ice for 15 min and centrifuged at 12,000 rpm, 4$^{o}$C for 10 min. Pellets were kept and then added 70% ethanol. After gentle mixing by inversion, the mixture was centrifuged at 12,000 rpm, 4$^{o}$C for 2 min. Washed pellets were air dried at room temperature. 100 µl of TE-Buffer pH 8.0 was suspended and then checked by

monitoring concentration at optical density 260 nm (OD 1.0 = 50 μg/ml), while the purity was checked by the absorbance ratio A260/A280 (Sambrook and Russell, 2001). DNA sample was run in 0.8% agarose gel electrophoresis with 1xTAE buffer pH 8.0.

## 2.3 Methods for *Escherichia coli* strains

### 2.3.1 General methods

#### 2.3.1.1 Gel electrophoresis

DNA sample were analysed by electrophoresis in 0.8.0% agarose gel. The agarose gels were run in 1X TAE buffer. 1 μl of DNA loading buffer was added to DNA samples, which were run at 100 volts for 60 min. The DNA samples were stained for 10 min in distilled water containing ethidium bromide at a final concentration of 1 ng/ml. A Geldoc® enabled DNA visualisation UV light.

#### 2.3.1.2 Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE)

The sample was treated with the sample buffer for SDS-PAGE and boiled for 5 min prior to application to the gel and then loaded to a slap gel (12% of separating gel and 5% of stacking gel). The gel was placed into electrophoresis chamber and then electrophoresis buffer was added over the gel. The electrolysis was performed at the constant current of 10 mA per slab. The gel was stained with Coomassie blue.

### 2.3.2 Molecular biology methods

#### 2.3.2.1 Preparation of competent cells

A single, well isolated colony of TOP10 (or strain BL21) was inoculated into 10 ml of LB media and incubated at 37°C overnight with shaking at 250 rpm on a rotary shaker. A 2 ml aliquot of the overnight culture was transferred to 100 ml of LB media and grown until the $OD_{600\ nm}$ was between 0.6-0.8. The cells were chilled for 5 min on ice followed by 4°C centrifugation at 3,000xg for 10 min. The pellet was resuspended in 10% glycerol. Aliquot of 40 µl were stored immediately at -80°C.

#### 2.3.2.2 Restriction digestion

Since the expression vector pET-19b contains *Nde*I and *Bam*HI restriction sites, these two restriction sites were then incorporated into the primer used for the amplification of *potD* gene. The vector and clone of the amplified *potD* gene were digested with *Nde*I and *Bam*HI. Approximately 20 µg of DNA was digested in 50 µl reaction volume, containing 1× *Bam*HI buffer (150 mM KCl, 10 mM Tris-HCl, 10 mM $Mg_2Cl$, 1 mM dithiothreitol), 100 µg/ml BSA, and 10 units of *Bam*HI and *Nde*I. The reaction was incubated at 37 ºC overnight. After digestion with *Nde*I and *BamHI*, the pET-19b was extracted with an alkaline lysis extraction. The DNA pellet was dissolved in 20 µl of TE buffer. The DNA concentration was estimated using 0.8% agarose gel electrophoresis under UV transilluminator by comparing with the λ/*Hin*dIII marker. The digested pET-19b was used for the cloning of *potD* gene.

**2.3.2.3 Ligation**

A suitable molecular ratio between vector and inserted DNA in a mixture of cohesive-end ligation is usually 1:3. To calculate the appropriate amount of PCR product (insert) used in ligation reaction, the following equation was used:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb sized of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

The 10 µl ligation reaction was composed of 1 µl of 10× T4 DNA ligase buffer, 1 unit of T4 DNA ligase, 10 ng of *Nde*I *and Bam*HI digested pET-19b and 4 ng of *Nde*I *and Bam*HI digested amplified *potD* gene. The reaction was incubated overnight at 16 ºC overnight. One microlitre of the ligation mixture was transformed into the competent *E. coli* TOP10.

**2.3.2.4 Electrotransformation**

Competent cells were gently thawed on ice. 40 µl of cell suspension was mixed well with 1 µl of the ligation reaction, and placed on ice for 1 minute. The cells were transformed by setting the GENE pulser apparatus (Bio-RAD) as follows: 25 µF, 200 Ω of the pulse controller unit, and 2.50 kV. After one pulse was applied, the cells were immediately resuspended with 1 ml of LB broth. The cell suspension was incubated at 37 ºC with shaking at 250 rpm for 45 minutes. The cell suspension was spread on the LB agar plate containing 100 µg/ml ampicillin and incubated at 37 ºC for an overnight. After incubation, colonies were randomly selected for plasmid isolation.

**2.3.2.5 Alkaline lysis extraction of plasmids**

A single, well-isolated colony of the transformed TOP10 (or BL21) strain was inoculated into 2 ml of LB media containing ampicillin and grown overnight with shaking at 250 rpm on rotary shaker. The entire culture was centrifuged for 30 s at 12,000 rpm. The pellet was resuspended in 100 µl of Solution I to which 20µg/ml RNase was added and incubated at room temperature for 5 min. The sample was placed on ice for 5 min, 200 µl of Solution II was added, shaken vigorously and further incubated on ice for 5 min. This was followed by the addition of 150 µl of Solution III and 5 min incubation on ice. After centrifuging for 5 min at 12,000 rpm, approximately 350 µl of the supernatant was transferred to a fresh microcentrifuge tube and extracted with 100 µl of 1:1 Phenol:Chloroform:Isoamyl alcohol(25:24:1), mix. The sample was centrifuged for 5 min at 12,000 rpm; the 300 µl upper phase was transferred to a new microcentrifuge tube. After that 180 µl of 100% isopropanol was added to the sample and incubated at room temperature for 10 min. The sample was centrifuged for 10 min at 12,000 rpm, then the supernatant was discarded and the pellet was washed with 800 µl of cold 70% ethanol and dried at 37$^{o}$C. The final pellet was resuspended in 50 µl of TE buffer. One µl of this sample was then checked by running on a 0.8% agarose gel in 1x TAE buffer.

**2.3.2.6 Isolation of recombinant plasmids**

The recombinant plasmid containing *potD* gene was selected by restriction enzyme digestion using *Nde*I *and Bam*HI. The digested plasmid was analyzed by 0.8% agarose gel electrophoresis. The clone containing the correct DNA fragment of approximately 1,150 bp for full-length of *potD* were selected. DNA

sequencing was performed to confirm the correct junction of the vector and the inserted DNA as well as the sequence of the *potD* gene.

#### 2.3.2.7 Induction and expression of PotD protein

After the correct expression clone was identified, a recombinant *potD* gene was used for transformation into the expression host. The expression hosts used in this experiment were *E. coli* strains BL21 (DE3) pLysS. A single colony was picked from the spread plate and inoculated in 10 ml LB containing 100 μg/ml of ampicillin in 125 ml Erlenmeyer flask. The culture was incubated with shaking at 37 ºC until the $OD_{600}$ reached 06-0.8. Consequently, cell culture was inoculated in the 500 ml of LB medium containing ampicillin. The culture was shaken at 37 ºC, when the optical density at 600 nm ($OD_{600}$) reached 0.6-0.8 (approximately 2-3 hours), induction of PotD was achieved by addition of isopropyl-D-thiogalactoside (IPTG) to a final concentration of 1 mM. One milliliter of culture medium was collected after induction at 0, 1, 2, 3, 6 hours. The pellets were collected by centrifugation at 5,000 rpm for 10 minutes at 4 ºC, and then lysed by adding cold lysis buffer (20 mM phosphate, pH 7.0, 3 mM EDTA). Both supernatant and the soluble protein pellet (or inclusion bodies) were analyzed by Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE).

#### 2.3.2.8 Ni-Sepharose column purification

The PotD-inclusion bodies were suspended in working buffer containing 8 M urea, 20 mM phosphate pH 7.4, 20 mM imidazole and 500 mM NaCl. Mixture was shaken at 4$^{o}$C and then purified with Ni-Sepharose column kit (His GraviTrap/His GraviTrap Kit, Amersham bioscience) used according to the

manufacturer's protocol. Briefly, Mixture sample was loaded onto nickel-chelated resin columns previously equilibrated with working buffer. The protein was eluted with buffer containing 8 M urea, 20 mM phosphate pH 7.4, 500 mM imidazole and 500 mM NaCl. Fractions (3 ml) were collected and analyzed by Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE) to identify the fractions containing PotD. The selected fractions were dialyzed extensively against phosphate-buffered saline pH 7.4, PBS (10 mM sodium phosphate pH 7.0 and 150 mM NaCl) to remove the imidazole, and the protein was stored at -20°C.

**2.3.2.9 Immuno blotting analysis**

First a polyacrylamide gel (SDS) has to be run to denaturable and separate the proteins in the sample according to their molecular mass. It is recommended to run two gels, staining one directly and using the other for immunoblotting. Proteins were transferred from the gel onto nitrocellulose membrane following instructions provided by the transfer system manufacturer. After the transfer, the gel can be stained with Coomassie blue stain to make sure that the transfer to the membrane has been completed. Membrane was immediately placed into the blocking buffer (phosphate buffer saline, 0.05% Tween-20 and 5% skim milk) for an overnight with shaking at 37$^{o}$C. Subsequently, the blot was incubated with primary antibody in the Antibody Binding Buffer (phosphate buffer saline, 0.05% Tween-20 and 1% skim milk) with shaking at 37°C for one hour. After incubation of the membrane with the primary antibody (1:3000), the blot was washed three times in PBST (phosphate buffer saline, 0.05% Tween-20 and 1% skim milk), each wash for 10 minutes. The blot was incubated with the secondary antibody

conjugated anti-rabbit immunoglobulin G (1:5000) for one hour with shaking at room temperature. After incubation of the secondary antibody, the blot was washed three times in PBST, each wash for 10 minutes. The blot was following the chemiluminescence alkaline phosphatase substrate instruction. The assay buffer contains 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 10 mM $MgCl_2$, 33 µl of BCIP and 88 µl of NBT. Membrane was transferred and incubated shaking with assay buffer.

# CHAPTER III

# RESULTS

## 3.1 Characterization of polyamine transport of *Synechocystis* cells

### 3.1.1 Protection against growth inhibition by polyamines

*Synechocystis* grown in the BG-11 medium supplemented with polyamines, namely putrescine and spermidine, resulted in a retardation of growth. The results shown in Figure 9 revealed that the addition of 0.5 mM putrescine did effect to normal growth up to 6 days. The addition of 1.0 mM and 2.0 mM putrescine caused similar growth pattern to that of 0.5 mM putrescine during the first 3 days. Slower growth occurred after 3 days with the apparent cessation of growth observed after 6 days. Subsequently, *Synechocystis* was grown in the medium containing 550 mM NaCl (Figure 10). It was observed that the growth of *Synechocystis* was more affected by 550 mM NaCl. Interestingly, an external concentration of putrescine at 0.5 mM supplemented into the medium could alleviate the effect of 550 mM NaCl with apparently normal growth up to 6 days. Conversely, the higher concentration of putrescine showed a large decrease of *Synechocystis* growth after 6 days (Figure 10). The results in Figure 11 showed the role of external concentration of spermidine on growth of *Synechocystis*. It was observed that the elevation of external concentration of spermidine from 0.5 mM to 2.0 mM resulted in the higher retardation on growth of *Synechocystis* after 3 days (Figure 11). The addition of 0.5 mM spermidine to the growth medium relieved the inhibition of growth in *Synechocystis* by 550 mM NaCl up to 3 days similar to that found in the presence of putrescine. This release of growth

inhibition was not observed at 1.0 mM and 2.0 mM spermidine presented in the medium. Moreover, after 3 days the cessation of growth was noticeable in these cases (Figure 12).

### 3.1.2 Time course of polyamine transport by *Synechocystis* cells

Polyamine transport by *Synechocystis* cells was determined at interval time for 30 min (Figure 13 and Figure 14). Changes in polyamine uptake activity could be observed in the presence of NaCl compared to control that disappearance of NaCl. The initial rate of uptake both by $^{14}$C-putrescine and $^{14}$C-spermidine were roughly first 5 min. Firstly, slight increase in putrescine uptake was observed in the presence of NaCl in range from 1 to 50 mM compared to control (Figure 13). The addition of higher concentration of NaCl caused similar putrescine uptake pattern to that of lower concentration during the first 3 min. Elevation of NaCl concentration from 150 to 550 mM resulted in decrease of putrescine uptake as shown in Figure 13. Likewise, in the presence of low concentration of NaCl ranging from 1 to 50 mM resulted in slight increase in spermidine transport Figure 14. The addition of higher concentration of NaCl caused similar spermidine uptake pattern to that of lower concentration during the first 3 min. The increasing concentration of NaCl from 150 to 550 mM effected to spermidine transport shown in Figure 14. These results suggested that a moderate concentration of NaCl enhanced the transport of polyamines.

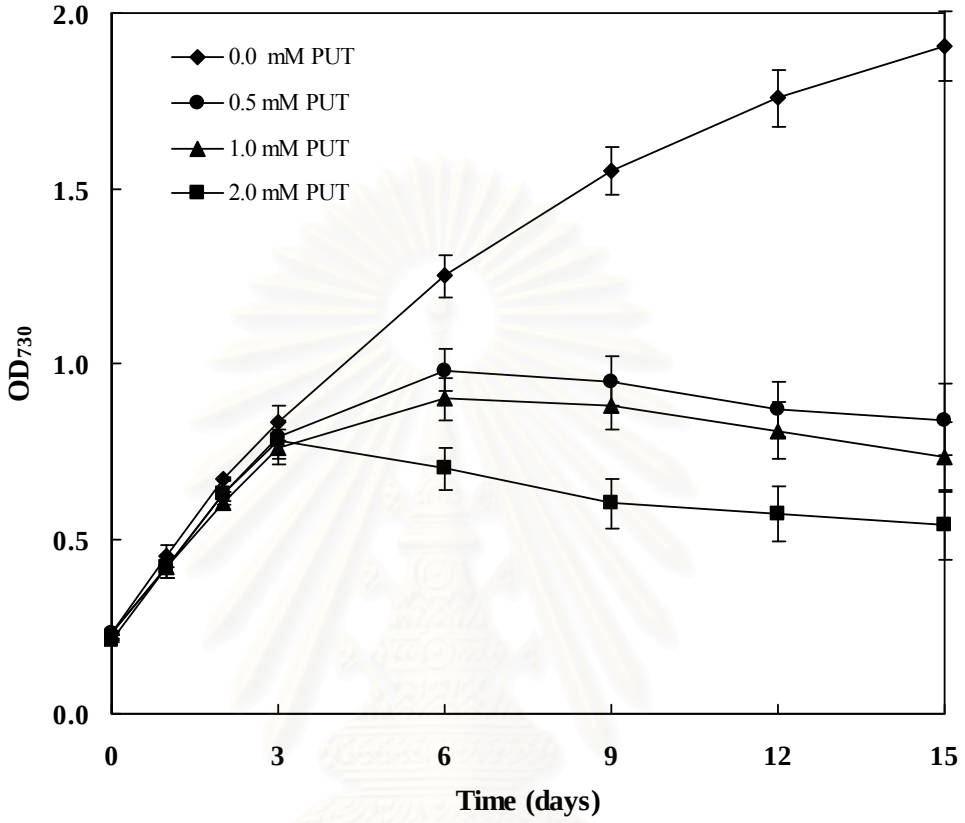


**Figure 9** Growth of *Synechocystis* sp. PCC 6803 in the presence of polyamine putrescine (PUT). Cells were grown under non-stress conditions. The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3.

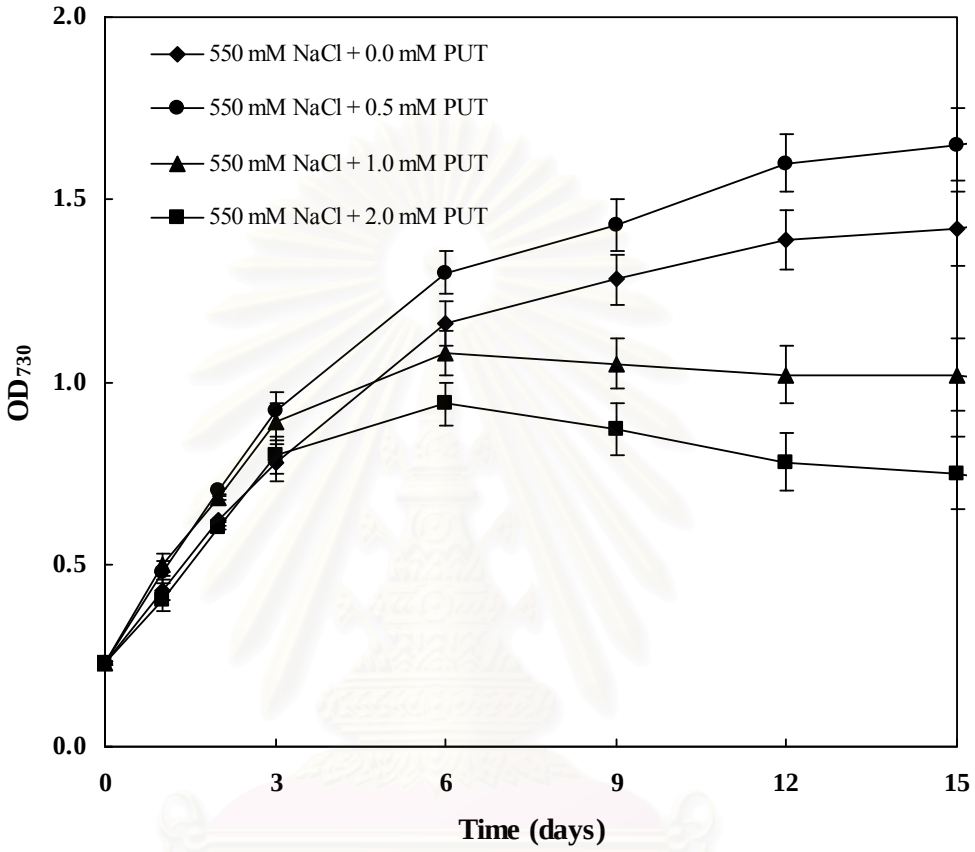


**Figure 10** Protection against growth inhibition by putrescine (PUT) in *Synechocystis* sp. PCC 6803. Cells were grown under salt stress (550 mM NaCl). The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3.

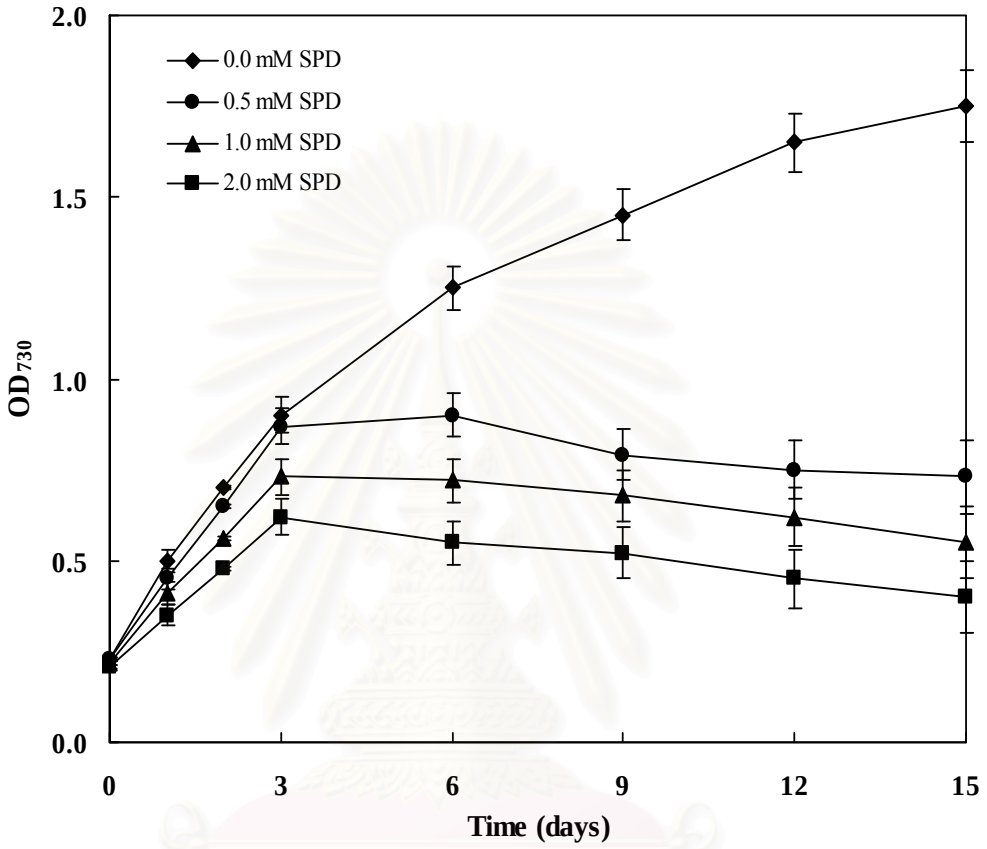


**Figure 11** Growth of *Synechocystis* sp. PCC 6803 in the presence of polyamine spermidine (SPD). Cells were grown under non-stress conditions. The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3.

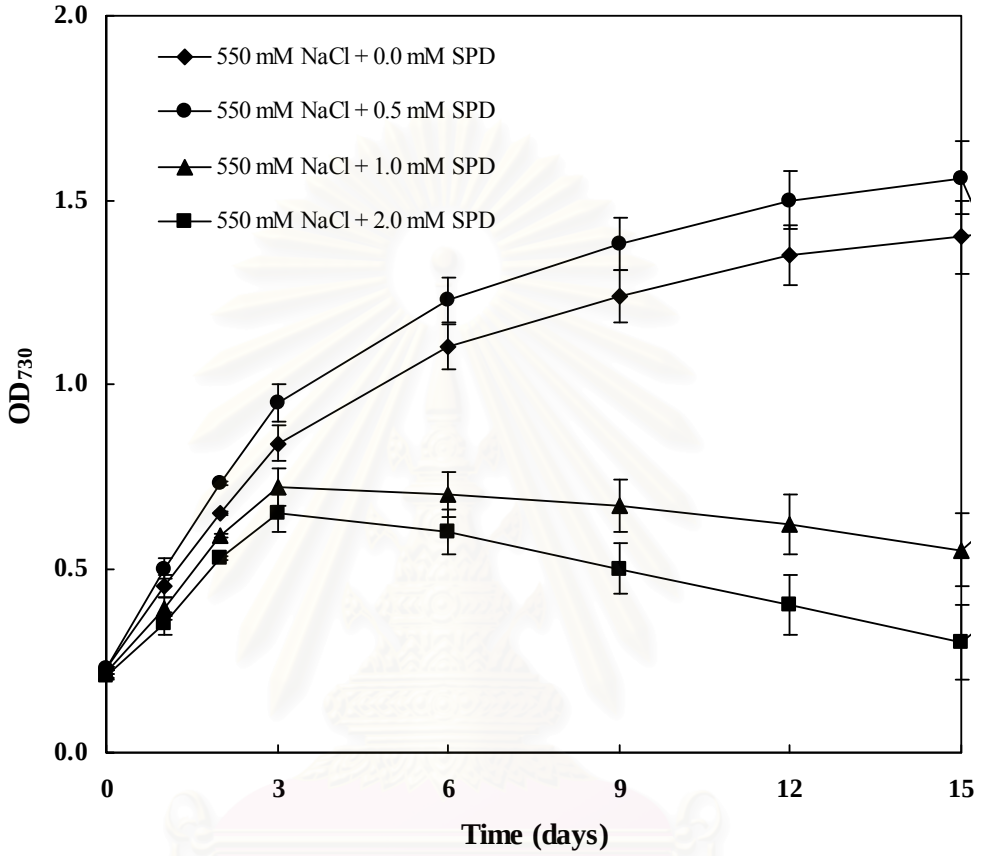


**Figure 12** Protection against growth inhibition by spermidine (SPD) in *Synechocystis* sp. PCC 6803. Cells were grown under salt stress (550 mM) NaCl. The data are means from three independent experiments with vertical bars representing standard errors of the means, n=3.

