ผลของภาวะกดดันไอออนิกและออสโมติกต่อชีวสังเคราะห์พอลิเอมีน และระบบสังเคราะห์ด้วยแสงในไซยาโนแบคทีเรีย *Synechocystis* PCC 6803

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EFFECTS OF IONIC AND OSMOTIC STRESSES ON POLYAMINE BIOSYNTHESIS AND PHOTOSYNTHETIC SYSTEMS IN A CYANOBACTERIUM *Synechocystis* PCC 6803

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การศึกษาถึงผลของความเข้มข้นของโซเดียมคลอไรด์และซอร์บิทอลในอาหารเลี้ยงเชื้อต่อปริมาณพอลิเอมีนและเอนไซม์สองชนิดที่เกี่ยวข้องกับการสังเคราะห์พอลิเอมีน คือ อาร์จินีนดิคาร์บอกซิเลส และเอสอะดิโนซิลเมไทออนีนดิคาร์บอกซิ-เลสในไซยาโนแบคทีเรียเซลเดี่ยว *Synechocystis* PCC 6803 พบว่าเมื่อเพิ่มความเข้มข้นของโซเดียมคลอไรด์ถึง 550 มิลลิโมลาร์ อัตราการเจริญของไซยาโนแบคทีเรียไม่เปลี่ยนแปลง อัตราการเจริญของไซยาโนแบคทีเรียลดลงเล็กน้อยที่ความเข้มข้นซอร์บิทอล 300 มิลลิโมลาร์ และไซยาโนแบคทีเรียไม่สามารถเจริญเติบโตได้ที่ความเข้มข้นตั้งแต่ 700 มิลลิโมลาร์ขึ้นไป ในไซยาโนแบคทีเรียอายุ 10 วัน ภาวะกดดันเนื่องจากเกลือและซอร์บิทอลมีผลต่อปริมาณพอลิเอมีนในรูปที่ละลายน้ำและไม่ละลายน้ำ ภาวะกดดันเนื่องจากเกลือจะเหนี่ยวนำการเพิ่มขึ้นของปริมาณพอลิเอมีนรวมในเซลล์โดยเฉพาะสเปอร์มีน ภาวะกดดันเนื่องจากออสโมติกเหนี่ยวนำให้ปริมาณพอลิเอมีนรวมในเซลล์เพิ่มขึ้นโดยเฉพาะปริมาณสเปอร์มิดีนเพิ่มขึ้นภายใต้ภาวะความเข้มข้นซอร์บิทอล 700 มิลลิโมลาร์ ปริมาณพอลิเอมีนภายในเซลล์จะเพิ่มขึ้นอย่างรวดเร็วในช่วง 4 วันแรกและลดลงในวันต่อมาของการเลี้ยงเป็นระยะเวลา 20 วัน สิ่งสำคัญของงานวิจัยนี้ก็คือสามารถตรวจพบสเปอร์มีนใน *Synechocystis* PCC 6803 ซึ่งพอลิเอมีนชนิดนี้ไม่เคยมีรายงานมาก่อนในไซยาโนแบคทีเรีย

แอคติวิตีของเอนไซม์อาร์จินินดิคาร์บอกซิเลสซึ่งมีผลต่อการสังเคราะห์พิวเทรสซีนไม่เปลี่ยนแปลงโดยภาวะกดดันเนื่องจากเกลือแต่จะลดลง 6 เท่าภายใต้ภาวะกดดันเนื่องจากความเข้มข้นซอร์บิทอล 700 มิลลิโมลาร์ เอนไซม์เอสอะดิโนซิลเม-ไทออนีนดิคาร์บอกซิเลสซึ่งสำคัญต่อการสังเคราะห์สเปอร์มิดีนและสเปอร์มีนก็ตอบสนองต่อภาวะกดดันเนื่องจากเกลือและซอร์บิทอลในทำนองเดียวกันกับเอนไซม์อาร์จินินดิคาร์บอกซิเลส การวิเคราะห์ด้วยวิธี RT-PCR พบว่าปริมาณ *ADC* mRNA ในเซลล์เพิ่มขึ้นภายใต้ภาวะกดดันเนื่องจากเกลือและซอร์บิทอล และที่สำคัญพบว่า *ADC* mRNA ในเซลล์มีอัตราการสลายตัวช้าลงภายใต้ภาวะกดดันทั้งสองแบบ จากฐานข้อมูลจีโนมของ *Synechocystis* sp. PCC 6803 ยังไม่สามารถตรวจพบลำดับยีนของ *SAMDC* เมื่อเปรียบเทียบยีนนี้ในสิ่งมีชีวิตอื่น

ทำการศึกษาผลของภาวะกดดันไอออนิกและออสโมติกต่อระบบสังเคราะห์ด้วยแสงโดยใช้เซลล์ *Synechocystis* PCC 6803 อายุ 10 วัน ภาวะความเข้มข้นโซเดียมคลอไรด์สูงไม่มีผลต่อแอคติวิตีของระบบแสงที่สองและระบบการสังเคราะห์ด้วยแสงทั้งหมด ขณะที่ภายใต้ภาวะความเข้มข้นซอร์บิทอลสูง (700 มิลลิโมลาร์) จะลดแอคติวิตีของระบบแสงที่สอง ที่สำคัญพบว่าความมืดทำให้แอคติวิตีของระบบแสงลดลง ปริมาณ *psbA* mRNA ลดลงเล็กน้อยภายใต้ภาวะกดดันเนื่องจากเกลือและลดลงมากภายใต้ภาวะกดดันเนื่องจากซอร์บิทอลโดยเฉพาะที่ความเข้มข้น 700 มิลลิโมลาร์ ปริมาณ *psaA* mRNA ลดลงภายใต้ภาวะกดดันเนื่องจากเกลือและให้ผลเช่นเดียวกันภายใต้ภาวะกดดันเนื่องจากซอร์บิทอล ภาวะกดดันเนื่องจากเกลือไม่มีผลต่อความคงตัวของ *psbA* mRNA ขณะที่ส่งผลให้ความคงตัวของ *psaA* mRNA ลดลง ในทางกลับกันภาวะกดดันเนื่องจากซอร์บิทอลลดความคงตัวของ *psbA* และ *psaA* mRNA ภาวะกดดันเนื่องจากเกลือไม่มีผลต่อปริมาณโปรตีน D1 D2 และโปรตีนระบบแสงที่หนึ่งขณะที่ปริมาณโปรตีน ndhF3 ลดลงในภาวะเดียวกัน การลดลงของปริมาณโปรตีน D1 D2 และ ndhF3 ถูกเหนี่ยวนำโดยภาวะกดดันเนื่องจากซอร์บิทอลขณะที่ปริมาณโปรตีนระบบแสงที่หนึ่งไม่เปลี่ยนแปลง โดยสรุปสามารถแบ่งโปรตีนได้สามกลุ่มตามปริมาณคงที่ภายใต้ภาวะกดดันที่ศึกษา ได้แก่ 1) โปรตีนระบบแสงที่สอง ลดลงภายใต้ภาวะกดดันออสโมติกเท่านั้น 2) โปรตีนระบบแสงที่หนึ่ง ไม่ถูกกระทบภายใต้ภาวะกดดันทั้งสองแบบ 3) โปรตีน ndhF3 ลดลงภายใต้ภาวะกดดันทั้งสองแบบ

หลักสูตร.....เทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต.....

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

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KEYWORD: IONIC STRESS / OSMOTIC STRESS / POLYAMINES / PHOTOSYNTHETIC SYSTEMS / CYANOBACTERIA / *Synechocystis* PCC 6803

SAOWARATH JANTARO : EFFECTS OF IONIC AND OSMOTIC STRESSES ON POLYAMINE BIOSYNTHESIS AND PHOTOSYNTHETIC SYSTEMS IN A CYANOBACTERIUM *Synechocystis* PCC 6803. THESIS ADVISOR : ASSOC. PROF. ARAN INCHAROENSAKDI, Ph.D., THESIS COADVISOR : ASSOC. PROF. PIRKKO MÄENPÄÄ, Ph.D., 150 pp. ISBN 974-17-3867-6.

Effects of various NaCl and sorbitol concentrations in the growth medium on polyamine content and on two enzymes of polyamine biosynthesis pathway, arginine decarboxylase (ADC) and S-adenosylmethionine decarboxylase (SAMDC), were investigated in a unicellular cyanobacterium *Synechocystis* sp. PCC 6803. *Synechocystis* cells showed no difference in the growth rate when the concentration of NaCl was raised up to 550 mM. Instead, the growth rate of the cells decreased at 300 mM sorbitol, and inhibition of growth occurred at or higher than 700 mM sorbitol. Salt and sorbitol stresses affected the soluble and insoluble polyamine titers in the *Synechocystis* cells at 10 days cultivation. Salt stress induced a moderate increase in the total cellular polyamine content, spermine in particular. Osmotic stress caused an apparent increase in the total cellular polyamine content with a marked increase of spermidine induced by 700 mM sorbitol. The cellular polyamine contents rapidly increased at early stage of cultivation of up to 4 days and decreased afterwards during growth for 20 days. Importantly, a low level of spermine, which so far has never been detected in cyanobacteria, could be found in *Synechocystis* sp. PCC 6803.

ADC, a key enzyme for putrescine synthesis, was unaffected by salt stress but showed a 6-fold increase of enzyme activity upon osmotic stress imposed by 700 mM sorbitol. SAMDC, another important enzyme for spermidine and spermine synthesis, responded to salt and osmotic stresses similarly to that observed for ADC. An analysis by reverse transcription-PCR revealed an increase of *ADC* mRNA level in cells under salt and osmotic stresses. Most importantly, the increase of *ADC* mRNA was partly attributed to its slower turnover rate under both stress conditions. The attempt to study the effect of salt and osmotic stresses on *SAMDC* mRNA level was unsuccessful since no putative gene for *SAMDC* could be identified in the genome of *Synechocystis* sp. PCC 6803 when comparing to known *SAMDC* gene sequences from other organisms.

Effects of ionic and osmotic stresses on photosynthetic systems were investigated using 10 days *Synechocystis* cells culture. High salt stress did not affect the photosystem II (PSII) activity whereas high sorbitol stress (700 mM sorbitol) completely inhibited both PSII activity and whole photosynthesis. Importantly, darkness was found to inactivate the photosynthetic oxygen evolution and this dark-inactivation was enhanced by a concomitant sorbitol stress. The abundance of *psbA* mRNA slightly decreased under salt stress and more under sorbitol stress, especially under 700 mM sorbitol condition. The abundance of *psaA* transcripts was also decreased under salt stress as well as under sorbitol stress. The stability of *psbA* mRNA was unaffected by salt stress whereas *psaA* mRNA stability was decreased. On the other hand, sorbitol stress decreased the stability of both *psbA* and *psaA* mRNAs. Salt stress had no effect on the amounts of D1, D2 and PSI proteins whereas the ndhF3 protein amount was slightly decreased. The decrease of D1, D2 and ndhF3 proteins were induced by sorbitol stress while no change of PSI protein level was observed under the same condition. In conclusion, three groups of proteins could be distinguished according to their steady-state amounts under the stress conditions studied: 1) PSII proteins are down regulated only under osmotic stress 2) PSI protein is not affected under any stress studied 3) ndhF3 protein is down regulated under both ionic and osmotic stresses.

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CONTENT

LIST OF TABLES

PAGE

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADC	Arginine decarboxylase
BCiP	Bromochloroindoyl phosphate
Bp	Base pair
BSA	Bovine serum albumin
Chl	Chlorophyll
Ci	Curie
cm	Centimeter
ºC	Degree Celsius
DAO	Diamine oxidase
DCBQ	2,6-Dichloro-*p*-benzoquinone
DFMA	α-Difluoromethylarginine
DFMO	α-Difluoromethylornithine
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetraacetic acid
kb	Kilobase
kDa	Kilodalton
g	Gram
h	Hour
l	Litre
LHC	Light-harvesting complex
mA	Milliampere
MBP	Maltose binding protein
min	Minute

μg	Microgram
μl	Microlitre
MGBG	Methylglyoxal(bis-guanylhydrazone)
ml	Millilitre
mM	Millimolar
M	Molar
nm	Nanometer
OD	Optical density
ODC	Ornithine decarboxylase
PAO	Polyamine oxidase
PCR	Polymerase chain reaction
PSI	Photosystem I
PSII	Photosystem II
Put	Putrescine
PMSF	Phenylmethylsulfonyl fluoride
RT-PCR	Reverse transcription - polymerase chain reaction
rpm	Revolution per minute
SAM	S-adenosylmethionine
SAMDC	S-adenosylmethionine decarboxylase
SDS	Sodium dodecyl sulphate
Spd	Spermidine
Spm	Spermine
TAE	Tris-acetate electrophoresis buffer
TBE	Tris-borate electrophoresis buffer
UV	Ultraviolet

Chapter I

Introduction

1.1 Salinity

Salinity is one of the most severe problems in worldwide agricultural production which can lead to change in development, growth, and productivity, and severe stress may threaten survival. Each organism displays a salinity tolerance range, which includes its optimal growth conditions, through larger ranges of salt concentrations (resistance range) may still be compatible with cell survival (reviewed by Joset *et al*., 1996). A high concentration of NaCl greatly reduces growth of both shoot and the root of plants (Greenway and Munns, 1980, Cheeseman, 1988, Katsuhara and Kawasaki, 1996). Crop plants are particularly limited in their tolerance range. Effects of salt stress have been examined in various salt-sensitive and -tolerant plants, including some crops (Cheeseman, 1988). However, only a few studies on plant acclimation to salt stress have been conducted such as a study about enhancement of salt tolerance in soybean with NaCl pretreatment and showed that some plants could acclimate to salt stress (Umezawa *et al*., 2000).

Exposure of cells to salt concentrations intracellularly threatens them via two deleterious effects, namely, increase of both the osmotic pressure and the ion concentration. The water potential decreases, leading to loss water by the cells, and simultaneously to influx of ions into the cytoplasm. Organisms that thrive in high salinity environment possess specific mechanisms to adjust their internal osmotic status (reviewed by Joset *et al*., 1996). One such mechanism that helps organisms to become more tolerant to salt stress is the accumulation of low molecular weight

osmolytes such as ectoine (Del Meral *et al*., 1994), glycine betaine (Incharoensakdi and Wutipraditkul, 1999), proline (Kavi Kishore *et al*., 1995, Lutts *et al*., 1999), glucosylglycerol (Hagemann and Erdmann, 1994) and plant growth regulator polyamines (Bouchereau *et al*., 1999). Furthermore, cells exposed to high salinity showed disturbances also on photosystem I (Allakhverdiev *et al*., 2000) and photosystem II (Lu and Vonshak, 2002, Allakhverdiev *et al*., 2000).

1.2 Polyamines

1.2.1 Physiology

Polyamines are naturally occurring aliphatic amines found in all organisms (Figure 1.1). The most common polyamines in plants are triamine spermidine (1,8-diamino-4-azaoctane), tetramine spermine (1,12-diamino-4,9-diazadodecane) and their diamine precursor putrescine (1,4 diaminobutane). Evidence gathered in recent years in plant systems supports their role as regulators of cell proliferation and differentiation, previously proposed for animal and bacterial cells (Heby, 1981, Marton and Morris, 1987). In addition to the much studied roles of polyamines in the regulation of cell division and morphogenesis in plants, they are known to affect the patterns of RNA and protein synthesis, membrane stability, and stress responses of plants. Polyamine metabolism has also been implicated in regulating the metabolism of ethylene and affecting nitrogen pools in plant cells (Walden *et al*., 1997).

Polyamines are basic molecules that are positively charged at physiological pH. They have been shown to bind strongly *in vitro* to negatively charged nucleic acids (Feurstein and Marton, 1989), acidic phospholipids (Tadolini *et al*., 1985) and many types of proteins, including numerous enzymes whose activities are directly

modulated by polyamine binding (Carley *et al*., 1983). These ionic interactions are important in regulating the structure and function of biological macromolecules, as well as their synthesis *in vivo* (Jacob and Stetler, 1989). Polyamines are also precursors in the synthesis of some secondary metabolites, such as tropane alkaloids and hydroxycinnamic acid amides (HCAs; Martin-Tanguy, 1997) which occur in several families of higher plants and are thought to be correlated with developmental phenomena (Scaramagli *et al*., 1999).

Polyamine titer increase dramatically during rapid growth in many plants systems, such as pollen germination and tube growth in tomato (Song *et al*., 2002), developing zygotic and somatic embryos of *Pinus radiata* (Minocha *et al*., 1999a), ovaries of tomato, and rapidly dividing tobacco cells in suspension culture, during crown gall-tumor development and embryogenesis of carrot suspension cells (Kaur-Sawhney *et al*., 1982a). In addition, the changes in the metabolism of polyamines are correlated with floral development, and their conjugates accumulate in shoot apices upon floral initiation (Aziz *et al*., 2001). Polyamines appear to inhibit senescence by preventing Chl, protein and RNA breakdown in leaves and by increasing macromolecular synthesis and mitotic activity in protoplasts (Kaur-Sawhney *et al*., 1982a and 1982b). In *Escherichia coli*, most spermidine exists as a spermidine-RNA complex, and about 40% and 50% of putrescine exists as a free form and a putrescine-RNA complex in cells, respectively. Under the conditions that the synthesis of specific proteins such as RNA replicase is stimulated by polyamines in a cell-free system, the amount of spermidine and putrescine bound to RNA was close to the value estimated in cells (Miyamoto *et al*., 1993). However, the polyamine mechanism in cyanobacteria is still not known.

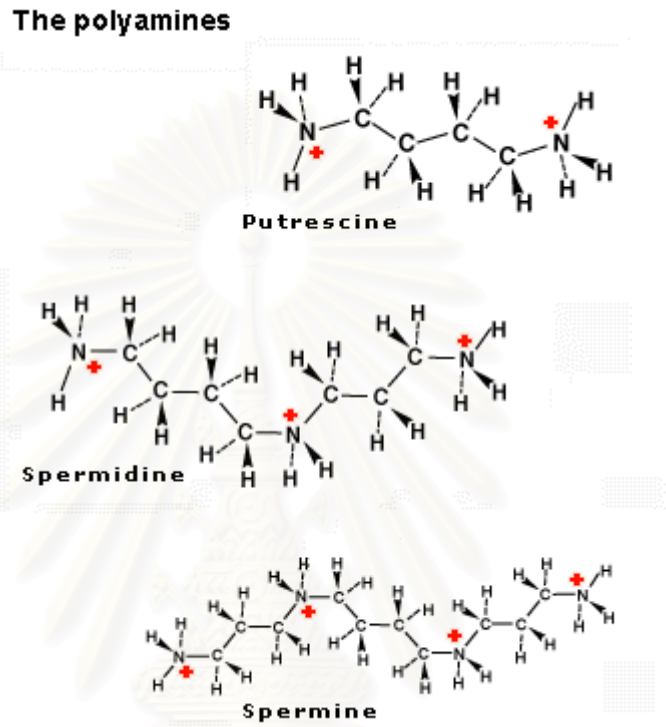

Figure 1.1 The polyamines ; putrescine (Put; $H_2N(CH_2)_4NH_2$), spermidine (Spd; $H_2N(CH_2)_3NH(CH_2)_4NH_2$) and spermine (Spm; $H_2N(CH_2)_3NH(CH_2)_4\,NH(CH_2)_3\,NH_2$) (http://www.biol.lu.se/zoofysiol/Cellprolif/Research/research_area_1.html).

1.2.2 Synthesis

Polyamine biosynthetic pathways in plants have been well elucidated and are similar to those in animals and microorganisms (Figure 1.2). In plants and bacteria, putrescine is formed either by direct decarboxylation of L-ornithine by the enzyme ornithine decarboxylase (ODC; EC 4.1.1.17), or by decarboxylation of arginine by arginine decarboxylase (ADC; EC 4.1.1.19) via agmatine and N-carbamoylputrescine intermediates. The distribution of these two enzymes in different organism species is regulated in a developmental and tissue specific manner (Robie and Minocha, 1989). In mammals and fungi, only one pathway (ODC reaction) leads to putrescine formation (Bey *et al*., 1987). In animals and plants have been reported that ornithine decarboxylase is located in both the cytoplasm and the nucleus (Voigt *et al*., 2000). As the arginine decarboxylase protein which widely appears in plants is localized in chloroplasts associated with the thylakoid membrane (Borrell *et al*., 1995). ADC activity increases have been reported for cell growth and embryogenesis, DNA synthesis and stress responses. On the other hand, ODC has been reported to be associated with proliferate growth and fruit development (Yoshida and Hirasawa, 1998). The mammalian ADC differs from ADC isoforms expressed in plants, bacteria, or *Caenorhabditis elegans* and is distinct from ODC (Regunathan and Reis, 2000).

Spermidine and spermine are synthesized by the sequential addition of an aminopropyl group onto putrescine and spermidine, respectively, in reactions catalyzed by the enzyme spermidine synthase (EC 2.5.1.16) and spermine synthase (EC 2.5.1.22). The aminopropyl group is donated by decarboxylated S-adenosylmethionine (SAM), which is produced by S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) In plants, SAM, aside from participating in

numerous transmethylation reactions, as it does in other organisms, is also a precursor of the plant hormone ethylene (Figure 1.2). A variety of other related compounds have been found in plants, including cadaverine (Cad). Caddiamine is synthesized predominantly as the result of lysine decarboxylase (LDC; EC 4.1.1.18) activity. This diamine is not as widely distributed as putrescine and is mainly found in Leguminoseae and in the flowers of Arum lilies (Smith and Meeuse, 1976). Moreover, some uncommon polyamines including branched pentamines, hexamines and heptamines have been detected in the extreme thermophilic bacteria (reviewed by Bagni and Tassoni, 2001).

Specific inhibitors for each of these enzymes have been used in many tissues to manipulate cellular polyamine metabolism. DL-α-difluoromethylornithine (DFMO) is a highly effective inhibitor of all animal ornithine decarboxylases while its effectiveness for plant ornithine decarboxylases is quite variable. DL-α-difluoromethylarginine (DFMA) and methylglyoxal bis - guanylhydrazone (MGBG) are generally quite effective in inhibiting the activities of arginine decarboxylase and S-adenosylmethionine decarboxylase, respectively, in most plant tissues. S-adenosylmethionine decarboxylase activity and transcript levels are known to increase in actively dividing tissues. On the other hand, due to its ability to inhibit cell division, MGBG has been widely used both in animal and plant cells for basic studies as well as for therapeutic applications in cancer treatment. Moreover, MGBG has almost in variably been reported to negatively affect *in vivo* and *in vitro* plant growth and development, such as, a reduced formation of oval-shaped cell aggregates on the explant surface and more frequent cases of nuclear extrusion in vegetative bud-forming tobacco (Scaramagli *et al*., 1999).