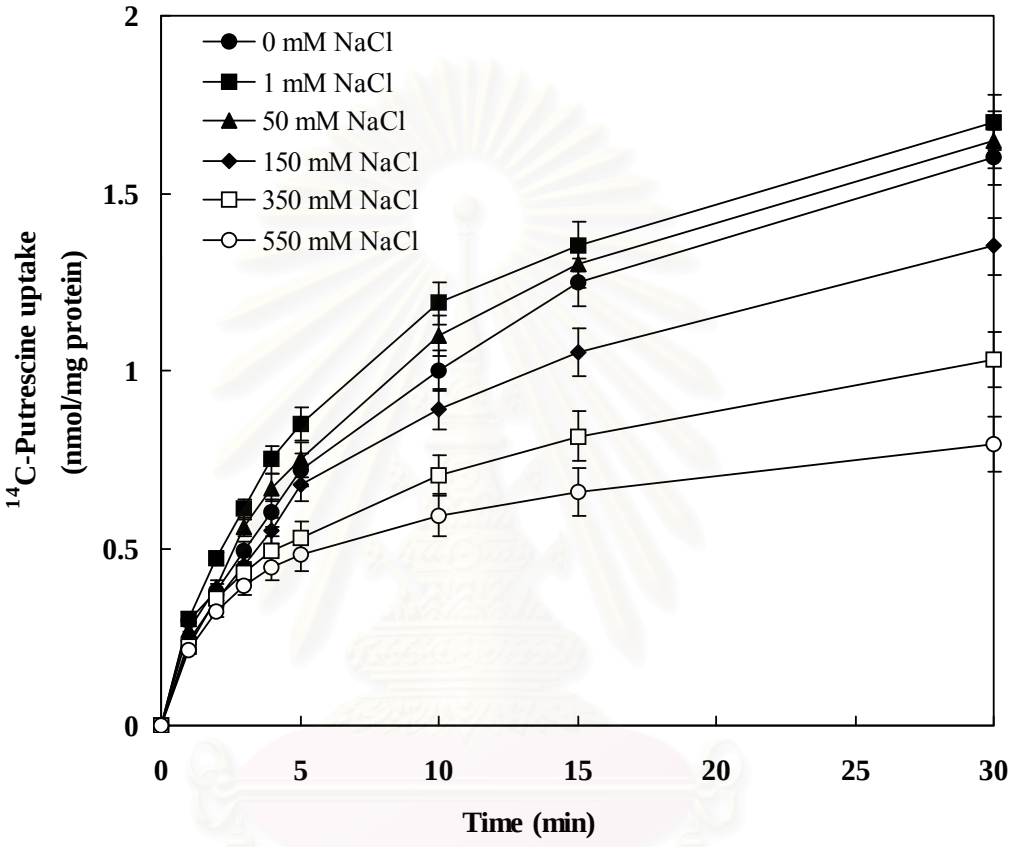


**Figure 13** Time intervals of putrescine uptake by *Synechocystis* sp. PCC 6803 in the absence and presence of varying concentration of NaCl. The data represents mean ± SE, n=3.

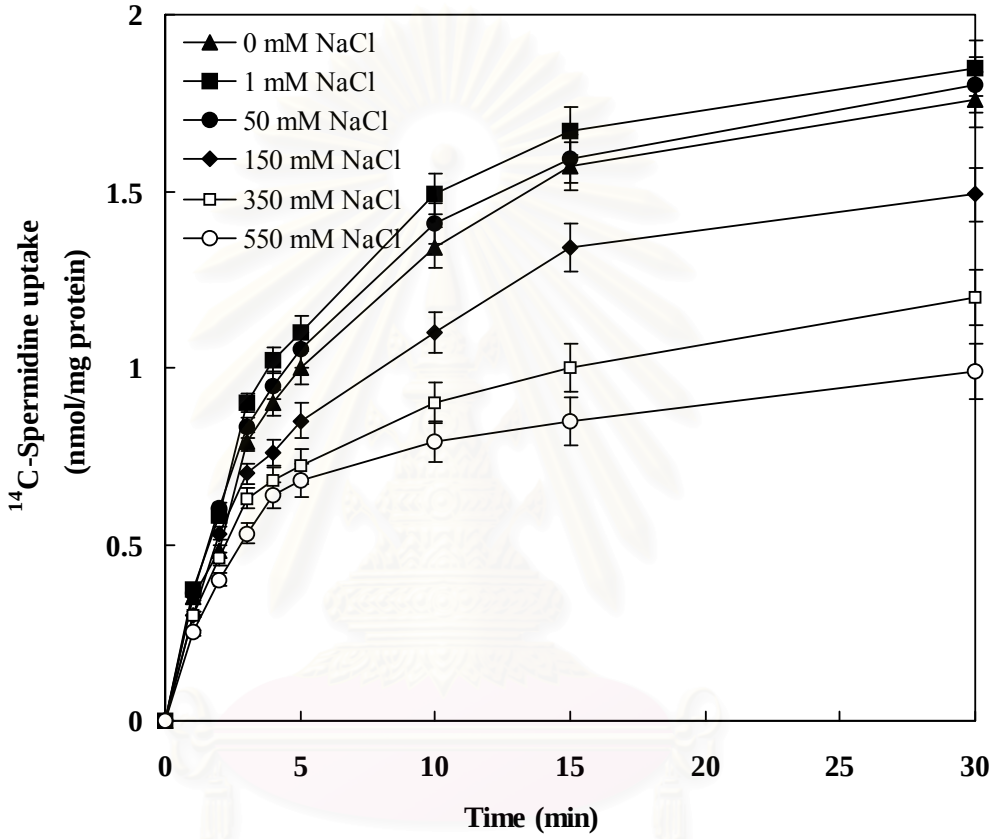


**Figure 14** Time intervals of spermidine uptake by *Synechocystis* sp. PCC 6803 in the absence and presence of varying concentration of NaCl. The data represents mean ± SE, n=3.

### 3.1.3 Saturation kinetics of polyamine uptake

Incubation of *Synechocystis* cells with varying each polyamine concentration from 0 to 500 μM resulted in saturable initial uptake (Figure 15 and Figure 16). A Lineweaver-Burk transformation yielded a straight line indicating that the uptake follows typical Michaelis-Menten kinetics. The apparent affinity constant ($K_m$) value for putrescine transport was 98 ± 10 μM and the maximal velocity ($V_{max}$) value was 0.33 ± 0.03 nmol/min/mg protein shown in Figure 15, while spermidine transport revealed the apparent affinity constant ($K_m$) value of 67 ± 9 μM and the maximal velocity ($V_{max}$) value of 0.45 ± 0.03 nmol/min/mg protein shown in Figure 16.

### 3.1.4 Influence of extracellular pH on polyamine transport

In addition to pH which can affect the transport reaction, this parameter must be considered and optimized in order to the transport reaction to be accurate and reproducible. Since polyamine carries a net positive charge, we therefore further tested whether the changes in external pH could affect its uptake. *Synechocystis* cells were incubated over a range of extracellular pHs and the initial rates of polyamines uptake was determined. Studies of putrescine transport of *Synechocystis* as a function of extracellular pH ranging from 4.0 to 9.0. The uptake assay was done with the modification using 100 mM Potassium acetate buffer pH 4.0-6.0, and 100 mM Tris-HCl buffer pH 7.0-9.0. The result showed that the optimal conditions for transport of putrescine were 7.0. Even though, the putrescine uptake was reduced with the decrease and increase of external pH, the uptake where alkaline conditions had more activity than that activity where acidic conditions shown in Figure 17. Additionally, spermidine transport among the pH values (pH 4.0-11.0) was tested. The uptake was

done with the modification using 100 mM Potassium citrate buffer pH 4.0-6.0, 100 mM Tris-HCl pH 7.0-8.0 and 100 mM Glycine-KOH pH 9.0-11.0. The highest uptake of spermidine transport of *Synechocystis* occurred at neutral pH of 8.0. Even though the uptake of spermidine decreased with the decrease and increase of external pH, the uptake where alkaline conditions had also more activity than that activity where acidic conditions shown in Figure 18. These results suggested that polyamine transport system was regulated by mechanisms related to their pH.

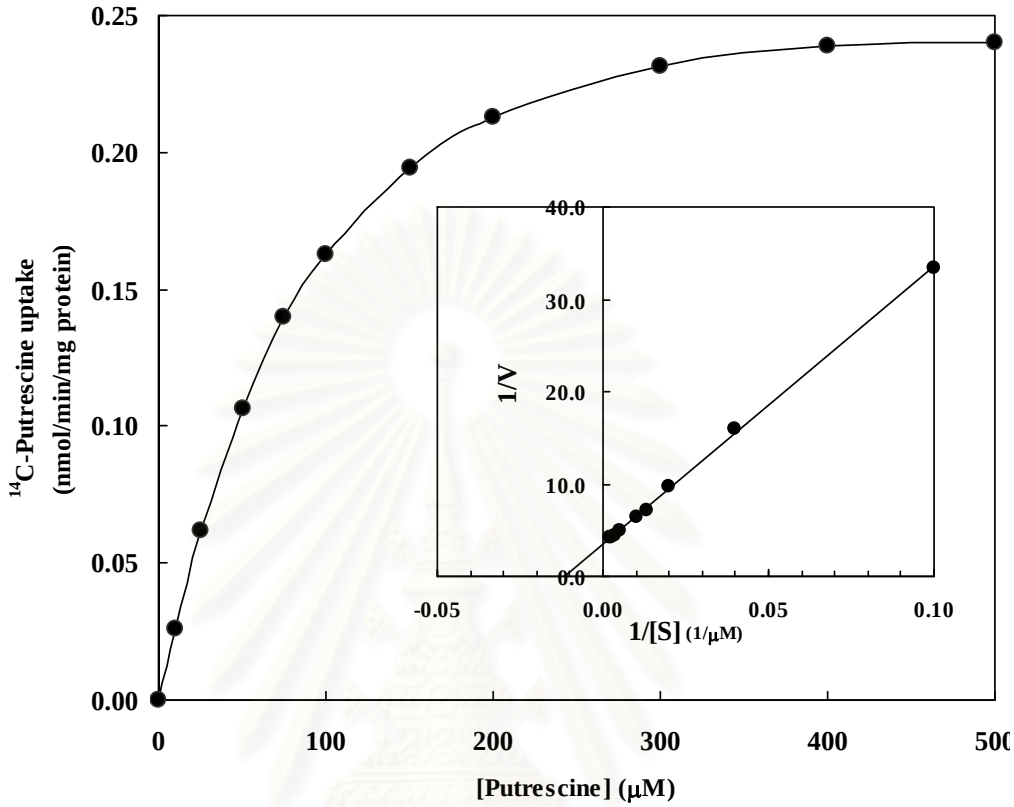


**Figure 15** Kinetics of putrescine uptake by *Synechocystis* sp. PCC 6803 incubated with 0-500 μM putrescine. Inset represents a Lineweaver-Burk transformation of the data. The line drawn is that derived from regression analysis of the data and the points shown are typical of this result from triplicate experiments.

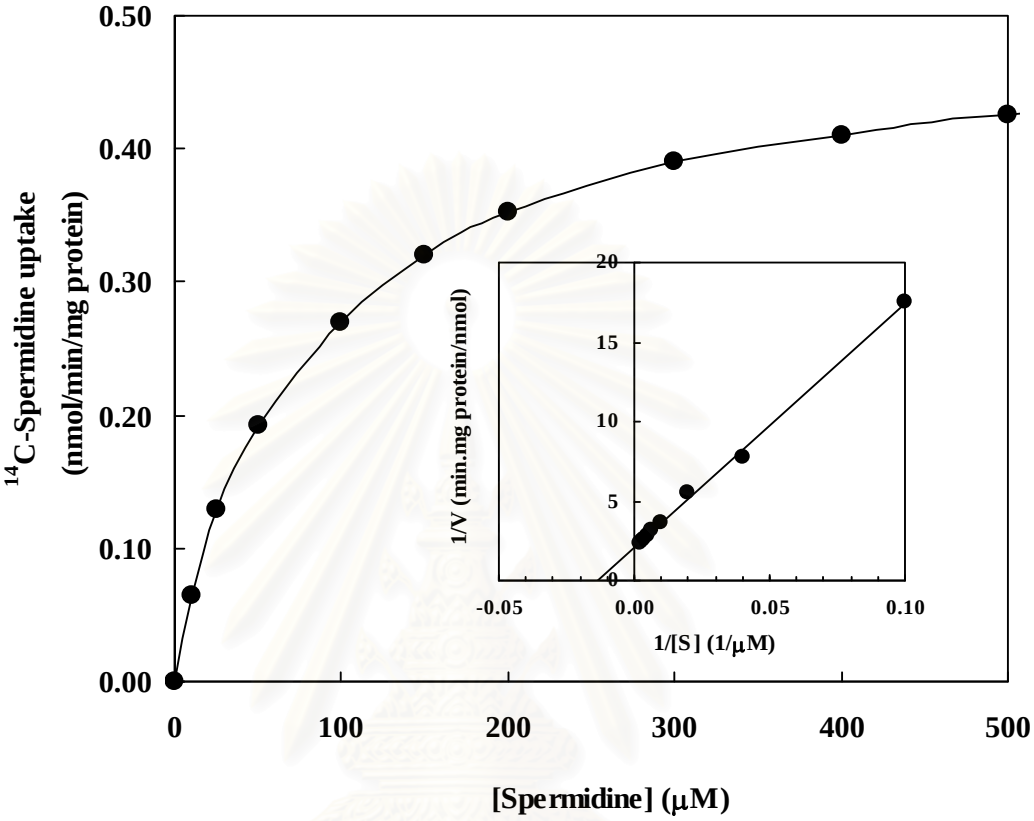


**Figure 16** Kinetics of spermidine uptake by *Synechocystis* sp. PCC 6803 incubated with 0-500 μM spermidine. Inset represents a Lineweaver-Burk transformation of the data. The line drawn is that derived from regression analysis of the data and the points shown are typical of this result from triplicate experiments.

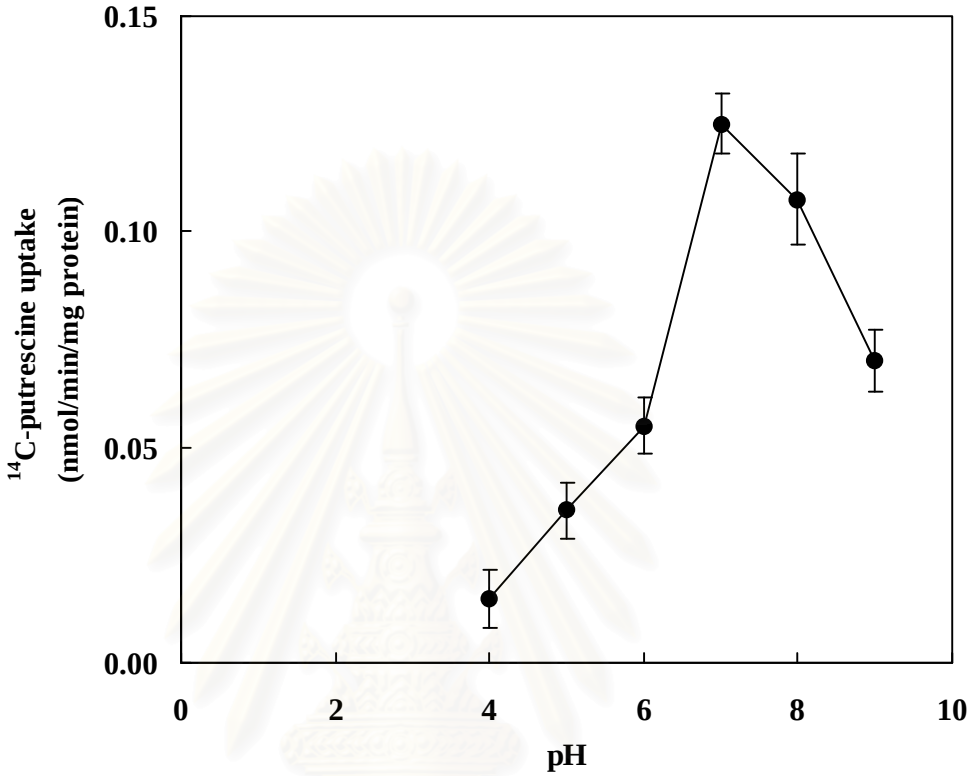


**Figure 17** pH response of putrescine transport in *Synechocystis* sp. PCC 6803. The uptake assay was done with the modification using 100 mM Potassium acetate buffer pH 4.0-6.0, and 100 mM Tris-HCl buffer pH 7.0-9.0. The data are means from three independent experiments with vertical bars representing standard errors of the means, n = 3.

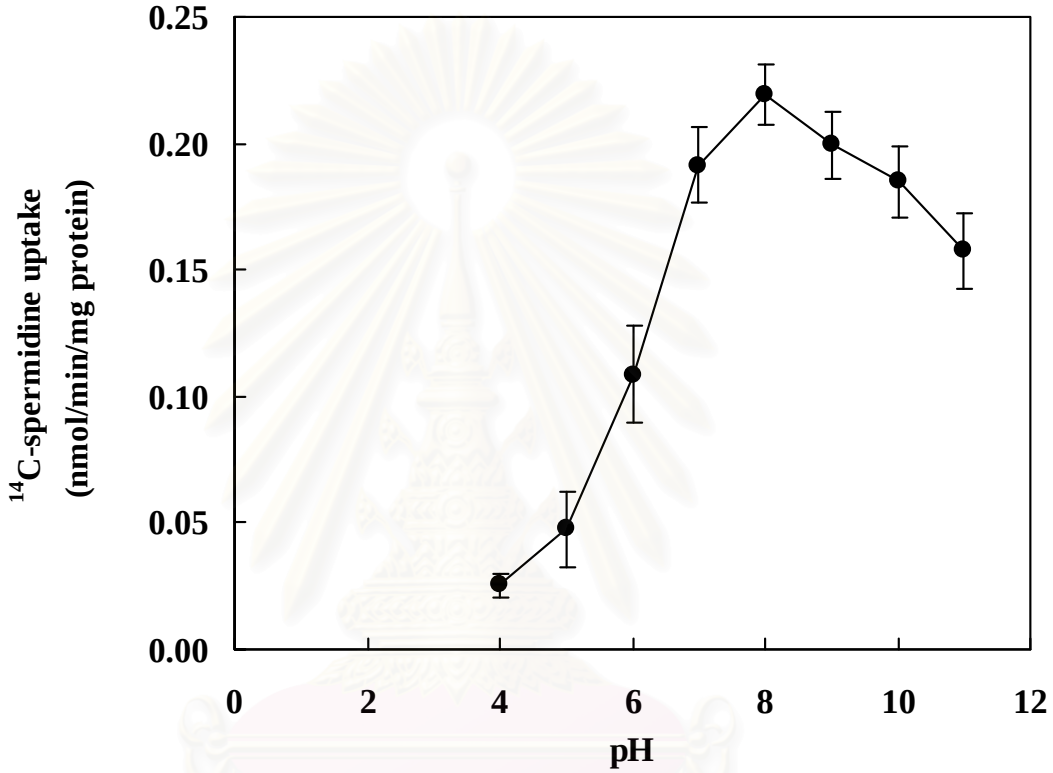


**Figure 18** pH response of spermidine transport in *Synechocystis* sp. PCC 6803. The uptake was done with the modification using 100 mM Potassium citrate buffer pH 4.0-6.0, 100 mM Tris-HCl pH 7.0-8.0 and 100 mM Glycine-KOH pH 9.0-11.0. The data shown are the means of three independent experiments with vertical bars representing standard errors of the mean, n=3.

### 3.1.5 Specificity of the polyamine transport

The specificity of polyamine uptake in intact cells was studied by addition of potential competitive substrates into the assay medium and following the rate of uptake. As shown in Table 3, agmatine, a biogenic amine formed by the decarboxylation of arginine, had no effect on putrescine and spermidine uptake. Lysine, a molecule comparable in size and charge with spermidine, basic amino acid asparagine, did not inhibit putrescine and spermidine uptake. Very slight inhibition of putrescine and spermidine uptakes occurred in the presence of ornithine, a precursor of polyamines, and other amino acids, namely alanine, cysteine, glycine, glutamic acid, serine, and valine *etc*. Cadaverine, a diamine found in plant but not as widely distributed as putrescine, did not inhibit putrescine and spermidine uptake. On the other hand, spermidine and spermine, which are structurally similar to putrescine but with additional amino groups, showed 40 and 60% inhibition putrescine transport, respectively. In the same way putrescine and spermine which are structurally similar to spermidine showed 40 and 50% inhibition, respectively.

We further assessed the transporter specificity through the competitive inhibition by measuring the spermidine uptake rate. The plot between inhibitor concentrations and the slopes obtained from double reciprocal plots of spermidine concentrations and uptake rates yielded the approximate value of inhibition constant, $K_i$. The $K_i$ values of putrescine and spermine were 291.6 and 431.6 μM which were about 4- and 6-fold higher than $K_m$ for spermidine. Inhibition of spermidine transport by either putrescine or spermine was found to be non-competitive (Figure 19), indicating that the uptake of spermidine does not share the same transport system with that for the uptake of putrescine or spermine.

**Table 3** Effect of polyamine analogues on the polyamine uptake of *Synechocystis* sp. PCC 6803

| **Analogues** | **Polyamine transport (%)** [a] | |
|---|---|---|
| | **Putrescine** | **Spermidine** |
| None | 100 ± 2 | 100 ± 2 |
| Agmatine | 98 ± 2 | 59 ± 6 |
| Asparagine | ND | 91 ± 5 |
| Alanine | 91 ± 4 | ND |
| Cadaverine | 88 ± 4 | 87 ± 7 |
| Cysteine | 66 ± 5 | ND |
| Glutamic acid | 85 ± 5 | ND |
| Glycine | 87 ± 3 | 89 ± 4 |
| Ornitine | 67 ± 5 | 87 ± 3 |
| Phenylalanine | 87 ± 3 | ND |
| Putrescine | ND | **62 ± 5** |
| Serine | 84 ± 3 | ND |
| Spermidine | **60 ± 5** | ND |
| Spermine | **40 ± 6** | **51 ± 6** |

[a]Cells were incubated in the mixture containing 1 mM unlabeled analogue and 50μM $^{14}$C-putrescine or $^{14}$C-spermidine. The data shown are the means of three independent experiments representing the percent uptake rate relative to the control rate which was 0.12 and 0.19 nmol/min/mg protein for putrescine and spermidine, respectively. (ND means no data)

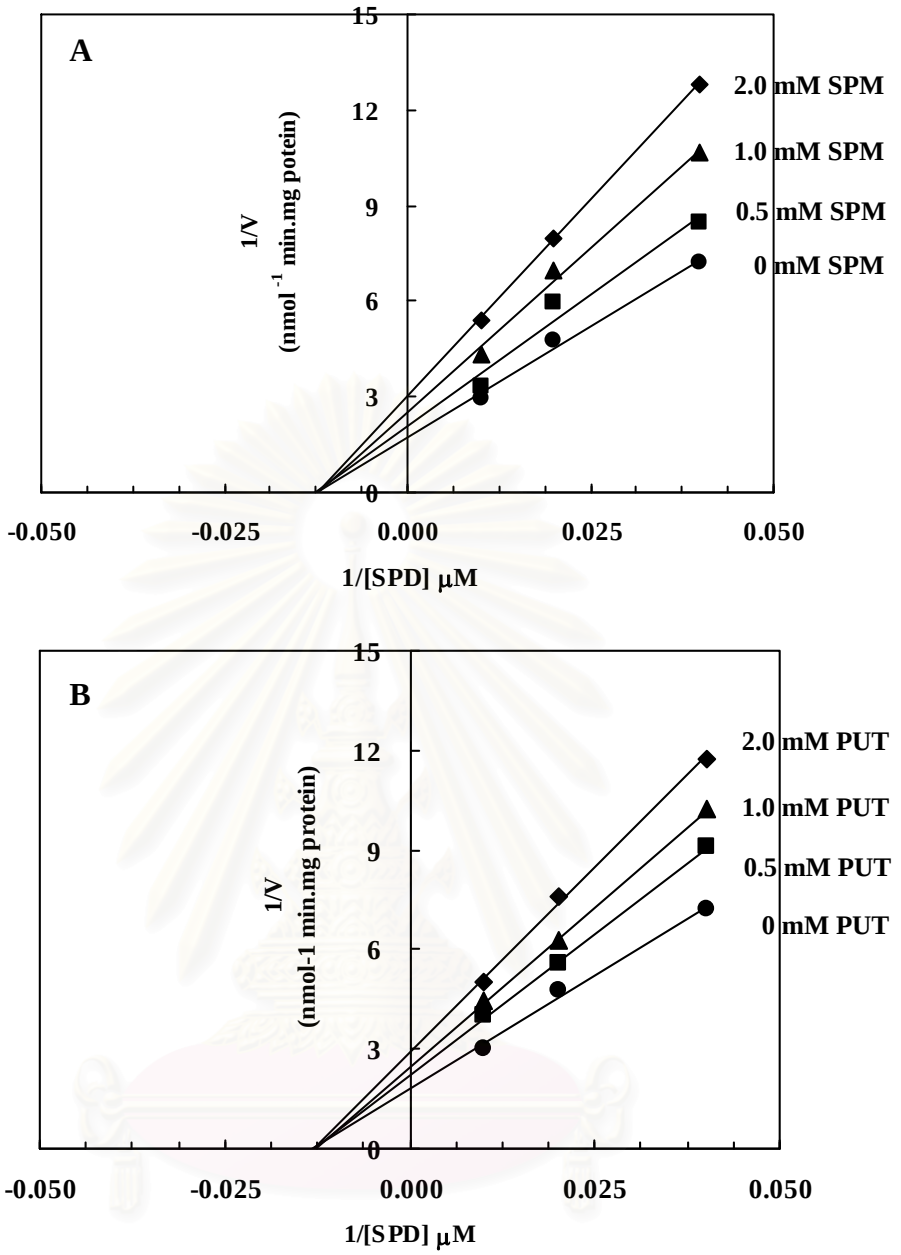


**Figure 19** Lineweaver-Burk plots for the inhibition of spermidine transport in *Synechocystis* sp. PCC 6803 by putrescine (A) and spermine (B). The inhibition kinetics were determined without inhibitors (●) and with three different concentrations; 0.5 mM (■), 1.0 mM (▲) and 2.0 mM (◆) of putrescine and spermine. Inhibition was found to be non-competitive with apparent $K_i$ values of 291.6 and 431.6 μM, respectively.