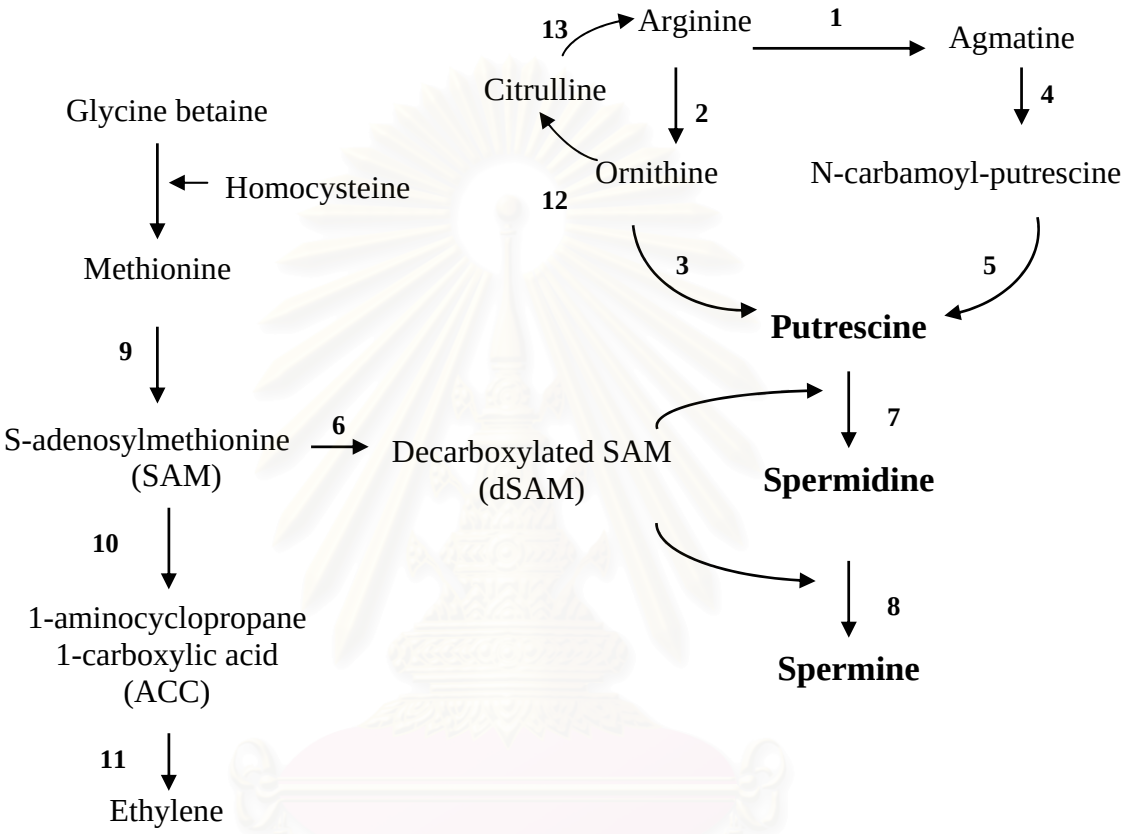

Figure 1.2 The pathway of polyamine synthesis (modified from Bouchereau *et al*., 1999). 1, Arginine decarboxylase (ADC); 2, Arginase; 3, Ornithine decarboxylase (ODC); 4, Agmatine iminohydrolase; 5, N-carbamoyl putrescine amidohydrolase; 6, S-adenosylmethionine decarboxylase (SAMDC); 7, Spermidine synthase; 8, Spermine synthase; 9, SAM synthase; 10, ACC synthase; 11, ACC oxidase; 12, Ornithine transcarbamylase; 13, Arginine synthase.

1.2.3 Degradation

As in the case of any plant growth regulator, the intracellular free polyamine pool does not only depend on its synthesis, but also on several other processes including polyamine degradation (oxidative deamination), polyamine conjugation and polyamine transport (reviewed by Bouchereau *et al*., 1999).

Polyamines are oxidatively deaminated by the action of amine oxidases (Figure 1.3). These enzymes include the copper proteins diamine oxidases (DAO; EC 1.4.3.6), defined on the basis of their higher substrate specificity towards diamines, and the flavoproteins polyamine oxidases (PAO; EC 1.5.3.3), which oxidize spermidine and spermine at their secondary amino groups. DAO, firstly found in Leguminosae apoplast (Smith and Barker, 1988) and present practically in all the monocots and dicots families tested, have a broad specificity oxidizing putrescine and other diamines (reviewed by Bagni and Tassoni, 2001). The DAO reaction products from putrescine are pyrroline, hydrogen peroxide and ammonia, while PAO yields pyrroline and 1,5-diabicyclononane, respectively, from spermidine and spermine, along with diaminopropane (Dap) and hydrogen peroxide. These oxidases are implicated in the production of free radicals and toxic aldehydes and in lignification processes of the cell wall (Angelini *et al*., 1993). Dap can be converted into *β*-alanine, whereas pyrroline can be further catabolized to γ-aminobutyric acid (GABA) in a reaction catalyzed by pyrroline dehydrogenase (PDH). The *γ*-aminobutyric acid is converted to succinic semialdehyde by a transaminase reaction, and then to succinate via an oxidation step, which is incorporated into the Krebs cycle. Thus, this pathway ensures the recycling of carbon and nitrogen from putrescine. Far from being only a means of eliminating cellular polyamines, the enzymes involved in polyamine catabolism and the products deriving from their action, have been demonstrated to be

involved in important physiological processes (reviewed by Bouchereau *et al*., 1999). Nevertheless, bacteria and fungi have different polyamines degradative enzymes (reviewed by Bagni and Tassoni, 2001).

In addition to polyamine degradation, ornithine decarboxylase (ODC), a key enzyme in polyamine biosynthesis, turns over rapidly with a half-life shortest among mammalian enzymes (Russell and Snyder, 1969). ODC is under negative feedback regulation by polyamines for the protection of cells from the adverse effects of excess polyamines. ODC degradation is itself regulated, and is accelerated by increases in levels of cellular polyamines. ODC is regulated at four different levels of gene expression, namely gene transcription, mRNA degradation, mRNA translation and enzyme degradation. In addition, ODC is specifically inhibited by antizyme, a unique regulatory protein of 26.5 kDa. Induced by polyamines, antizyme reversibly binds to ODC with a high affinity inhibiting enzyme activity and rendering the enzyme susceptible to proteolysis (reviewed by Hayashi and Murakami, 1995).

1.2.4 Conjugation

Plant polyamines may occur as free molecules but also as conjugates to small molecules (Bagni and Tassoni, 2001) like phenolic acids (conjugated forms) and also to various macromolecules like proteins (bound forms) (reviewed by Martin-Tanguy, 1997).

1.2.4.1 Conjugated forms

Polyamines are conjugated by the formation of an amide linkage, utilizing esters of CoA for the provision of the activated carboxyl groups (Negrel, 1989). The most common conjugated polyamines are those that are covalently linked

to cinnamic acids. Conjugated polyamines, such as hydroxycinnamic acid amides, have been observed in higher plants and are correlated with developmental phenomena. They accumulate in roots and shoots, upon floral initiation in tobacco. Polyamine content increases during all three organogenic programs, especially during meristemoid formation and up to the protrusion of the first organs (Scaramagli *et al*., 1999). Putrescine mainly forms monomers (PCA-soluble fraction) with coumaric acid, caffeoyl acid or feruloyl acid. These conjugates are of particular importance both for the regulation of polyamine concentration inside the cell, and for their interaction with cell wall components. In fact hydroxycinnamic acid bridges, through ester-ester linkages, different cell wall polymers, essentially hemicellulose and lignin (reviewed by Bagni and Tassoni, 2001). As all developmental programs, there is a possibility that balance between the levels of free and conjugated polyamines may contribute to growth regulation and play a role during morphogenesis. Aliphatic amines (putrescine, spermidine and spermine) appear as water-soluble forms, whereas conjugated with aromatic amines as well as aliphatic amines that use each terminal amino group to bind cinnamic acid are water-insoluble (reviewed by Martin-Tanguy, 1997). It was in fact clarified that only polyamines in the free form are translocated and that conjugated polyamines have no effect on cell division process. A role of hydroxycinnamic acid amide conjugates in defence mechanism against biotic and abiotic stress has been reported (reviewed by Bagni and Tassoni, 2001).

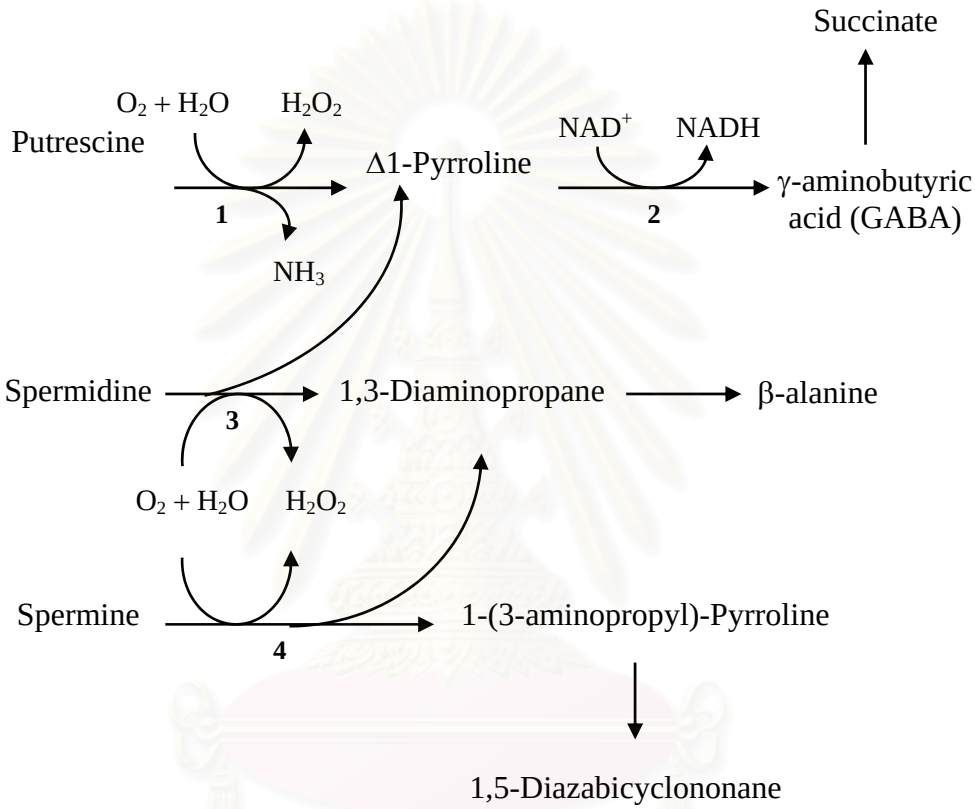

Figure 1.3 The pathway of polyamine degradation in plants. 1, Diamine oxidase; 2, Pyrroline dehydrogenase; 3 and 4, Polyamine oxidase (Bouchereau *et al*., 1999).

1.2.4.2 Bound forms

The delocalised positive charges of polyamines can provide the electrostatic linking to charged protein and/or phospholipids and nucleic acids making their effect more complicated. Thus polyamines can bridge elements of membrane and cytoskeletal net work and impart rigidity to biological membranes. Polyamine-binding proteins have been identified in a wide range of organisms including mammals, yeasts, and bacteria. The interaction between polyamines and membranes is suggested to be an intermediate in important cellular events such as membrane fusion (Schuber, 1989) and transmission of receptor-mediated signals (Koenig *et al*., 1983). The peptidoglycans, which are essential for both cell surface integrity and normal cell growth, formed covalent linkage to polyamines in *Anaerovibrio lipolytica* (Hirao *et al*., 2000).

Post-translational covalent linkage of polyamines to proteins is catalyzed by a class of enzymes known as transglutaminase (EC 2.3.2.13), which have been localized both intra- and extra-cellularly (Folk, 1980). Transglutaminases are calcium-dependent enzymes capable of linking polyamines to glutamine residues and may thus cross-link proteins. Having an active site cysteine, transglutaminases change activity when treated with N-ethylmaleimide. The cross-linking of proteins through covalent attached polyamines makes tissues more stable and resistant to both proteolysis and physical degradation (Votyakova *et al*., 1999). In addition, the observation that the number of polyamine linkages occurring in the chloroplast is enhanced in the presence of light (Dondini *et al*., 2000).

1.2.5 Transport

In animal cells, uptake of polyamines can increase during hormonal stimulation and cell proliferation. Furthermore, although different polyamines appear to share the same transport systems, multiple polyamine uptake systems exist (reviewed by Igarachi and Kashiwaki, 1999). Polyamine transport across the plasmalema is energy-dependent and calcium is involved in the corresponding mechanism (Antognoni *et al*., 1995).

In *Escherichia coli*, there are three polyamine transport systems (Figure 1.4). Uptake is mainly catalyzed by two polyamine-uptake systems: one is a putrescine-specific system and the other is spermidine-preferential system. Both systems are ABC (ATP Binding Cassette) transporters consisting of a substrate-binding protein in the periplasm, two channel-forming proteins and a membrane-associated ATPase that is involved in energy supply. The operon for the putrescine-specific uptake encodes four proteins : PotF (a substrate binding protein), PotG (an ATPase), and PotH and PotI (channel-forming proteins). The operon for the spermidine-preferential uptake system encodes PotA (an ATPase ; M_r 43,000), PotB and PotC (channel-forming proteins ; M_r 31,000 and 29,000 respectively) and PotD (a substrate-binding protein) (reviewed by Igarashi and Kashiwagi, 1999). The PotD protein is a periplasmic binding protein and consists of 348 amino acids, corresponding to a molecular mass of 39 kDa (Sugiyama *et al*., 1996). The third polyamine transport system involves the PotE protein. PotE can catalyze both the uptake and excretion of putrescine. Uptake of putrescine by PotE is dependent on the membrane potential. Substrate specificity of putrescine uptake by PotE is strict. In contrast, the excretion of putrescine is catalyzed by a putrescine/ornithine antiporter activity of PotE. The exchange ratio between putrescine and ornithine is 1:1. The excretion of putrescine is increased by carbonyl

cyanide m-chlorophenylhydrazone, which inhibits the membrane potential dependent reuptake of putrescine and extinguishes the hyperpolarization caused by putrescine excretion (reviewed by Igarashi and Kashiwagi, 1999). Nevertheless, PotE is not involved in growth- or osmodependent putrescine transport in defined minimal medium in *E. coli* (Schiller *et al*., 2000). In animal cells, although polyamines are synthesized in the extramitrochondrial space, they are also present within the mitochondria. Spermine is transported bidirectionally across the inner membrane both in rat liver and in heart mitochondria. The influx, which occurs electrophoretically, is dependent on a high transmembrane potential and exhibits a nonlinear current/voltage relationship (Toninello *et al*., 1992).

Genes for several kinds of putative polyamine transport systems have been described in bacteria in which the whole genome has been sequenced (Table 1.1). In *Synechocystis* PCC 6803, only a PotD-like protein is detectable. The PotD-like protein may share channel-forming proteins and membrane-associated ATPase with other uptake systems. Another possibility is that the similarity between *E. coli* and *Synechocystis* genes may be too low to detect equivalents of the Pot genes (reviewed by Igarashi and Kashiwagi, 1999).

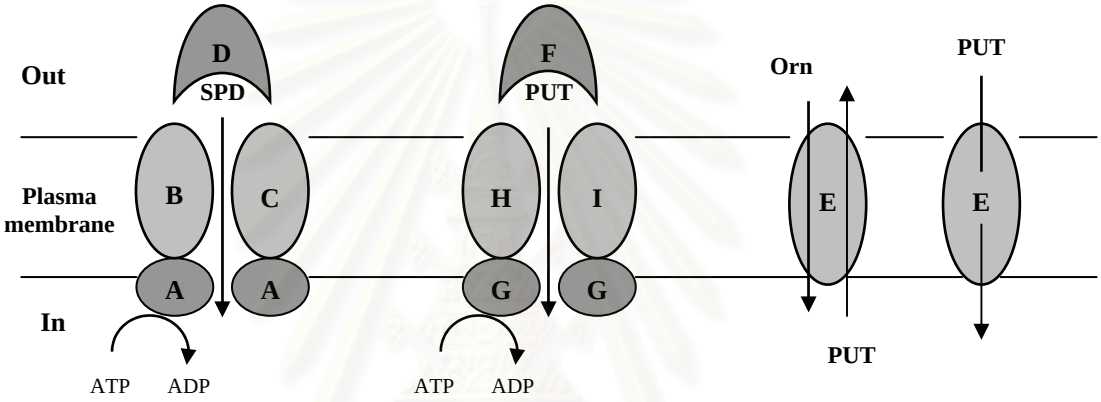

Figure 1.4 Polyamine transport systems in *E. coli*. ATPases (A, PotA and G, PotG), the substrate-binding proteins (D, PotD and F, PotF), channel-forming proteins (B, PotB ; C, PotC ; H, PotH and I, PotI) and the PotE protein (E) (Igarashi and Kashiwagi, 1999).

Table 1.1 Genes for polyamine transport

Bacterium or archaeon	**Size of chromosome (kbp)**	**Number of genes code**	**Gene for 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 PCC6803	3573	3169	*potD*
Treponema pallidum	1138	1031	*potABCD*

Source : Igarashi and Kashiwagi (1999)

1.2.6 Stress factors

In higher plants, polyamine metabolism is responsive to wide array of environmental stress conditions (reviewed by Bouchereau *et al*., 1999). Polyamine levels are an integral part of the response mechanisms of plants to various stresses (Santa-Cruz *et al*., 1997a), such as nutritional, osmotic (Flores and Galston, 1984, Fugihara and Yoneyama, 1993, Botella *et al*., 2000), salinity (Lovett and Watts, 1995, Aziz *et al*., 1998), heat and drought, light (Kaur-Sawhney *et al*., 1982b, Moysset *et al*., 2002), as well as chilling temperature (Szalai *et al*., 1997).

Polyamines may contribute to the osmotic and excess ion adaptation by maintaining a proper cation-anion balance and by stabilizing membranes at high external salinity. One of the possible mechanisms of saline resistance was observed to be due to the highly increased Spd and Spm against the low increase in Put. Alternatively, the salt sensitivity could be due to high increase of Put and incapacity to maintain high levels of Spd and Spm (Krishnamurthy and Bhagwat, 1989). Put accumulation is very often characteristic of a stress response. Although this accumulation could play a protective role in the cell, it has also been reported that Put excess may have some negative effects (Walladino *et al*., 1996). The endogenous levels of polyamines may serve as markers for different phases of the growth response under NaCl concentrations (Krishnamurthy and Bhagwat, 1989). However, no clear relationship was observed between the mean levels of salinity resistance and the endogenous concentrations of Spd or Spm (Lefevre *et al*., 2001).

The osmotica with widely different assimilation routes, such as sorbitol (Tiburcio *et al*., 1986), mannitol (Santa-Cruz *et al*., 1997b), sucrose, glycerol (Fujihara and Yoneyama, 1993), polyethylene glycol (Erdei *et al*., 1996, Lefevre *et al*., 2001), all induce a rise in Put. These changes are coincident with measurable

signs of stress, such as wilting and protein loss (reviewed by Bouchereau *et al*., 1999). On the basis of osmotic strength, NaCl, KCl, sucrose, or glycerol induced similar decreases in cellular homospermidine (homo-Spd) content in the soybean rhizobia *Phizobium fredii* P220. Homo-Spd, an analogue of triamine Spd, is an organic polycation detected ubiquitously in the soil environment and its occurrence has been demonstrated in a wide variety of microorganisms. Subsequently, the cellular levels of homo-Spd in strain P220 may be regulated by mechanisms related to their pH and osmotic tolerance (Fujihara and Yoneyama, 1993). A highly salt-tolerant strain, Mg^{2+} and homo-Spd, a major polyamine in *Rhizobium*, might be closely associated with osmoregulation, since the cellular levels of Mg^{2+} and homo-Spd were also regulated critically in response to the external medium osmolarity (Fujihara and Yoneyama, 1994). The decline of Spd and Spm levels beyond 30 min applied stress in tolerant rice callus, showed a shift towards the production and accumulation of the higher molecular mass rare polyamines, NorSpd and NorSpm. The pattern of accumulation of uncommon polyamines under heat stress in the tolerant cultivar's callus is consistent with that observed in heat-tolerant cotton (Roy and Ghosh, 1996).

1.3 Photosynthetic light reactions

Cyanobacteria, which show to remarkable capacities to adapt to varying environmental conditions, are the only prokaryotes performing oxygenic photosynthesis and probable ancestors of chloroplasts (reviewed by Joset *et al*., 1996). In cyanobacteria, the composition of thylakoid components, including phycobilisomes, is controlled by photosynthetic light conditions. This phenomenon was first found as the chromatic adaptation of red algae (Fujita and Murakami, 1987).

Thylakoid membranes display a lipid composition similar to both that of their plasma membrane counterparts and of plant thylakoids (Joset *et al*., 1996). Light is captured by extramembraneous phycobilisomes (Figure 1.5). Phycobilisomes are antenna pigment-complexes found in cyanobacteria and the eukaryotic red algae. They contain phycobiliproteins, in which bilin chromophores are covalently attached to proteins. The biliproteins are organized into rods attached to cores. The pigments of the light-harvesting complex are in close contact with one another, facilitating rapid energy transfer via the 43 kDa and 47 kDa chlorophyll *a* binding proteins to the reaction center of PSII. The stoichiometry of the two photosystems, PSI and PSII, is variable in cyanophytes and in developing chloroplasts of flowering plants. The variability serves to maintain the efficiency of photosynthesis (Fujita *et al*., 1995). The ratio PSI/PSII is high in cells growth under light absorbed by phycobiliprotein, but it is low in cells grown under light absorbed by Chl *a* (Fujita and Murakami, 1987).

1.3.1 Photosystem II

Photosystem II (PSII) is a membrane-embedded multiprotein complex performing light-catalyzed oxidation of water and reduction of plastoquinone in thylakoid membranes of cyanobacteria and plants. This complex is the major producer of oxygen in the biosphere. Upon excitation with light, the reaction center chlorophyll (P680) of PSII release electrons, which move via electron acceptors including pheophytin and two plastoquinone molecules (Q_A and Q_B) on the acceptor side of PSII (Figure 1.5). The oxidized reaction center is subsequently reduced by electrons from water via a cluster of four manganese ions and a redox-active tyrosine (Tyr) residue on the donor side of this photosystem.

In cyanobacteria, PSII consists of approximately 20 different protein subunits (reviewed by Ikeuchi, 1992). At the core of PSII is a heterodimer of two homologous polypeptides D1 and D2. Closely associated with them are other intrinsic and extrinsic membrane proteins, namely cytochrome b_{559}, CP47, CP43, and a number of smaller polypeptides (Figure 1.5). There is a remarkable difference in the extrinsic proteins associated with and functioning in the oxygen-evolving PSII complex between cyanobacteria and higher plants. In red algae, three proteins of 33, 20, and 12 kDa are present as extrinsic proteins functioning in maintaining the stability and activity of the oxygen-evolving complex (Enami *et al*., 1998). The water-splitting complex in cyanobacteria consists of the 33 kDa protein (Burnap *et al*, 1989), a cytochrome c_{550} and a 9-12 kDa protein (Shen *et al*., 1992). Although light is required for PSII biogenesis and function, it also damages PSII, the main target being the reaction center protein D1 (Ohad *et al*., 1984). D1 is rapidly turned over *in vivo* during a damage-repair cycle after excitation of the reaction center with light while D2 is relatively stable (Aro *et al*, 1993). The D1 and D2 reaction center proteins are encoded by the *psbA* and *psbD* genes, respectively (Cyanobase : www.kazusa.or.jp/cyano/Synechocystis).

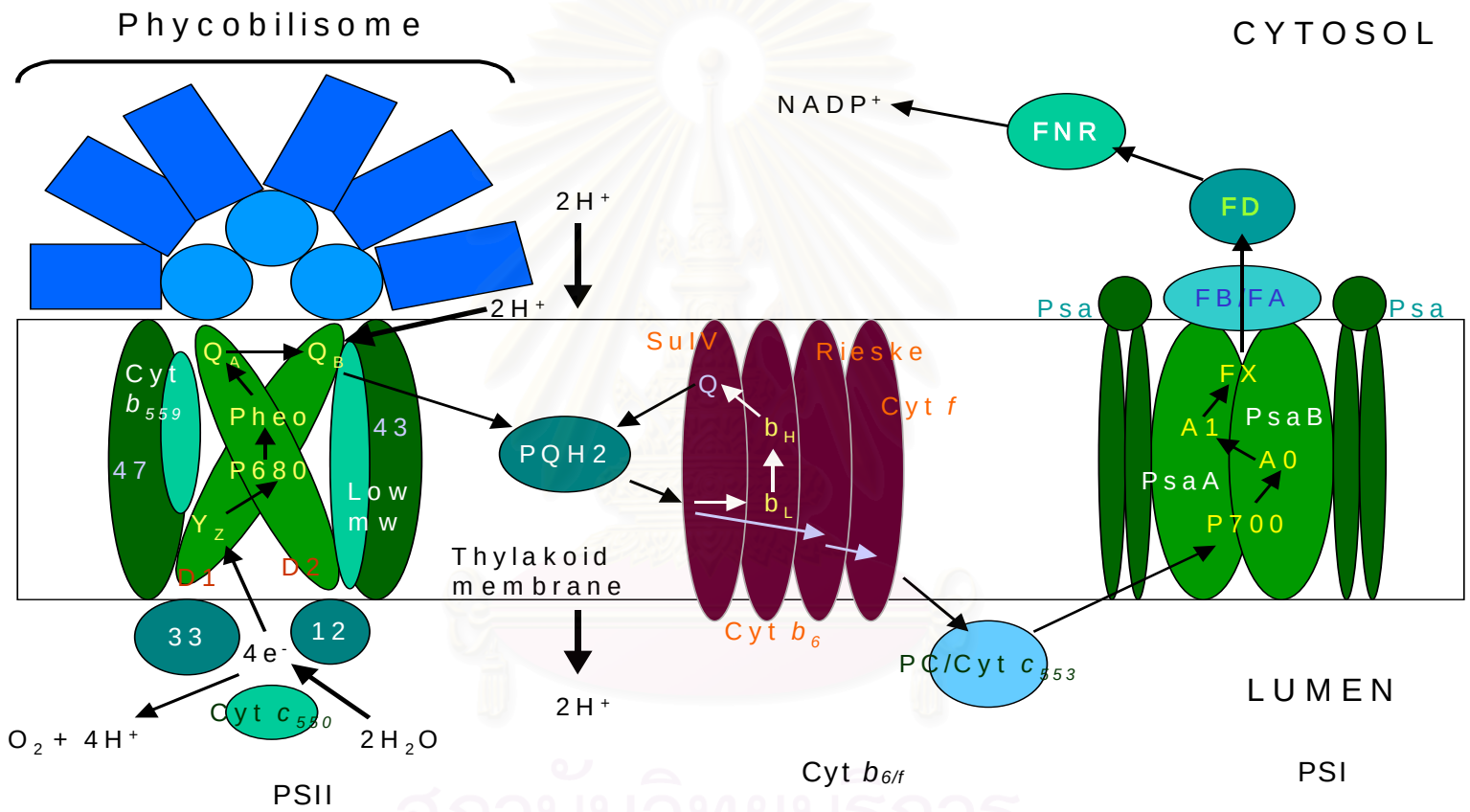

Figure 1.5 A schematic view of photosynthetic electron and proton flow in the thylakoid membrane of cyanobacteria (modified from Mulo, 1998).

Figure 1.5 depicts a schematic view of photosynthetic electron and proton flow in the thylakoid membrane of cyanobacteria. Thin arrows show the transfer of electrons through the photosynthetic protein complex PSII, Cyt $b_{6/f}$ and PSI. Thick arrows show the transfer of protons. The D1 and D2 proteins form a reaction center heterodimer, which ligates all redox active components of PSII. The 43 and 47 kDa Chlorophyll a binding proteins (43 and 47, respectively), the α and β subunits of cytochrome b_{559} (Cyt b_{559}) and several low molecular weight proteins (low mw) are also components of PSII. The oxygen evolving complex is composed of cytochrome c_{550} (Cyt c_{550}) and the 33 and 12 kDa proteins (33 and 12, respectively). The redox-active components of PSII include Tyr161 of the D1 protein (Y_Z), the reaction center chlorophylls (P680), primary electron acceptor pheophytin (Pheo), and the primary and secondary plastoquinone electron acceptors (Q_A and Q_B). The phycobilisome antenna, which *in vivo* is shared with two PSII complexes, absorbs light energy. Plastoquinol (PQH_2) mediates electron transfer from PSII to Cyt $b_{6/f}$ complex, which is composed of subunit IV (SuIV), cytochrome b_6 (Cyt b_6), cytochrome *f* (Cyt *f*) and Rieske Fe-S protein (Rieske). Componets of the Q-cycle include plastoquinone bound to Cyt $b_{6/f}$ complex (Q) and low- and high-potential *b* hemes in cytochrome b_6 protein (b_L and b_H, respectively). Plastocyanin or cytochrome c_{553} (PC/Cyt c_{553}) mediate electron flow from Cyt $b_{6/f}$ complex to PSI. PsaA and PsaB are the heterodimer-forming reaction center proteins of PSI, and Psa depicts other membrane-spanning and extrinsic PSI proteins. Redox-active components of PSI include a chlorophyll *a* molecule (A0), which serves as a primary electron acceptor in PSI, phylloquinone electron acceptor (A1) and 4Fe-4S centers (FX and FB/FA). Soluble ferredoxin (FD) and ferredoxin $NADP^+$ reductase (FNR) mediate electron transfer to the stromal NADP.

1.3.2 Cytochrome b_6/f complex

The cytochrome b_6/f complex is a large multisubunit protein with several prosthetic groups. The complexes contain four essential proteins including two *b*-type hemes (Cyt b_6), one *c*-heme (Cyt *f*), a single chlorophyll *a* molecule (subunit IV) and a high-potential 2Fe-2S center (Rieske protein) (Bald *et al*., 1992, Huang *et al*., 1994). The mechanism of electron and protons flow through the cytochrome b_6/f complex has been known as the Q cycle, the oxidized Rieske protein accepts an electron from reduced plastoquinone (QH_2) and transfers it to Cyt *f*. Subsequently, transfers an electron to the blue-colored copper protein plastocyanin (PC). In the absence of copper, plastocyanin is replaced by cytochrome c_6 in cyanobacteria without any change in photosynthetic capacity (Zhang *et al*., 1992). Cytochrome b_6/f complex serves both linear and cyclic electron transfer and is indispensable also for respiration in cyanobacteria (Schere, 1990) (Figure 1.5).

The ATP is synthesized by a large (400 kDa) enzyme complex known as the coupling factor, ATPase or ATP synthase. This enzyme consists of two parts: a hydrophobic membrane-bound protein called CF_0 and a portion that sticks out into the stroma cells CF_1. CF_0 appears to be a channel across the membrane through with protons can pass, while CF_1 is the portion of the complex that actually synthesizes ATP. Protons pumped across the membrane by the cytochrome b_6/f complex or protons produced by water oxidation must move laterally up to reach ATP synthase.

1.3.3 Photosystem I

Photosystem I (PSI) is a plastocyanin-ferredoxin oxidoreductase driven by light energy. PSI receives electrons from PSII through plastoquinone, cytochrome b_6/f and plastocyanin in a linear electron flow, and it finally reduces ferredoxin and NADP.

This electron transfer is coupled with the formation of ATP. PSI is also involved in cyclic electron transfer which produces only ATP without the participation of PSII (reviewed by Hihara and Sonoike, 2001).

PSI exists in thylakoid membranes as a supramolecular complex composed of more than ten different proteins. After absorption of a photon, the reaction center chlorophyll of PSI, P700, donates an electron to the primary electron acceptor, A0, which is a chlorophyll *a* molecule. Thereafter, the electron is transferred via phylloquinone, A1, to iron-sulfur centers (FX and FB/FA) and finally, to ferredoxin (Figure 1.5). The phylloquinone is vitamin K1 which binds to the large subunit(s) of PSI complexes and functions as a secondary electron acceptor (Iwaki *et al*., 1992). The soluble flavoprotein ferredoxin-NADP reductase serves to reduce $NADP^+$ to NADPH, thus completing the sequence of noncyclic electron transport that begins with the oxidation of water (Figure 1.5). The *psaA* and *psaB* genes, which encode the reaction center subunits of PSI, exist tandem and are co-transcribed in the chloroplast genome of vascular plants and in the cyanobacterial genome (reviewed by Hihara and Sonoike, 2001).

1.3.4 Stress factors

It is well known that photosynthetic systems in higher plants are most sensitive to high temperature, water and salt treatment (Falk *et al*., 1996). Upon salt stress (0.3 M NaCl) for 5 days of *Amaranthus tricolor* which has leaves with green, yellow and red regions, the levels of Chl, PSI, PSII, ribulose 1,5-bis phosphate carboxygenase and oxygenase in the green region decreased by about 20-35 % whereas those in the non-green regions remained almost at the same levels. The reduced levels of Chl *b* would

cause almost the absence of light harvesting complex II (LHCII) and the significant decrease of PSI and PSII contents (Wang *et al*., 1999).

Strong light impairs the activity of the photosynthetic apparatus, in particular that of photosystem II (PSII), via a process known as photodamage or photoinhibition (Aro *et al*., 1993). The effects of osmotic and ionic stresses are involved in the NaCl-induced inactivation of the photosynthetic machinery in the cyanobacterium *Synechococcus* sp. PCC 7942. Incubation of cyanobacteria cells in 0.5 M NaCl induced a rapid and reversible decline and subsequent slow and irreversible loss of the oxygen-evolving activity of photosystem II and the electron transport activity of PSI (Allakhverdiev *et al*., 2000). In cyanobacterial *Spirulina platensis*, salinity stress induced a decrease in oxygen evolution activity, which correlated with the decrease in the quantum yield of PSII electron transport. Phycocyanin content decreased significantly as well as the efficiency of electron transfer from Q_A^- to Q_B. The results suggest that salt stress inhibited the electron transport at both donor and acceptor sides of PSII, resulted in damage to phycobilisome and shifted the distribution of excitation energy in favor of PSI (Lu and Vonshak, 2002). PSII was supposed to be more sensitive than PSI to almost all kinds of stresses, in particular to light (Hihara and Sonoike, 2001). The degradation rate of the D1 polypeptide regulates its own synthesis at translational level (Tyystjärvi *et al*., 1996). However, the effect of salt stress on PSII has still been controversial in cyanobacteria (Lu and Vonshak, 2002). At low temperature, *Synechocystis* growth at 20 ºC caused inhibition of PSI activity and increased degradation of the PSI reaction center proteins PsaA and PsaB, while no significant changes were found in the level and activity of photosystem II (Zak and Pakrasi, 2000).

Mulo *et al*. (1998) demonstrated that the disruption of a *spe*-like open reading frame (ORF) encoding arginine decarboxylase, which involved in the biosynthesis of polyamines, downstream from the *psbA-2* gene encoding D1 protein of PSII in the chromosome of *Synechocystis* 6803 resulted in reduced dark stability of *psbA-2* transcripts. This disruption also decreased the Spd content of the cells. The results were suggesting that polyamines might play a role in the stability of the *psbA-2* mRNA.

1.4 *Synechocystis* sp. PCC 6803

Using a combination of biochemical characters together with morphological difference, cyanobacteria can be classified as the Procaryota, Division of Cyanophyta and Cyanophyseae class. Single-celled blue-green algae range in size from about 0.6 μm to well over 30 μm in their largest dimension (for review see Whitton, 2002). The bacteria and cyanobacteria lack mitochondria, true vacuoles and endoplasmic reticular. There is no membrane bounded chloroplast; in cyanobacteria the photosynthetic lamellae are usually distributed in the peripheral cytoplasm. Cells of cyanobacteria are surrounded by the cytoplasmic membrane, the cell wall which contains an outer membrane and a peptidoglycan layer, and in many cases a glycocalyx layer (Figure 1.6). The outer membrane functions more as a passive molecular sieve, whereas the cytoplasmic membrane serves as a true selective permeability barrier (Gantt, 1994).

Among prokaryotes, cyanobacteria are the only organisms to engage in oxygenic photosynthesis. *Synechocystis* sp. PCC 6803 is a unicellular non-nitrogen (N_2) fixing cyanobacterium and a ubiquitous in fresh water. It has been one of the

most popular organisms for genetic and physiological studies. The entire genome of *Synechocystis* sp. PCC 6803 was sequenced in 1996 which was the first phototrophic organism to be fully sequenced (www.kazusa.or.jp/cyano/). *Synechocystis* has been defined as a unicellular coccoid, or spherical cyanobacterium (Figure 1.6) lacking gas vacuoles or a sheath. They devide by binary fission at two or three successive plans (reviewed by Ikeuchi and Tabata, 2001). There are four culture substrains of *Synechocystis* including PCC, ATCC, GT (Glucose-Tolerant) and Kazusa, all of which were derived from the Berkeley strain 6803, which was isolated from fresh water in California by R. Kunisawa (Stanier *et al*., 1971) (Figure 1.7).

The cellular genome was originally deduced to be 3,573,470 bp long (Figure 1.8). A total of 3167 potential-protein coding genes have been assigned to the genome of *Synechocystis* (Table 1.2). The strain *Synechocystis* sp. PCC 6803 has the advantage of being naturally competent for transformation by foreign DNA, allowing research of unknown genes through gene disruption and insertion mutational analysis. Also, since the strain can carry out photo-heterotrophic growth without loss of viability, the photosynthetic related genes can be disrupted to investigate their contribution to the photosynthetic pathways (Nakamura *et al*., 2000). *Synechocystis* cells also acclimate to various kinds of environmental stress by regulating the expression of numerous stress-inducible genes. The results showed clear evidence that salt stress and hyperosmotic stress regulate different sets of genes, although expression of some genes was induced in common by both kinds of stress (Kanesaki *et al*., 2002). Moreover, DNA microarray analysis is a novel technique examined the temporal program of gene expression during acclimation under stresses (Hihara *et al*., 2001, Kanesaki *et al*., 2002).

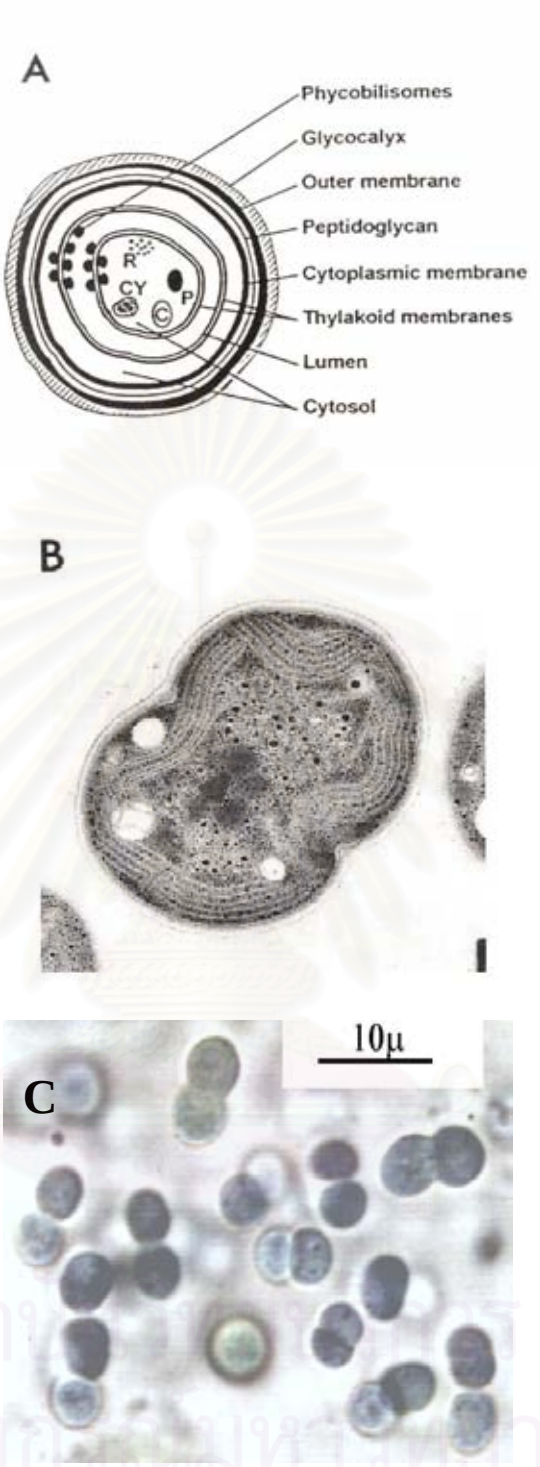

Figure 1.6 Ultrastructure of a cyanobacterial cell (Mulo, 1998). A. Schematic representation of a thin section of a cyanobacteria cell (R, ribosomes; P, polyphosphate granule; C, carboxysome; CY, caynophycin granule). B. Thin section of *Synechocystis* sp. PCC 6803. C. *Synechocystis* cells.

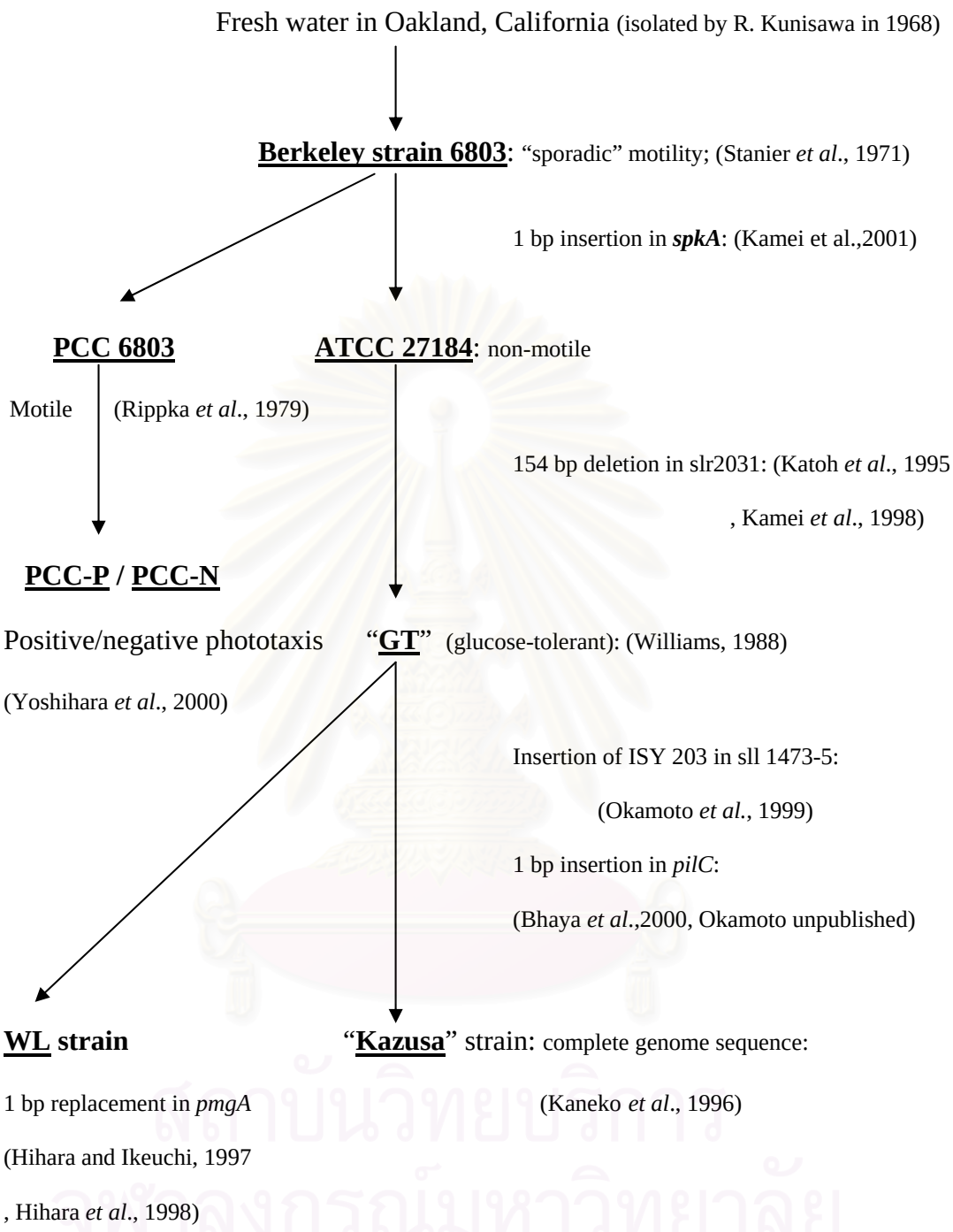

Figure 1.7 Strain history of *Synechocystis* sp. PCC 6803 (Ikeuchi and Tabata, 2001).

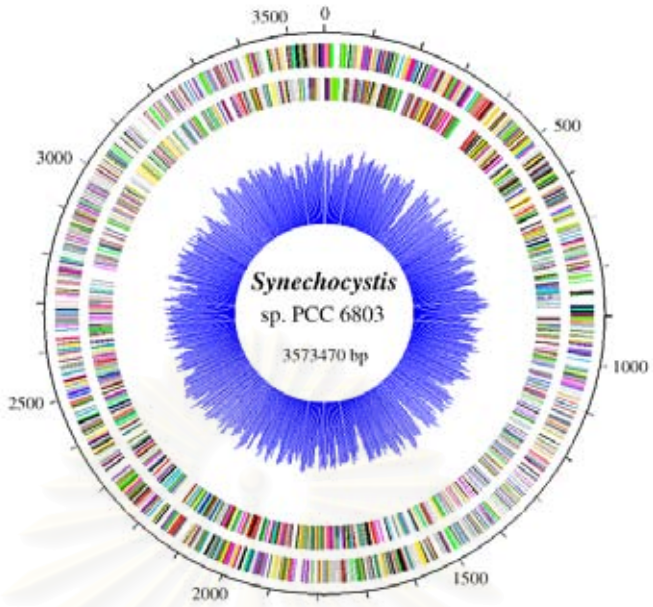

Figure 1.8 The cellular genome of *Synechocystis* sp. PCC 6803 according to Cyanobase (www.kazusa.or.jp/cyano/Synechocystis).

Table 1.2 Functional categories of the products of putative protein encoding genes in *Synechocystis* sp. PCC 6803 (Ikeuchi and Tabata, 2001)

Category	Gene number
Amino acid biosynthesis	84
Biosynthesis of cofactors, prosthetic groups and carriers	108
Cell envelope	64
Cellular processes	62
Central intermediary metabolism	31
Energy metabolism	86
Fatty acid, phospholipid and sterol metabolism	35
Photosynthesis and respiration	131
Nucleic acid metabolism	38
General regulatory functions	147
DNA replication, recombination and repair	49
Transcription	24
Translation	144
Transport and binding proteins	158
Other categories	255
Function unknown	1751
Total	3167

Cyanobacteria owe the ubiquitous distribution to remarkable capacities to adapt to varying environment conditions. Subsequently, 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 factor as well as for solving agricultural problems (reviewed by Joset *et al*., 1996).

1.5 OBJECTIVES OF THIS RESEARCH

1) To investigate the effects of ionic and osmotic stresses on polyamine contents and key polyamine biosynthetic enzyme activities in *Synechocystis* cells.
2) To study the expression of arginine decarboxylase gene under stress conditions.
3) To investigate the effects of ionic and osmotic stresses on photosynthetic systems in *Synechocystis*.