### 3.1.6 Effect of metabolic inhibitors, ionophores and ATPase inhibitors on polyamine transport

To evaluate the role of ATP and proton motive force in energizing the uptake of polyamines, the effects of some inhibitors on polyamines uptake were investigated. As shown in Table 4, *N*-ethylmaleimide (NEM) and *p*-chloromercurisulfonic acid (PCMS), which are the protein structure modifiers, markedly reduced both of putrescine and spermidine uptakes. The inhibitors for ATP formation, sodium arsenate and sodium fluoride also decreased both uptakes activity suggesting the requirement of ATP for polyamine transport. The role played by electrochemical gradient on polyamine transport was assessed by disrupting the transmembrane potential. Potassium cyanide, an inhibitor of the electron transport chain caused effective inhibition of polyamine uptake. Transport uncouplers such as gramicidin D and dinitrophenol, which dissipate proton motive force, could significantly inhibit spermidine and putrescine uptake to a similar extent by about 40-45 % and 60-65 %, respectively. Valinomycin, an ionophore which permeabilises the membrane to potassium and has been proposed to affect the $\Delta\psi$, strongly inhibited both polyamine uptakes. Similarly strong inhibition was also observed by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and nigericin.

*N, N'* dicyclohexylcarbodiimide (DCCD), a specific inhibitor for the $H^+$-ATPase, and orthovanadate, an inhibitor of P-type ATPase also effectively inhibited both of spermidine and putrescine uptake activities. Furthermore, these uptakes were inhibited by amiloride, a potent inhibitor of many $Na^+$-coupled transport systems including $Na^+/H^+$ antiporter. Reagents, which interfere with the transmembrane sodium ion gradient (monensin, a sodium ionophore; ouabain, an inhibitor of the plasma membrane $Na^+/K^+$-ATPase) caused an effective reduction of both systems.

Judging from the sensitivity of polyamine transport to various types of inhibitors, this system was ATP-dependent requiring proton motive force with the contribution of both ΔpH and Δψ.

### 3.1.7 Effect of external NaCl and sorbitol on polyamine transport

In order to find out whether the polyamine transport in *Synechocystis* is affected by the external either NaCl or sorbitol, the effect of either on uptake was extensively studied. The results revealed that both of putrescine and spermidine uptake activities were very sensitive to NaCl and sorbitol. Firstly, increasing the concentration of NaCl up to 10 mM resulted in the stimulation of putrescine uptake compared to the control. When the concentration of NaCl was higher than 50 mM, putrescine uptake was dramatically declined (Figure 20). The same result increasing the concentration of sorbitol up to 20 mM, putrescine uptake was significantly increased. However, elevation of sorbitol higher than 100 mM also drastically reduced putrescine uptake (Figure 21). Secondly, increasing the concentration of NaCl up to 5 mM did stimulate spermidine uptake compared to the control, nevertheless the reduction of spermidine uptake was detected when the concentration of NaCl was higher than 50 mM (Figure 22). Likewise, increasing the concentration of sorbitol up to 10 mM resulted in increase of spermidine uptake. Increasing the concentration of sorbitol higher than 100 mM did considerably affect to spermidine uptake (Figure 23).

**Table 4** Effect of metabolic inhibitors on the polyamine transport of *Synechocystis* sp. PCC 6803 [a]

| Inhibitors | Concentration | Polyamine transport[a] (%) | |
|---|---|---|---|
| | | Putrescine | Spermidine |
| None | - | 100 ± 2 | 100 ± 2 |
| Chloramphenicol | 1 μg/ml | 63 ± 5 | 64 ± 9 |
| Nigericin | 10 μM | 44 ± 5 | 60 ± 13 |
| Valinomycin | 10 μM | 9 ± 3 | 48 ± 4 |
| Amiloride | 100 μM | 11 ± 4 | 57 ± 8 |
| CCCP | 100 μM | ND | 63 ± 3 |
| DCCD | 100 μM | 26 ± 5 | 68 ± 7 |
| Gramicidin D | 100 μM | 42 ± 5 | 56 ± 1 |
| Monensin | 100 μM | 37 ± 5 | 50 ± 2 |
| Sodium ionophore | 100 μM | ND | 57 ± 5 |
| Sodium arsenate | 1 mM | 13 ± 2 | 70 ± 7 |
| KCN | 1 mM | 39 ± 7 | 43 ± 6 |
| DNP | 1 mM | 32 ± 6 | 56 ± 6 |
| NaF | 1 mM | 26 ± 2 | 51 ± 8 |
| NEM | 1 mM | 38 ± 5 | 48 ± 13 |
| Ouabain | 1 mM | 20 ± 5 | 53 ± 17 |
| PCMS | 1 mM | 20 ± 5 | 45 ± 10 |
| Orthovanadate | 1 mM | 25 ± 2 | 54 ± 7 |
| Sodium azide | 1 mM | ND | 56 ± 2 |

[a]Cells were preincubated with inhibitors for 30 min before the addition of 50 μM $^{14}$C- labeled polyamine to initiate the uptake as described in Materials and methods. The data shown are the means of three independent experiments representing the percent uptake rate relative to the control rate which was 0.12 ± 0.02 and 0.19± 0.02 nmol/min/mg protein for putrescine and spermidine, respectively. (ND means no data)

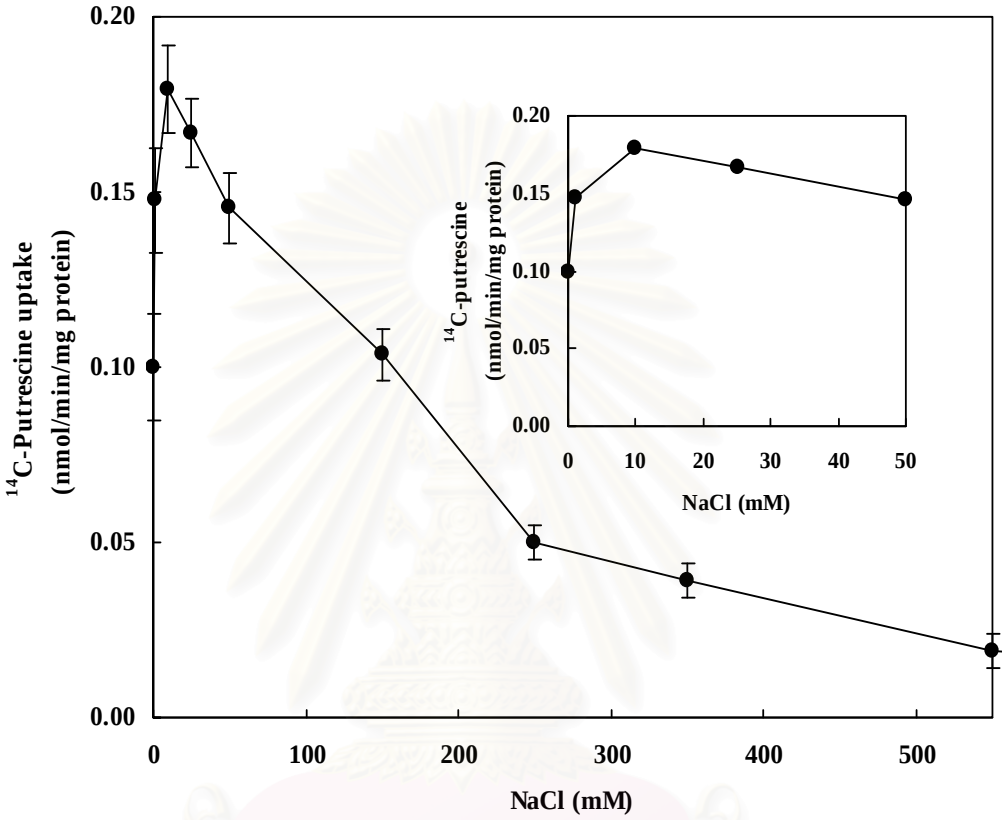


**Figure 20** Effect of external NaCl on putrescine uptake by *Synechocystis* cells. Initial uptake rates were determined in the presence of increasing NaCl concentration from 0-550 mM. The data are means from three independent experiments with vertical bars representing standard errors of the means, n = 3.

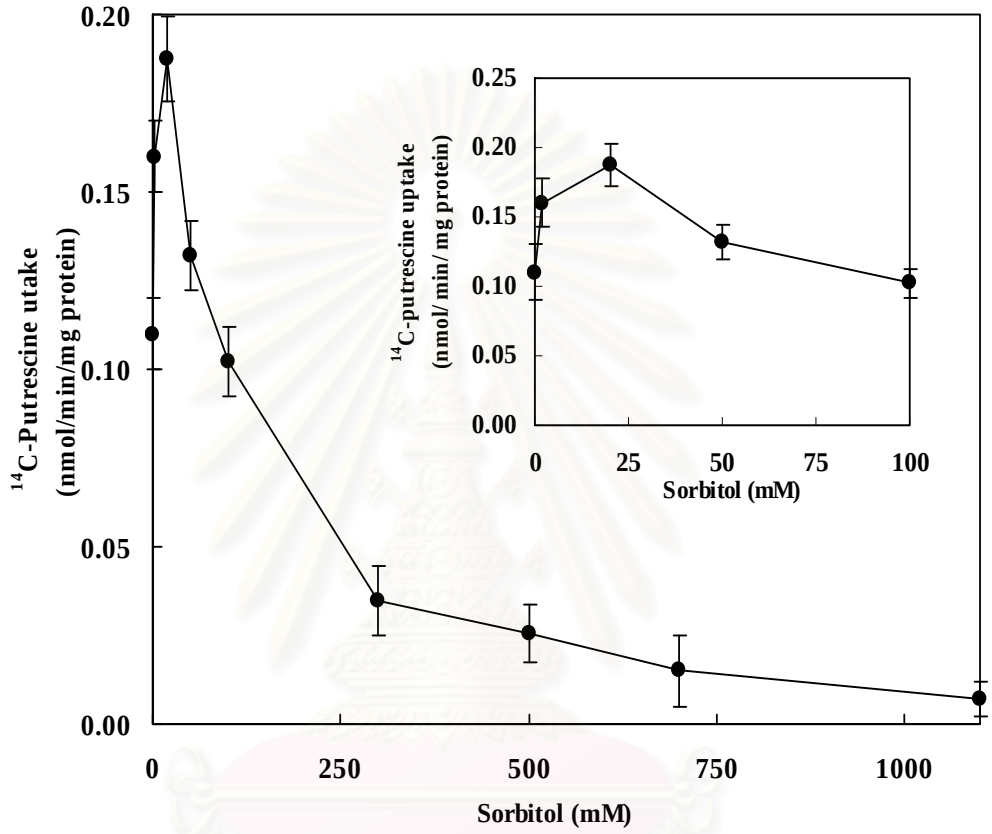


**Figure 21** Effect of external sorbitol on putrescine uptake by *Synechocystis* cells. Initial uptake rates were determined in the presence of increasing sorbitol concentration from 0-1100 mM. The data are means from three independent experiments with vertical bars representing standard errors of the means, n = 3.

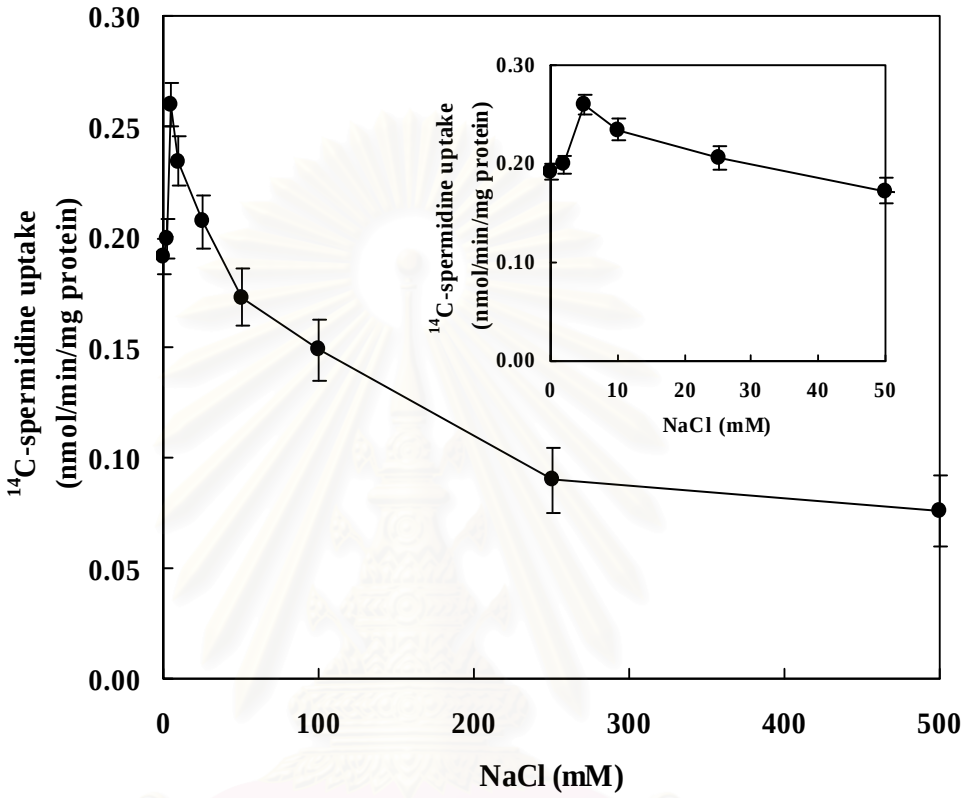


**Figure 22** Effect of external NaCl on spermidine uptake by *Synechocystis* cells. Initial uptake rates were determined in the presence of increasing NaCl concentration from 0-500 mM. The data are means from three independent experiments with vertical bars representing standard errors of the means, n = 3.

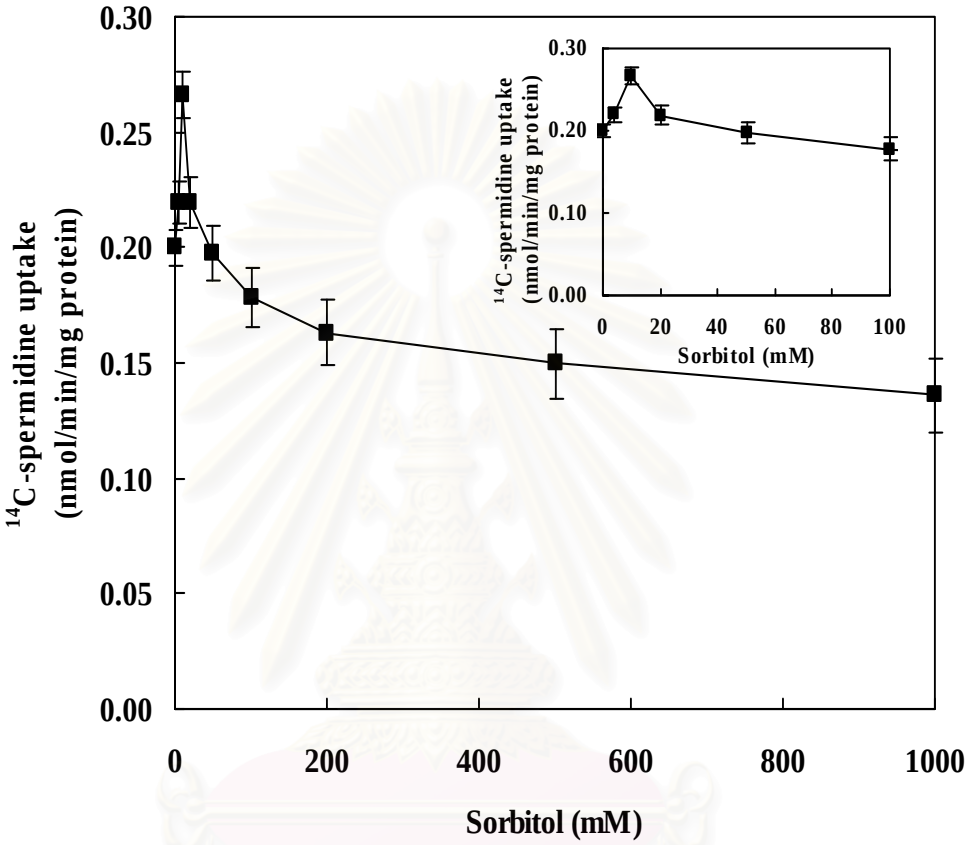


**Figure 23** Effect of external sorbitol on spermidine uptake by *Synechocystis* cells. Initial uptake rates were determined in the presence of increasing sorbitol concentration from 0-1000 mM. The data are means from three independent experiments with vertical bars representing standard errors of the means, n = 3.

### 3.1.8 Stimulation of polyamine uptake by moderate osmotic upshift

In order to determine whether the polyamine transport in *Synechocystis* is affected by the osmotic upshift, the effect of external osmolality generated by either NaCl or sorbitol on uptake was extensively examined. Both of putrescine and spermidine uptake activities were very sensitive to osmotic upshift. Firstly, increasing the osmolality generated by either NaCl or sorbitol up to 20 mosmol/kg resulted in about 2-fold increase of putrescine uptake compared to the control (Figure 24). When the osmolality was higher than 20 mosmol/kg the extent of the stimulation of the uptake was reduced. Moreover, the osmotic upshift higher than 100 mosmol/kg caused a progressive decline in putrescine uptake. Similar observation was shown by spermidine transport is that a small increase of the osmolality up to 10 mosmol/kg resulted in about 1.5-fold increase of spermidine uptake compared to the control. Elevation of osmotic upshift higher than 10 mosmol/kg caused a progressive decline in the extent of the stimulation of the uptake (Figure 25). The osmolality higher than 100 mosmol/kg caused a drastic reduction of uptake activity. It is noteworthy that the uptake of polyamine responded to changes in osmolality with similar pattern regardless of the source generating the osmotic upshift.

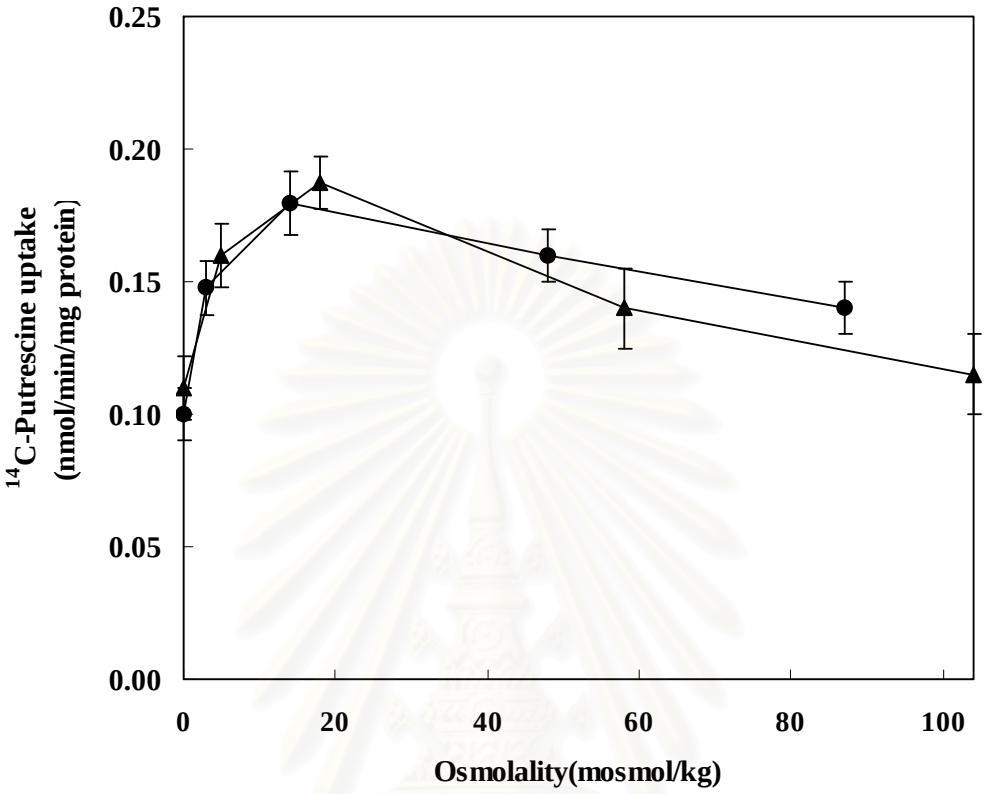


**Figure 24** Effect of external osmolality on putrescine uptake by *Synechocystis* sp. PCC 6803. Initial uptake rates were determined in the presence of increasing osmolality generated by NaCl (●) or sorbitol (▲). The data are means from three independent experiments with vertical bars representing standard errors of the means, n = 3.

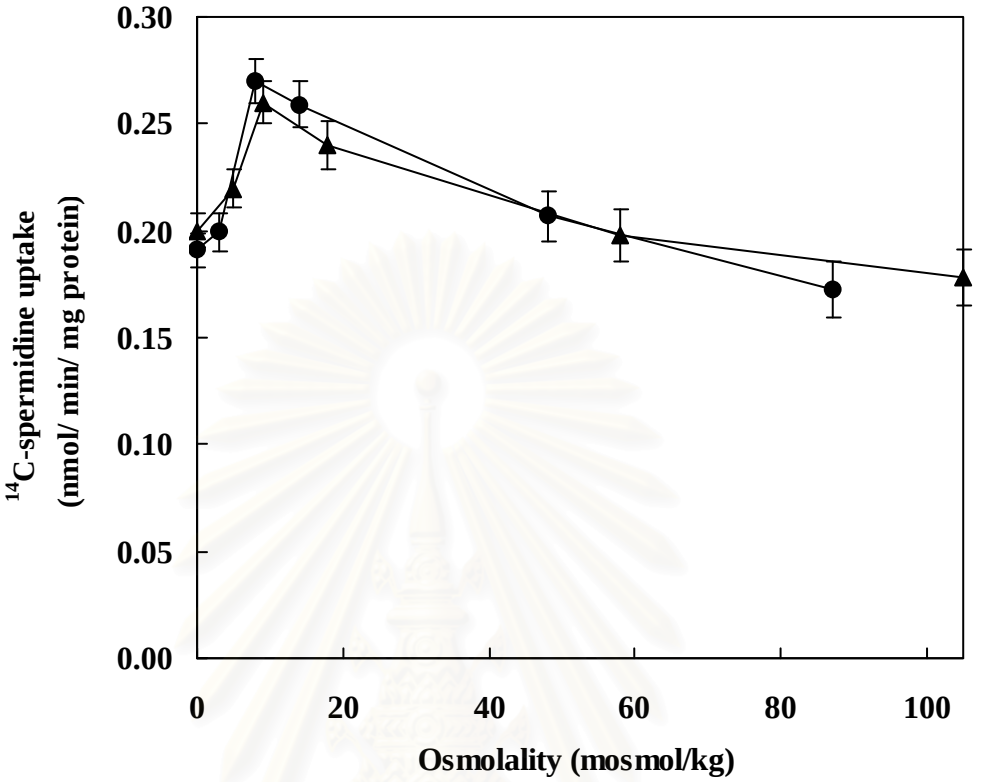


**Figure 25** Effect of external osmolality on spermidine uptake by *Synechocystis* sp. PCC 6803. Initial uptake rates were determined in the presence of increasing osmolality generated by NaCl (●) or sorbitol (▲). The data are means from three independent experiments with vertical bars representing standard errors of the means, n = 3.

### 3.1.9 Light quality and the induction of polyamine transport

Photosynthetic cyanobacteria must maintain a metabolic homeostasis despite daily variations in incident light. Not only does light provide substrate for photosynthesis, but a large number of developmental events are also responsive to light cues. To determine light dependence of polyamine transport, pre-incubated *Synechocystis* cells under light, darkness and photosynthetic inhibitor, DCMU, were further used for transport assay. In the presence of white light during uptake, most pre-incubated cells were able to uptake putrescine more noticeable than that uptake under darkness (Table 5). Obviously, DCMU did not exhibit the inhibition of putrescine transport under the exposure of white light even if the concentration was increased to 5-fold. Similarly, in the presence of whit light during uptake, pre-incubated cells could transport spermidine more noticeable than that uptake under darkness. In the same way DCMU did not inhibit the spermidine uptake under the exposure of white light (Table 6). These results suggest that energy from light during uptake may be sufficient to sustain the changes of polyamine transport system.

**Table 5** Effect of photosynthesis inhibitor and darkness on putrescine uptake in *Synechocystis* sp. PCC 6803.

| Pre-incubation | $^{14}$C-Putrescine uptake[a] (nmol/ min/ mg protein) | |
|---|---|---|
| | White light | Darkness |
| 120 min white light | 0.121 ± 0.003 | 0.079 ± 0.003 |
| 120 min dark | 0.119 ± 0.003 | 0.075 ± 0.002 |
| 120 min white light + 10 μM DCMU | 0.113 ± 0.002 | 0.069 ± 0.002 |
| 120 min white light + 50 μM DCMU | 0.111 ± 0.005 | 0.061 ± 0.003 |

[a]The putrescine uptake was measured in intact cells with 50 μM $^{14}$C-putrescine in the presence and in the absence of white light. The data represent the average of triplicate measurement.

**Table 6** Effect of photosynthesis inhibitor and darkness on spermidine uptake in *Synechocystis* sp. PCC 6803.

| Pre-incubation | $^{14}$C-Spermidine uptake[a] (nmol/ min/ mg protein) | |
|---|---|---|
| | White light | Darkness |
| 120 min white light | 0.191 ± 0.005 | 0.133 ± 0.005 |
| 120 min dark | 0.187 ± 0.003 | 0.129 ± 0.002 |
| 120 min white light + 10 μM DCMU | 0.181 ± 0.002 | 0.124 ± 0.002 |
| 120 min white light + 50 μM DCMU | 0.172 ± 0.005 | 0.114 ± 0.003 |

[a]The spermidine uptake was measured in intact cells with 50 μM $^{14}$C-spermidine in the presence and in the absence of white light. The data represent the average of triplicate measurement.