CHAPTER II

MATERIALS AND METHODS

Materials

2.1 Equipments

Balances	METTLER PJ360 Delta Range® GWB
	METTLER AT261 Delta Range® GWB
Centrifuge	Jouan MR 1812
	SORVALL® ULTRA PRO 80
	HERMLE Z233 MK
Electrophoresis Unit	BIO-RAD PROTEAN® II xi Cell
C-18 column	250mm× 4.6mm Model LUNA 5 μm i.d.,
	Phenomenex, USA
Hybridiser	TECHNE Hybridiser HB-1D
HPLC	Hewlett Packard series 1050, Japan
Laminar flow	BVT-124 International Scientific Supply, Thailand
Oxygen electrode	Clark-type oxygen electrode, Hansatech
Light source unit	Perkeo S250 Zeiss IKON
PCR apparatus	PERKIN ELMER DNA Thermal Cycler, Japan
pH meter	ORION model 420A, USA
Power supply	BIO-RAD POWER PAC 1000
	BIO-RAD Model 1000/500
Scintillation counter	Pharmacia LKB Wallac, Reckbeta 1218, England
Spectrophotometer	SPECTRONIC® GENESYS™2
	Jenway UV/VIS 6400, USA

Vortex	Model K-550-GE, Scientific Industries, USA
Water bath	THERMOMIX® B B.BRAUN

2.2 Chemicals

Acetic acid	BDH, England
Acetone	Merck, Germany
Acrylamide	Merck, Germany
Agarose	Promega Corporation, USA
Ammonium persulfate	Merck, Germany
DL-[1-^{14}C]-arginine	Amersham Bioscienes, Sweden
Benzoyl chloride	Sigma, USA
Brilliant blue	Sigma, USA
Chloroform	Merck, USA
DCBQ	Sigma, USA
DMSO	Sigma, USA
Diethyl ether	Merck, USA
DTT (Dithiothreitol)	Sigma, USA
EDTA	Sigma, USA
Ethanol	Scharlau Chemie S.A., Spain
Ethidium bromide	Sigma, USA
Ferricyanide	Sigma, USA
Formadehyde 37%	Merck, Germany
Glycerol	Scharlau Chemie S.A., Spain
Glycine	Sigma, USA
Glyoxal	Sigma, USA

1,6-Hexanediamine	Sigma, USA
HEPES	Sigma, USA
Isoamylalcohol	Sigma, USA
Isopropanol	Sigma, USA
Leupeptin	Sigma, USA
Mercaptoethanol	BIO-RAD, USA
Methanol	Scharlau Chemie S.A., Spain
Perchloric acid	Merck, USA
Pyridoxal-5-phosphate	Sigma, USA
Phenol	Merck, Germany
PMSF	Sigma, USA
Putrescine	Sigma, USA
Rifampicin	Sigma, USA
L-[1-^{14}C]-SAM	Amersham Biosiences, Sweden
Silver nitrate	Sigma, USA
Sodium bicarbonate	BDH, England
Sodium chloride	APS, Australia
Sodium dodecyl sulfate	Sigma, USA
Sodium thiosulfate	Sigma, USA
Sorbitol	Sigma, USA
Spermidine	Sigma, USA
Spermine	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
Xylene cyanol FF	Sigma, USA

2.3 Supplies

AIS™ The Analytical Imaging Station Operations program : Imaging Research Inc. Ontario, Canada

Hybond-N membrane	Amersham Biosciences
Millipore Utltrafree-DA	Millipore Coorperation, USA
Nylon membrane filter	0.45 micron, 1.3 mm i.d., Sartorius, Germany
Immobilon-P membrane	Millipore Coorperation, USA
Whatman 3MM paper	Whatman International, England
X-ray film	X-Omat XK-1, Eastman Kodak, USA

2.4 Kit

Chemiluminescence kit	BioRad, USA
1 kb DNA Ladder	BioLabs, New England
RNA Ladder, High Range	MBI Fermentas, Germany
DyNAzyme™ DNA Polymerase kit	FINNZYMES, Finland
PCR amplification kit	MBI Fermentas, Germany
Prestained Protein Marker, Broad range	BioLabs, New England
Prime-a-Gene® Labeling System	Promega Corporation, USA

Ready-To-Go You-Prime First-Strand Beads kit : Amersham Biosciences

RQ1 Rnase-Free Dnase Promega Corporation, USA

2.5 Primers and probes

Table 2.1 Sequences of the primers and probes

Target gene	Name	Primers	Length in pairs
16S rRNA	forward-16s reverse-16s	5′-AGTTCTGACGGTACCTGATGA-3′ 5′-GTCAAGCCTTGGTAAGGTTCT-3′	521
ADC	forward-*adc* reverse-*adc*	5′-ACCTTATTTTGCCATTAATGC-3′ 5′-CCGTACCCCCAACATGGG-3′	557
psaA	forward-*psaA* reverse-*psaA*	5′-GGATCGGATGCTGCGCCAC-3′ 5′-CTAGCCAATGGAAAGACTGC-3′	751
psbA	forward-*psbA* reverse-*psbA*	5′-AGTCAGTTCCAATCTGAAC-3′ 5′-TAATTGGCAACTGTCGTCC-3′	360

2.6 Organism

Synechocystis sp. PCC 6803 wild type strain was obtained from the Laboratory of Plant Physiology and Molecular Biology, Department of Biology, University of Turku, Finland.

Methods

2.7 Culture conditions

Synechocystis cells were grown in BG-11 medium (Appendix 1) under continuous light at 50 μmol m^{-2} s^{-1} at 32 °C. Growth was monitored by measuring the optical density at 730 nm with Spectronic® Genesys™ 2 spectrometer (Figure 2.1). For ionic and osmotic stress conditions, NaCl and sorbitol were added into the medium solution by changing the final concentrations of NaCl from 0 to 550 mM and

sorbitol from 0 to1100 mM, respectively. Each experiment was repeated two times and each treatment was run in three replicates. The harvested cells will be used for further analysis.

Figure 2.1 Ten days old stock culture of *Synechocystis* sp. PCC 6803.

2.8 Polyamine Biosynthesis

2.8.1 Polyamine determination

The *Synechocystis* cells were extracted in 5 % cold $HClO_4$ at a ratio of about 100 mg/ml $HClO_4$. After extraction for 1 hour in an ice bath, samples were centrifuged at 5,000*g* for 20 min. Polyamines were analyzed by high performance liquid chromatography (HPLC) (Flores and Galston, 1982). The supernatant and pellet fractions were used to derivatize and quantify the total polyamines. The derivatization was carried out with benzoyl chloride using 1,6-hexane-diamine as an internal standard. One ml of 2 N NaOH was mixed with 500 μl of $HClO_4$ extract and 10 μl benzoyl chloride. The mixture was vigorously vortexed and incubated for 20 min at room temperature. The reaction was terminated by adding 2 ml of saturated NaCl. Subsequently, the benzoyl-polyamines were extracted with 2 ml of cold dietyl ether. Finally, 1 ml of the ether phase were evaporated to dryness under the stream of

warm air and redissolved in 1 ml of methanol. Authentic polyamine standards (Sigma Chemical Co.) were prepared similarly to cell samples.

The polyamine contents were analyzed by HPLC with a C-18 reverse phase column and a UV-VIS detector at 254 nm. A mixture of methanol-water was used as mobile phase. The solvent system ran at gradient from 50 to 80 % methanol for 25 min, and regeneration to the initial state with 50 % methanol, for 5 min with a flow rate of 0.7 ml min^{-1} (Appendix 2).

2.8.2 Enzyme biochemical radioassay

The activities of ADC and SAMDC were determined according to the method of Minocha *et al*. (1999b). *Synechocystis* cells (100 mg wet weight) were placed in the polystyrene disposable tubes (Figure 2.2). The extraction buffer for ADC contained 50 mM Tris-HCl (pH 8.4), 0.5 mM pyridoxal-5-phosphate, 0.1 mM EDTA and 5 mM dithiothreitol (DTT). For SAMDC, the buffer contained 100 mM potassium phosphate (pH 7.5), 3 mM putrescine and 1 mM DTT. The reaction mixture of 300 μl contained 250 μl extraction buffer and 50 μl 12 mM arginine containing 3.7 kBq of DL-[1-^{14}C]-arginine or 1.2 mM SAM containing 3.7 kBq of L-[1-^{14}C]-SAM, respectively. A 0.5 × 2 cm^2 piece of Whatman 3 MM filter paper soaked with 2 N KOH was placed in each tube fitted with a rubber cap to trap liberated $^{14}CO_2$. After incubation at 37 °C for 60 min (30 min for SAMDC), 500 μl 0.5 N H_2SO_4 was injected to terminate the reaction. After an additional incubation for 30 min, the filter papers were removed into 2 ml scintillation fluid and counted for radioactivity. Enzyme activity was expressed as nmol $^{14}CO_2$ liberated mg $protein^{-1}$ min^{-1}(Appendix 3). Protein content was estimated by the method of Bradford using bovine serum albumin as a standard (Bradford, 1976) (Appendix 4).

Figure 2.2 Reaction chamber used for enzyme biochemical assay.

2.8.3 RNA preparation

The *Synechocystis* cells were harvested at the logarithmic growth stage by centrifugation (4000*g*, 10 min) and the cell pellet was immediately frozen in liquid nitrogen. The total RNA was extracted by the hot phenol method (Mohamed and Jansson, 1989). Pellet was thawed and resuspended in 1 ml resuspension buffer (Appendix 5) on ice. After centrifugation for 5 min at 12,000 rpm (4 ºC) in eppendorf, pellet was resuspened in 250 μl resuspension buffer. Added 75 μl of 250 mM EDTA, pH 8.0 and incubated on ice for 5 min. Added 375 μl lysis buffer (Appendix 5) and incubated at 65 ºC for 3 min. Hot phenol (65 ºC) was added into the reaction mixture and incubated for 3 min. Cooling down the solution on a freeze aluminium block for 5-10 sec and centrifuged at 12,000 rpm, 5 min at room temperature. The over phase was taken and repeated adding hot phenol. The mixture was extracted once with equal volume of phenol/chloroform/isoamylalcohol (25:24:1) mixed gently and centrifuged at 12,000 rpm for 5 min at room temperature. The RNA was precipitated by adding 1/10 volume of 3 M sodium acetate buffer, pH 6.0 and 2.5 volume cold ethanol (-20

ºC), incubated at –20 ºC for 30 min. After incubation, centrifuged at 14,000 rpm for 10 min (4 ºC). The pellet was washed with 70 % ethanol, centrifuged at 12,000 rpm for 2 min. After the pellet was dried, resuspened in 100 μl RNA storage buffer (Appendix 5). Kept at freezer (–80 ºC) until used. To determine concentration and purity of RNA, sample was diluted with storage buffer and checked by measuring the OD at 260 nm and ran 0.6 % agarose gel (Appendix 6) in TBE buffer (Appendix 7).

To remove chromosomal DNA contamination, RNA samples were treated with RQ1 RNase-free DNase (Promega Corporation). The reaction mixture contained 50 μg RNA, 10×buffer (5 μl) and RQ1 RNase-free DNase (5 μl) and adjusted with Milli-Q water to a total volume of 50 μl. After an incubation at 37 ºC for 1 h, added 5 μl RQ1 RNase-free DNse and incubated in the same condition for 2 h. The mixture was added 200 μl of 40 mM Tris-HCl, pH 8.0 and extracted with 250 μl phenol/chloroform/isoamylalcohol (25:24:1). After centrifugation at 14,000 rpm for 2 min (4 ºC), the over phase was taken and added 1/10 volume of 3 M Na-acetate, pH 5.2 and 0.6 volume of isopropanol, incubated at –20 ºC for 30 min. Centrifuged at 14,000 rpm for 10 min (4 ºC). The pellet was washed with 70 % ethanol and centrifuged at 12,000 rpm for 2 min and dried. After an addition of 25 μl RNA-storage buffer (Appendix 5), the solution was checked RNA concentration by measuring OD at 260 nm.

2.8.4 RT-PCR

Total RNAs prepared from *Synechocystis* cells as describes in 2.8.3 were diluted with Milli-Q water to final concentration of 0.5 μg in total volume 30 μl. After heating at 65 °C for 10 min, the samples were chilled on ice for 2 min. The cDNAs were synthesized using the Ready-To-Go You-Prime First-Strand Beads kit without

mixing. Added 1μl specific 3′ primer (Table 2.1) and 2 μl Milli-Q water. Two primers specific to two *adc* genes [slr1312 (*adc1*) and slr0662 (*adc2*)] designed from the Cyanobase sequence (Figure 2.3). The beads in reaction tube were dissolved at room temperature for 1 min. The solution was mixed by gently vortexing or pipetting up and down and incubated at 37 °C for 1 h.

PCRs (Appendix 9) were performed using reverse-transcribed (RT) samples and primers specific to ADC genes designed on the basis of sequence data from Cyanobase (Table 2.1). The initial denaturation for 5 min was done at 95 °C, followed by 30 cycles for 1 min denaturation at 95 °C, 1 min of primer annealing at 55 °C and 1 min of synthesis at 72 °C. The PCR products were checked on a 0.6 % agarose gel in 1× TAE buffer (Appendix 8). Quantification was carried out using AIS^{TM} The Analytical Imaging Station program (Imaging Research Inc.).

2.8.5 Half-life of *ADC* transcripts

Synechocystis cell culture (10 μg chl/ml) was recovered for 1-2 h under growth condition and incubated under growth-light conditions in the presence of rifampicin (500 μg/ml), the inhibitor of transcriptional initiation. Aliquots were taken after 0, 15, 30, 60 and 120 min of incubation, frozen immediately in liquid nitrogen. RT-PCR was performed as described in section 2.8.4.

2.8.6 SDS-polyacrylamide gel electrophoresis and Western blottng

Total proteins for enzyme assays were extracted from *Synechocystis* cells (Appendix 10). Analytical and preparative SDS-PAGE (Appendix 11) of proteins extracts were performed. Proteins were denatured at 65 °C for 5 min in SDS sample buffer containing Laemmli solution (Appendix 11) and 10 % 2-mercaptoethanol.

```
0662   ATGGA AGGGC AGTCA ATCGA ACTAG AACTA AGTGT CATGG CAATG CCGGA GTTAA TCGAC   60
1312   ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- -----

0662   AGTAC TGAAG CAGGC CATAC CGCCG GGGTG AAAAC TGATT CCAAT CCCCA GGCGA TCGCC   120
1312   -ATGG GGGAA GAACC TGTGC CGGCG GATAA AGCAT TAGGC AAGAA ATTCA AGAAA A----   55
         *    * *   *  *    * * ** ** *      *  *  *        *     **  *  *

0662   CAAGA CCGTC GTTGG ACCAT TGACG ACAGC GAAAA CCTCT ACCGC ATCAC TGGTT GGGGG   180
1312   -AAAA TGCCT CCTGG AGCAT TGAAG AAAGC GAAGC TCTGT ACCGG GTTGA GGCCT GGGGG   114
        ** *          *** * *** *** * * *** ***    ** * ****   *     *  * *****

0662   GAACC TTACT TTTCC ATTAA TGCGG CCGGC CATGT GACCG TTTCT CCCCA GGCTG ACCAT   240
1312   GCACC TTATT TTGCC ATTAA TGCCG CTGGT AACAT AACCG TCTCT CCCAA CGGCG ATCGG   174
       * *** *** * ** ** ***** *** * * **   *  *  **** * *** *** * *  * * * *

0662   GGGGG AGCGT TGGAT TTGTA CGAAC TGGTT AAGGG TTTGA GGCAA AGAAA TATTG GCTTG   300
1312   GGCGG TTCGT TAGAT TTGTT GGAAC TGGTG GAAGC CCTGC GGCAA AGAAA GCTCG GCTTA   234
       ** **   *** * *** ****   **** ****  * *    **  ***** *****   * * ****

0662   CCTTT ACTGT TGCGC TTTTC TGATA TTCTG GCTGA CCGCA TCAAT CGCCT CAATG CGGCC   360
1312   CCCCT ATTAA TTCGT TTTTC CGATA TTTTG GCCGA TCGCC TAGAG CGATT GAATA GTTGT   294
       **  * * *   * **  *****  **** ** ** ** **  ***  *  *  **  *  ***

0662   TTTGC CCGGG GCATT GCCCG TTACC GCTAT CCCAA CACCT ACCGG GGGGT TTATC CCATT   420
1312   TTTGC CAAGG CGATC GCCCG TTACA ATTAC CCCAA CACCT ATCAG GCGGT TTATC CGGTC   354
       ***** *  **   **  ***** ****    **  ***** ***** * * * * *** ***** *  *

0662   AAGTG CAACC AGCAT CGCCA CATTG TGGAA TCCCT GGTGC GCTAC GGCAC TCCCT ATAAT   480
1312   AAATG TAACC AGCAA CGACA TCTGG TGGAA GCCCT GGTTC GCTTT GGGCA AACTT CCCAG   414
       ** **  **** **** ** **   * * *****  **** *** * ***  **      * *    *

0662   TTTGG CTTGG AAGCC GGTTC TAAAC CGGAG TTGAT GATTG CCCTG GCTAT GCTCC AACCC   540
1312   TGTGG ATTGG AGGCA GGTTC CAAAC CGGAA TTGAT GATTG CCCTC GCAAC TCTAC CACCT   474
       * ***  **** * **  ***** **** **** ***** ***** ****  ** *  ** *  ***

0662   CAGGA GAACC CAGAG CCGGA TCAAC AAAAT CAGCC TTTAC TAATT TGTAA TGGTT ATAAA   600
1312   CCCTT AGACC ---GT CAGGA CAAGC ATACC AAGCC CCTAA TCATT TGTAA TGGCT ACAAA   531
       *      ***       * ***   * * * *   ****   **  * *** ***** *** * * ***

0662   GACCG GGAAT ATATT GAAAC CGCCT TGCTA GCCCG TCGTC TGGGG CATCG GCCGA TTATT   660
1312   GACCA GGATT ATCTA GAAAC AGCTC TGTTA GCCAA ACGCT TAGGC CATCG TCCCA TCATC   591
       ****  *** * ** *  ***** **   ** ** ***    **  * **  *****  ** * * **

0662   GTGGT GGAGC AGGTA GCGGA GGTGG CCTTG GCCAT CGAAA TTTC- CAGCA ATCTG GGCAT   719
1312   ATCAT TGAAC AACTA CGGGA ACTGG AATGG GTACT ACACA TCTCT CAGCA GT-TA AACAT   650
         *  *  ** * *   **  ***   * * *   * * * **  *****  * *    ***

0662   TAAGC CAATT TTGGG GGTAC GGGCC AAACT GAGTA CCCAG GGCAT GGGCC GTTGG GGCAT   779
1312   TAAAC CCATG TTGGG GGTAC GGGCC CGGTT AAGTT GTCAG TCGCT CAAAT CCTCG GAAAT   710
       *** * * **  ***** ***** *****     *  ***    ***      *         * * *  **

0662   TTCCA CTGGC ----- -GATC GGGCT AAATT TGGTT TAACC ATCCC GGAAA TGTTG ACGGC   833
1312   TTCCT CTGGC AACGG CGATC GGGCT AAGCT TGGTC TGACC ATGCC GGACA TTGTG ACCGT   770
       ****  *****       **** ***** **  * **** * *** ** ** *** * *  ** ** *

0662   GATCG AGCAA CTGCG CCGAG CTGAT ATGTT GGACA GTCTG CAATT GCTCC ATTTT CACAT   893
1312   AATTC ATCGT CTAGA GGAAA ATAAT TGTCT CGATT GCCTG AAAAT GCTTC ATTTT CATCT   830
        **   * *   **      *   * **     *  **   * ***  ** * *** * ***** **  *

0662   CGGTT CCCAG ATCTC TTCCA TCTCT GTGAT CAAAG AAGCG ATGAC GGAAG CCAGC CAAAT   953
1312   GGGAA CGCAA GTTTC GGACA TTGCT CTGAT TAAAG AAGCT ATGCG GGAAG CCAGC CAACT   890
        **   * **   * **    ** *  **  **** **** ****  ***   ***** ***** *** *

0662   TTTTG TCCAG TTGGC CAAGC TGGGG GCCAA TATGC GCTAC CTGGA TGTGG GGGGC GGACT   1013
1312   CTATG TGGAA TTGGT CAAGC TGGGG GCAAA AATGC GCTAC CTCAA TGTGG GGGGC GGTTT   950
        * ** *  *  **** ***** ***** ***** ** **  **** ***** **  * ***** **  *

0662   AGGGG TGGAC TACGA CGGTT CCAAA ACCAA TTTTT ACGCT TCCAA AAATT ACAAC ATCCA   1073
1312   GGCGG TGGAT TATGA CGGTT CCAAG ACCAA CTATC CCGCC TCGAA AAATT ACAAC ATGCA   1010
        * ** **** ** ** ***** **** ***** * *  *** ** ** ***** ***** ** **

0662   GAATT ACGTT AATGA TGTGA TTTCG GCAGT GCAGG ATGCC TGTGT GGCCG CTGAA GTGCC   1133
1312   AAACT ACGCC AACGA CATTG TGGCG GCCAT TCAAG ATGCC TGTGA GCTTG GGCAG GTTTC   1070
        ** * ***  ** **   *   *  ** **  *  ** * ***** ****  *   *  *  **  *

0662   CTGTC CTGTG CTGAT TAGTG AAAGC GGCCG GGCGA TCGCC AGCCA TCAGT CAGTA CTCAT   1193
1312   CCCTC CCATT CTTGT GAGCG AAAGT GGGCG GGCAA TTATG GCCCA TCAGT CGGTG CTAGT   1130
       *  ** *  * **  *  ** * **** ** ** *** * *    *** ***** * **  **  *

0662   TTTTG ATGTT GTGGC CACCA ATGAC ATTAA TCCCC CCTTG CCTAA GGTGA AGGGC AAAGA   1253
1312   TTTTG ATGTG CTAGG CAGCA ACCAA ACAGG CTTCA GTGAA CCCCA TCCCC CCGAT GAAAA   1190
       ***** ****   * *  ** ** *  *  *       *        **  *          *   ** *

0662   ---CC ATGCC ATTTT GCGTA ATTTG ATGGA AACCT GGGAA ACCAT TACGG TGGAT AATTA   1310
1312   TGCCC ATCCC CTGCT AAAAA ATCTC TGGGA ATGTT ACGAA ACAAT TACAG CGGAA CAATA   1250
          ** ** **  *  *     * ** *   *** *   *  *** ** ** *** *  ***   * **

0662   CCAAG AGGCG TACCA TGATG TGGAA CAGTT TAAAA CTGAA GCCAT TAGTT TATTT AACTT   1370
1312   CCAGG AGCAA TACCA TGATG CTCTG CAATT AAAGA CGGAG GCTAG TAGTC TTTTT AACTT   1310
       *** * **    ***** *****       ** **  ** * * **  ** *  ****  * *** *****

0662   TGGTT ATTTA GGTCT GAAAG AAAGG GCTAA AGCAG AGGAG CTTTA TTGGG CTTGT TGCCG   1430
1312   CGGCT ATTTA AGTCT GACGG AACGG GGACA AGCAG AGCAA ATTCA CTGGG CTTGT TGTCG   1370
        ** * *****  **** **  * ** ** *   * ***** ** *   ** *  **** ***** ** **

0662   CAAGA TTTTA CAAAT TTGCC GTCAG CAAGA ATACG TCCCC GATGA TTTGG AAAAT TTGGA   1490
1312   TAAAA TTTTT GAAAT CACCA GACAA CTAGA GTATA TTCCC GAAGA TTTTC AAGCG CTGGA   1430
        ** * ****   ****    *  * **  * ***  **   * *** ** ** ***   **    ****
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```
0662   AGTTA ACTTG GCTTC TATTT ACTAC GCCAA TATGT CGGTG TTTCA GTCGG CCCCG GATTC   1550
1312   TAAAA TAATG ACCGA TATTT ATTAC GTTAA CTTAT CGGTT TTCCA GTCAG CACCG GAATC   1490
           *    **  *    ***** * *** *  **   * * ****  ** ** *** * * *** ** **

0662   CTGGG CGATC GATCA ACTTT TCCCG ATTAT GCCCA TCCAC CGTTT GGATG AAGAA CCCAC   1610
1312   CTGGT CTTTA GATCA ACTTT TTCCC ATTTT GCCCA TTCAC CATCT CAATG AGAAA CCTAG   1550
       ****  *  *  ***** ***** * **  *** * ***** * *** * * *   *** *  ** ** *

0662   CCAGC GGGGC ATTCT GGCGG ATATT ACCTG CGACA GTGAT GGCAA AATTG ACCAA TTTAT   1670
1312   TCAAA GGGTG ATTTT AGCCG ATTTA ACCTG TGACA GTGAT GGTAA AATTG ACCGT TTTAT   1610
        **   ***   *** *  ** * ** *  *****  **** ***** ** ** ***** ***   *****

0662   TGACC TGCGG GATGT CAAAT CAGTA TTGGA ATTGC ATCCT TTAAT AGAAG TGCAT CAGCC   1730
1312   TGACC TGTGG GATGT CAAGT CATAC CTAGA AGTTC ACCCC CTAGA AAATG ----- -----   1660
       ***** ** ** ***** *** * **     * ** * * * * **   **   * * *

0662   AGGGA CTCCC CCCAG GGTGG AACCC TATTA TTTGG GCATG TTTTT GGTGG GGGCT TACCA   1790
1312   ----- ----- ---AC GGCA- ATCCT TACTA TTTAG GTATG TTTTT AGTCG GTGCT TACCA   1706
                     *  **    * **  ** ** *** * * *** *****  ** * * *** *****

0662   AGAAA TTATG GGTAA TTTAC ATAAT TTATT TGGGG ACATC AATGT GGTGC ATATT CAGAT   1850
1312   AGAAA TTATG GGCAA TTTAC ATAAT TTATT TGGTG ACATT AATGT AGTTC ACATT GCCAC   1766
       ***** ***** ** ** ***** ***** ***** *** * ****  *****  ** * * ***    *

0662   GAATC CGAAA GGTTA CCAAA TTGAA CATTT AGTGA GGGGG GATAC CATCG CCGAA GTGTT   1910
1312   TACTC CCCAA GGTTA TCAGA TTGAA TCGGT GGTGC GGGGA GATAC CATGA CGGAG GTTTT   1826
        * ** *  ** *****  ** * *****     *  ***  ****  ***** ***   * **  ** **

0662   GGGCT ATGTG CAGTA CGATC CCGAA GATTT GCTGG AAAAT ATGCG CCGCT ACTGT GAACA   1970
1312   GGGTT ATGTT CAGTA CGATT CTGAT GATTT ACTCG AAGGC CTGCG GCGTC ATACG GAGTT   1886
       *** * ****  ***** ****  * **  *****  ** * **     ****  **   *    **

0662   GGCCA TGGAA GATAA ACGTA TGAGC TTGGA GGAAG CCCAA TTATT GCTGG AAAAT TACGA   2030
1312   AGCCT TGAGC AATGG ACAAA TTACC CTGGA GGAAT CTCGG CGCTT ATTGG AAGAT TATGA   1946
        ***  **     **   **  * * * *  **** ****  * *      **   *** ** ** ** **

0662   GCGCA GTTTG TTGCA ATACA CCTAC CTCAA GCCCA CTT-- CAGGC ATCCA CACCA GCTAA   2088
1312   GCAAA GTTTA CGGCG CTACA CCTAT CT-AA GTTGA TCTGG TAGAA AAAAA CAATG GTTGG   2005
       **  * ****    **   **** **** **** *    *    *    **   *   * **    * *

0662   ATTGA CGATG GTGGG GAATG GTAGA GCATT ACAAA ATATT CAAAT CTCCT GTTCT CAATT   2148
1312   ATTC- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- -----   2009
       ***

0662   CAGAA CAAAT TCGCC TCTTT GCCTA TTTGC TTAAG CGCTT ATGGG 2193
1312   ----- ----- ----- ----- ----- ----- ----- ----- -----
```

forward primer : **5′-ACC TTA TTT TGC CAT TAA TGC-3′**

reverse primer : **5′-CCG TAC CCC CAA CAT GGG-3′**

Figure 2.3 Alignment of arginine decarboxylase nucleotide sequences from Cyanobase of *Synechocystis*. 0662 : slr0662 (*adc2*), 1312 : slr1312 (*adc1*), Star symbols represented homology area. Two designed primers producing 557 bp of PCR product were used in RT-PCR analysis.

The separating gel was 12 % including 4 M urea. Electrophoresis was performed in a BIO-RAD apparatus at a constant current of 7 mA per gel for overnight. The SDS-gel could be stained for checking (Appendix 12). The electrophoretically resolved proteins by 12 × 16 cm SDS-polyacrylamide gels were transferred onto Immobilon-P membrane (Millipore) by blotting solution. Blotting was done at 154 mA for 1 h. Nitrocellulose blots were incubated with first (overnight at 4 °C or 2 h at room temperature) and second (2 h at room temperature) antibodies, diluted 1:2000 (anti-MBP-ADC), 1:5000 (anti-rabbit IgG), in PBS containing 1 % (w/v) BSA and 0.5 % w/v Tween-20. Visualization of antigen-antibody complexes was performed by alkaline phosphatase-conjugated second antibody and the BCiP (0.05 mg/ml) color development reaction in 100 mM diethanolamide pH 9.6 and 0.1 mg/ml nitroblue tetrazolium. The polyclonal ADC antibodies were kind gifts from Prof. Kalliopi A. Roubelakis-Angelakis, Department of Biology, University of Crete, Greece.

2.9 Photosynthetic systems

2.9.1 Oxygen evolution measurements

For each measurement, an aliquot of cell culture containing 10 μg chlorophyll was spun down and resuspended in 1 ml of fresh BG-11 medium. The cell solution was recovered under growth conditions for 1-2 h. PSII activity was measured as oxygen evolution (Appendix 14) under saturating white light with a Clark-type oxygen electrode (Hansatech) at 35 ^{o}C. The 0.5 mM DCBQ (2,6-dichloro-*p*-benzoquinone) (Appendix 13) was used as the electron acceptor, and 0.5 mM ferricyanide (Appendix 13) added to keep the acceptor in the oxidized form. Photosynthetic capacity was measured as light-saturated rate of oxygen evolution in

the Clark-type O_2 electrode by supplementing the growth medium with 0.6 mM bicarbonate (Appendix 13).

2.9.2 Northern blot analysis

Synechocystis cells were harvested at the logarithmic growth stage by centrifugation (4000 × *g*, 10 min), and RNA was isolated as described in section 2.8.3. Northern blotting was performed using standard method (Sambrook and Russell, 2001). The 10 μg RNAs were diluted with Milli-Q water to 10 or 15 μl. After the addition of 30 or 45 μl glyoxal denaturation mix (Appendix 15), the reaction mixture was incubated for 60 min at 50 ºC. Before samples were loaded to the 1.2 % agarose gel in sodium phosphate buffer pH 6.8 (Appendix 15) followed by adding 2 μl glyoxal dye mix (Appendix 15). Running the gel was conducted at 100 volts for 2 - 4 h. Subsequently, separated RNAs were transferred from an agarose gel to Hybond-N nylon membrane (Amersham) (Appendix 16), facilitated by the upward flow of 6 × SSC buffer (Appendix 15) to wet the 3 MM filter papers. Blotting was left at least 12 h. The membrane was exposed under UV-light for 5 min and deglyoxylated at 80 ºC for 2 h. After incubation, the membrane was soaked in 5 % acetic acid and dyed with RNA-membrane dye (Appendix 15), washed with Milli-Q-water.

Prehybridization and hybridizaton were performed at 60 – 65 ºC for 2 h in prehybridizing solution (Appendix 15) and 100 μg/ml Herring sperm DNA. DNA probes were radiolabelled with α-^{32}P-CTP using a multiprime DNA labelling kit (Appendix 17). The following probes were used: the entire coding region of the *psbA*-2 gene (amplified with PCR), *psaA* gene and ribosomal RNA gene of *Synechocystis* sp. PCC 6803. The membrane was hybridized with denatured probes for over night. After hybridization, the membrane was washed with 4 × SSC for 5 min and incubated

shaking in 0.1 % SDS / 0.1 × SSC at 45 - 50 °C for 5 min. Subsequently, wrapped a membrane with plastic foil and established an autoradiograph by exposing the membrane for 24 - 48 h to X-ray film at –70 °C.

2.9.3 Half-lives of *psbA* and *psaA* transcripts

Synechocystis cell culture (10 μg chl/ml) was incubated under growth-light conditions in the presence of rifampicin (500 μg/ml), the inhibitor of transcriptional initiation. Aliquots were taken after 0, 15, 30, 60 and 120 min of incubation, frozen immediately in liquid nitrogen until used. RNA extraction and Northern blot analysis from these samples were done as described in section 2.9.2.

2.9.4 Western blot analysis of photosynthetic proteins

Thylakoid samples were extracted from *Synechocystis* cells using STNE buffer containing 10 mM Tris-HCl (pH 8.0), 0.4 M sucrose, 10 mM NaCl and 20 mM Na-EDTA, pH 8.0 (Appendix 18) followed by the determination of chlorophyll content (Appendix 19). Analytical and preparative SDS-PAGE of thylakoid extracts were performed (Appendix 11). Thylakoid samples were denatured at 70°C for 5 min in SDS sample buffer containing Laemmli solution and 10 % 2-mercaptoethanol. The separating gel was 15 % including 6 M urea. Electrophoresis was performed in a BIO-RAD apparatus at a constant current of 7 mA per gel for overnight. The electrophoretically resolved proteins by 12 × 16 cm SDS-polyacrylamide gels were transferred onto Immobilon-P membrane by blotting solution (Appendix 20). Blotting was done at 154 mA for 1 h followed by washing in 1 × TBS (Appendix 20) and blocking in blocking solution (Appendix 20) for 1 h at room temperature. After washing twice with TTBS (Appendix 20) for 3 min, the membrane was incubated

with primary antibody for overnight and washed with TTBS for 5 min, 5 times. After that, the membrane was incubated for 2 h with alkaline phosphatase-conjugated second PSI and PSII antibodies to locate the protein. Antibodies against the D1 polypeptide (raised against N-terminal amino acids 58-86), D2 polypeptide (raised against amino acids 230-245, PSI and ndhF3 were kindly provided by Prof. Eva-Mari Aro, Department of Biology, University of Turku, Finland.

CHAPTER III

RESULTS

3.1 Effects of NaCl and sorbitol on growth of *Synechocystis* sp. PCC 6803

Growth of *Synechocystis* cells under various concentrations of NaCl and sorbitol was investigated. The presence of NaCl in the growth medium at increasing concentrations up to 550 mM had no effect on the growth rate of *Synechocystis* compared to that in the absence of NaCl (Figure 3.1A). Similar growth rates were observed for cells grown in the presence of increasing concentrations of sorbitol up to 100 mM. Slight inhibition of growth occurred at 300 mM sorbitol whereas no growth was observed at 700 mM or higher (Figure 3.1B), suggesting that the osmotic effect exerted by sorbitol is highly growth-inhibitory to *Synechocystis* cells. It should be pointed out that 700 mM sorbitol treatment did not cause cell death. Although no growth was observed in cells treated with 700 mM sorbitol for 10 days, the cells were fully viable and started to grow normally when shifted to unstressed conditions.

3.2 Polyamine biosynthesis in *Synechocystis* sp. PCC 6803 under salt and osmotic stresses

3.2.1 Cellular polyamine contents under salt stress

Polyamine contents in the cells of *Synechocystis* after growth for 10 days in different concentrations of NaCl is shown in Figure 3.2. Soluble Put content slightly increased under high 550 mM NaCl (Figure 3.2A) whereas insoluble-Put content was unchanged under this condition (Figure 3.2B). The level of Spd in both soluble and insoluble decreased under 50, 150 and 350 mM NaCl stress conditions,

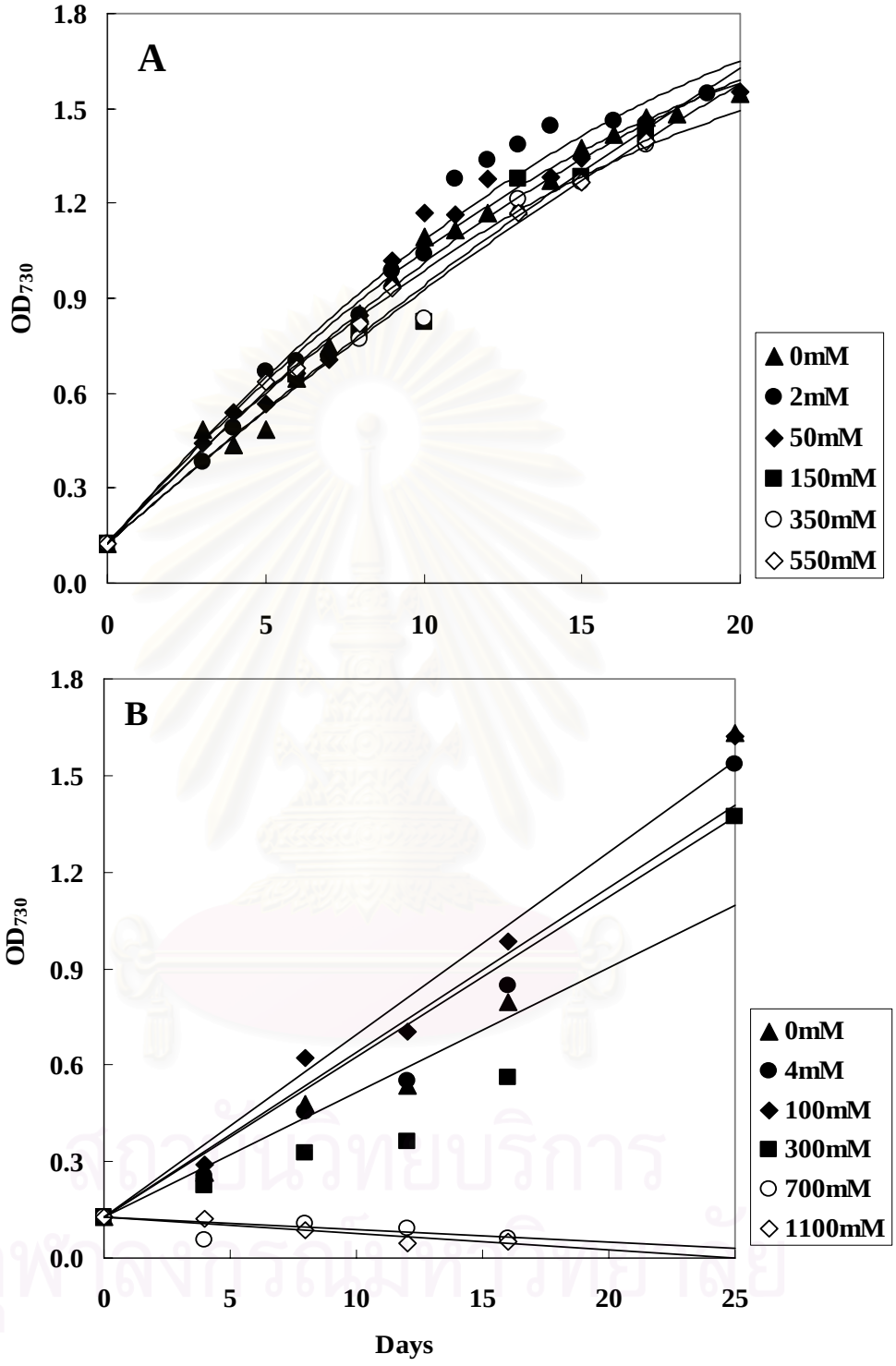

Figure 3.1 Growth curves for *Synechocystis* sp. PCC 6803 grown under different concentrations of NaCl (A) and sorbitol (B). The inset key provides actual concentrations of NaCl and sorbitol added to the growth medium. The data presented are typical of the results from triplicate experiments.

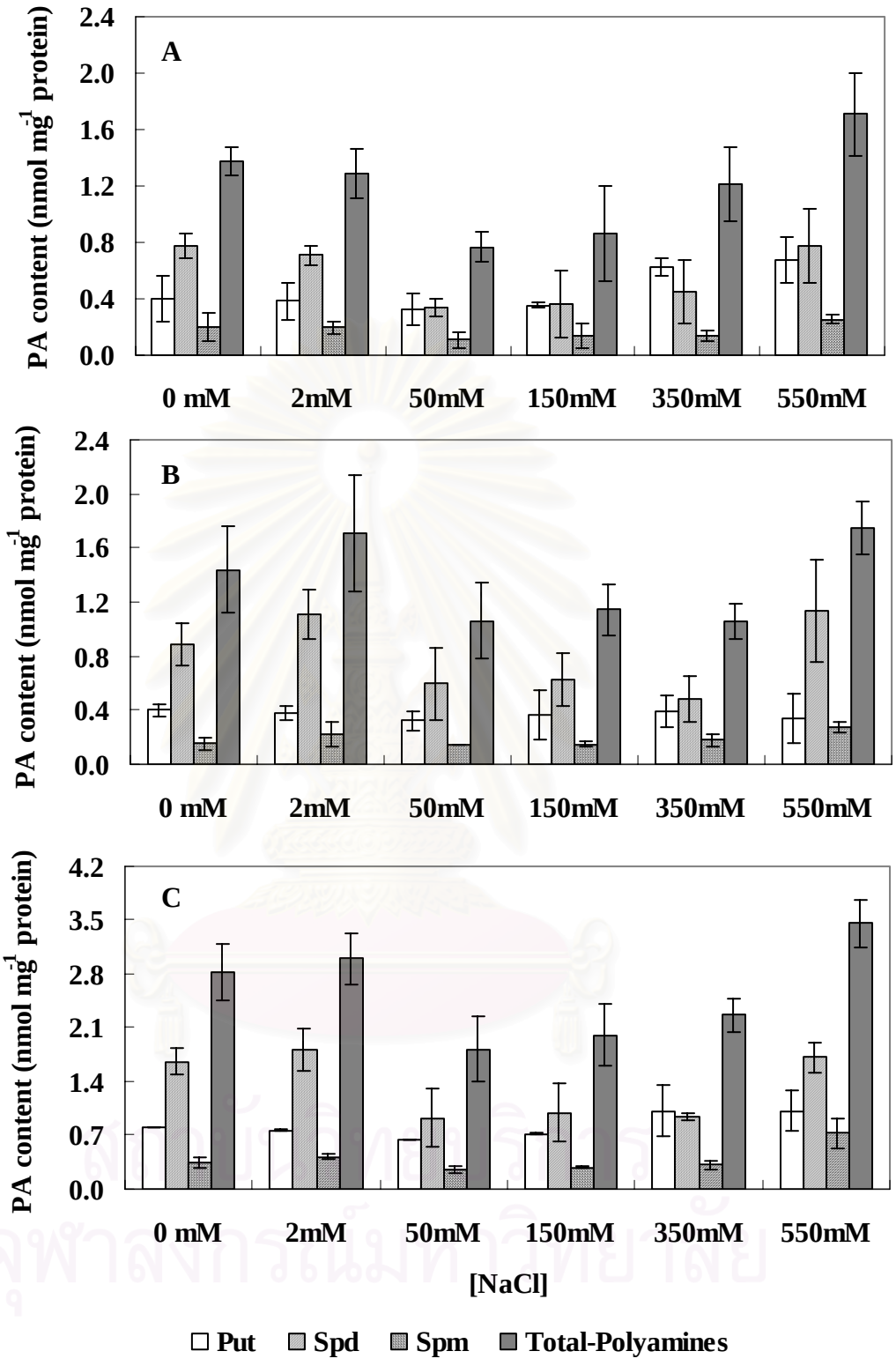

Figure 3.2 Effects of different concentrations of NaCl on polyamine contents of *Synechocystis* sp. PCC 6803. A, Soluble polyamine fraction ; B, Insoluble polyamine fraction and C, Total soluble and insoluble polyamine fraction. Cells were grown for 10 days in the growth medium containing various concentrations of NaCl. The data represent mean ± SE, n = 3.

except for 550 mM NaCl stress condition. The level of Spm in both soluble and insoluble fractions was slightly increased under 550 mM NaCl. The results of total polyamine fraction including soluble and insoluble fractions were also presented in Figure 3.2C. A moderate increase in total cellular polyamine level, specifically Spm, was observed upon salt stress imposed by 550 mM NaCl (Figure 3.2C). The contents of Put and Spd were unaffected by salt stress.

3.2.2 Time course of polyamine levels under salt stress

Polyamine contents in the cells of *Synechocystis* were determined at intervals during 20 days under 2 mM and 550 mM NaCl conditions (Figures 3.3 – 3.6). The soluble Put levels under 550 mM NaCl stress condition clearly showed higher than their soluble levels under 2 mM NaCl condition. Soluble Put contents under 550mM NaCl showed the highest amount of 3.79 nmol/10^9cells at 4 days and subsequently decreased afterwards (Figure 3.3A). On the other hand, insoluble Put fractions under 550 mM NaCl showed lower amounts than that under 2 mM NaCl (Figure 3.3B). Consequently, under 550 mM NaCl stress, the soluble Put contents were higher than insoluble Put fractions. The total amounts of soluble and insoluble Put fractions are depicted in Figure 3.3C. Total Put contents in *Synechocystis* cells under 2 mM and 550 mM NaCl stress gave highest amounts at 4 days for 13.12 and 26.73 nmol/10^9cells, respectively and subsequently decreased later.