### 3.2 Transcriptional profiling of *Synechocystis potD*

#### 3.2.1 Effect of environmental factors on *Synechocystis potD* transcript levels

To reveal if transcription of the *potD* gene is regulated by environmental stresses, we first determined the effect of light quantity on the expression of the *potD* gene. The *Synechocystis* sp. strain PCC 6803 cultures were transferred from growth light to darkness followed introducing the cells to light after dark adaptation. Subsequently, we also investigated the effect of salinity, hyperosmotic, temperature stresses and nutrient deficiency on the expression of the *potD* gene. RT-PCR analyses of total RNA revealed two main RNA species recognized by the *potD*- and 16s RNA-specific primers: an ~530-bp *potD* transcript and an ~*521*-bp 16srRNA transcript.

According to our results, *Synechocystis* sp. strain PCC 6803 cells transferred from growth light to darkness for short-term (18-h) and long-term (3 days) incubation revealed that the steady-state transcriptional levels of *potD* gene were apparently remained unaltered during both short-term and long-term stresses (Figure 26 and Figure 27). Interestingly, 3-days-dark-adapted cells shifted to continuous light (50 μmol photons/ms) for 5-min photoperiod showed that the transcriptional levels of *potD* gene accumulated rapidly by 160 ± 32% compared to the level of growth light condition (Figure 27). Conversely, 18-h-dark-adapted cells showed unaltered transcript levels within 5-min of light induction. Furthermore, to determine the effect of osmolality generated by salt or sorbitol on the expression of *potD* transcript, cells were exposed to NaCl and sorbitol for 3 days (long-term stress). The steady-state *potD* transcripts were observed to slightly increase by 140 ± 14% and 168 ± 27%, respectively (Figure 27). In contrast, a 5-min incubation of both NaCl and sorbitol stresses (short-term) was not effect on the transcriptional levels of the *potD* gene (Figure 26). Moreover, *Synechocystis* cells were transferred to non-optimal growth temperature (18$^{o}$C and

42°C) for 18 h and 3 days. It was found that the *potD* transcripts were not affected within short-term incubation (18) (Figure 26), while long-term incubation (3 days) was affected on the transcriptional levels of *potD* gene (Figure 27). These transcriptional levels were slightly increased by 150 ± 20% under 18°C while decrease by 34 ± 8% under 42°C compared to the level of growth light condition (Figure 26). The effects of nutrient deficiencies and changes in nitrogen source on *potD* transcription were further studied. Iron deficiency did not affect the transcriptional levels of *potD* gene both for 18 h and 3 days of nutrient limitation (Figure 26 and Figure 27). $NH_4Cl$ used as nitrogen source instead of nitrate in BG-11 showed slight affect on the transcriptional levels of *potD* after 3 days by 148 ± 31% (Figure 27) while the short-term environmental stress (18 h) did not have any effect on the steady-state transcriptional levels of *potD* gene (Figure 26).

### 3.2.2 Effect of high temperature on the stability of *potD* mRNA

To test whether half lives of *potD* mRNA levels caused by high temperature stresses were the consequence of the stability of the transcript, RT-PCR analysis of the cells after treatment with rifampicin was performed. As shown in Figure 28, a significant level of *potD* mRNA was sequentially decreased after incubation with rifampicin in cells under both growth light and high temperature stresses. The decreased accumulation of the *potD* transcripts was not a result of a decreased stability of the transcript, but merely refers to an altered transcription activity by high temperature stress.

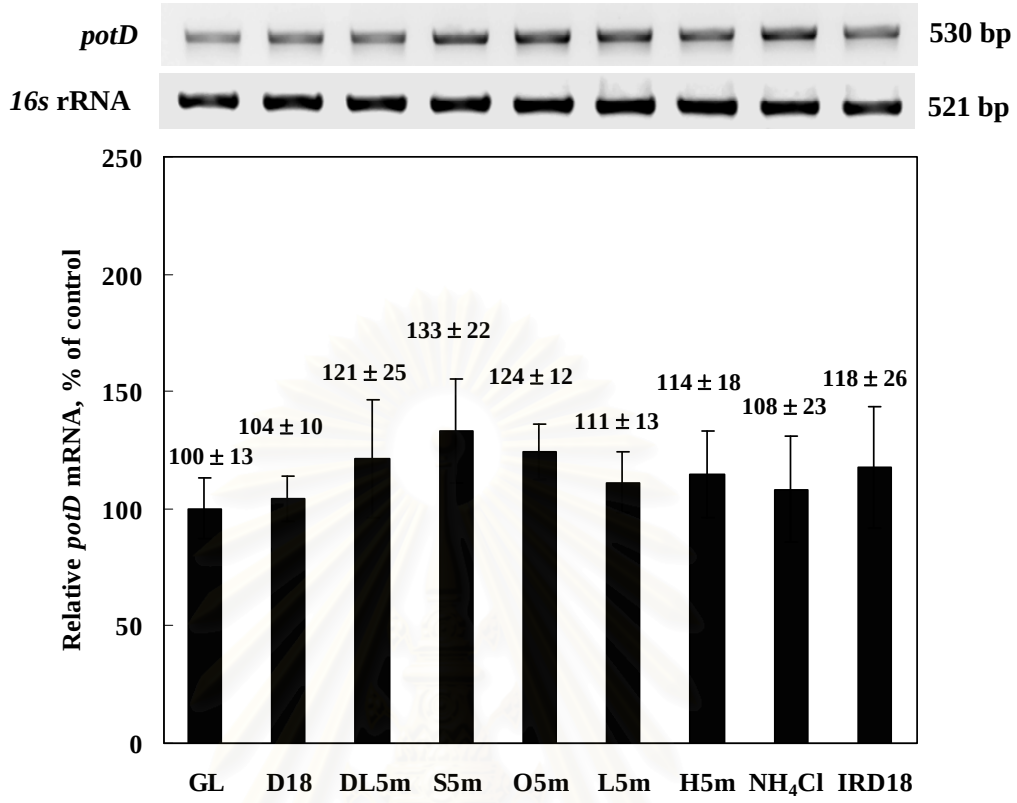


**Figure 26** Steady-state transcript levels of the *Synechocystis potD* under short-term environmental stress conditions. The cells were grown in BG-11 medium at 32°C with continuous light 50 µmol photons/$m^2$/s (GL). Cells were adapted to darkness (D18) for 18 h followed by light induction for 5 min (DL5m). During salt and hyperosmotic stress treatments cells were incubated in the presence of 550 mM NaCl (S5m) and 300 mM D-sorbitol (O5m), respectively for 5 min. High temperature (H5m) and low temperature (L5m) incubation lasted for 5 min at 42°C and 18°C, respectively. Ammonium chloride ($NH_4Cl$) and iron deficiency (IRD18) were induced for 18-h. The data represent the average of three independent experiments with n = 3 for each experiment, ± SE.

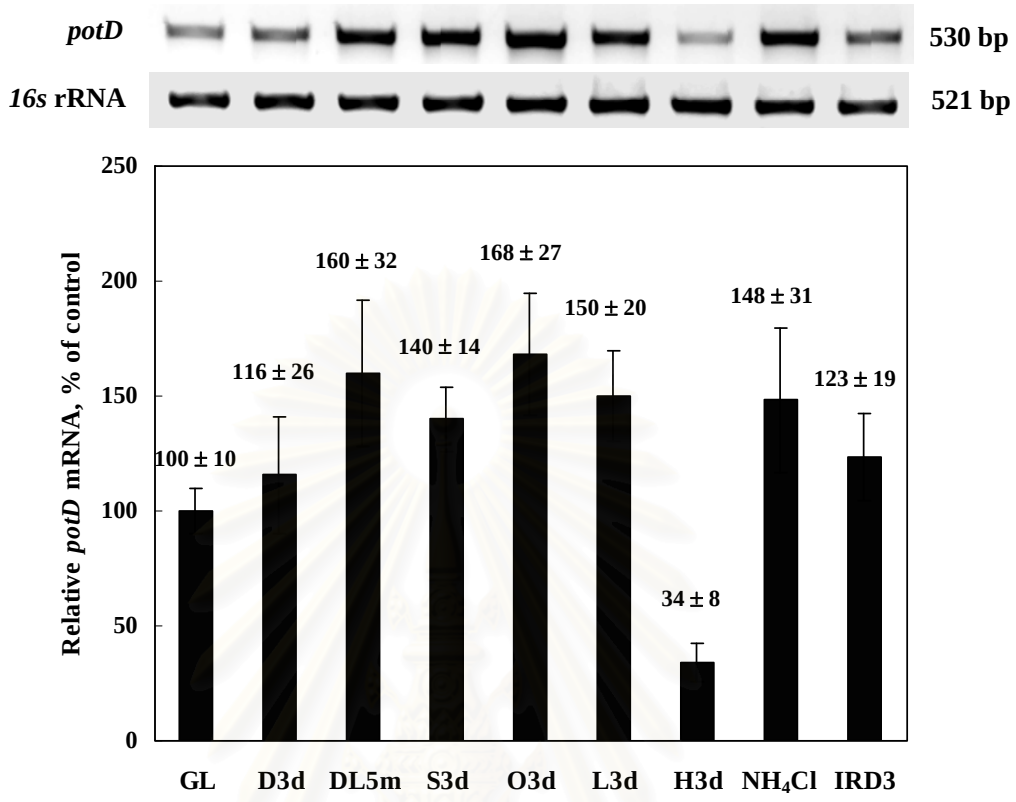


**Figure 27** Steady-state transcript level of *Synechocystis potD* under long-term environmental stress conditions. The cells were grown in BG-11 medium at 32°C with continuous light 50 µmol photons/$m^2$/s (GL). Cells were adapted to darkness (D3d) for 3 d followed by light induction for 5 min (DL5m). During salt and hyperosmotic stress treatments cells were incubated in the presence of 550 mM NaCl (S3d) and 300 mM D-sorbitol (O3d), respectively for 3 d. High temperature (H3d) and low temperature (L3d) incubation lasted for 3 d at 42°C and 18°C, respectively. Ammonium chloride ($NH_4Cl$) and iron deficiency (IRD3) were induced for 3 d. The data represent the average of three independent experiments with n = 3 for each experiment, ± SE.

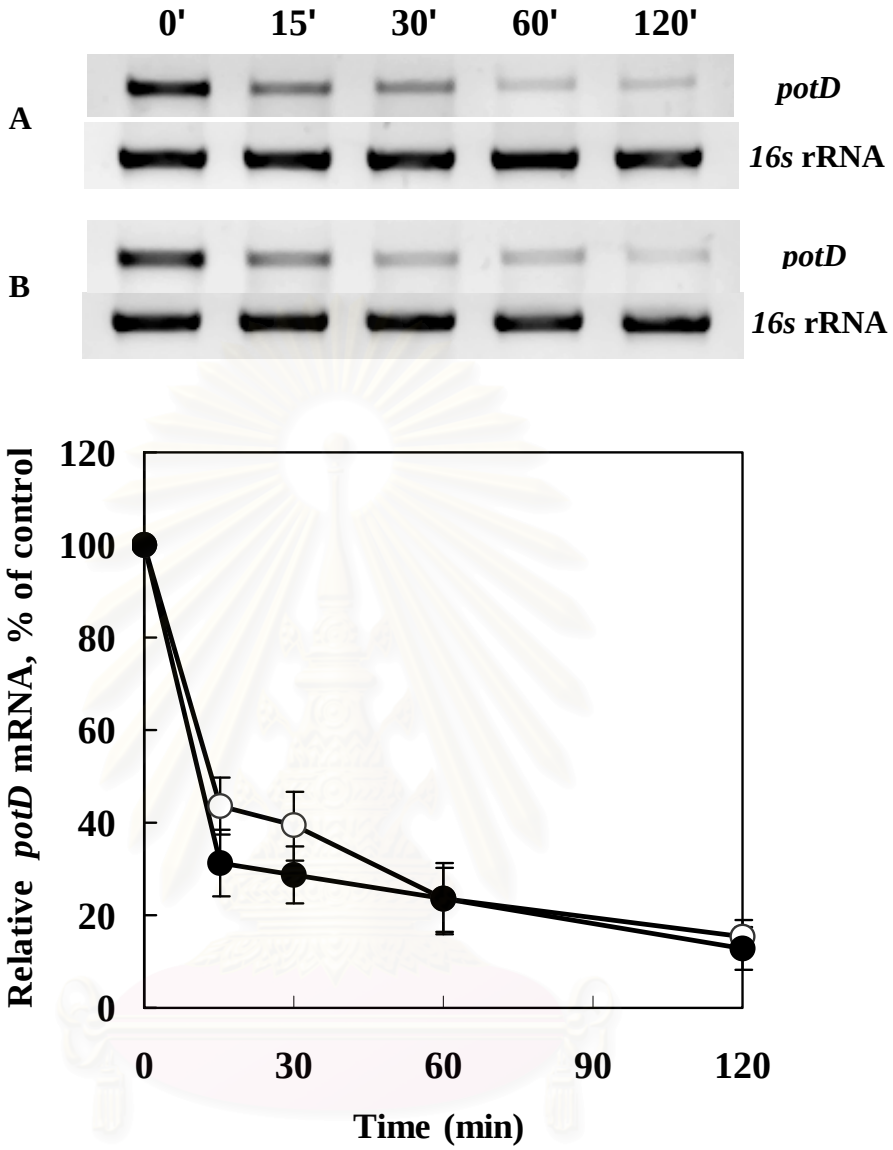


**Figure 28** Influence of high temperature treatment on the half-lives of *Synechocystis potD* mRNA. Total RNA was isolated from cell grown under control condition, (A; open circle) and from cells treated at high temperature (42$^{o}$C), (B; solid circle) in the presence of rifampicin (500 μg/ml), an inhibitor of transcriptional initiation. Aliquots were taken after 0, 15, 30, 60 and 120 min of incubation, frozen immediately in liquid nitrogen and subjected to RT-PCR analysis.

## 3.3 Cloning and Characterization of recombinant plasmid

### 3.3.1 Amplification of the *potD* gene from *Synechocystis* chromosomal DNA

The PCRs were performed using *Synechocystis* chromosomal DNA and DNA polymerase (DyNAzyme$^{TM}$ DNA Polymerase kit). The initial pre-denaturation at 95$^{o}$C for 5 min was done, followed by 34 cycles of denaturation at 95$^{o}$C for 1 min, varying annealing at 50$^{o}$C, 53.2 $^{o}$C, 55.5 $^{o}$C, 60.8 $^{o}$C, 63.5 $^{o}$C, 66 $^{o}$C or 68 $^{o}$C, respectively for 1 min and extension at 72$^{o}$C for 1.30 min, followed by final extension at 72$^{o}$C for 2 min. The intensities of PCR products were analyzed by electrophoresis and autoradiography of a 0.8% agarose gel in 1x TAE buffer as shown in Figure 29.

### 3.3.2 Construction of a pGEM-T easy recombinant plasmid containing the *potD* gene

The recombinant plasmid containing the *potD* gene was organized. Firstly, PCR product containing *Nde*I and *Bam*HI restriction sites from the previous step was purified using NucleoSpin® Extract II. The fragments were ligated together with commercial cloning plasmid pGEM-T easy vector at 4$^{o}$C. The ligation reaction was transformed into the *E. coli* Top10 cells. The recombinant clone was selected on LB agar plate containing ampicilin and X-Gal. The white colonies were randomly selected and cultured in LB broth media containing ampicillin overnight. The cultures were subjected to plasmid extraction and digestion with *Nde*I and *Bam*HI restriction enzymes to prove the correction of recombinant plasmid. Subsequently, these reactions were analyzed by 0.8% agarose gel electrophoresis. The result showed that

the recombinant plasmid, pGEMpotD, contained a *potD* gene of an approximated size of 1.15 kb (Figure 30).

### 3.3.3 Construction of the pET-19b recombinant plasmid containing the *potD* gene

The pGEM-T easy recombinant plasmid the inserted DNA was then subcloned into expression vector, pET19b, at the position of *Nde*I and *Bam*HI sites. Beginning with digestion of recombinant plasmid, this plasmid was digested overnight by restriction enzymes, *Nde*I and *Bam*HI. Also, the pET19b expression vector was digested overnight with *Nde*I and *Bam*HI restriction enzymes. Both reactions were separated on 0.8% agarose gel electrophoresis. The target band was eluted from gel and purified by using NucleoSpin® Extract II. The ligation mixture was transformed into the *E. coli* Top10 cells. The transformants were randomly selected for a plasmid extraction. After an extraction, the plasmids from each clone were digested with *Nde*I and *Bam*HI restriction enzymes and then analyzed by 0.8% agarose gel electrophoresis to confirm the presence of inserted fragment. The analysis revealed that the recombinant plasmid, pETpotD, contained a *potD* gene of an approximated size of 1.15 kb (Figure 31).

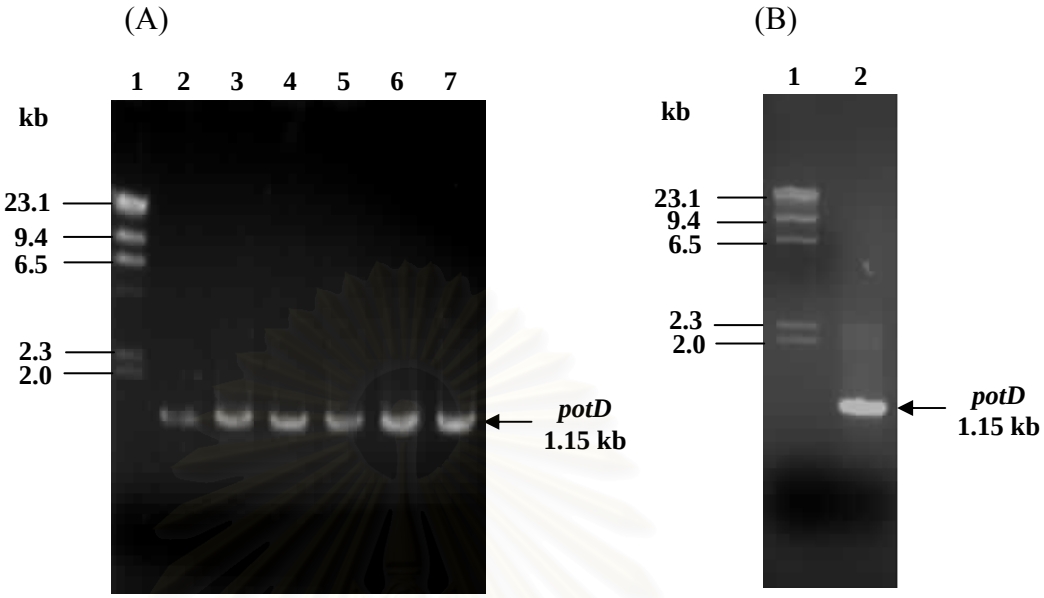


**Figure 29** (A) Optimization of annealing temperature to reduce bias cause by a primer mismatch in PCR was electrophoresed on 0.8% agarose gel. Lane 1: 50 ng of Lambda *Hin*d III marker; Lane 2- Lane 7: *potD* PCR product corresponding to 1.15 kb. Reaction was performed in the same cycling condition using different annealing temperature, namely 50$^{o}$C, 53.2 $^{o}$C, 55.5 $^{o}$C, 60.8 $^{o}$C, 63.5 $^{o}$C, 66 $^{o}$C and 68 $^{o}$C, respectively.

(B) PCR Purification product using NucleoSpin® Extract II was electrophoresed on 1% agarose gel. Reaction was performed in the same cycling condition using annealing temperature at 55$^{o}$C. Lane 1: 50 ng of Lambda *Hin*d III marker and Lane 2: *potD* PCR product corresponding to 1.15 kb.

### 3.3.4 Sequence analysis of recombinant plasmid

To confirm whether the inserted fragment was correctly *potD* gene, both pGEMpotD and pETpotD clones were subjected to DNA sequencing using universal primer according to Materials and Methods at Bioscience Unit (BSU), National Science and Technology Development Agency (NSTDA), Bangkok, Thailand. After reaching the sequence of this *potD* gene, this sequence was compared to the *slr0401* gene in CyanoBase (http://bacteria.kazusa.or.jp/cyanobase/) by using the ClustalW program. The comparison result revealed that the *potD* gene was a homologue to *slr0401* gene with 100% homology as shown in Figure 32.

(A) (B)

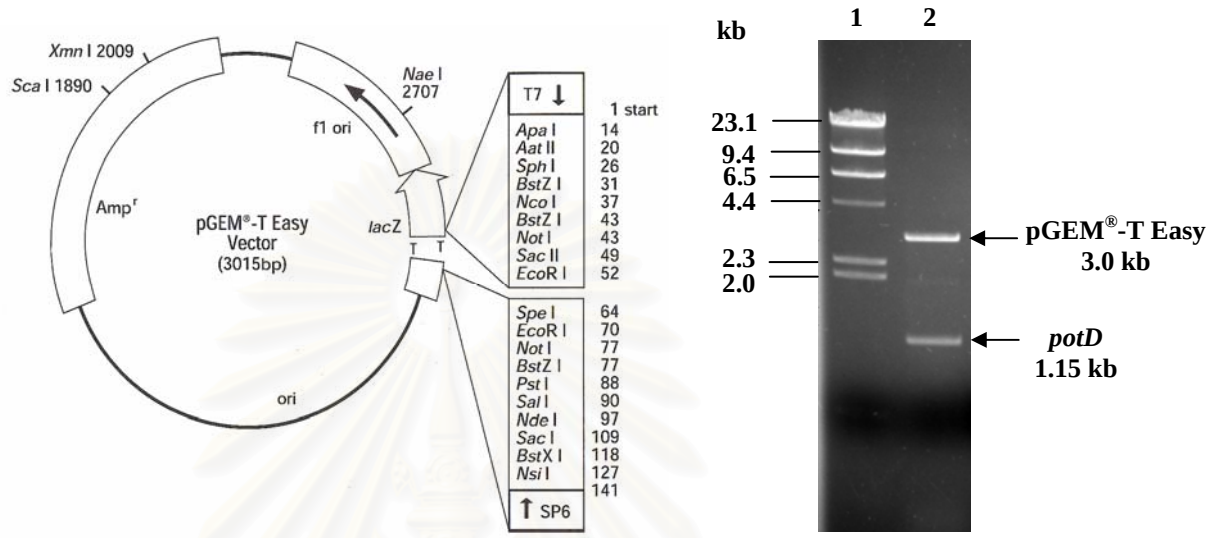


**Figure 30** (A) pGEM®-T Easy Vector circle map and cloning/expression region (B) Analysis of recombinant plasmid, namely pGEMpotD, digested with *Nde*I and *Bam*HI was electrophoresed on 1% agarose gel.

Lane 1: 50 ng of Lambda *Hin*dIII marker

Lane 2: pGEMpotD double digestion with *Nde*I and *Bam*HI

(A) (B)

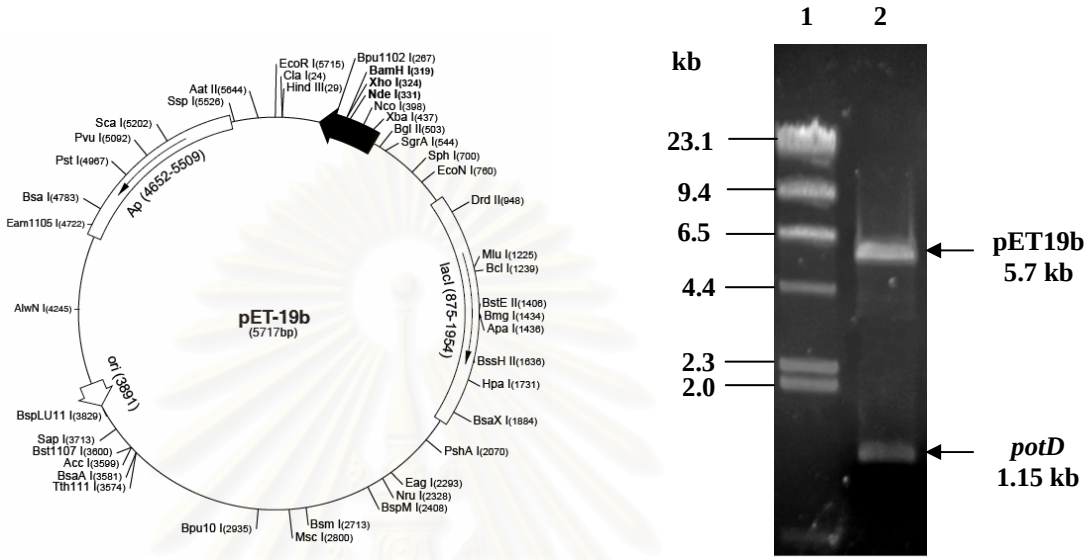


**Figure 31** (A) pET-19b Vector circle map and cloning/expression region

(B) Analysis of recombinant plasmid, namely pETpotD, digested with *Nde*I and *Bam*HI was electrophoresed on 1% agarose gel.