Figure 3.4A, B and C shows the soluble, insoluble and total Spd contents, respectively. The soluble Spd levels under 550 mM NaCl stress condition were higher than that under 2 mM NaCl condition. Soluble Spd contents under 550mM NaCl showed the highest amount of 20.74 nmol/10^9 cells at 4 days and subsequently decreased afterwards (Figure 3.4A). For insoluble Spd fractions under 550 mM NaCl,

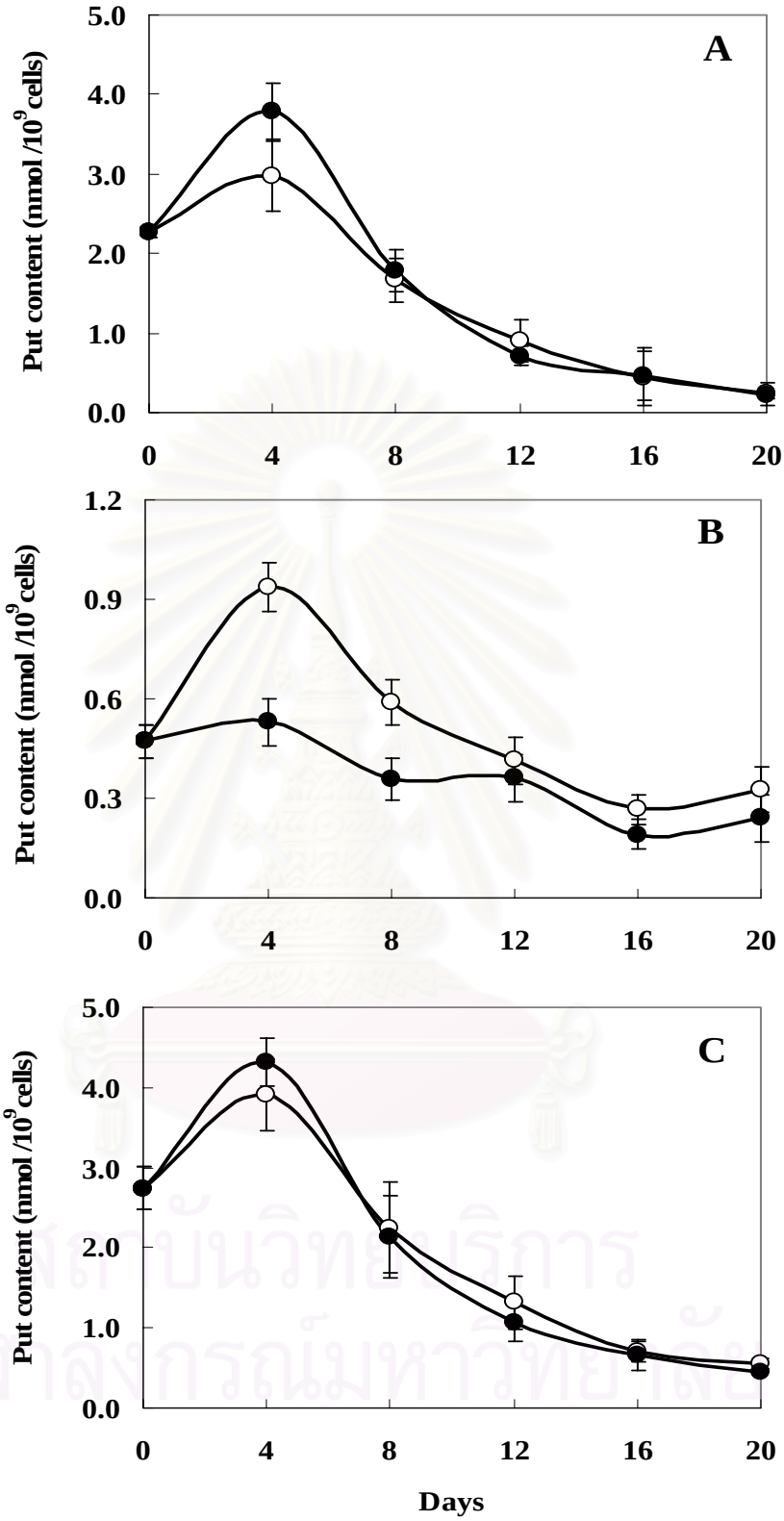

Figure 3.3 Time course of putrescine contents in *Synechocystis* sp. PCC 6803 under salt stress. A, soluble Put fraction; B, insoluble Put fraction and C, total Put content containing soluble and insoluble Put fractions. Open circle, 2mM NaCl; Closed circle, 550mM NaCl. The data represent Mean ± SE, n = 3.

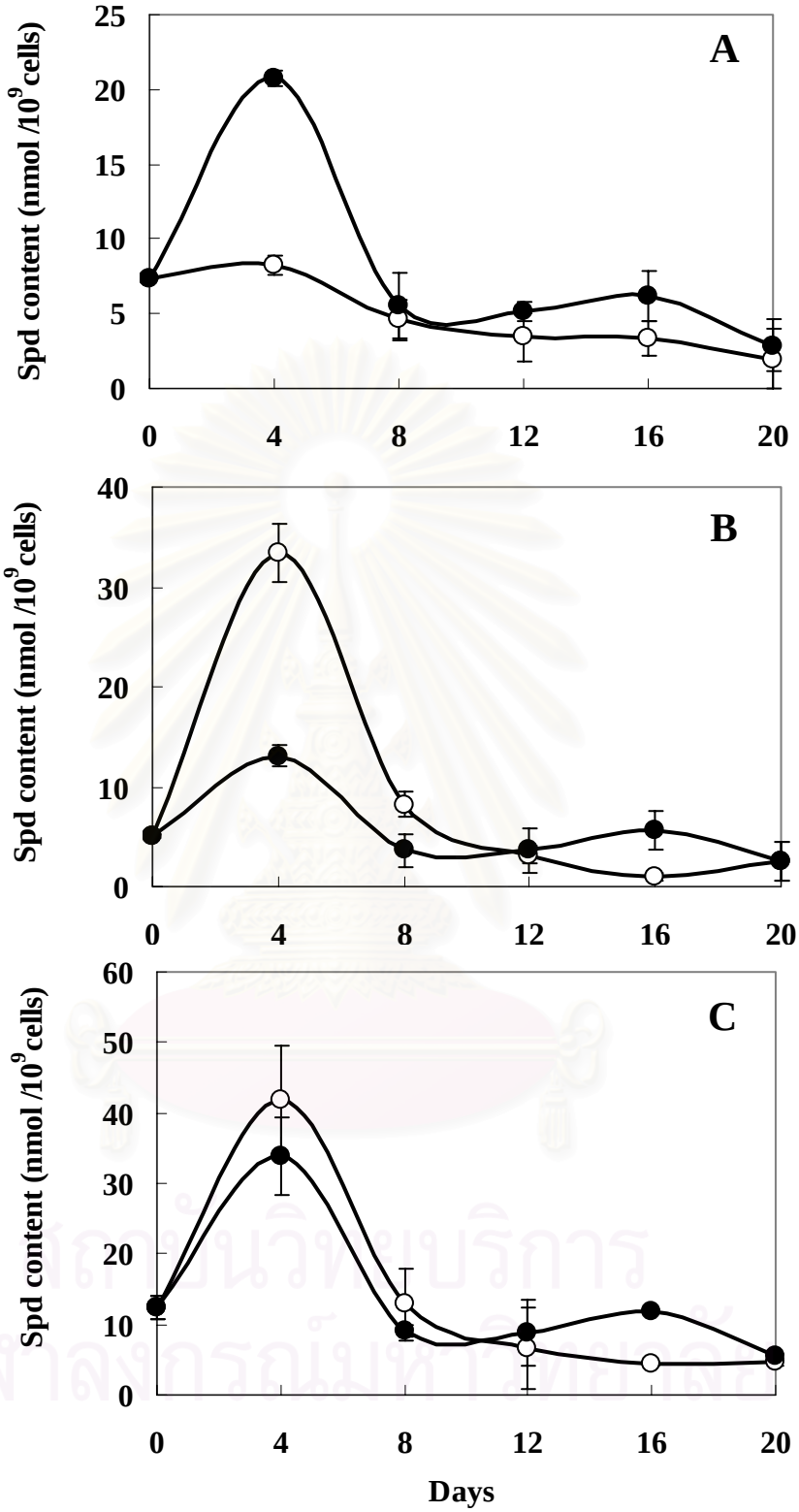

Figure 3.4 Time course of spermidine contents in *Synechocystis* sp. PCC 6803 under salt stress. A, soluble Spd fraction; B, insoluble Spd fraction and C, total Spd content containing soluble and insoluble Spd fractions. Open circle, 2mM NaCl; Closed circle, 550mM NaCl. The data represent Mean ± SE, n = 3.

their contents were lower than that under 2 mM NaCl (Figure 3.4B). The insoluble Spd content gave the highest amount of 33.44 nmol/10^9 cells under 2 mM NaCl at 4 days (Figure 3.4B). The total amounts of soluble and insoluble Spd fractions are presented in Figure 3.4C. Total Spd contents in *Synechocystis* cells under 2 mM and 550 mM NaCl stress gave highest amounts at 4 days for 41.71 and 33.78 nmol/10^9 cells, respectively and subsequently decreased later.

The soluble Spm contents were the highest amount at the start of cultivation and showed a decrease (Figure 3.5A). The level of soluble Spm under 550 mM NaCl condition was higher than that under 2 mM NaCl condition. On the other hand, the insoluble Spm showed the highest content of 4.95 nmol/10^9cells at 4 days under 2 mM NaCl condition (Figure 3.5B). The total amounts of soluble and insoluble Spm fractions are presented in Figure 3.5C. Total Spm contents in *Synechocystis* cells under 2 mM and 550 mM NaCl stress gave highest amounts at the start of cultivation and subsequently decreased later.

Figure 3.6 shows the time course of total polyamine contents for soluble fractions (A), insoluble fractions (B) and total soluble and insoluble fractions (C), respectively. The total soluble polyamine content clearly showed the highest amount of 26.73 nmol/10^9cells under 550 mM NaCl condition at 4 days (Figure 3.6A). On the other hand, total insoluble polyamine fractions under 550 mM NaCl contained lower amount than that under 2 mM NaCl (Figure 3.6B). The results suggested that under salt stress, the contents of total soluble polyamine under 550 mM NaCl stress are higher than the total insoluble polyamine. The total insoluble polyamine content under 2 mM NaCl showed the highest amount of 39.33 nmol/10^9 cells at 4 days. The total cellular polyamine content showed the highest amount of 52.44 nmol/10^9 cells under 2 mM NaCl at 4 days.

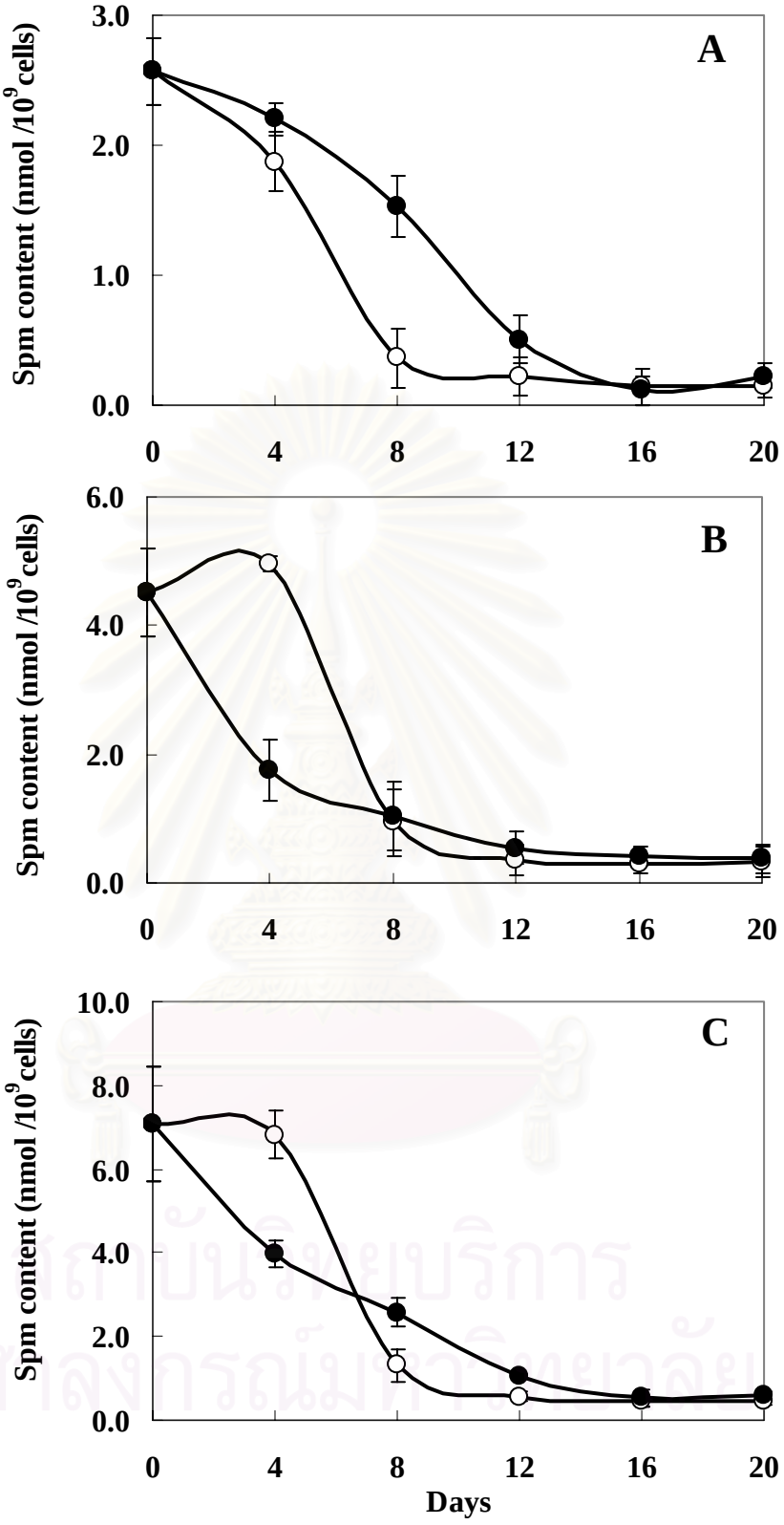

Figure 3.5 Time course of spermine contents in *Synechocystis* sp. PCC 6803 under salt stress. A, soluble Spm fraction; B, insoluble Spm fraction and C, total Spm content containing soluble and insoluble Spm fractions. Open circle, 2mM NaCl; Closed circle, 550mM NaCl. The data represent Mean ± SE, n = 3.

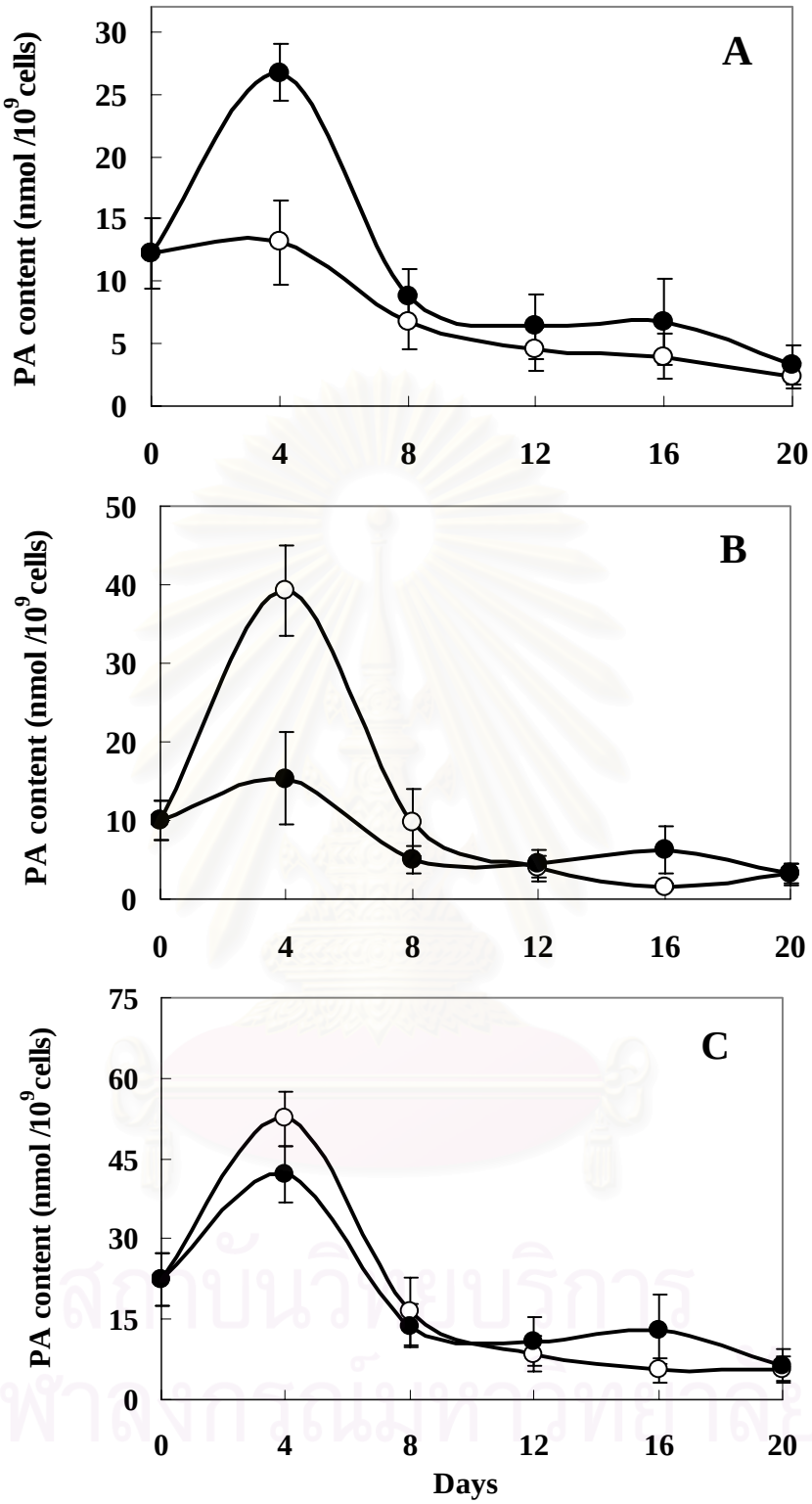

Figure 3.6 Time course of total polyamine contents in *Synechocystis* sp. PCC 6803 under salt stress. A, soluble polyamine fraction; B, insoluble polyamine fraction and C, total polyamine content containing soluble and insoluble polyamine fractions. Open circle, 2mM NaCl; Closed circle, 550mM NaCl. The data represent Mean ± SE, n = 3.

3.2.3 Cellular polyamine contents under sorbitol stress

Polyamine contents in the cells of *Synechocystis* after growth for 10 days in different concentrations of sorbitol are shown in Figure 3.7. Soluble Put and Spm showed the increases under 300 mM sorbitol while their decreases were observed under 700 mM and 1100 mM sorbitol conditions. Soluble Spd content was unaffected by sorbitol stress (Figure 3.7A). Insoluble Put, Spd and Spm contents were not affected under 0-300 mM sorbitol conditions while insoluble Put and Spd contents increased under 700 and 1100 mM sorbitol conditions (Figure 3.7B).

On the other hand, osmotic stress imposed by 300 mM sorbitol resulted in a 4-fold increase in total cellular Spm and a 3-fold increase in total cellular Spd when imposed by 700 mM sorbitol (Figure 3.7C). The level of Spm at 700 mM sorbitol decreased and approached the level observed in the unstressed cells. The Put content also increased with the treatment of 4 mM sorbitol and no further increase was evident even at higher concentration of sorbitol. Overall results also showed an apparent increase in the total cellular polyamine content upon osmotic stress imposed by sorbitol. It is worthy of mentioning here that Spm could be detected in *Synechocystis* cells whereas it is absent in most prokaryotic organisms that have been studied so far (Kashiwagi and Igarashi, 1988 ; Nakabachi and Ishikawa, 2000). Only recently, some thermophilic eubacteria have been found to contain Spm (Hamana *et al*., 2001).

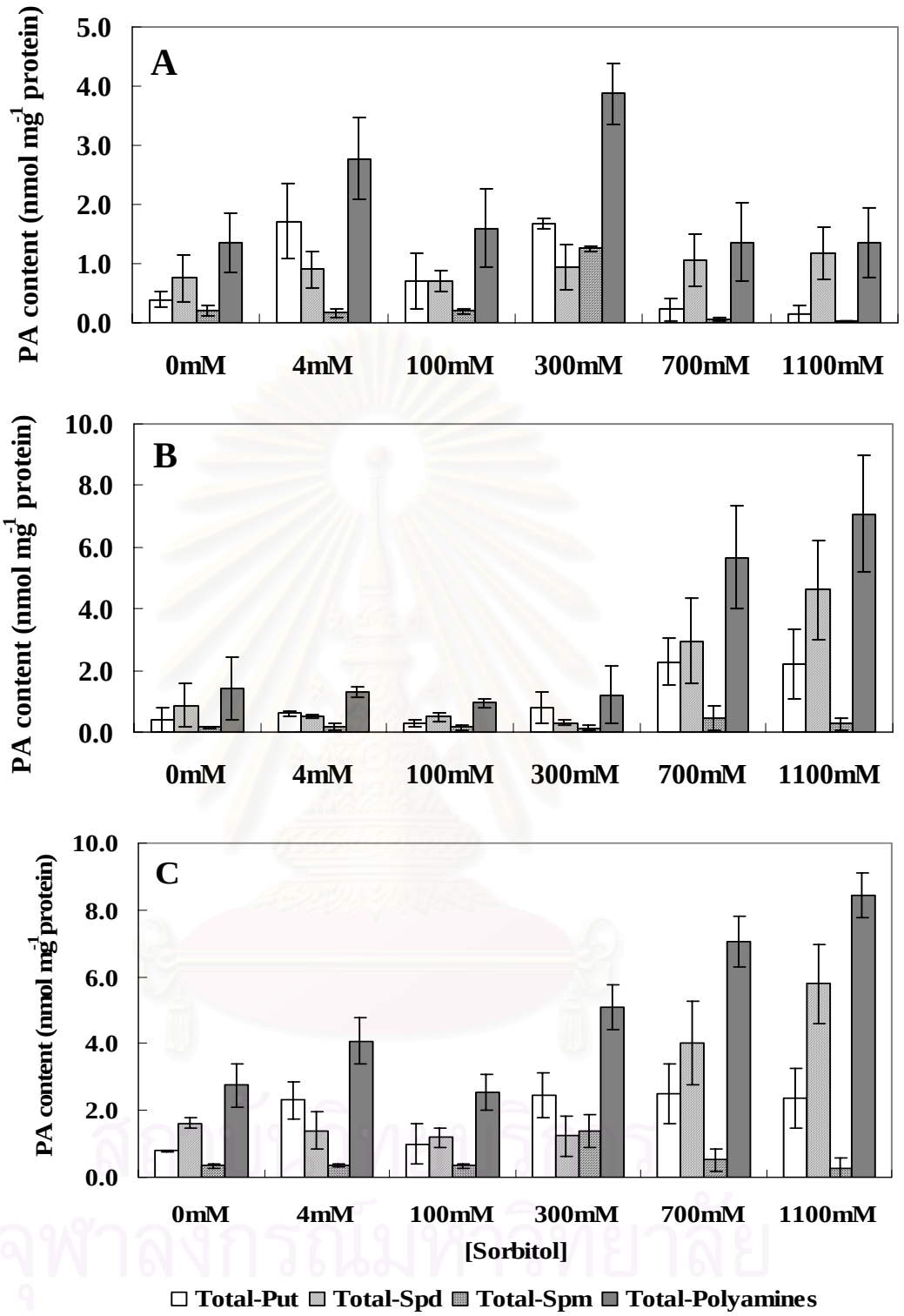

Figure 3.7 Effects of different concentrations of sorbitol on polyamine contents of *Synechocystis* sp. PCC 6803. A, Soluble polyamine fraction ; B, Insoluble polyamine fraction and C, Total soluble and insoluble polyamine fraction. Cells were grown for 10 days in the growth medium containing various concentrations of sorbitol. The data represent Mean ± SE, n = 3.

3.2.4 Time course of polyamine levels under sorbitol stress

Polyamine contents in the cells of *Synechocystis* were determined at intervals during 20 days under 0 mM and 300 mM sorbitol conditions (Figures 3.8 – 3.11). The level of soluble Put under 300 mM sorbitol stress condition was higher than that under unstressed condition. Soluble Put contents under 300 mM sorbitol showed the highest amount of 21.38 nmol/10^9cells at 4 days and subsequently decreased afterwards (Figure 3.8A). On the other hand, insoluble Put content under 300 mM sorbitol was not different from that under unstressed condition. The insoluble Put contents were 6.90 and 6.50 nmol/10^9 cells under 0 mM and 300 mM sorbitol, respectively, at 4 days (Figure 3.8B). The total amounts of soluble and insoluble Put fractions are shown in Figure 3.8C. Total Put contents in *Synechocystis* cells under 0 mM and 300 mM sorbitol showed highest amounts at 4 days for 20.89 and 27.88 nmol/10^9cells, respectively and subsequently decreased later.

Figure 3.9A, B and C shows the time course of soluble, insoluble and total Spd contents under sorbitol stress, respectively. The soluble Spd level under 300 mM sorbitol stress condition was higher than that under unstressed condition. Soluble Spd contents under 300 mM sorbitol showed the highest amount of 5.58 nmol/10^9cells at 8 days and subsequently decreased afterwards (Figure 3.9A). For insoluble Spd fractions under 0 mM and 300 mM sorbitol, the highest amounts was observed at start of cultivation and decreased later (Figure 3.9B). The total amounts of soluble and insoluble Spd fractions are shown in Figure 3.9C. Total Spd contents in *Synechocystis* cells under 300 mM sorbitol stress showed the highest amount at 4 days of 11.56 nmol/10^9cells while the total Spd contents under unstressed condition showed the highest content (10.40 nmol/10^9cells) at start of cultivation and decreased later.