Lane 1: 50 ng of Lambda *Hin*dIII marker

Lane 2: pETpotD double digestion with *Nde*I and *Bam*HI

```
SeqA Name      Len(nt)  SeqB Name      Len(nt)  Score

=====================================================
1    slr0401   1155     2    PotD      1155     100
=====================================================

slr0401  ATGAATTTACCCTGCTATTCCCGCCGCCATTTTCTCCAGTCCGCCGCCGCTGTCGGCCTT 60
potD     ATGAATTTACCCTGCTATTCCCGCCGCCATTTTCTCCAGTCCGCCGCCGCTGTCGGCCTT 60
         ************************************************************

slr0401  GCCACGGGTCTAGGAGGCTGTTGGCCTGGCCAGGGCAGTAGCAAAGAAGTGCAGTTTTTG 120
potD     GCCACGGGTCTAGGAGGCTGTTGGCCTGGCCAGGGCAGTAGCAAAGAAGTGCAGTTTTTG 120
         ************************************************************

slr0401  AACCGTTCCCTGCCGCCCCAGTTAATTAACCAATTTCAGCGGCAGTTTCCCCAAGCTAAG 180
potD     AACCGTTCCCTGCCGCCCCAGTTAATTAACCAATTTCAGCGGCAGTTTCCCCAAGCTAAG 180
         ************************************************************

slr0401  GAATTAAATTTCAAGGCGATCGCCAATCTGCAGAGTTTGTTCGACAATTTACAAACCTGG 240
potD     GAATTAAATTTCAAGGCGATCGCCAATCTGCAGAGTTTGTTCGACAATTTACAAACCTGG 240
         ************************************************************

slr0401  CACAATGCCCAAAATGCAGATACTTCCAAGGGACTGTGGGGCAGTGGATCGAGGGCAAAT 300
potD     CACAATGCCCAAAATGCAGATACTTCCAAGGGACTGTGGGGCAGTGGATCGAGGGCAAAT 300
         ************************************************************

slr0401  CAGACCCCAAAAGCCCAGTTGGTCACCGGGGGAGATTTATGGTTGGAACGGGTAATCAAA 360
potD     CAGACCCCAAAAGCCCAGTTGGTCACCGGGGGAGATTTATGGTTGGAACGGGTAATCAAA 360
         ************************************************************

slr0401  GAAAAGCTCATTCAACCCTTTGTCCCCGACCAATTGAGCCAATGGTCTAGTCTGCCTCCA 420
potD     GAAAAGCTCATTCAACCCTTTGTCCCCGACCAATTGAGCCAATGGTCTAGTCTGCCTCCA 420
         ************************************************************

slr0401  CGGTGGCAATTGTTGGGGCAACGCAATGACCAAGGCTTACCGGATCAAAGCGGCAAAATT 480
potD     CGGTGGCAATTGTTGGGGCAACGCAATGACCAAGGCTTACCGGATCAAAGCGGCAAAATT 480
         ************************************************************

slr0401  TGGGCTGTTCCCTACCGTTGGGGGCCCACCATGATTATTTATCGCCAACAACCATTTGCT 540
potD     TGGGCTGTTCCCTACCGTTGGGGGCCCACCATGATTATTTATCGCCAACAACCATTTGCT 540
         ************************************************************

slr0401  GATTTAGGTTGGCAACCGACCGATTGGTCTGACCTCTGGCGGCCAGAGTTAAAGCAACGC 600
potD     GATTTAGGTTGGCAACCGACCGATTGGTCTGACCTCTGGCGGCCAGAGTTAAAGCAACGC 600
         ************************************************************

slr0401  ATTGCTCTGGTGGATGATCCCAGGGAGGCGATCGGTTTGACTCTGAAAAAGCTGGGTTAT 660
potD     ATTGCTCTGGTGGATGATCCCAGGGAGGCGATCGGTTTGACTCTGAAAAAGCTGGGTTAT 660
         ************************************************************

slr0401  TCCTACAATGTCACCAACCCCAAAGCTGTTAATGCCCTACCTTCCGCTTTGCAAGAATTG 720
potD     TCCTACAATGTCACCAACCCCAAAGCTGTTAATGCCCTACCTTCCGCTTTGCAAGAATTG 720
         ************************************************************

slr0401  GCGACCCAAGTGAAGTTTTACAACAGTAAATATTATCTCCAAGCTCTACTCAATAAAGAT 780
potD     GCGACCCAAGTGAAGTTTTACAACAGTAAATATTATCTCCAAGCTCTACTCAATAAAGAT 780
         ************************************************************

slr0401  GTATGGTTAGCAGTGGGCTGGTCAAACCAAATTATTCCTCTGCTGAGAAATCAGTCAGAA 840
potD     GTATGGTTAGCAGTGGGCTGGTCAAACCAAATTATTCCTCTGCTGAGAAATCAGTCAGAA 840
         ************************************************************

slr0401  CTCAGGGCGGTTATTCCTAGTTCCGGTACATCCCTCTGGGCTGATCTCTGGACTATGCCC 900
potD     CTCAGGGCGGTTATTCCTAGTTCCGGTACATCCCTCTGGGCTGATCTCTGGACTATGCCC 900
         ************************************************************
```

```
slr0401  GCTGAGATGGAGGCGGCTGAAATTACCTATGAATGGTTAAATTTTTCCGCCCAGCCTTCC 960
potD     GCTGAGATGGAGGCGGCTGAAATTACCTATGAATGGTTAAATTTTTCCGCCCAGCCTTCC 960
         ************************************************************

slr0401  AGTCTAGAGCAGATGGCAACTTTCAGTAATGCCCTGGCGGTGCCCTATCAAAACCTGGAG 1020
potD     AGTCTAGAGCAGATGGCAACTTTCAGTAATGCCCTGGCGGTGCCCTATCAAAACCTGGAG 1020
         ************************************************************

slr0401  GTTTCCGCCCTGAGCACTAACCCTTTATTGGCTTTTTCACCGGAGTTACTGGAGCGTTGT 1080
potD     GTTTCCGCCCTGAGCACTAACCCTTTATTGGCTTTTTCACCGGAGTTACTGGAGCGTTGT 1080
         ************************************************************

slr0401  GAATTTCTCGCGCCCCTGGACATTGCCACCGTCAACCAATATAAGCAACTCTGGCAAACC 1140
potD     GAATTTCTCGCGCCCCTGGACATTGCCACCGTCAACCAATATAAGCAACTCTGGCAAACC 1140
         ************************************************************

slr0401  ATGCGGAGTGCTTAG 1155
potD     ATGCGGAGTGCTTAG 1155
         ***************
```

**Figure 32** Nucleotide sequence alignment of *slr0401* gene encoding periplasmic polyamine-binding protein of ABC transporter from *Synechocystis*. The *potD* cloning sequence and *slr0401* gene from CyanoBase were indicated by red and black letter, respectively.

### 3.3.5 Expression of the recombinant protein, His-PotD, in the *Escherichia coli* expression system

To express the His-PotD protein in *E. coli* system, the recombinant plasmid, pET19b_PotD, was transformed into the *E. coli* strain BL21 (DE3) pLysS. The transformant cells were cultured in LB medium containing 100 μg/ml ampicillin. IPTG was used as an inducer for activating the protein expression. After induction with 1 mM IPTG, one millilitre of culture medium was collected after induction at 0, 1, 2, 3, 6 hours. The solubilized cells and the soluble proteins were analyzed by 12% SDS-PAGE. The result shown in Figure 33 illustrated that recombinant *Synecocystis* PotD protein was expressed in *Escherichia coli* strain BL21 (DE3) pLysS system and formed an inclusion body. The expected size for the recombinant protein was 46 kDa.

### 3.3.6 Purification of recombinant His-PotD protein

The PotD-inclusion bodies were purified by Ni-Sepharose column. The His-PotD protein was eluted with buffer containing 8 M urea, 20 mM phosphate pH 7.4, 500 mM imidazole and 500 mM NaCl. Fractions (3 ml) were collected and were dialyzed extensively against phosphate-buffered saline pH 7.0 to remove the imidazole. The protein was solubilized in 1X loading dye and then analyzed by 12% SDS-PAGE. Coomassie blue stained gel was shown in Figure 34. The expected size for the recombinant protein was 46 kDa.

### 3.3.7 Imuno blotting of His-PotD

Recombinant purified PotD was analyzed by immuno blotting analysis using a monoclonal antibody raised against His-tag at a dilution of 1:3,000 and a secondary antibody conjugated anti-rabbit immunoglobulin G a dilution of 1:5,000 to confirm the molecular weight and purity (Figure 35). After incubation of the secondary antibody, membrane protein was developed by addition of substrate for chemiluminescence alkaline phosphatase. During exposure for 20 minutes, the signal corresponding to the specific antibody-antigen reaction was apparently visualized. The expected band for the recombinant protein appears at 46 kDa.

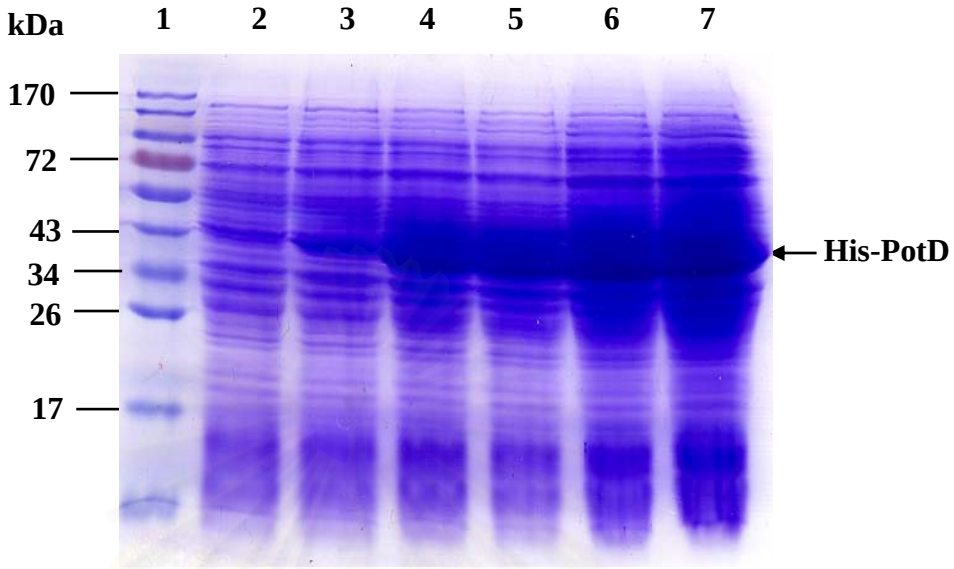


**Figure 33** Expression of recombinant PotD protein from *E. coli* was induced by addition of isopropyl-D-thiogalactoside (IPTG) to a final concentration of 1 mM. One milliliter of culture medium was collected after induction at 0, 1, 2, 3, 6 hours. Coomassie blue–stained 12% SDS-polyacrylamide gel. Lane 1: 1 μg of molecular size ladder (in Kilodalton); Lane 2: *E. coli* cells contained plasmid pET19b (No induction); Lane 3-Lane 7: *E. coli* cells contained recombinant plasmid pETpotD induced for 0, 1, 2, 3, 6 hours, respectively.

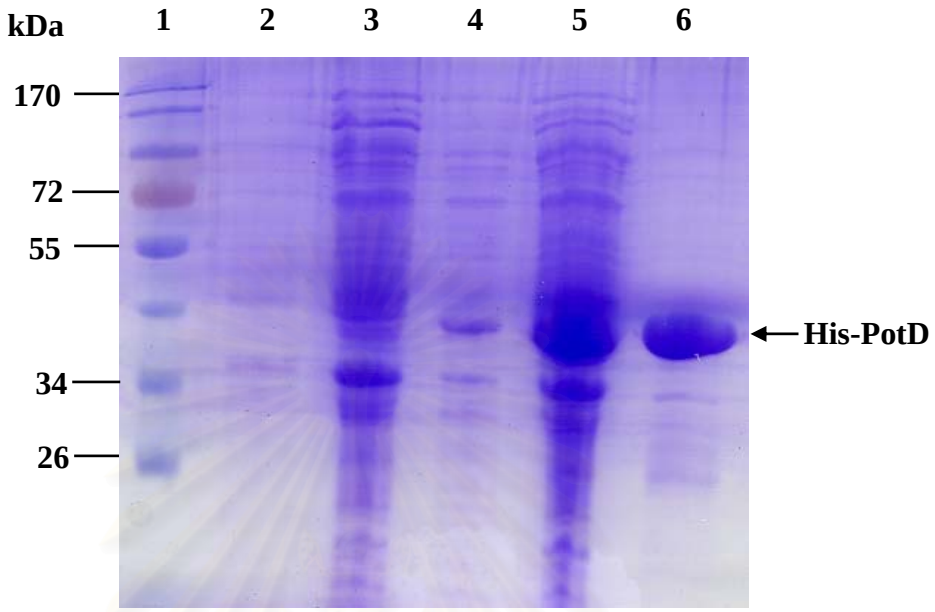


**Figure 34** Purification of recombinant PotD protein from *E. coli*. Coomassie blue–stained 12% SDS-polyacrylamide gel. Lane 1: 1 µg of molecular size ladder (in Kilodalton); Lane 2: *E. coli* cells (No induction); Lane 3: *E. coli* cells (Induction for 6 h); Lane 4: *E. coli* cells contained recombinant plasmid pETpotD (No induction); Lane 5: *E. coli* cells contained recombinant plasmid pETpotD (Induction for 6 h). Lane 6: 10 µg of purified His-PotD (approximately 46 kDa) using Ni-sepharose column eluted with 500 mM imidazole.

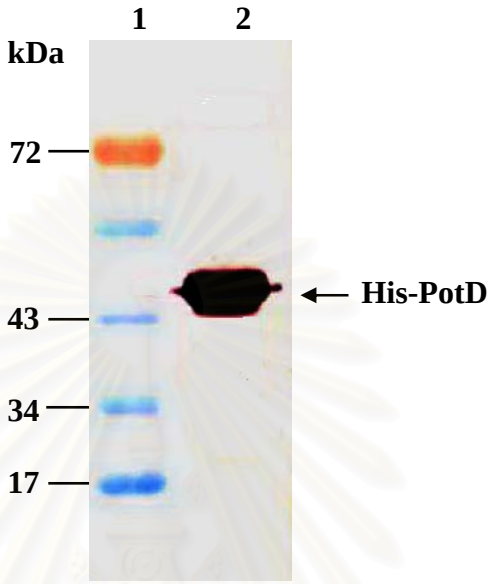


**Figure 35** Immunoblot analyses showing 20 µg of non-purified His-tagged PotD protein (approximately 46 kDa) probed with monoclonal anti-$His_6$-antibody (Lane 2). Lane 1: 1 µg of molecular size ladder (in Kilodalton). This was analyzed with Coomassie blue-stained on 12% SDS-polyacrylamide gel.

### 3.4 Structural profile of *Synechocystis* PotD protein

#### 3. 4. 1 Sequence alignment of PotD proteins

The sequence comparison includes the structures of *E. coli* PotD and PotF and the sequences of spermidine/putrescine-binding periplasmic proteins from *Synechocystis*, *Trichodesmium erythraeum*, *Nostoc sp.* PCC 7120, *Thermosynechococcus elongatus* and *Synechococcus sp.* PCC 7942. When compared to the *Synechocystis* PotD sequence, the other cyanobacteria sequences showed a sequence identity between 38-47%, but when compared to the the *E. coli* PotD, the cyanobacteria sequences were less similar (24 % identity or less). The sequences are most different in the N- and C-terminal parts. In N-terminus there is a ~20 amino acid insertion, which is present in *Synechocystis*, *Trichodesmium erythraeum* and *Nostoc sp. PCC7120* sequences, but lacks from in *E. coli* PotD and PotF, as well as in *Thermosynechococcus elongatus* and *Synechococcus sp. PCC7942*. The *E. coli* PotF sequence is markedly longer than the other sequences. Although the sequence similarity is low, the ABC transporter subunits are members in the same structural family and have been shown to share the overall fold (Sugiyama *et al.*, 1996). Aminoterminal signal sequences were predicted for the *Synechocystis* PotD as well as for the *E. coli* PotD and PotF by SignalP 3.0 Server program. (Bendtsen *et al.*, 2004). The result showed that the *E. coli* PotD and PotF contained signal sequences, while *Synechocystis* did not as shown in Figure 36.

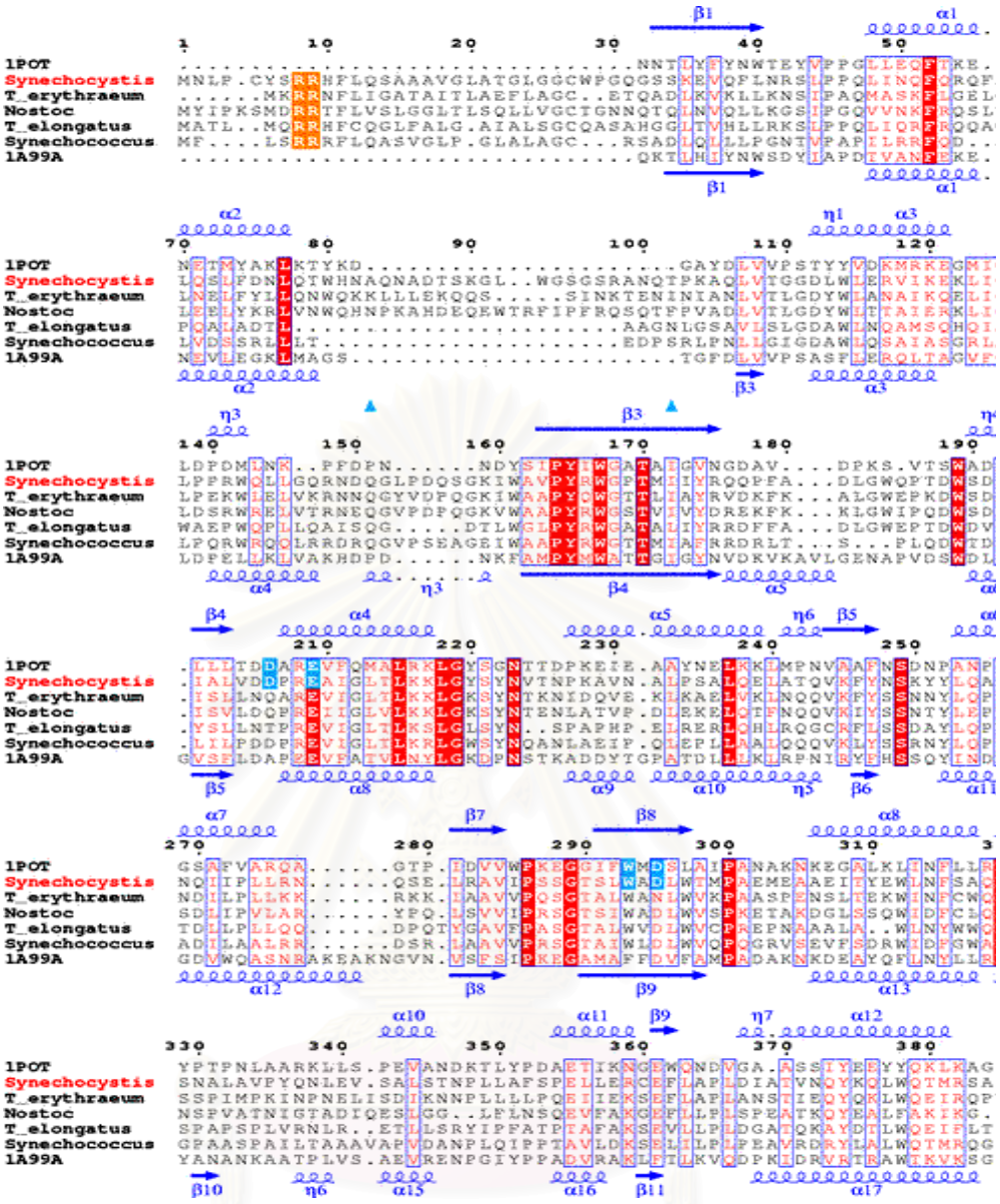


**Figure 36** Sequence alignments of spermidine/putrescine binding periplasmic proteins. The alignment of *E. coli* PotD (1POT) and PotF (1A99) is based on structural super position. The amino acid numbering in the alignment is according to *Synechocystis* PotD sequence. The secondary structure elements *E. coli* PotD and PotF structures are indicated above and below the alignment, respectively. The identical residues are indicated by red background and the similar residues by red letters boxed with blue frames, respectively. The loop (Ala83-Thr102) excluded from the model of *Synechocystis* PotD is indicated by turquoise arrows. The five residues that are conserved in the active sites of *E.coli* and *Synechocystis* PotD are highlighted turquoise. The twin-arginins of the TAT-signal are indicated by an orange box. The organisms are referred to as: 1POT (the structure of *E. coli* PotD), Synechocystis (*Synechocystis* sp. PCC 6803), T_erythraeum (*Trichodesmium erythraeum*), Nostoc (*Nostoc* sp. PCC 7120), T_elongatus (*Thermosynechococcus elongatus*), Synechococcus (*Synechococcus* sp. PCC 7942) and 1A99 (the structure of *E. coli* PotF).

### 3.4.2 Structural modelling of PotD

The structural model of *Synechocystis* PotD was constructed based on the known crystal structure of *E. coli* PotD (Figure 37). The sequence comparison between the crystal structure of *E. coli* PotD and the sequence of *Synechocystis* PotD was refined manually. Since *Synechocystis* PotD sequence is longer than *E. coli* PotD, the N-terminal part was excluded from the model. The loop area Ala83-Thr102 could not be modeled reliably because of the lack of template. The sequence identity between *E. coli* PotD and *Synechocystis* PotD was based on the sequence alignment used for modelling, 22.7%, whereas *E. coli* PotF had a lower sequence identity (under 20%).

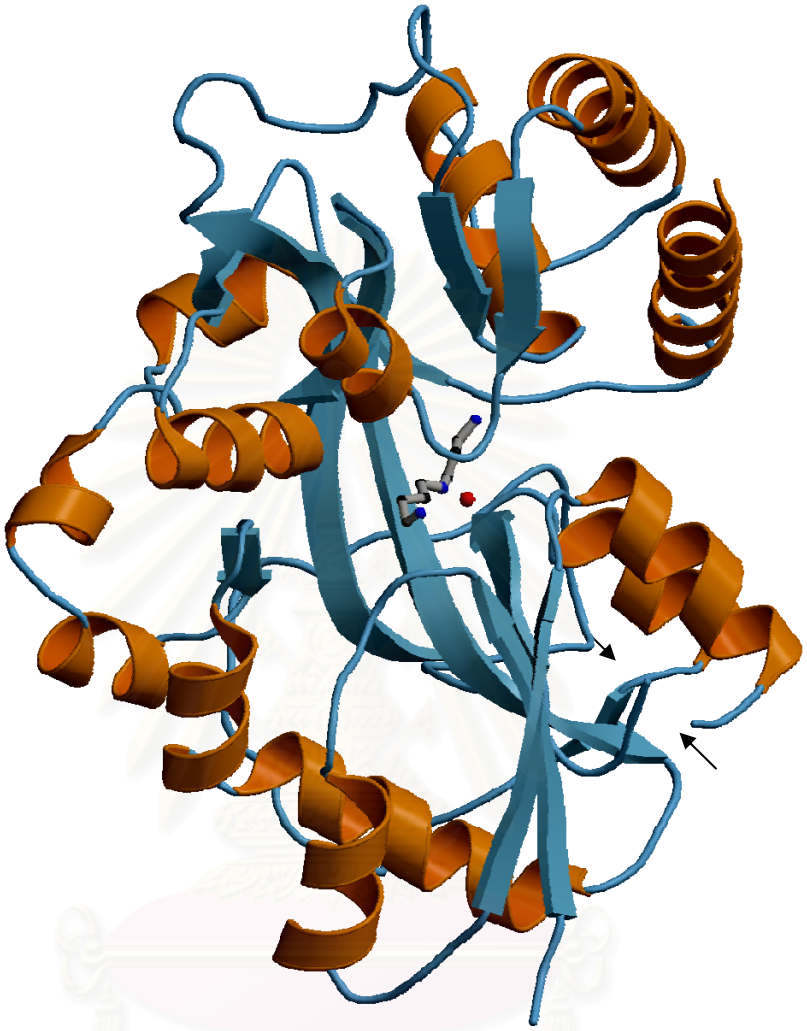

**Figure 37** The overall structure of *Synechocystis* PotD. The N-terminal domain is at the bottom and the C-terminal domain at the top. The loop Ala83-Thr102 that is missing from the model is indicated by arrows. The spermidine ligand is represented in ball-and-stick. α-helices are shown in orange and β-sheets in blue, respectively.

# CHAPTER IV

# DISCUSSION

In this work I have demonstrated the existence of a transport system for polyamine in *Synechocystis* sp. PCC 6803. As previous study showed that *Synechocystis* were able to accumulate polyamines after exposure to long-term osmotic stresses (Jantaro *et al*., 2003). However, the synthesis of polyamines requires investment of organic carbon and metabolic energy. It is likely that the portion of cell carbon and energy diverted for polyamine synthesis is at least partially responsible for the observed reduction in the growth rate of cells under osmotic stress. Therefore, the ability to accumulate polyamines via the uptake from the growth medium may be advantageous in terms of the adaptive response to high salinity environment. The data in Figure 9 and Figure 10 indicated that both exogenous putrescine and spermidine could be transported into *Synechocystis* cells and furthermore they could act as growth promoters under high salt conditions. These results suggest the role of polyamines as a compatible solutes in this cyanobacterium. Nevertheless, the protective role by polyamines against inhibition of growth under hyperosmotic stress was not as efficient as that by glucosylglycerol, suggesting that polyamines per se are not an osmoprotectant (Ferjani *et al*., 2003; Mikkat *et al*., 1997). The levels of putrescine and spermidine accumulated in *Synechocystis* cells after salt and osmotic stress treatments were not sufficient to account for any osmotic importance (Jantaro *et al*., 2003). However, the addition of exogenous putrescine and spermidine higher than 1.0 mM was inhibitory to the growth of *Synechocystis* cells. This could be ascribed to the toxic effects caused by the accumulation of polyamines. It is possibly caused from oxidation of

polyamines which can generate toxic compounds such as acrolein (Sakata *et al*., 2003). Similarly, it was observed that the growth of *Anacystis nidulans* was markedly inhibited by higher concentration of spermidine (Ramakrishna *et al*., 1978). Polyamines were able to disrupt several metabolic functions within the cells by inhibition of protein synthesis accompanied by an irreversible dissociation of ribosomes (Ramakrishna *et al*., 1978). The cytotoxic effects were observed in *Chlamydomonas reinhardtii* when exogenous putrescine was higher than 1.5 mM (Theiss *et al*., 2004).

The polyamine transport system in *Synechocystis* sp. PCC 6803 was saturable displaying typical Michaelis-Menten type kinetics (Figure 15 and Figure 16). *Synechocystis* cells exhibited a low affinity ($K_m$ 98 μM) for putrescine transport (Raksajit *et al.*, 2006). Also, the transport showed a low affinity ($K_m$ 67 μM) for spermidine, which is in contrast to a rather high affinity demonstrated for *E. coli* with the $K_m$ value of 1.4 μM (Kashiwaki *et al*., 1986). Low affinity for both putrescine and spermidine transport indicated slow uptake of polyamines by *Synechocystis* cells. This was corroborated by the observations that a rather long period of exposure to putrescine and spermidine was required for growth inhibition and growth stimulation to take place in the presence of both 1.0 and 0.5 mM for putrescine and spermidine, respectively (Figure 9 and Figure 10).

Importantly, we therefore found that the changes in external pH could affect polyamine uptake. The highest uptake for putrescine into *Synechocystis* cells occurred at neutral pH of 7.0, while spermidine transport retained high activity at alkaline pH 8.0 similar to that of sea water red alga *Ulva rigida* (Badini *et al*., 1994). Drastic decrease of both putrescine and spermidine uptake activity occurred when the pH

became acidic (Figure 17 and Figure 18). The dependence of polyamine uptake on the extracellular pH was previously reported in *Leishmania* (Basselin *et al*., 2000) and also in Wistar rats (Kobayashi *et al*., 1992). It is worth noticing that the transport systems for spermidine and putrescine in *Synechocystis* actually are quite different with regard to the responses to alterations in extracellular pH. Moreover, an increase in pH up to 10.0 resulted in only a slight decrease of spermidine transport. In contrast, a sharp drop in putrescine transport was observed when the pH was increased from 7.0 to 8.2.

Uptake system specific for spermidine in *Synechocystis* is active transport. Both putrescine and spermidine transport is highly inhibited by various energy generation inhibitors (Table 4) demonstrating the requirement of energy for the transport. The coupling mechanism between $Na^+$ electrochemical gradient and different kinds of transport systems has been extensively studied earlier. Partial $Na^+$-dependency has been interpreted as indicating the presence of two transporters, one $Na^+$-dependent, and the other $Na^+$-independent (Parys *et al*., 1990; Van Den Bosch *et al*., 1990). The dependency on $Na^+$ has been reported for the transport of choline and nitrate into the halotolerant cyanobacterium *Aphanothece halophytica* (Incharoensakdi and Karnchanatat, 2003; Incharoensakdi and Wangsupa, 2003; Incharoensakdi and Laloknam, 2005). $Na^+$-gradient has been shown to act as a major source of energy, coupling with the active transport for polyamine in mouse cells (Rinehart and Chen, 1984). Moreover, the dependency on $Na^+$ has been reported for the transport of potassium and bicarbonate into *Synechocystis* (Matsuda *et al*., 2004, Shibata *et al*., 2002).

The addition of sodium ionophores, namely amiloride and monensin, significantly diminished putrescine and spermidine uptake, suggesting $Na^+$-gradient

involvement for polyamine transport of into *Synechocystis* cells. DCCD, an $H^+$-ATPase inhibitor, also inhibited polyamine transport. Similar effect was observed with ionophores, namely valinomycin, CCCP, as well as the electroneutral $K^+/H^+$ antiporter, nigericin. It was likely that the uptake of polyamines was dependent upon the proton motive force with the contribution of both ΔpH and Δψ for the uptake. Similar evidence has been reported in *S. cerevisiae* (Kakinuma *et al*., 1992) and *E. coli* (Kashiwagi *et al*., 1986) and *Drosophila melanogaster* (Romero-Calderon and Krantz 2006). Moreover, preincubation of cells with chloramphenicol (100 μg/ ml) resulted in a significant decrease of both putrescine and spermidine transport (Table 4). This suggests that the transport appeared to be dependent on *de novo* synthesis of a transport protein or protein(s) regulating the activity of preexisting transport protein(s).

The present study revealed that spermidine and spermine could significantly inhibit the uptake of putrescine by 60% and 40%, respectively in *Synechocystis* cells. Similar observations were reported for the uptake of putrescine by a green alga *Chlamydomonas reinhardtii* (Theiss *et al*., 2002). However, in contrast to *C. reinhardtii* the transport of putrescine by *Synechocystis* cells was influenced by inhibition of protein synthesis. On the other hand, no significant inhibition of putrescine transport by various amino acids was observed suggesting a distinctly different transport system for putrescine and amino acids (Table 3). Furthermore, it was observed that putrescine and spermine were able to significantly inhibit the uptake of spermidine in *Synechocystis* cells by 40% and 50%, respectively (Table 4). The $K_i$ of putrescine and spermine is also greater (4-6 fold) than the $K_m$ of spermidine indicating a higher affinity of spermidine to the transporter. Kinetics study of inhibition of spermidine uptake showed that putrescine and spermine were non-

competitive inhibitors suggesting different transporters for different polyamines in *Synechocystis* (Figure 19). Similar observations were reported for the uptake of spermidine by *Leishmania* (Basselin *et al.*, 2000). On the other hand, spermidine transport was not significantly affected by various amino acids thus differing from other cell types (Rinehart and Chen, 1984), suggesting a distinctly different transport system for spermidine and amino acids in *Synechocystis*. Taken together, it appears that *Synechocystis* cells possess highly specific transport system for the uptake of spermidine.

Many reports suggest that polyamines are involved in plant stress response including ionic homeostasis within cells disturbed by osmotic stress (Bouchereau *et al.*, 1999). We have shown here that the accumulation of putrescine and spermidine in *Synechocystis* sp. PCC 6803 in the presence of low concentration of NaCl and sorbitol was a result of an osmotic effect rather than an ionic effect (Figure 24 and Figure 25). This was based on the results showing that sorbitol with no ionic effect also produced similar pattern of stimulation of polyamine uptake seen for NaCl. It is noted that a marginal level of putrescine and spermidine uptake could be detected in the absence of osmotic upshift. Without stress this low level of putrescine and spermidine taken up might serve as substrate for metabolism. Increasing the osmolality resulted in the enhancement of the uptake rate since cells require putrescine and also spermidine to thrive better against osmotic upshift. However, at much higher osmolality a decline in putrescine and spermidine uptake was detected which might be due to less energy available for transport as a consequence of impaired metabolic function. All these results suggested that polyamine transport in *Synechocystis* was efficient at moderate osmolalities. This was similarly reported in *E. coli* (Munro *et al.*, 1974 and 1972). Previous finding revealed that the hyperosmotic stress induced excretion of putrescine

in *Escherichia coli* mutants (Munro and Sauerbier, 1973) and could activate proline transporter (Milner *et al*., 1988). Worth mentioning here is that the slight stimulation of putrescine and spermidine uptake by both NaCl and sorbitol could not be ascribed to osmotic or salt stress effect. The increased uptake might reflect a better physiological state of *Synechocystis* cells rather than direct effect on the uptake system. The increase in putrescine and also spermidine uptake are not be related to salt or osmotic acclimation since no parallel increase in the uptake was observed with the increase in the external osmolality. Indeed, a progressive decline in putrescine uptake was evident at greater than 50 mM NaCl and 100 mM sorbitol.

Photosynthetic bacteria must acclimate to changing light intensity, osmolarity, temperature and nutrient availability in their environment by strictly regulating the expression of distinct sets of genes, as shown in *Synechocystis* (Murata and Suzuki, 2005). In the present work, we have focused on the uncharacterized environmental regulation of the *potD* gene at the transcriptional level. Since, light is not only energy source, but also a signal in regulation of cyanobacterial gene expression (Hübschmann *et al*., 2005, Gill *et al*., 2002, Hihara *et al*., 2001), effect of light on the steady state transcript amounts of *Synechocystis potD* gene under was studied. According to the results, the transcript amounts of *potD* gene showed no apparent change in respond to light (Figure 26 and Figure 27). Interestingly, the transcript amounts were noticed to increase markedly after shifting 3-d dark-adapted cells to light to 5 min, while 18-h dark-adapted cells showed no change. These results show that light is necessary to activate the transcription of *potD* gene after a long period of darkness. Similar observation was found in the uptake activity of putrescine and spermidine (Table 5 and Table 6). The responsiveness of photosynthesis genes to white light has recently become obvious in a study of *Synechocystis* global gene expression profile (Huang *et*

*al*., 2002). Furthermore, the transcript amounts of polyamine biosynthesis gene (*adc*) under light-to-dark transition were observed to remain unchanged in *Synechocystis* (Jantaro *et al*., 2005).

Recent findings on the genome-wide responses of gene expression of *Synechocystis* to high osmolarlity stresses were studied (Kanesaki *et al*., 2002, Marin *et al*., 2004). The signal regulations by salt stress were perceived as distinct signals through the Hik-Rre systems (Shoumskaya *et al*., 2005) which may respond by direct osmolarity-induced activation of the transport or by enhanced expression of the corresponding gene. In the present study, the effect of osmolarlity on the steady-state transcriptional levels of *potD* gene was investigated. The transcript amounts of *potD* gene were noticed to increase significantly after exposure to long-term salt and osmotic stresses, but no response for short-term stresses. Corresponding to our previous results, the accumulation of cellular polyamine levels was increased after exposure to long-term salt and osmotic stresses in *Synechocystis* (Jantaro *et al*., 2003). These results suggest that *Synechocystis* acclimated to long-term osmolarlity stress by accumulating polyamine and regulating the transcript amounts of *potD* gene.

Non-optimal temperature, either low or high temperature, is an effective abiotic factor, which is known to influence polyamine metabolism in plants (Bouchereau *et al*., 1999). The expression of certain cold-inducible genes in *Synechocystis* was perceived as distinct signals through the histidine kinase system (Suzuki *et al.*, 2001). Chilling stress (22$^{o}$C) was observed to enhance the transcriptional levels of gene encoding sulfate transport ATP-binding system in *Synechocystis* (Inaba *et al*., 2003). Additionally, heat stress (42$^{o}$C) was noticed to markedly increase the specific activity of polyamine biosynthetic enzyme (ADC) even

though did not affect on the steady-state transcript amounts of ADC gene in *Synechocystis* (Jantaro *et al*., 2005). Based on our study, non-optimal temperatures did not affect the steady-state transcript amounts of *pot*D gene within 5 min but cause a greatly altered transcript amounts after long period incubation (3 days). During exposure to chilling stress (18$^{o}$C), adapted-cells displayed a remarkable increase in transcript amounts of *potD* gene while to heat stress (42$^{o}$C) showed a draatic decrease in transcript amounts. These results also indicated that long-period time of non-optimal temperature could affect the expression of *potD* gene.

In addition to nutrient availability, deficiency of nourishing has been studied in cyanobacteria (Schwarz *et al.,* 2005). A full-genome microarray of the photosynthetic cyanobacterium *Synechocystis* sp. PCC 6803 genes were transcriptionally regulated by iron deficiency (Singh *et al.,* 2003, Odom *et al.,* 1993). Previously, it has been shown that the *slr1295* (*futA1*), *slr0513* (*futA2*), and *slr1319* genes encoding putative substrate binding proteins for iron transporter of *Synechocystis* were expressed at high levels in both iron replete and iron-deprived cells, with the amount of transcripts higher in iron-deprived cells(Katoh *et al.*, 2001). On the other hand the *sll1878* (*futC*) and *slr0327* (*futB*) genes of *Synechocystis* were expressed constitutively in iron-replete cells. In the present study, iron deficiency did not effect to the transcript amounts of *potD* gene both of short- and long-term stresses (Figure 26 and Figure 27). Furthermore, the effects of nitrogen starvation on the abundance of pigment molecules in *Synechocystis* have been well documents (Richaud *et al*., 2001). The results decrease in chlorophyll and phycobilisome content leads to dramatic change in cell color. Nitrate is the major source of nitrogen for cyanobacteria. Nitrogen deficiency was noticed to significantly enhance the transcript

amounts of *glnA* and *glnN* genes after 12-h limitation in *Synechocystis* (Reyes et al. 1997). Furthermore, it has been demonstrated that in the plasma membranes of *Synechococcus* sp. PCC 6301 and PCC 7942, formerly *A. nidulans* and *A. nidulans* R2 (Maduefio *et al*., 1988, Omata *et al*., 1989, Sivak *et al*., 1989), a polypeptide of 45-48 kDa drastically changes in amount, in response to variation in the nitrogen source. This protein constitutes major part of the plasma membranes in nitrate-grown cells and is usually lacking in ammonia-grown cells. Thus, it was suggested that the 45-kDa protein has a role in nitrate uptake. Further evidence for this role was provided by Omata *et al*., (1989), who cloned its gene, *nrtA* from *Anacystis nidulans* R2, and constructed a defined insertional mutant (M45) that lacked this protein. The M45 mutant has been shown to take up nitrate by typical passive diffusion kinetics, which required approximately a 40-time higher concentration of nitrate for growth than the wild type. Sequencing of the *nrtA* gene revealed that the corresponding protein is mostly hydrophilic and contains only two hydrophobic amino acid segments that could be membrane spanning (Omata, 1995); thus it is unlikely that the 45-kDa polypeptide is the transport protein. Further studies led to the identification of three more genes, *nrtB, nrtC* and *nrtD,* required for nitrate transport in *Synechococcus* PCC 7942. The deduced amino acid sequences of NrtB, NrtC and NrtD proteins (Omata, 1995) indicated that the nitrate transporter is a member of theABC (ATP-binding-cassette) super family of active transporters, also denoted as shock-sensitive transporters or four-component periplasmic transport systems (Higgins *et al*., 1990). It was suggested that the 45-kDa protein (NrtA) is the substrate-binding protein, thereby making the cyanobacterial nitrate transporter unique in having a substrate-binding protein that is membrane bound (Omata, 1995) instead of the usual bacterial periplasmic, hydrophilic protein. However, there is no biochemical evidence for the

involvement of ATP hydrolysis in nitrate transport in cyanobacteria. No phosphorylated polypeptides were found in plasma membranes exposed for 5 min to ammonia and the nitrate transport was completely inhibited. Therefore, it seems that ammonia elicited an effective control of nitrate transport through reversible phosphorylation/dephosphorylation, in addition to the control caused by nitrate at the transcriptional level. According to present results, change the N-source from nitrate to $NH_4Cl$ was observed to rapidly increase the expression of *potD* transcripts after 3 days limitation whilst not affect on the transcriptional levels of *potD* gene after 18 h. These results suggest that the transcriptional regulation of *potD* gene may be involved in response to changes in nitrate availability after long period starvation; this *potD* gene may play an important role in nitrogen metabolism in this cyanobacterium. In conclusion, our results suggest that the steady-state transcript amounts of *potD* gene were markedly regulated by a wide spectrum of long-term environmental stresses.

The transport of polyamines in bacterial cells has long been studied, but so far in *Synechocystis* the transport system is not well characterized. A PotD subunit has been identified, but so far no other subunits belonging to the transport complex have been found in *Synechocystis*. It has previously been reported that PotD from *E. coli* share no significant sequence similarity with other periplasmic substrate-binding proteins, but that they all share a similar fold of repeated α-β-α units (Sugiyama *et al.*, 1996). In this study we have found that *Synechocystis* PotD exhibits significant sequence similarity with other cyanobacterial periplasmic substrate-binding proteins identified to date, but lower similarity to *E. coli* PotD. Based on the earlier published information about all the periplasmic substrate-binding proteins having a similar structural fold, we were able to model the *Synechocystis* PotD using the known crystal

structure of *E. coli* as the template. The model is based on the crystal structure of the ligand-binding form of *E. coli* PotD. All periplasmic substrate-binding proteins have a similar tertiary structure, consisting of two domains. The proteins have been shown to change conformation from closed to open when binding the substrate (Spurlino *et al.*, 1991). The structure of the closed conformation, without a ligand, has not been solved so far. The active site has been shown to exist in a central cleft between the two domains, which also correlates with our model of *Synechocystis* PotD. Based on sequence alignments and our structural model the active site is well conserved. The ligand specificity, based on the model, appears to correlate with *E. coli* PotD in having preference for spermidine, but also binding putrescine. It has previously been found that residues Glu171, Trp255, Asp257 in the active site of *E. coli* PotD participate more strongly than other amino acids in the binding of spermidine. The sequence alignments and our structural model shows that the corresponding residues Glu209, Trp293, Asp295 in *Synechocystis* PotD are conserved and this indicates that they participate most strongly in the binding of spermidine in the active site.

We made multiple database searches in order to identify candidates for the channel-forming proteins PotB or PotC in *Synechocystis* genome, but were unable to find any candidates with a significant similarity. Since there seem not to be any sequences corresponding to the channel-forming proteins in *Synechocystis*, we speculate that PotD in fact might share the channel forming subunits (and the ATPase) with another transport system, as suggested also earlier (Igarashi and Kashiwagi, 1999). Another aspect is that *E.coli* PotB and PotC share a too low sequence similarity with *Synechocystis*, to be able to retrieve any similar sequences by BLAST search. The multiple sequences corresponding to the ATPase protein PotA that were retrieved from *Synechocystis* genome in BLAST searches all showed a very

close sequence identity, and based on sequence comparison solely we were not able to reliably identify a candidate for polyamine transport PotA.

Regulation of the polyamine transport protein D (PotD) at translational levels in *Synechocystis* have not been characterized earlier so far. In *pneumococcus*, PotD protein contributes to virulence in a murine sepsis model, may be a potential target for antimicrobial therapy on vaccine candidate (Ware *et al*., 2006), and possesses a characteristic Gram-positive signal peptide (Ware *et al*., 2005). Cellular location of polyamine transport protein PotD was described by Shah *et al.* (2006b). They had demonstrated that PotD was processed by secretory pathway. In contrast, *Synechocystis* PotD has no amino terminal signal sequence, suggesting that it has no processing by the general secretory pathway and subsequently extracellular location which is not similar to that shown in *Streptococcus pneumoniae*. The recombinant *Synechocystis* PotD protein has been characterized in *E. coli* system (Figure 33 and Figure 34). The recombinant *Synechocystis* His-PotD, which includes histidine tag at N-terminal, could be expressed in *Escherichia coli* system and it was shown to be overproduced after addition of 1 mM IPTG for 6 h. The expression profile was analyzed by SDS-PAGE and immunoblotting using an anti His-tag monoclonal antibody at a dilution of 1:3,000 to confirm the molecular weight (Figure 35). The recombinant protein had an expected molecular mass of 46 kDa. Similarly, the recombinant *Pneumoniae* PotD had high specificity to monoclonal anti-His and polyclonal antiserum against PotD with apparent molecular weight of 41 kDa (Shah *et al.*, 2006a; 2006b).

**Future perspectives**; the results I have obtained in this thesis have given rise to many new studies. For example, it would be interesting to characterize mutants of PotD protein in *Synechocystis*; would the lack of this protein affect the function of

polyamine transport?, to further assess the regulation of polyamine transport at translational levels in *Synechocystis* by using specific polyclonal PotD antibody, to use bioinformatics to search for still unknown proteins related function to PotD protein, and to use molecular analysis and proteomics tools to predict crystal structure of PotD.

# CHAPTER V

# CONCLUSION

The present study of the polyamine transport into a moderately salt tolerant cyanobacterium *Synechocystis* sp. PCC 6803 has revealed the following findings:

1. Exogenous polyamines were transported into *Synechocystis* cells and they could act as growth promoters in the presence of high salt concentration.
2. The transport of polyamine was shown to be saturable following typical Michaelis-Menten kinetics with an apparent affinity constant ($K_m$) value of 98 and 67 μM, the maximal velocity ($V_{max}$) value of 0.33 and 0.45 nmol/min/mg protein for putrescine and spermidine, respectively.
3. The polyamine uptake was pH-dependent and ATP-dependent requiring proton motive force with the contribution of both ΔpH and Δψ.
4. The *Synechocystis* cells possess highly specific transport system for the uptake of polyamines.
5. The inhibition kinetics of spermidine transport by putrescine and spermine was found to be non-competitive with $K_i$ values of 292 and 432 μM respectively.
6. The moderate external osmolality generated by either NaCl or sorbitol caused an increased polyamine transport.
7. The steady-state transcript amounts of the *potD* gene were under regulation of a wide spectrum of long-term environmental stresses, namely light intensity, salt, osmotic, temperature and nutrient availability even if no short-term regulation occurred under the same conditions.

8. The most significant decrease in accumulation of the *potD* transcripts under high temperature was not a result of a decreased stability of the transcript, but merely reflected an altered transcription activity under high temperature stress.
9. The recombinant *Synechocystis* His-PotD protein could be expressed in *Escherichia coli* strain BL21 (DE3) pLysS system.
10. Immunoblot analysis with monoclonal Anti-His antibody showed a single protein band of 46 kDa.
11. The sequence identity between *Synechocystis* PotD and *Escherichia coli* PotD was only 24 %, but the overall fold and the active site were found to be well conserved.