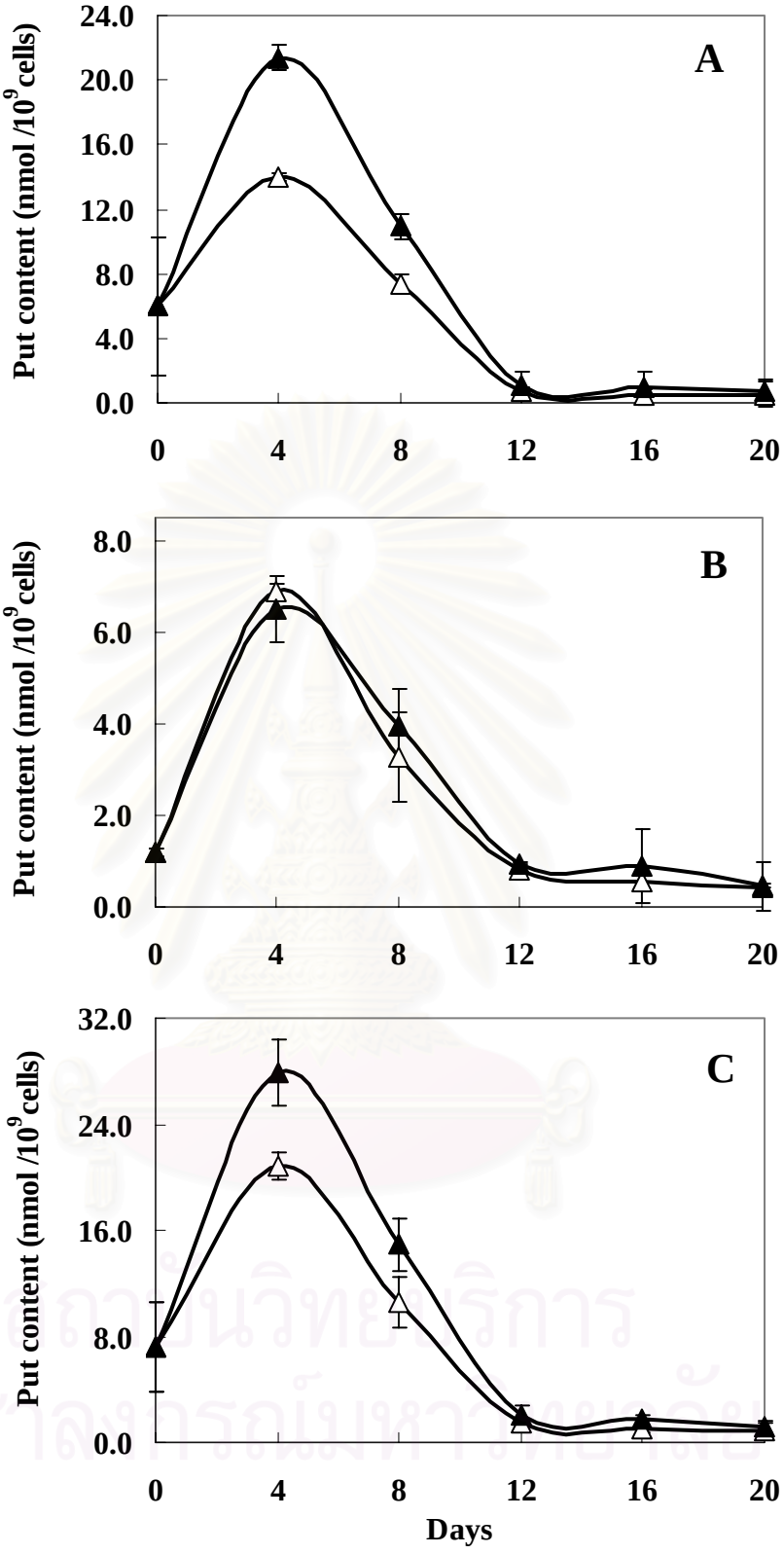

Figure 3.8 Time course of putrescine contents in *Synechocystis* sp. PCC 6803 under sorbitol stress. A, soluble Put fraction; B, insoluble Put fraction and C, total Put content containing soluble and insoluble Put fractions. Open triangle, 0 mM sorbitol; Closed triangle, 300 mM sorbitol. The data represent Mean ± SE, n = 3.

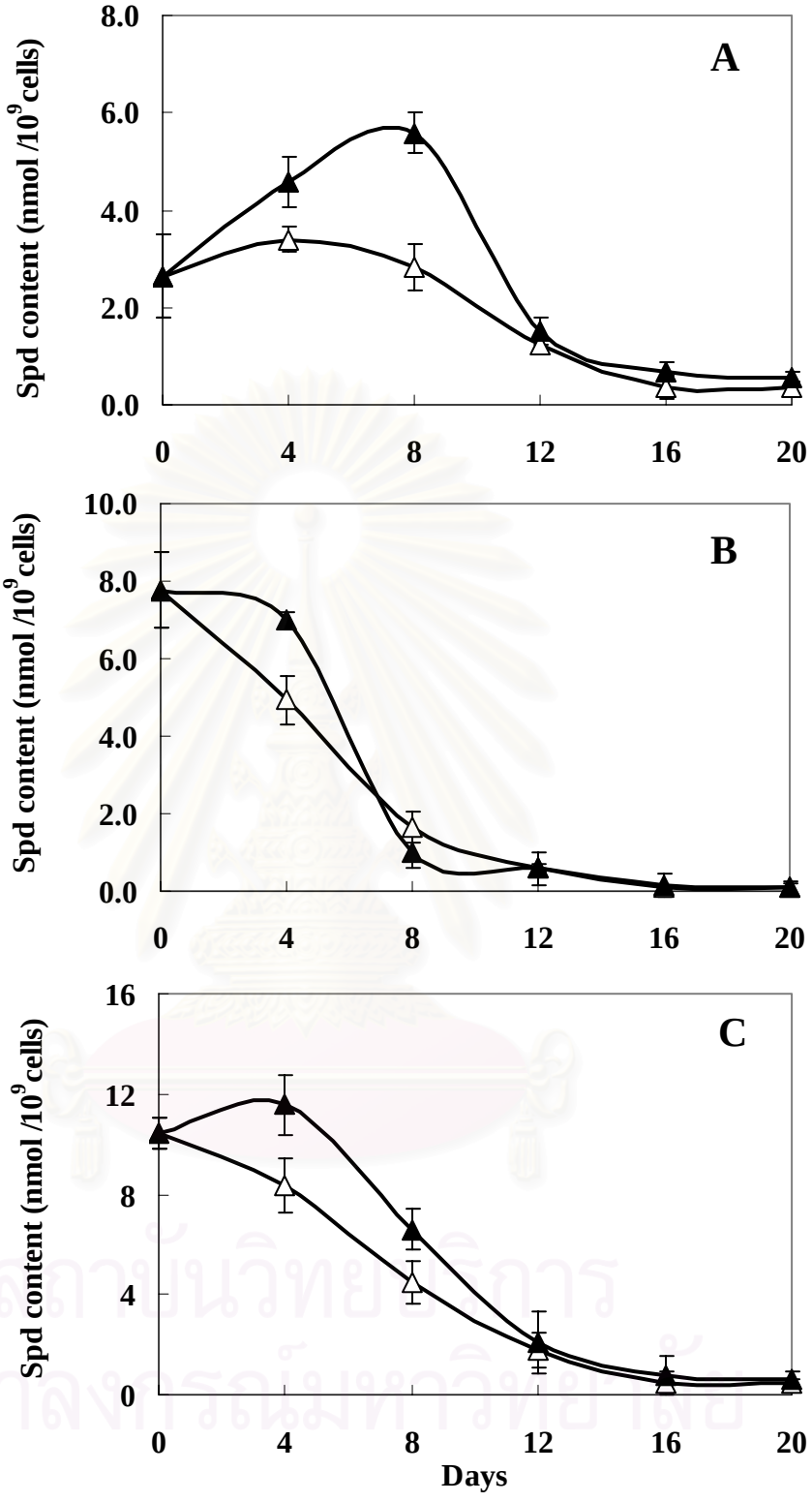

Figure 3.9 Time course of spermidine contents in *Synechocystis* sp. PCC 6803 under sorbitol stress. A, soluble Spd fraction; B, insoluble Spd fraction and C, total Spd content containing soluble and insoluble Spd fractions. Open triangle, 0 mM sorbitol; Closed triangle, 300 mM sorbitol. The data represent Mean ± SE, n = 3.

The time course of soluble, insoluble and total soluble and insoluble Spm fractions under sorbitol stress is presented in Figure 3.10. The soluble Spm contents showed the highest amounts of 3.01 and 5.06 nmol/10^9cells under 0 and 300 mM sorbitol conditions, respectively, at 8 days and subsequently decreased afterwards (Figure 3.10A). The level of soluble Spm under 300 mM sorbitol condition was higher than that under unstressed condition. The insoluble Spm showed the highest content of 0.90 nmol/10^9cells under 300 mM sorbitol condition while the insoluble Spm content showed 0.58 nmol/10^9cells under unstressed condition at 4 days (Figure 3.10B). Figure 3.5C depicts the time course of total amounts of soluble and insoluble Spm fractions. Total Spm contents in *Synechocystis* cells under 300 mM sorbitol stress was higher than that under unstressed condition. The total Spm content under 300 mM sorbitol showed the highest level of 5.85 nmol/10^9cells at 8 days while the total Spm content under unstressed condition showed the highest level of 3.58 nmol/10^9cells at 4 days followed by that of 3.52 nmol/10^9cells at 8 days.

Figure 3.11 shows the time course of total polyamine contents under sorbitol stress. The total soluble polyamine content showed the highest amount of 29.69 nmol/10^9cells under 300 mM sorbitol condition at 4 days (Figure 3.11A). Coincidently, total insoluble polyamine fractions under 300 mM sorbitol showed the highest amount at 4 days with higher level than that under unstressed condition (Figure 3.11B). Similarly, the total cellular polyamine content showed the highest amount of 44.08 nmol/10^9cells under 300 mM sorbitol at 4 days and again with higher level than that under unstressed condition.

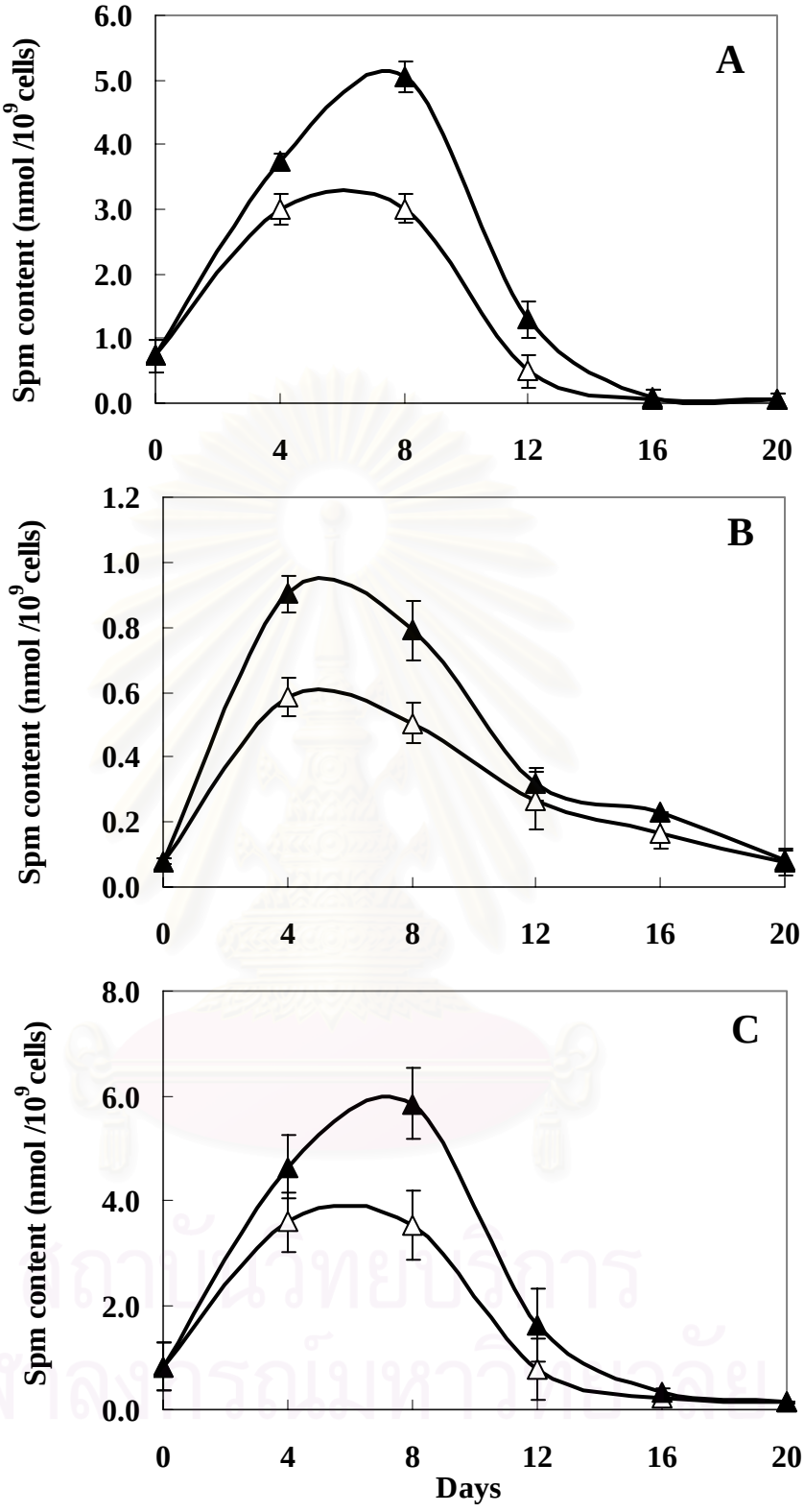

Figure 3.10 Time course of spermine contents in *Synechocystis* sp. PCC 6803 under sorbitol stress. A, soluble Spm fraction; B, insoluble Spm fraction and C, total Spm content containing soluble and insoluble Spm fractions. Open triangle, 0 mM sorbitol; Closed triangle, 300 mM sorbitol. The data represent Mean ± SE, n = 3.

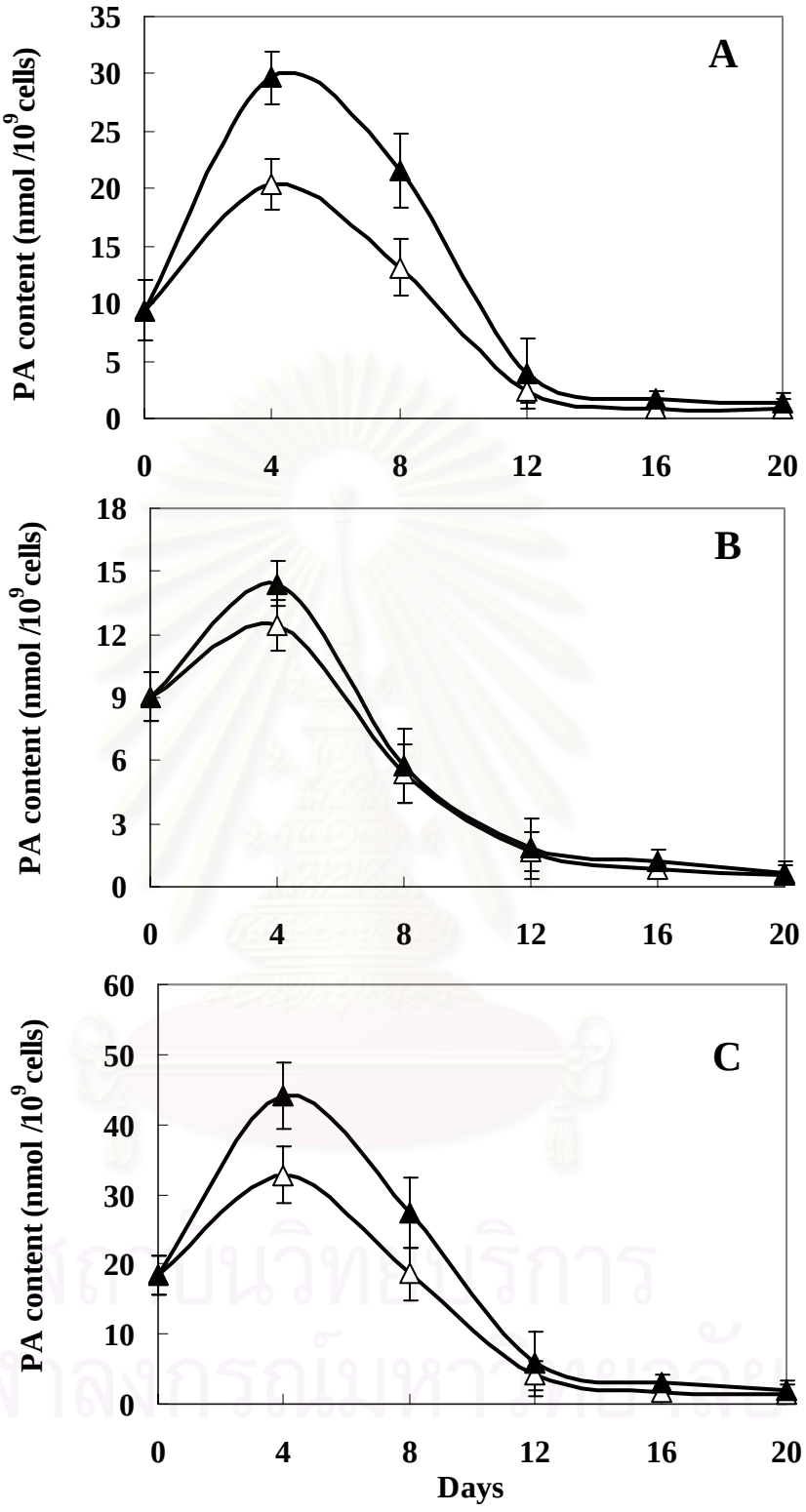

Figure 3.11 Time course of total polyamine contents in *Synechocystis* sp. PCC 6803 under sorbitol stress. A, soluble PA fraction; B, insoluble PA fraction and C, total PA content containing soluble and insoluble Spm fractions. Open triangle, 0 mM sorbitol; Closed triangle, 300 mM sorbitol. The data represent Mean ± SE, n = 3.

3.3 Effects of salt and osmotic stresses on ADC and SAMDC activities

3.3.1 Effect of salt stress on ADC activity

Synechocystis cells grown for 10 days in different concentrations of NaCl were assayed for ADC activity. Salt stress imposed by 550 mM NaCl caused no apparent changes in enzyme activity (Figure 3.12A). The effect of stage of cell growth on ADC activity was conducted by exposing cells in medium containing 2 mM and 550 mM NaCl for 20 days (Figure 3.12B). The ADC activity under 2 mM NaCl condition showed highest value of 1.26 nmol$^{14}CO_2$ min^{-1} mg^{-1} protein at 4 days while the activity under 550 mM NaCl condition showed the value of 1.20 – 1.25 nmol$^{14}CO_2$ min^{-1} mg^{-1} protein at 12 - 16 days.

3.3.2 Effect of sorbitol stress on ADC activity

Osmotic stress imposed by sorbitol resulted in an increase of ADC as shown in Figure 3.13A. A marked increase of ADC activity occurred upon treatment with 700 mM sorbitol, although no change of the activity was detected at 300 mM sorbitol. The enzyme activity under 700 mM sorbitol stress was about 6-fold of that under unstressed condition. The effect of stage of cell growth on ADC activity was conducted by exposing cells in medium containing 0 mM and 300 mM sorbitol for 20 days (Figure 3.13B). The ADC activity under unstressed and 300 mM sorbitol stress conditions showed the highest value of 0.58 and 0.72 nmol$^{14}CO_2$ min^{-1} mg^{-1} protein at 4 days respectively.

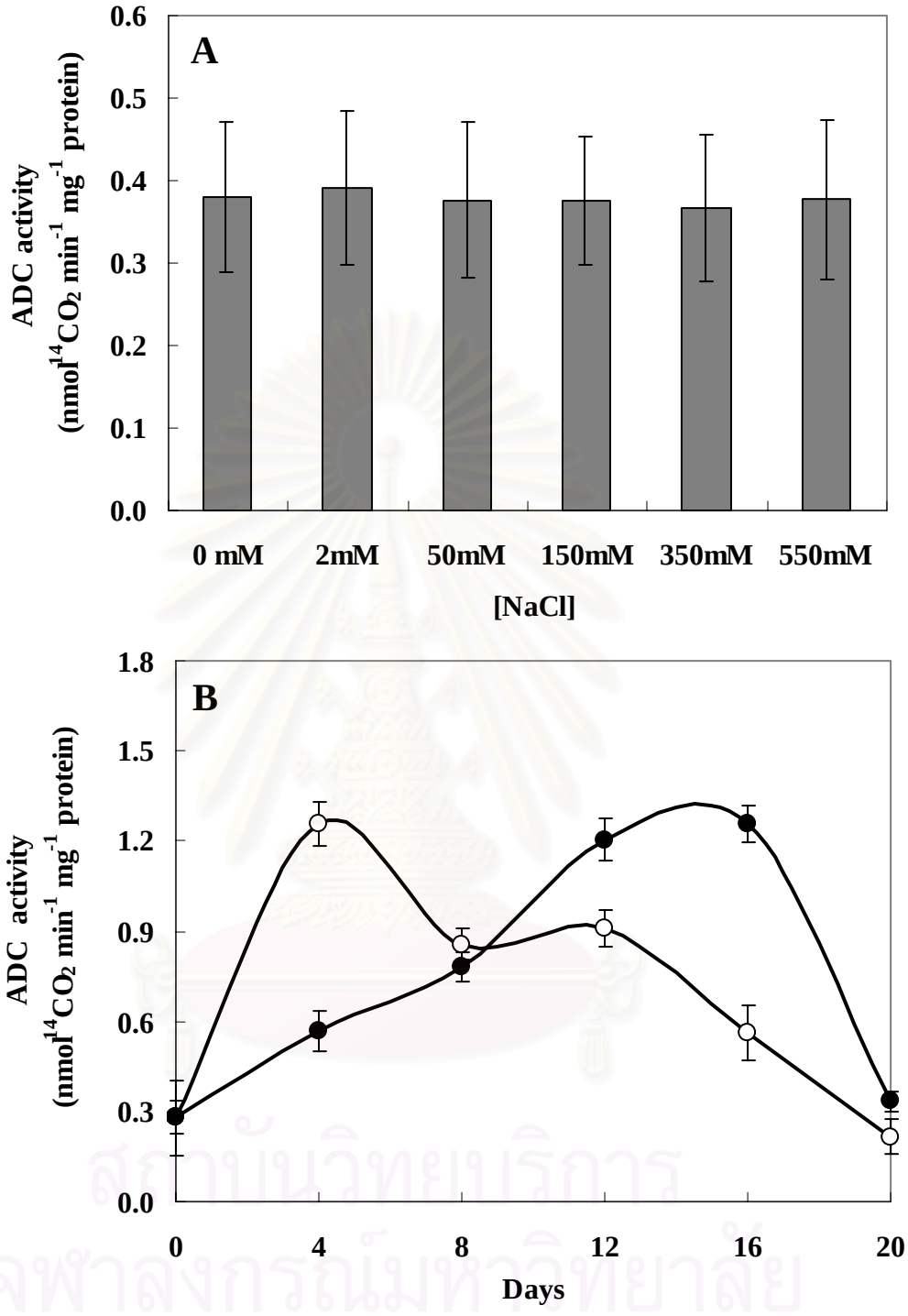

Figure 3.12 Effect of different concentrations of NaCl (A) and stage of cell growth (B) on ADC activity of *Synechocystis* sp. PCC 6803. A, Cells were grown for 10 days in the growth medium containing various concentrations of NaCl. The enzyme activities were assayed by monitoring the $^{14}CO_2$ released from the reaction using DL-[1-^{14}C]-arginine. Symbols in B : open circle (2 mM NaCl) and closed circle (550 mM NaCl). The data represent Mean ± SE, n = 3.