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# APPENDICES

# APPENDIX A

## BG-11 medium

**BG-11 medium (1,000 ml)**

| | Solid medium | Liquid medium |
|---|---|---|
| $H_2O$ | 947 ml | 967 ml |
| Becto-agar | 15 g | - |
| 100 x BG-FPC* | 10 ml | 10 ml |
| 189 mM $Na_2CO_3$ | 1 ml | 1 ml |
| 175 mM $K_2HPO_4$ | 1 ml | 1 ml |
| 6 mg/ml Feric ammonium citrate | 1 ml | 1 ml |
| 1 M TES | 10 ml | - |
| 30% $Na_2S_2O_3$.5H2O | 10 ml | - |
| 1 M Hepes-NaOH, pH 7.5 | 20 ml | 20 ml |

**100xBG-FPC***

| | 100 ml |
|---|---|
| $NaNO_3$ | 14.96 g |
| $MgSO_4.7H_2O$ | 0.75 g |
| $CaCl_2.2H_2O$ | 0.36 g |
| Citric acid | 0.065 g |
| 0.5M Na-EDTA | 55.4 ml |

*After autoclaved, add 10 ml of 1000xTrace metal

**1000xTrace metal mix (1,000 ml) ****

| | 1000 ml |
|---|---|
| $H_3BO_3$ | 2.86 g |
| $MnCl_2.4H_2O$ | 1.81 g |
| $ZnSO_4.7H_2O$ | 0.221 g |
| $Na_2MoO_4.2H_2O$ | 0.390 g |
| $CuSO_4.5H_2O$ | 0.080 g |
| $Co(NO_3)_2.6H_2O$ | 0.049 g |

** Sterile filtrate, store at 4 $^oC$

# APPENDIX B

## Bradford protein determination

| Bradford stock solution |
|---|
| 100 ml 95% ethanol |
| 200 ml 88% phosphoric acid |
| 350 mg Serva Blue G |
| Stable indefinitely at room temperature |

| Bradford working buffer |
|---|
| 425 ml distilled water |
| 15 ml 95% ethanol |
| 30 ml 88% phosphoric acid |
| 30 ml Bradford stock solution |
| Filter through Whatman No. 1 paper, Store at room temperature. Usable for several week, but may need to be refiltered |

**Assay**

1. Prepare protein solution 10 μl into tube
2. Add 1 ml Bradford working buffer and vortex
3. Read $OD_{595}$ after 10 min but before 1 h

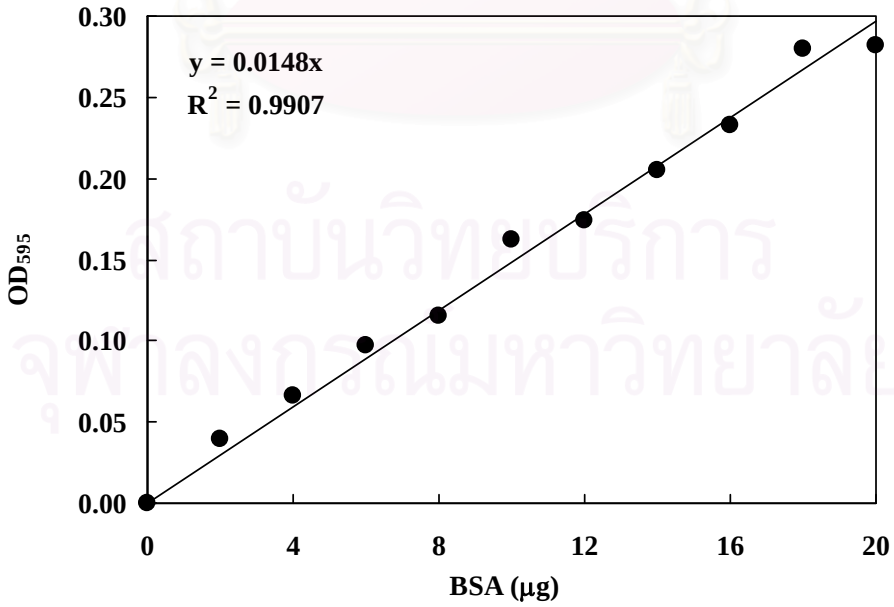


**Figure A. 1** Standard curve of BSA

# APPENDIX C

## Scintillation fluid preparation

**Standard curve of $^{14}C$**

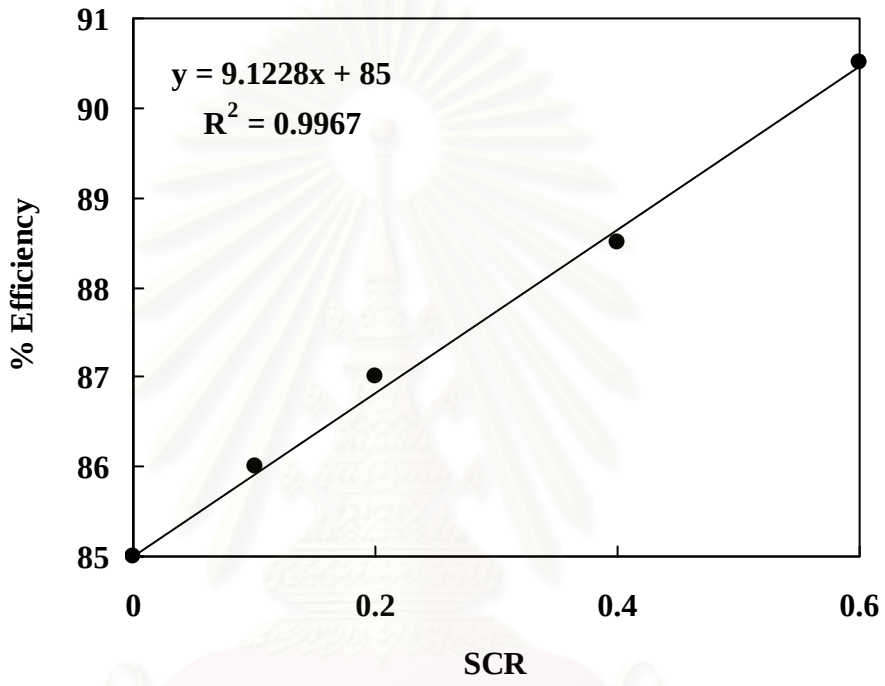


**Figure A.2** Standard curve of $^{14}C$

**Scintillation fluid (1,000 ml) 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 D

## PCR amplification protocol

### PCR amplification mixture

| | | |
|---|---|---|
| 10x PCR buffer* | 5.0 | µl |
| 5 mM dNTP mix | 2.5 | µl |
| Primer 5' (5 pmol/µl) | 5.0 | µl |
| Primer 3' (5 pmol/µl) | 5.0 | µl |
| cDNA or DNA | X | µl |
| MQ-water | 32-X | µl |
| DyNAzyme DNA polymerase** (2unit/µl) | 0.5 | µl |
| Total | 50.0 | µl |

** DyNAzyme$^{TM}$II is a thermostable DNA polymerase, has a half-life of 2.5 h at 96$^{o}$C.

### Program running PCR

| | | | |
|---|---|---|---|
| 95$^{o}$C | 2 | min (Pre denaturation time) | |
| 95$^{o}$C | 1 | min (Denaturation time) | 34 cycle |
| 55$^{o}$C | 1 | min (Annealing time) | |
| 72$^{o}$C | 1.30 | min (Extension time) | |
| 72$^{o}$C | 2 | min (Final Extension cycle) | |
| 4$^{o}$C | for ever | | |

### * 10x PCR buffer

100 mM Tris-HCl buffer pH 8.8 at 25$^{o}$C

15 mM $MgCl_2$, 500 mM KCl

1% Triton X-100

# APPENDIX E

## Preparation and Examination 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. TAE electrophoresis buffer (100 ml) was prepared
3. 0.8 g of agarose was weighed and put into the TAE 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/ml and 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.
10. 6xloading dye was added in each sample and then samples were loaded into the slot well.
11. The lid of the gel tank was closed and then the electrical status was adjusted to 80 volts
12. The electric current was turned off and the gel was removed into UV-light machine to see their bands

# APPENDIX F

## TAE buffer

**Working solution**

1X: 0.04 M Tris-acetate

0.01 M EDTA

**Concentrated stock solution (per liter)**

50X: 242 g Tris base

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA (pH 8.0)

# APPENDIX G

## TBE buffer

**Working solution**

0.5X: 0.045 M Tris-borate

0.001 M EDTA

**Concentrated stock solution (per liter)**

5X: 54 g Tris base

27.5 g Boric acid

20 ml 0.5 M EDTA (pH 8.0)

# APPENDIX H

## RNA extraction buffers

### Resuspension buffer

0.3 M sucrose

10 mM sodium acetate buffer pH 4.5

### Lysis buffer

2% SDS

10 mM sodium acetate buffer pH 4.5

### RNA storage buffer

20 mM sodium phosphate buffer pH 6.5

# APPENDIX I

## LB medium

### Liquid media, composition per 1 litre

| | | |
|---|---|---|
| Bacto tryptone | 10 | g |
| NaCl | 10 | g |
| Yeast extract | 5 | g |

### Solid media, composition per 1 litre

| | | |
|---|---|---|
| Bacto tryptone | 10 | g |
| NaCl | 10 | g |
| Yeast extract | 5 | g |
| Agar | 15 | g |

All compositions were dissolved together with 800 ml of distilled water; and then the mixture was adjusted to pH of 7.0 with 6 M NaOH. The total volume of solution was then adjusted to 1 litre with deionized water. The medium was sterilized by autoclaving at 15 Ib/in2 for 15 minute.

# APPENDIX J

## Polyacrylamide Gel Elctrophoresis

### Stock solutions

#### 30% (w/v) Acrylamide, 0.8% (w/v) bis-acrylamide, 100 ml

| | | |
|---|---|---|
| Acrylamide | 29.2 | g |
| N,N'-methylene-bis-acrylamide | 0.8 | g |
| Distilled water | 100 | ml |

The mixture was stirred until completely dissolved

#### 1.5 M Tris-HCl buffer pH 8.8, 100 ml

| | | |
|---|---|---|
| Tris (hydroxymethyl)-aminomethane | 18.17 | g |
| Distilled water | 100 | ml |

The mixture was adjusted pH to 8.8 with concentrated HCl slowly

#### 2.0 M Tris-HCl buffer pH 8.8, 100 ml

| | | |
|---|---|---|
| Tris (hydroxymethyl)-aminomethane | 24.2 | g |
| Distilled water | 100 | ml |

The mixture was adjusted pH to 8.8 with concentrated HCl slowly

**0.5 M Tris-HCl buffer pH 6.8, 100 ml**

| | | |
|---|---|---|
| Tris (hydroxymethyl)-aminomethane | 6.06 | g |
| Distilled water | 100 | ml |

The mixture was adjusted pH to 6.8 with concentrated HCl slowly

**1 M Tris-HCl buffer pH 6.8, 100 ml**

| | | |
|---|---|---|
| Tris (hydroxymethyl)-aminomethane | 12.1 | g |
| Distilled water | 100 | ml |

The mixture was adjusted pH to 6.8 with concentrated HCl slowly

**10% SDS, 100 ml (stored at room temperature)**

| | | |
|---|---|---|
| SDS | 10 | g |
| Distilled water | 100 | ml |

**10% Ammoniumpersulfate (APS), 1 ml**

| | | |
|---|---|---|
| APS | 0.1 | g |
| Distilled water | 100 | ml |



**0.5 % Bromophenol blue**

| | | |
|---|---|---|
| Bromophenol blue | 0.05 | g |
| Distilled water | 100 | ml |

**Solution B (SDS-PAGE)**

| | | |
|---|---|---|
| 2 M Tris-HCl buffer pH 8.8 | 75 | ml |
| 10% SDS | 4 | ml |
| Distilled water | 21 | ml |

**Solution C (SDS-PAGE)**

| | | |
|---|---|---|
| 1 M Tris-HCl buffer pH 6.8 | 50 | ml |
| 10% SDS | 4 | ml |
| Distilled water | 46 | ml |

**Non-denaturing PAGE**

**12% separating gel**

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



**5% stacking gel**

| | | |
|---|---|---|
| 30% acrylamide solution | 1.67 | ml |
| 1.5 M Tris-HCl buffer pH 8.8 | 2.50 | ml |
| Distilled water | 5.8 | ml |
| 10% APS | 50 | μl |
| TEMED | 5 | μl |

**Sample buffer**

| | | |
|---|---|---|
| 0.5 M Tris-HCl buffer pH 6.8 | 1 | ml |
| Glycerol | 8.0 | ml |
| 0.5% Bromophenol blue | 0.5 | ml |
| Distilled water | 5.8 | ml |

**Electrophoresis buffer, 1000 ml (25 mM Tris, 192 mM glycine)**

| | | |
|---|---|---|
| Tris (hydroxymethyl)-aminomethane | 3.0 | g |
| Glycine | 14.4 | g |
| Distilled water | 1000 | ml |

**SDS- PAGE**

**12% separating gel**

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



**5% stacking gel**

| | | |
|---|---|---|
| 30% acrylamide solution | 1.67 | ml |
| Solution | 2.50 | ml |
| Distilled water | 5.8 | ml |
| 10% APS | 50 | µl |
| TEMED | 5 | µl |

**Sample buffer**

| | | |
|---|---|---|
| 0.5 M Tris-HCl buffer pH 6.8 | 0.6 | ml |
| Glycerol | 8.0 | ml |
| 0.5% Bromophenol blue | 0.5 | ml |
| 10% SDS | 2.0 | ml |
| 2-mercaptoethanol | 0.5 | ml |
| Distilled water | 5.8 | ml |

* The ratio of sample and sample buffer is 4:1. The mixture was heated for 5 min in boiling water before loading to the gel.

**Electrophoresis buffer, 1000 ml (25 mM Tris, 192 mM glycine)**

| | | |
|---|---|---|
| Tris (hydroxymethyl)-aminomethane | 3.0 | g |
| Glycine | 14.4 | g |
| SDS | 1.0 | g |
| Distilled water to | 1000 | ml |

# APPENDIX K

## Chlorophyll content determination

1. The 795 µl of 80% acetone was added into the microcentrifuge tube.
2. 5 µl of cell suspensions was added.
3. The mixture was then vortexed for 2-3 min
4. Centrifugation at 10,000xg for 5 min at 4$^{o}$C
5. Measurement the optical density at 663 nm
6. Calculation of Chlorophyll content by

   [OD663 x 12.7 x (800/5)]/1000 =…….. µg ChlA/ µl

   Coefficient constant is 12.7

# APPENDIX L

## Reagents for alkaline lysis

**Solution I (100 ml)**

5.0 ml 1.0 M Glucose

2.5 ml 1.0 M Tris-HCl, pH 8.0

2.0 ml 0.5 M EDTA, pH 8.0

After autoclave 20 µg/ml of RNase was added and stored at 4$^{o}$C

**Solution II (25 ml)**

0.5 ml 10 M NaOH

1.25 ml 20% SDS

**Solution III (500 ml)**

147 g potassium acetate

57.5 ml glacial acetate

Autoclave and store at 4$^{o}$C

**TE buffer (500 ml)**

5 ml 1 M Tris-HCl, pH 8.0

1 ml 0.5 M EDTA

Autoclave and store at room temperature

# APPENDIX M

## Western Blotting Reagents

**10X Chemiluminescene detective solution**

100 mM Tris-HCl buffer pH 9.5

100 mM NaCl

10 mM $MgCl_2$

(used time = 1X)

100 mg/ml (33 µl/10 ml) BCIP

50 mg/ml (88 µl/10 ml) NBT

**10X TBS (Tris-buffer-saline)**

200 mM Tris-HCl buffer pH 7.5

5 M NaCl

**TTBS**

1X TBS

0.05% Tween-20

**Blocking solution**

5% Skim milk in TBS

**Antibody buffer**

1% Skim milk in TTBS

# APPENDIX N

## Detection reagent for western blotting

1 ml of 100 mM Tris-HCl pH 9.5

1 ml of 100 mM NaCl,

0.5 ml of 10 mM $MgCl_2$,

33 µl of BCIP (5-Bromo-4-chloro-3-indolyl phosphate, 74 mg/ml)

88 µl of NBT (*p*-Nitro blue tetrazolium chloride, 5 mg/ml)

Detection reagent for western blotting should be freshly prepared and used within 30 min. When the bands are of the desire intensity, the membrane was washed with deionized water twice.

# APPENDIX O

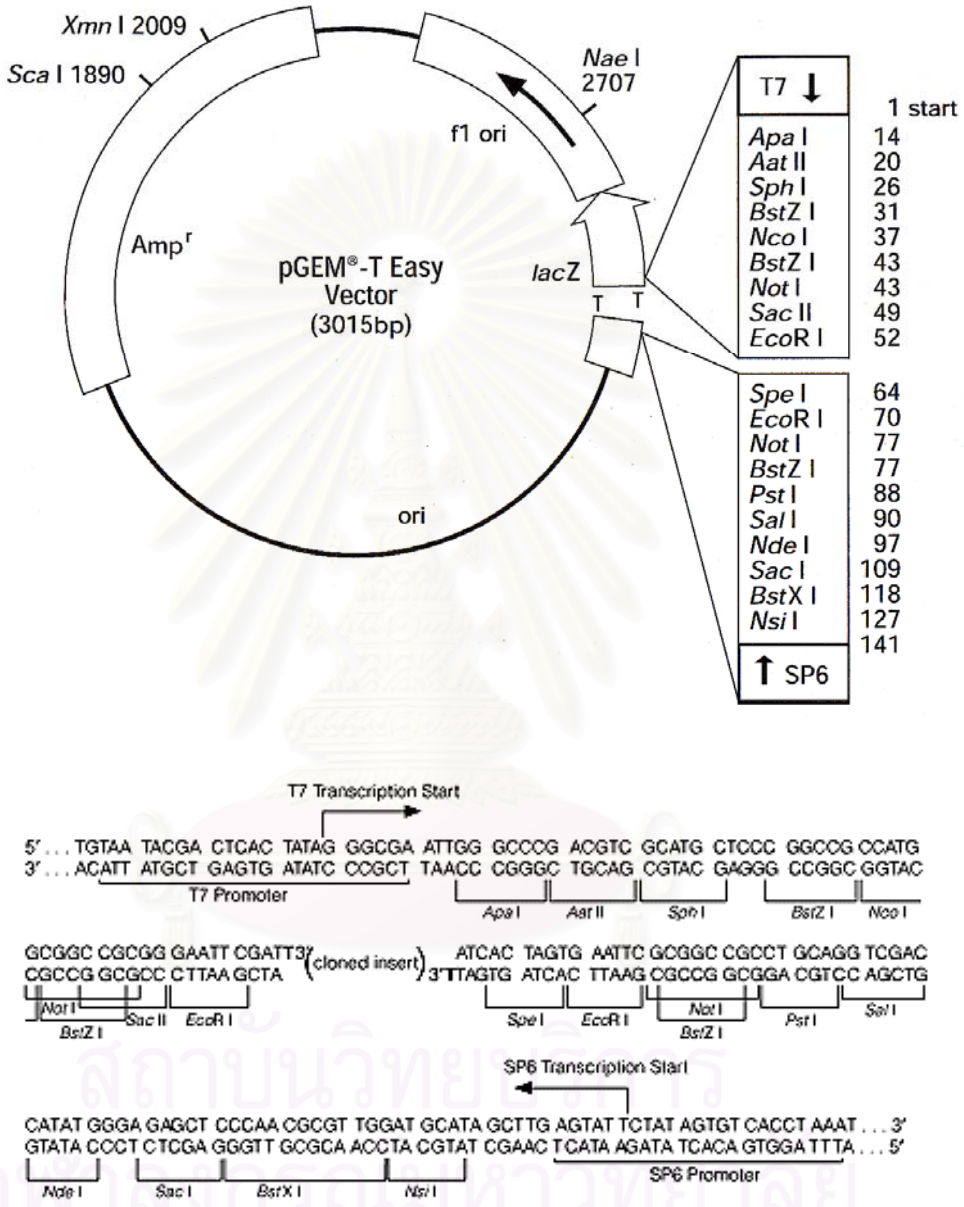


**Figure A.3** pGEM®-T Easy Vector circle map and cloning/expression region

# APPENDIX P

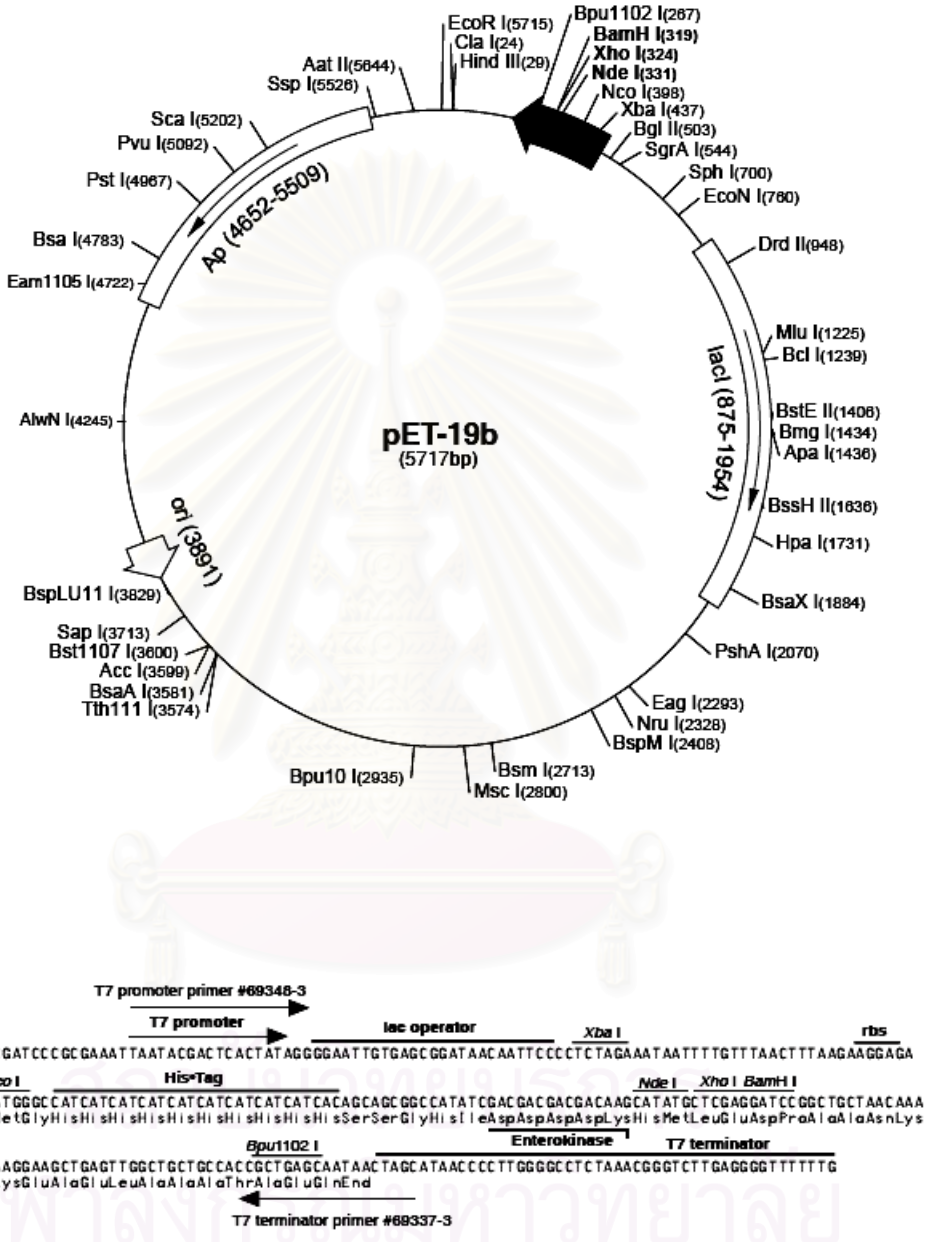


**Figure A.4** pET-19b Vector circle map and cloning/expression region

# APPENDIX Q

## PERSONAL INFORMATION

### FIELD OF RESEARCH INTEREST

My dissertation focused on polyamine transport in cyanobacterium *Synechocystis* sp. PCC 6803 under environmental stresses. Methodologically, I am familiar with biochemical laboratory techniques such as radioactivity assays, RT-PCR, mutant construction, RNA and DNA extraction, DNA cloning, protein expression, Northern blot, Western blot, FPLC, HPLC, thylakoid membrane preparation, plasma-membrane isolation, enzyme purification including gel-filtration, ion-exchange chromatography, and Microsoft Office as well as Photoshop Adobe and CorelDraw programs.

### SCHOLARSHIPS

June 2003 - May 2007

Academic scholarship from Thailand Research Fund to the Royal Golden Jubilee Ph.D. program, Bangkok, Thailand

January 2008-July 2008

Academic fellowship from CIMO through Department of Biology, Laboratory of Plant Physiology and Molecular Biology, University of Turku, Finland

### ACADEMIC EXPERIENCES

1. The 17$^{th}$ FAOBMB Symposium/2$^{nd}$ IUBMB Special Meeting/7th A-IMBN Conference Genomics and Health in the 21$^{st}$ Century at The Imperial Queen's Park Hotel, Bangkok, Thailand 22-26 November 2004. (Secretary general)
2. The Examination for Radiation Protection Course Level II. Office of Atoms for Peace Ministry of Science and Technology, Bangkok Thailand 17-28 July 2006 (Proceeding).
3. The 32$^{nd}$ Congress on Science and Technology of Thailand Science and Technology for Sufficiency Economy to celebrate the 60$^{th}$ Anniversary of His Majesty the King's Accession to the Throne, Queen Sirikit National Convention

Center, Bangkok Thailand 10-12 October 2006. (Proceeding) “The Role of Putrescine for Osmoregulation in Cyanobacterium *Synechocystis* sp. PCC 6803”

4. The 11th Biological Sciences Graduate Congress. (Explorations Towards the Improved Quality of Life, Sustainable Development, and Secured Future) Faculty of Science, Chulalongkorn University, Thailand. December 15-17, 2006.
5. RGJ-Ph.D. Congress VIII, Jomtein Palm Beach Resort Pattaya, Chonburi. April 20-22, 2007. (Proceeding) “Transcriptional Regulation of Periplasmic Polyamine-Binding Protein as Responses to Environmental Signals in *Synechocystis* sp. PCC 6803’’.
6. The 33rd Congress on Science and Technology of Thailand Science and Technology for Global Sustainability, Walailak University, Nakhon Sri Thammarat, Thailand 18-20 October 2007 (Proceeding). “Changes in Spermidine Uptake in Response to Abiotic Stress in Cyanobacterium *Synechocystis* sp. PCC 6803”.
7. The 12th Biological Sciences Graduate Congress (Science Empowering life). Institute of Biological Sciences, University of Malaya, Kuala Lumpur, Malaysia December 17-19, 2007 (Proceeding) “Postgenomic studies of Polyamine Transport Protein D (PotD) in Cyanobacterium *Synechocystis* sp. PCC 6803”.

**PUBLICATIONS**

1. Wiangnon, K., **Raksajit, W.** and Incharoensakdi, A. (2007) Presence of a plasma membrane $Na^+$-ATPase in the halotolerant cyanobacterium *Aphanothece halophytica*. *FEMS Microbiol. Lett.* **270**, 139-145
2. **Raksajit, W.**, Mäenpää, P. and Incharoensakdi, A. (2006) Putrescine transport in a cyanobacterium *Synechocystis* sp. PCC 6803. *J. Biochem. Mol. Biol.* **39**, 394-399.
3. **Raksajit, W.**, Yodsang, P., Mäenpää, P. and Incharoensakdi, A. Characterization of spermidine transport system in a cyanobacterium *Synechocystis* sp. PCC 6803. (Revised)
4. Brandt, A.M., **Raksajit, W.**, Mulo, P., Incharoensakdi, A., Salminen, T., and Mäenpää, P. Postgenomic studies of *potD* encoding the putative periplasmic substrate-binding subunit of a polyamine transporter complex in *Synechocystis* sp. PCC 6803. (Revised)

**REFERENCES**

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*Journal of Biochemistry and Molecular Biology*, Vol. 39, No. 4, July 2006, pp. 394-399

JOURNAL OF Biochemistry and Molecular Biology

# Putrescine Transport in a Cyanobacterium *Synechocystis* sp. PCC 6803

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*Received February 8 2006, Accepted April 3 2006*

**The transport of putrescine into a moderately salt tolerant cyanobacterium *Synechocystis* sp. PCC 6803 was characterized by measuring the uptake of radioactively-labeled putrescine. Putrescine transport showed saturation kinetics with an apparent $K_m$ of 92 ± 10 μM and $V_{max}$ of 0.33 ± 0.05 nmol/min/mg protein. The transport of putrescine was pH-dependent with highest activity at pH 7.0. Strong inhibition of putrescine transport was caused by spermine and spermidine whereas only slight inhibition was observed by the addition of various amino acids. These results suggest that the transport system in *Synechocystis* sp. PCC 6803 is highly specific for polyamines. Putrescine transport is energy-dependent as evidenced by the inhibition by various metabolic inhibitors and ionophores. Slow growth was observed in cells grown under salt stress. Addition of low concentration of putrescine could restore growth almost to the level observed in the absence of salt stress. Upshift of the external osmolality generated by either NaCl or sorbitol caused an increased putrescine transport with an optimum 2-fold increase at 20 mosmol/kg. The stimulation of putrescine transport mediated by osmotic upshift was abolished in chloramphenicol-treated cells, suggesting possible involvement of an inducible transport system.**

**Keywords:** Cyanobacterium, Osmotic activation, Putrescine transport, *Synechocystis* sp. PCC 6803

## Introduction

Living organisms have evolved strategies for the maintenance of metabolism subject to fluctuations in osmotic strength. The response to hyperosmotic stress can be separated into two main processes. First, the reduction of the water potential after hyperosmotic shock causes the cell to lose water and shrink. Consequently, this process is counteracted by an increase in organic solutes entering the cells along chemical gradients (Wood, 1999; Heide *et al*., 2001). Many organisms that thrive in high salinity use two strategies to acquire high intracellular amounts of compatible solutes: *de novo* synthesis and/or transport from the surroundings. Salt-loaded cells accumulate compatible solutes, which are low molecular weight compounds that do not interfere with cell metabolism, such as carnitine (Fraser and O'Byrne, 2002), choline (Incharoensakdi and Karnchanatat, 2003), ectoine (Jebbar *et al*., 1997), glycine betaine (Incharoensakdi and Wutipraditkul, 1999), proline (Dunlap and Csonka, 1985) and polyamines (Flores and Galston, 1984).

Polyamines, represented by putrescine, spermidine and spermine, play important roles in all aspects of cellular processes such as growth, development and biosynthesis (Tabor and Tabor, 1985; Bouchereau *et al*., 1999; Thomas and Thomas, 2001). Uptake systems specific for polyamines are reported in both prokaryotic and eukaryotic cells (Rinehart and Chen, 1984; Igarashi and Kashiwagi, 1999; Tassoni *et al*., 2002). The properties of three polyamine uptake systems were characterized by cloning the genes for these systems in *Escherichia coli* (Igarashi *et al*., 2001). The first system which is spermidine-preferential consists of PotA, PotB, PotC and PotD proteins whereas the second system which is putrescine-specific consists of PotF, PotG, PotH and PotI proteins. Both systems are classified as ABC [ATP binding cassette]-type transporters. The third system is involved in the excretion of putrescine by a putrescine-ornithine antiporter activity consisting of only the PotE protein. The importance of polyamine uptake by living cells is far from clear, since all organisms have enzymes able to synthesize them and maintain optimum levels for their metabolism. Indeed, most of studies for the uptake of polyamine were carried out in *Escherichia coli*. There are a few scattered reports on putrescine transport in cyanobacteria. Perhaps the earliest one was the study in *Anacystis nidulans* where the mechanism of putrescine transport was passive diffusion and ion trapping within the cells (Guarino and Cohen, 1979).

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*Synechocystis* sp. PCC 6803, which is a moderately salt-tolerant cyanobacterium, can accumulate glucosylglycerol as an osmoprotective compound by *de novo* synthesis (Mikkat *et al*., 1997; Ferjani *et al*., 2003). Previously, we reported that the effect of long-term salt and osmotic stresses resulted in the increase of the cellular putrescine contents of this cyanobacterium (Jantaro *et al*., 2003). In the present study, we have investigated putrescine transport in *Synechocystis* sp. PCC 6803 and found that putrescine transport was energy dependent, specific for polyamines and could be stimulated by moderate osmotic upshift.

## Materials and Methods

**Organism and culture conditions.** Axenic cells of *Synechocystis* sp. PCC 6803 were grown photoautotrophically in BG-11 medium at 30°C under continuous illumination (warm white fluorescent tubes) of 50 μE/m$^2$/s. The cultivation was performed in cotton-plugged 250-ml conical flasks on a rotatory shaker at 160 rpm. The growth rate was monitored by measuring the optical density of the culture at 730 nm with a Spectronic® Genesys™2 spectrophotometer.

**Transport assays.** Cells at late log phase were harvested by centrifugation (8,000 × *g*, 10 min, 4°C), washed twice with 50 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid(Hepes)-KOH buffer pH 7.6 containing 0.4% glucose and suspended in the same buffer to yield a protein concentration of 0.1 mg cell protein/ml. The cell suspension (95 μl) was preincubated at 37°C for 5 min. The uptake experiment was initiated by the addition of 5 μl [methylene-$^{14}$C] putrescine (Radiochemical Centre) with a specific activity of 2 mCi/mmol at a final concentration of 50 μM. After incubation, the cells were rapidly collected on membrane filters (cellulose acetate, 0.45 μm pore size; Millipore Corp.). The filters were washed twice with 1 ml of cold buffer containing 1 mM putrescine to remove the adsorbed [methylene-$^{14}$C] putrescine. The amount of amine adsorbed to the cell surface and the filter was less than 0.1% of the added amine under these experimental conditions. The radioactivity on the filter was determined with a liquid scintillation counter. Initial putrescine uptake rates were determined from the linear increase of uptake and are expressed as nanomoles of putrescine taken up per minute per milligram protein. Cellular protein was determined by the method of Bradford (1976) using bovine serum albumin as a standard. Cells were lysed by the addition of the Bradford reagent. After centrifugation, the supernatant was measured for optical density at 595 nm.The osmolality of the uptake assay medium was measured with a Wescor vapor pressure osmometer model 5520. For the assay of inhibition by substrate analogs, cells were added to a mixture of labeled substrate and 20-fold excess of unlabeled analogs. In inhibitory assays, cells were preincubated with the inhibitor for 30 min at 37°C before the addition of [methylene-$^{14}$C] putrescine.

## Results

**Protection against growth inhibition by putrescine.** Growth of *Synechocystis* cells in the medium containing 550 mM NaCl was not as rapid as that in the absence of NaCl (Fig. 1). Addition of 0.5 mM putrescine could alleviate the effect of salt stress with apparently normal growth up to 6 days. The addition of 1 mM putrescine caused similar growth pattern to that of 0.5 mM putrescine during the first 3 days. Slower growth occurred after 3 days with the apparent cessation of growth observed after 6 days.

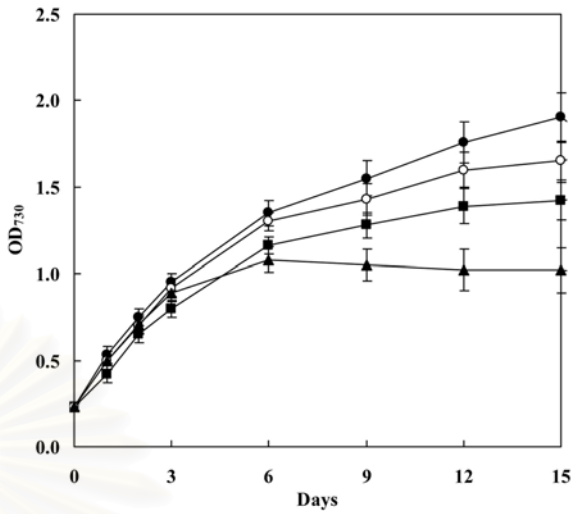


**Fig. 1.** Growth promoting effect of putrescine on salt-stressed *Synechocystis* sp. PCC 6803. Cells were grown under normal condition (●), salt stress condition by the addition of 550 mM NaCl (■), 550 mM NaCl plus 0.5 mM putrescine (○), 550 mM NaCl plus 1.0 mM putrescine (▲). The data are means from three independent experiments with vertical bars representing standard errors of the means, n = 3.

**Kinetics of putrescine uptake.** Incubation of *Synechocystis* cells with increasing concentration of putrescine up to 500 μM resulted in a saturable initial uptake rate (Fig. 2). A Lineweaver-Burk transformation of the data yielded a straight line typical of Michaelis-Menten kinetics. The apparent affinity constant ($K_m$) value of 92 ± 10 μM and the maximal velocity ($V_{max}$) value of 0.33 ± 0.05 nmol/min/mg protein were obtained.

**Effect of external pH on putrescine uptake.** Since putrescine carries a net positive charge, we therefore further tested whether the changes in external pH could affect its uptake. Among the three pH values tested, highest uptake occurred at neutral pH of 7.0 (Fig. 3). Alkaline condition yielded a higher rate of putrescine uptake than acidic condition. The initial rates of putrescine uptake in 100 mM Tris-maleate or Tris-HCl buffer at pH 5.5, 7.0, and 8.2 were estimated to be 0.024 ± 0.002, 0.167 ± 0.004, and 0.080 ± 0.004 nmol/min/mg protein, respectively.

**Specificity of putrescine uptake.** The specificity of putrescine uptake in intact cells was studied by addition of various amine

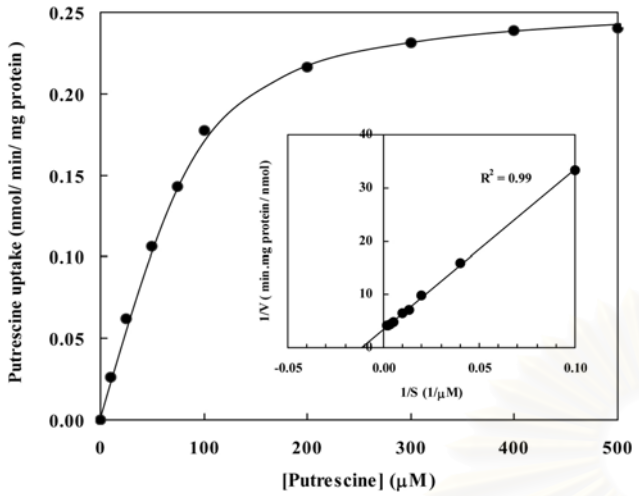


**Fig. 2.** Kinetics of putrescine uptak by *Synechocystis* sp. PCC 6803 incubated with 0-500 μM putrescine. Initial rates were determined with 1 min incubation time. Inset represents a Lineweaver-Burk transformation of the data. The line drawn is that derived from regression analysis of the data and the points shown are typical of this result from triplicate experiments.

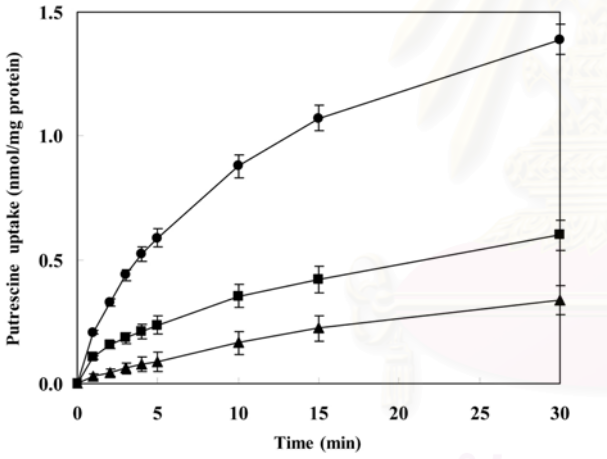


**Fig. 3.** Dependence of putrescine uptake on external pH. Putrescine uptake assay was done with the modification using 100 mM Tris-maleate for pH 5.5 (▲) and 100 mM Tris-HCl for pH 7.0 (●) and pH 8.2 (■). The data are means from three independent experiments with vertical bars representing standard errors of the means, n = 3.

analogs into the assay medium and following the rate of uptake. As shown in Table 1, agmatine, a putrescine precursor, had no effect on putrescine uptake. Slight inhibition of putrescine uptake occurred in the presence of alanine, glycine, glutamic acid, and serine. In contrast, spermidine and spermine, which are structurally similar to putrescine but with additional amino groups, showed 40 and 60% inhibition, respectively.

**Inhibition of putrescine uptake by metabolic inhibitors.** To determine whether putrescine uptake was energy-dependent, the effects of some inhibitors on the uptake activity were

**Table 1.** Effect of putrescine analogs on the putrescine uptake of *Synechocystis* sp. PCC 6803[a]

| Analog compound | Concentration | Putrescine uptake (%) |
|---|---|---|
| None | | 100 ± 2 |
| Agmatine | 1 mM | 98 ± 2 |
| Alanine | 1 mM | 91 ± 4 |
| Glycine | 1 mM | 87 ± 3 |
| Glutamic acid | 1 mM | 85 ± 5 |
| Serine | 1 mM | 84 ± 3 |
| Spermidine | 1 mM | 60 ± 5 |
| Spermine | 1 mM | 40 ± 6 |

[a]Cells were incubated in the mixture containing 1 mM unlabeled analog and 50 μM [Methylene-$^{14}$C] putrescine. Initial rates were determined with 1 min incubation time. The data shown are the means of three independent experiments representing the percent uptake rate relative to the control rate which was 0.12 nmol/min/mg protein.

**Table 2.** Effect of metabolic inhibitors on the putrescine uptake of *Synechocystis* sp. PCC 6803[a]

| Inhibitor | Concentration | Putrescine uptake (%) |
|---|---|---|
| None | | 100 ± 2 |
| *N*-Ethylmaleimide | 1 mM | 38 ± 5 |
| *p*-Chloromercurisulfonic acid | 1 mM | 20 ± 5 |
| Sodium arsenate | 1 mM | 13 ± 2 |
| Sodium fluoride | 1 mM | 26 ± 2 |
| Potassium cyanide | 1 mM | 39 ± 7 |
| Gramicidin D | 10 μM | 42 ± 5 |
| 2,4-Dinitrophenol | 1 mM | 36 ± 6 |
| *N,N'*-Dicyclohexylcarbodiimide | 80 μM | 26 ± 5 |
| Valinomycin | 10 μM | 9 ± 3 |
| Amiloride | 50 μM | 11 ± 4 |

[a]Cells were preincubated with inhibitors for 30 min before the addition of 50 μM [methylene-$^{14}$C] putrescine to initiate the uptake with 1 min incubation as described in Materials and Methods. The data shown are the means of three independent experiments representing the percent uptake rate relative to the control rate which was 0.12 nmol/min/mg protein.

studied. Results summarized in Table 2 show that *N*-ethylmaleimide and *p*-chloromercurisulfonic acid, which modify the protein structures, were effective inhibitors of putrescine uptake. The inhibitors for ATP formation, sodium arsenate and sodium fluoride also reduced the uptake activity suggesting the requirement of ATP for putrescine uptake. Interference of electron transport by potassium cyanide resulted in effective inhibition of putrescine uptake. Transport uncouplers such as gramicidin D and dinitrophenol, which dissipate proton motive force, could significantly inhibit putrescine uptake to a similar extent by about 60-65%. *N, N'*

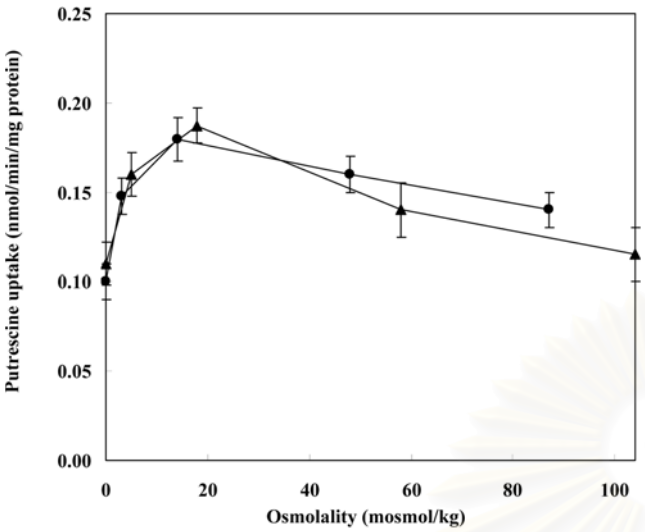


**Fig. 4.** Effect of external osmolality on putrescine uptake by *Synechocystis* sp. PCC 6803. Initial uptake rates (1 min incubation) were determined in the presence of increasing osmolality generated by NaCl (●) or sorbitol (▲). The data are means from three independent experiments with vertical bars representing standard errors of the means, n = 3.

dicyclohexylcarbodiimide, an ATPase inhibitor causing a reduction in pH gradient, also effectively inhibited putrescine uptake. Valinomycin, an ionophore collapsing the electrical potential with a reduction in ψ, strongly inhibited putrescine uptake. Similarly strong inhibition of putrescine uptake was also observed with amiloride, an inhibitor of $Na^+/H^+$ exchanger. All these results collectively indicate that the proton motive force with the contribution of both pH and ψ (electrical or membrane potential), plays an important role in putrescine transport in *Synechocystis* cells.

**Stimulation of putrescine uptake by osmotic upshift.** When testing the effect of osmotic upshift on putrescine uptake, increasing osmolality generated by either NaCl or sorbitol up to 20 mosmol/kg caused about 2-fold increase of putrescine uptake compared to the control (Fig. 4). When the osmolality was higher than 20 mosmol/kg the extent of the stimulation of the uptake was reduced. Moreover, the osmotic upshift higher than 100 mosmol/kg caused a progressive decline in putrescine uptake (data not shown). It is noteworthy that the uptake of putrescine responded to changes in osmolality with similar pattern regardless of the source generating the osmotic upshift.

**Induction of putrescine uptake.** Since NaCl-imposed osmotic upshift led to an increase of initial putrescine uptake rate, we investigated whether protein synthesis was involved in the stimulation of putrescine uptake. Pretreatment of cells in the presence of 50 mM NaCl with chloramphenicol dramatically lowered putrescine uptake rate, i.e. it was decreased to 50% of that without chloramphenicol pretreatment (Table 3). Cells

**Table 3.** Effect of chloramphenicol on putrescine uptake of *Synechocystis* sp. PCC 6803 [a]

| Condition | Concentration | Putrescine uptake (%) |
|---|---|---|
| Control | | 100 ± 2 |
| NaCl | 50 mM | 142 ± 3 |
| Chloramphenicol | 100 μg/ml | 63 ± 5 |
| NaCl + Chloramphenicol | 50 mM, 100 μg/ml | 74 ± 4 |

[a]Cells with or without NaCl were preincubated with chloramphenicol for 30 min before the addition of 50 μM [methylene-$^{14}$C] putrescine to initiate the uptake with 1 min incubation as described in Materials and Methods. The data shown are the means of three independent experiments. One hundred percent represents the uptake rate of 0.12 nmol/min/mg protein.

pretreated with chloramphenicol in the absence of NaCl also showed considerable reduction in the rate of putrescine uptake suggesting the involvement of protein synthesis in the uptake process.

## Discussion

Here we have described the existence of a transport system for putrescine in *Synechocystis* sp. PCC 6803. In a previous study, we demonstrated that *Synechocystis cells* were able to accumulate polyamines after exposure to long-term osmotic stresses (Jantaro *et al*., 2003). However, the synthesis of polyamines requires an investment of organic carbon and metabolic energy. It is likely that the portion of cell carbon and energy diverted for polyamine synthesis is at least partially responsible for the observed reduction in the growth rate of cells under osmotic stress. Therefore, the ability to accumulate polyamines via the uptake from the growth medium may be advantageous in terms of the adaptive response to high salinity environment. The data in Fig. 1 indicated that exogenous putrescine could be transported into *Synechocystis* cells and could act as growth promoter in the presence of high salt concentration. These results seem to suggest the role of putrescine as a compatible solute in this cyanobacterium. Nevertheless, the protective role by putrescine against inhibition of growth under hyperosmotic stress was not as efficient as that by glucosylglycerol, suggesting that putrescine per se is not an osmoprotectant (Ferjani *et al*., 2003; Mikkat *et al*., 1997). Indeed, we previously showed that the levels of putrescine accumulated in *Synechocystis* cells after salt and osmotic stress treatments were not sufficient to account for any osmotic importance (Jantaro *et al*., 2003). Furthermore, high concentration of intracellular putrescine is detrimental to the growth of cells since the oxidation of polyamines can generate toxic compounds such as acrolein (Sakata *et al*., 2003). It is worth mentioning here that the addition of exogenous putrescine higher than 1.0 mM was

inhibitory to the growth of *Synechocystis* cells. Similar results were reported for *Chlamydomonas reinhardtii* where cytotoxic effects were observed when exogenous putrescine was higher than 1.5 mM (Theiss *et al*., 2004). The results in Fig. 1 clearly showed that the growth of *Synechocystis* cells was inhibited after 6 days in the presence of 1 mM putrescine. This could be ascribed to the toxic effects caused by the accumulation of intracellular putrescine. Previously, it has been shown in *Anacystis nidulans* that exposure of cells to 150 μM putrescine resulted in cell death within 3 h concomitant with high intracellular putrescine concentration of 100 mM (Guarino and Cohen, 1979).

The putrescine transport system in *Synechocystis* sp. PCC 6803 was saturable displaying typical Michaelis-Menten type kinetics (Fig. 2). *Synechocystis* cells exhibited a low affinity ($K_m$ 92 μM) for putrescine transport, which is in contrast to a rather high affinity for *E. coli* with the $K_m$ value of 1.4 μM (Kashiwaki *et al*., 1986). Low affinity for putrescine transport indicated the slow uptake of putrescine by *Synechocystis* cells. This was corroborated by the observations that rather long period of exposure to putrescine was required for growth inhibition and growth stimulation to manifest in the presence of 1.0 and 0.5 mM putrescine, respectively (Fig. 1). The transport system for putrescine in *Synechocystis* sp. PCC 6803 is an active transport. Putrescine transport is highly inhibited by various energy generation inhibitors (Table 2) suggesting the requirement of energy for the transport system. The coupling mechanism between $Na^+$ electrochemical gradient and transport systems has been extensively studied. Previously, we have shown that a halotolerant cyanobacterium *Aphanothece halophytica* contains $Na^+$-dependent choline and nitrate transport systems (Incharoensakdi and Karnchanatat, 2003; Incharoensakdi and Wangsupa, 2003; Incharoensakdi and Laloknam, 2005). $Na^+$-gradient has been shown to be a major source of energy, coupling with the active transport for polyamine in mouse cells (Rinehart and Chen, 1984). Moreover, the dependency on $Na^+$ has been reported for the transport of potassium and bicarbonate into *Synechocystis* PCC 6803 (Matsuda *et al*., 2004; Shibata *et al*., 2002). Although we observed a reduction of putrescine uptake in the presence of amiloride, a $Na^+$-gradient dissipator, it is premature to suggest the involvement of $Na^+$-gradient in the transport of putrescine into *Synechocystis* cells. The ionophores, valinomycin and 2,4-dinitrophenol and DCCD, an ATPase inhibitor, strongly inhibited the uptake of putrescine, suggesting that the energy required is provided by electron transport in the cytoplasmic membrane through a proton motive force, with the hydrolysis of ATP. Previously, proton motive force-dependent transport of polyamine has been shown in *Saccharomyces cerevisiae* (Kakinuma *et al*., 1992) and *E. coli* (Kashiwaki *et al*., 1986).

The present study revealed that spermidine and spermine could significantly inhibit the uptake of putrescine in *Synechocystis* cells (Table 1) suggesting the same transport system for these three polyamines. Similar observations were reported for the uptake of putrescine by a green alga *Chlamydomonas reinhardtii* (Theiss *et al*., 2002). However, in contrast to *C. reinhardtii* the transport of putrescine by *Synechocystis* cells was influenced by inhibition of protein synthesis (Table 3). On the other hand, no significant inhibition of putrescine transport by various amino acids was observed suggesting a distinctly different transport system for putrescine and amino acids.

*Synechocystis* cells appeared to possess putrescine transport system with optimal activity around neutral pH (Fig. 3). This was in agreement with a previous study in a parasitic protozoan, *Leishmania mexicana* Promastigote (Basselin *et al*, 2000).

We have shown here that the accumulation of putrescine in *Synechocystis* sp. PCC 6803 in the presence of low concentration of NaCl and sorbitol was a result of an osmotic effect rather than an ionic effect. This was based on the results showing that sorbitol with no ionic effect also produced similar pattern of stimulation seen for NaCl (Fig. 4). It is noted that a marginal level of putrescine uptake could be detected in the absence of osmotic upshift. Without stress this low level of putrescine taken up might serve as nutrient for metabolic function. Increasing the osmolality resulted in the enhancement of the uptake rate since cells require putrescine to better thrive against osmotic upshift. However, at much higher osmolality a decline in putrescine uptake was detected which might be due to less energy available for transport as a consequence of impaired metabolic function. All these results suggested that putrescine transport in *Synechocystis* was efficient at moderate osmolalities. This was similarly reported in *E. coli* (Munro *et al*., 1974). Worth mentioning here is that the slight stimulation of putrescine uptake by both NaCl and sorbitol could not be ascribed to the osmotic or salt stress effect. The increased uptake might reflect a better physiological state of *Synechocystis* cells rather than the direct effect on the uptake system. The increase in putrescine uptake would not be related to salt or osmotic acclimation since no parallel increase in the uptake was observed with the increase in the external osmolality.

Results in Table 3 also indicated that protein synthesis is required for the functional putrescine transport since it was significantly inhibited by chloramphenicol. Future work on the characterization of genes as well as the transporters involved in putrescine uptake in *Synechocystis* sp. PCC 6803 is needed for a better understanding of the putrescine transport system.

**Acknowledgments** This work was supported by the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (PHD/0115/2546) to W. Raksajit and A. Incharoensakdi and partly by the Academy of Finland to P.Mäenpää.

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# BIOGRAPHY

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