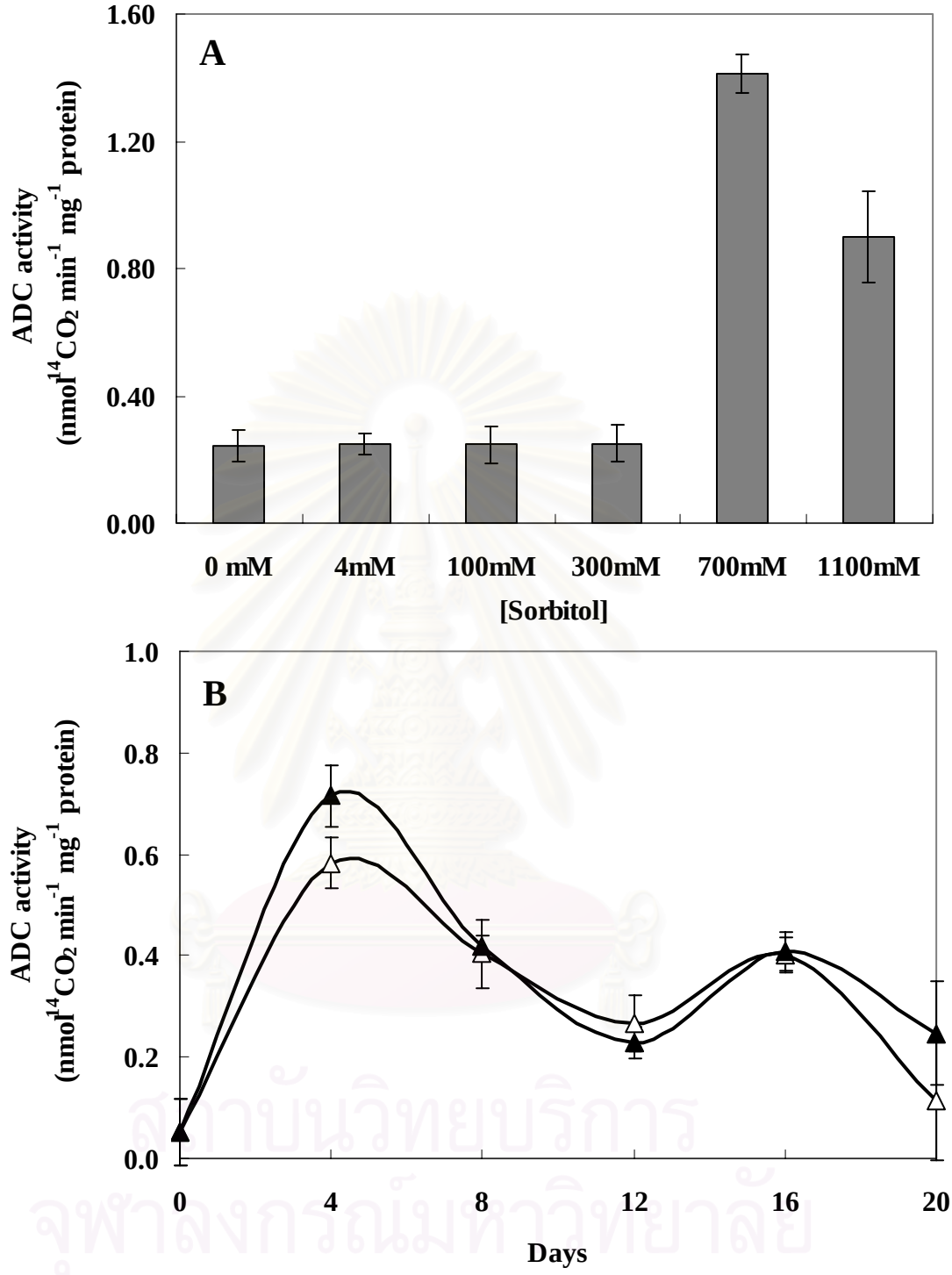

Figure 3.13 Effect of different concentrations of sorbitol (A) and stage of cell growth (B) on ADC activity of *Synechocystis* sp. PCC 6803. A, Cells were grown for 10 days in the growth medium containing various concentrations of sorbitol. The enzyme activities were assayed by monitoring the $^{14}CO_2$ released from the reaction using DL-[1-^{14}C]-arginine. Symbols in B : open triangle (0 mM sorbitol) : and closed triangle (300 mM sorbitol). The data represent Mean ± SE, n = 3.

3.3.3 Effect of salt stress on SAMDC activity

Salt stress imposed by 550 mM NaCl caused no apparent changes in SAMDC activity (Figure 3.14A). It is notable that the activity of ADC in unstressed *Synechocystis* cells was about 3 to 6-fold of that observed for SAMDC. Effects of stage of cell growth on SAMDC activity under salt stress are presented in Figure 3.14B. The highest SAMDC activity was observed at the start of cultivation and decreased later. Overall results showed that SAMDC activities under 550 mM NaCl condition were higher than those under 2 mM NaCl condition.

3.3.4 Effect of sorbitol stress on SAMDC activity

Osmotic stress imposed by sorbitol resulted in increased SAMDC activities as shown in Figure 3.15A. The treatment with 4, 100 and 300 mM sorbitol caused only a moderate increase in SAMDC activity, whereas treatment with 700 mM sorbitol resulted in a marked increase of enzyme activity. Furthermore, the stimulatory effect of 700 mM sorbitol on ADC and SAMDC activity was in parallel, i.e. ADC and SAMDC enzyme activities showed an increase of 6-fold when compared to that in unstressed conditions (Figures 3.13A, 3.15A). The effects of stage of cell growth on SAMDC activity under 0 and 300 mM sorbitol are depicted in Figure 3.15B. The highest SAMDC activity under 300 mM sorbitol was 0.49 nmol $^{14}CO_2$ min^{-1} mg^{-1} protein at 4 days while its activity under unstressed condition was 0.06 nmol $^{14}CO_2$ min^{-1} mg^{-1} protein. Obviously, unstressed *Synechocystis* cells showed only marginal SAMDC activity.

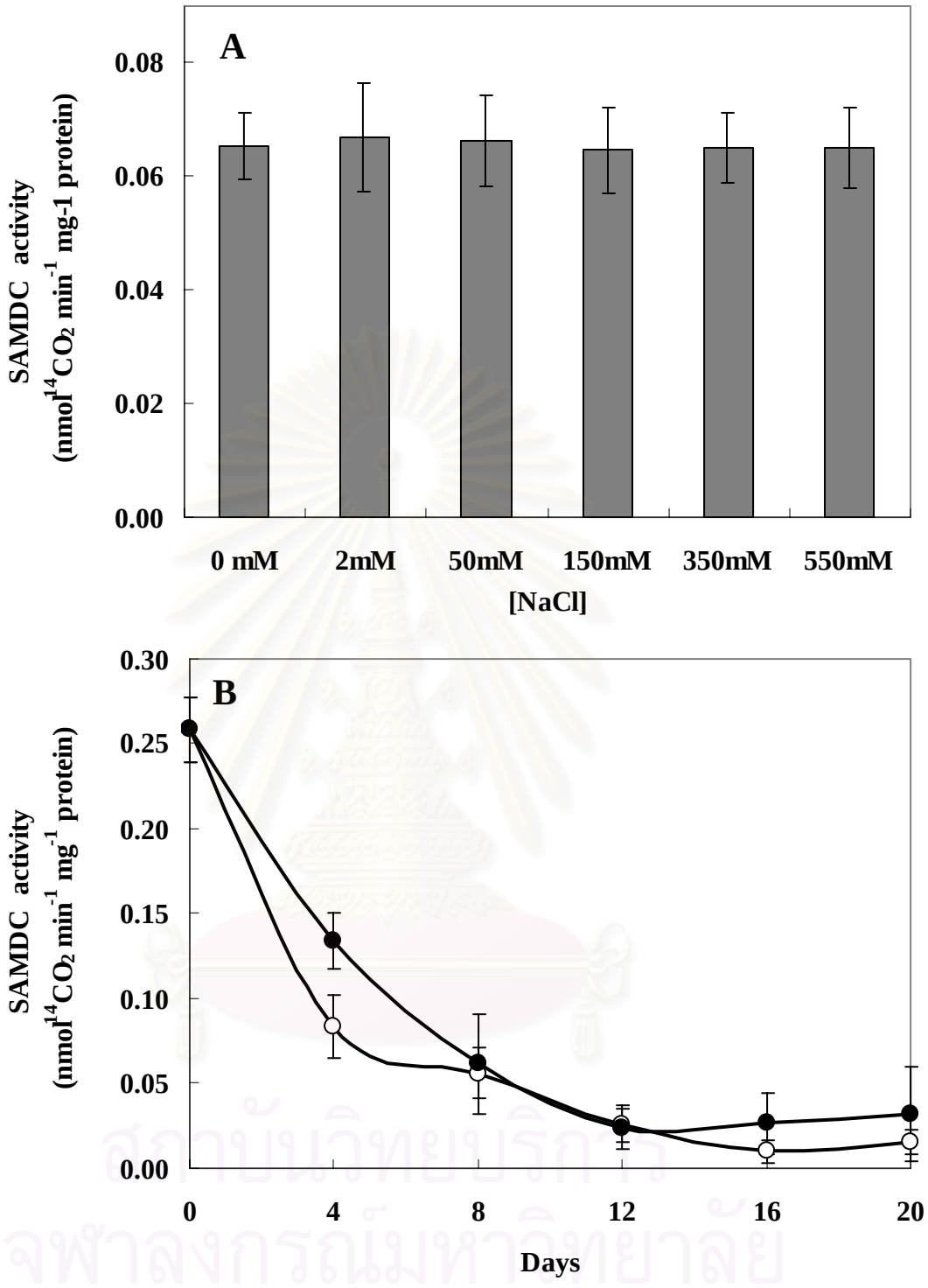

Figure 3.14 Effect of different concentrations of NaCl (A) and stage of cell growth (B) on SAMDC activity of *Synechocystis* sp. PCC 6803. A, Cells were grown for 10 days in the growth medium containing various concentrations of NaCl. The enzyme activities were assayed by monitoring the $^{14}CO_2$ released from the reaction using L-[1-^{14}C]-S-adenosylmethionine. Symbols in B : open circle (2 mM NaCl) and closed circle (550 mM NaCl). The data represent Mean ± SE, n = 3.

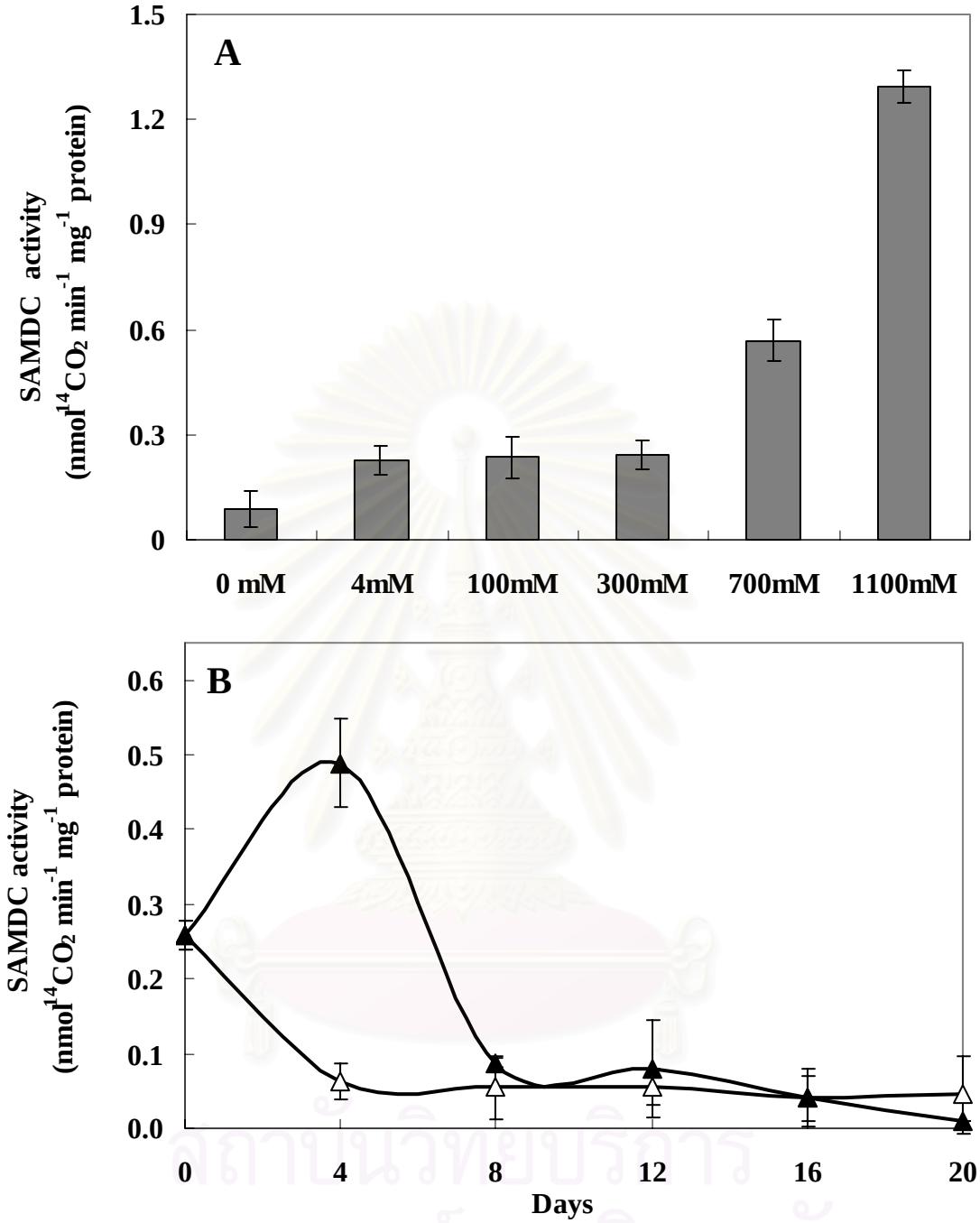

Figure 3.15 Effect of different concentrations of sorbitol (A) and stage of cell growth (B) on SAMDC activity of *Synechocystis* sp. PCC 6803. A, Cells were grown for 10 days in the growth medium containing various concentrations of sorbitol. The enzyme activities were assayed by monitoring the $^{14}CO_2$ released from the reaction using L-[1-^{14}C]-S-adenosylmethioine. Symbols in B : open triangle (0 mM sorbitol) and closed triangle (300 mM sorbitol). The data represent Mean ± SE, n = 3.

3.4 Effects of salt and osmotic stresses on *ADC* mRNA levels

Total RNAs, extracted from *Synechocystis* cells grown for 10 days in various NaCl and sorbitol concentrations, were reverse-transcribed. The abundance of *ADC* mRNA was highest in 550 mM NaCl-treated cells (Figure 3.16). For the sorbitol-treated cells, an increase of *ADC* mRNA was observed when the concentration of sorbitol was increased. The effect of salt stress on the increase of *ADC* mRNA appeared to be more profound than did the effect of osmotic stress. The results for 16S rRNA in samples derived from the same amount of total RNAs were also included which represented an internal standard.

3.5 Effects of salt and osmotic stresses on the stability of *ADC* mRNA

To test whether the increased *ADC* mRNA levels caused by salt and osmotic stresses were the consequence of the increased transcriptional activity or increased stability of the transcript, the RT-PCR analysis of the cells after treatment with rifampicin was performed. As shown in Fig. 3.17C, D and E significant level of *ADC* mRNA was detected after 120 min incubation in cells under both salt and osmotic stresses. In contrast, the unstressed cells and low salt stress (2mM NaCl) conditions showed no detectable *ADC* mRNA after 120 min incubation (Fig. 3.17A and B), suggesting an increased stability of *ADC* mRNA under stress conditions. The half-life of *ADC* mRNA under unstressed and 2 mM NaCl conditions were determined to be 73 and 75 min respectively while *ADC* mRNA under 550 mM NaCl, 4 mM sorbitol and 700 mM sorbitol treatments were more than 2 h (Figure 3.18).

3.6 Effects of salt and sorbitol stresses on ADC protein

The Western blotting of ADC protein is shown in Figure 3.19A. Incubation of Western blots with the antibody immunodetected a single protein band with an apparent molecular mass of 18 kDa. Moreover, two bands of ADC protein from spinach could be detected (60 and 23 kDa). The spinach sample was added as a positive control because the ADC antibody used is produced against a plant ADC protein. ADC protein moderately decreased under salt stress and sorbitol stress (Figure 3.18B).

3.7 Effects of salt and sorbitol stresses on photosynthetic oxygen evolution

Synechocystis cells grown for 10 days under continuous light were measured for photosynthetic oxygen evolution under salt and sorbitol stresses. Figure 3.20A demonstrates that the PSII activity showed no apparent changes under salt stress conditions comparing with unstressed treatment. On the other hand, the PSII activity increased under 300 mM sorbitol condition while no activity was observed under 700 mM sorbitol condition. The photosynthetic capacity was unaffected by salt or sorbitol stresses (Figure 3.20B).

After incubation in darkness for 3 h, the photosynthetic oxygen evolution was detected as demonstrated in Figure 3.21. The PSII activity and photosynthetic capacity of cells treated in darkness were lower than these under light treatment. Dark treatment markedly decreased PSII activity under 300 mM sorbitol stress (Figure 3.21A). Similarly, the photosynthetic capacity decreased under 300 mM sorbitol stress (Figure 3.21B). At 700 mM sorbitol, no PSII activity nor photosynthetic capacity was observed.

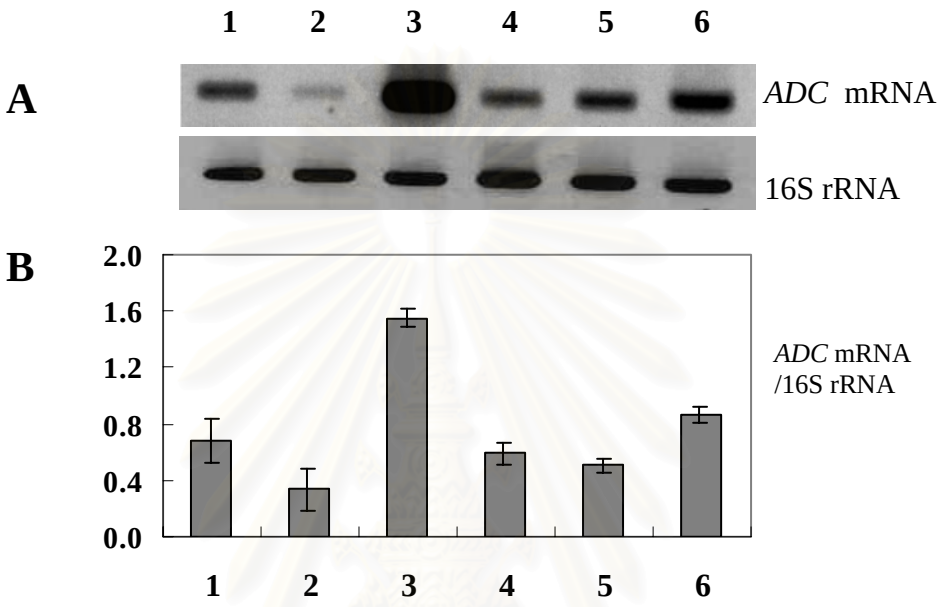

Figure 3.16 (A) RT-PCR analysis of *ADC* mRNA levels in *Synechocystis* sp. PCC 6803 grown in different concentrations of NaCl and sorbitol. Total RNA was isolated from 10 d cells grown in medium containing no addition : lane 1, 2 mM NaCl : lane 2, 550 mM NaCl : lane 3, 4 mM sorbitol : lane 4, 300 mM sorbitol : lane 5, 700 mM sorbitol : lane 6. The relative abundance of 16s rRNA is also shown. Quantification was carried out using AIS™ program. (B) The ratio (*ADC* mRNA/rRNAs) ± SE, n = 3.

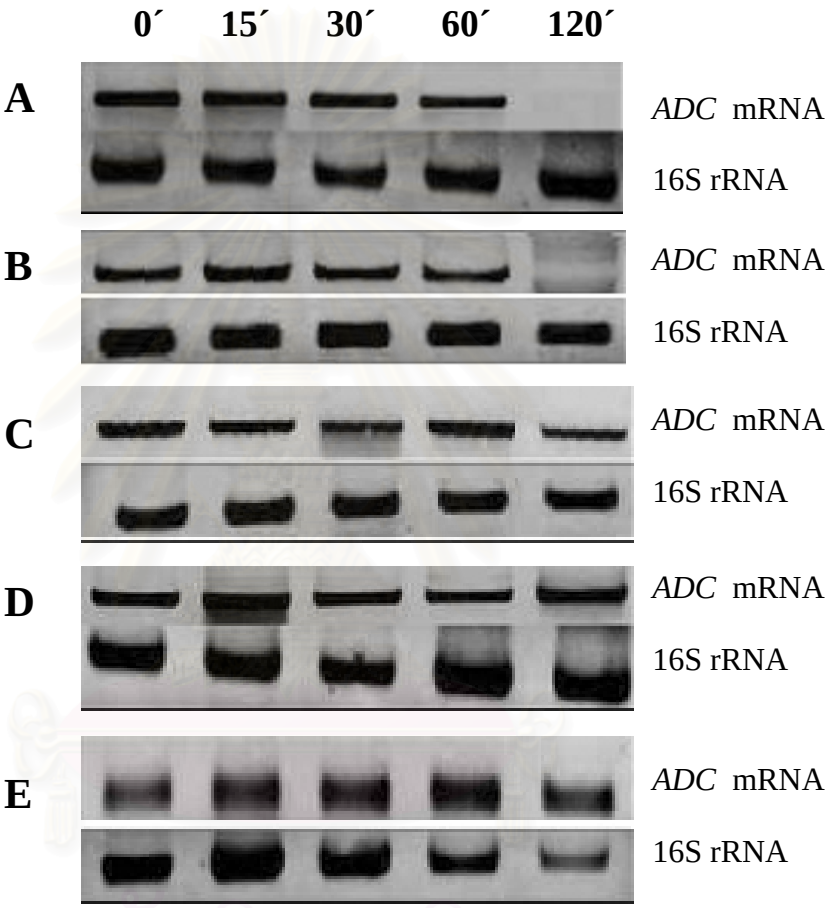

Figure 3.17 Stability of *ADC* mRNA in *Synechocystis* sp. PCC 6803 grown in high concentrations of NaCl and sorbitol. RT-PCR analysis was done using 10 d cells grown in medium containing no addition (A), 2 mM NaCl (B), 550 mM NaCl (C), 4 mM sorbitol (D) and 700 mM sorbitol (E). Total RNA was isolated from cells after further incubation with rifampicin (500 μg ml^{-1}) at the times indicated (min). Quantification was carried out using AIS^{TM} program.

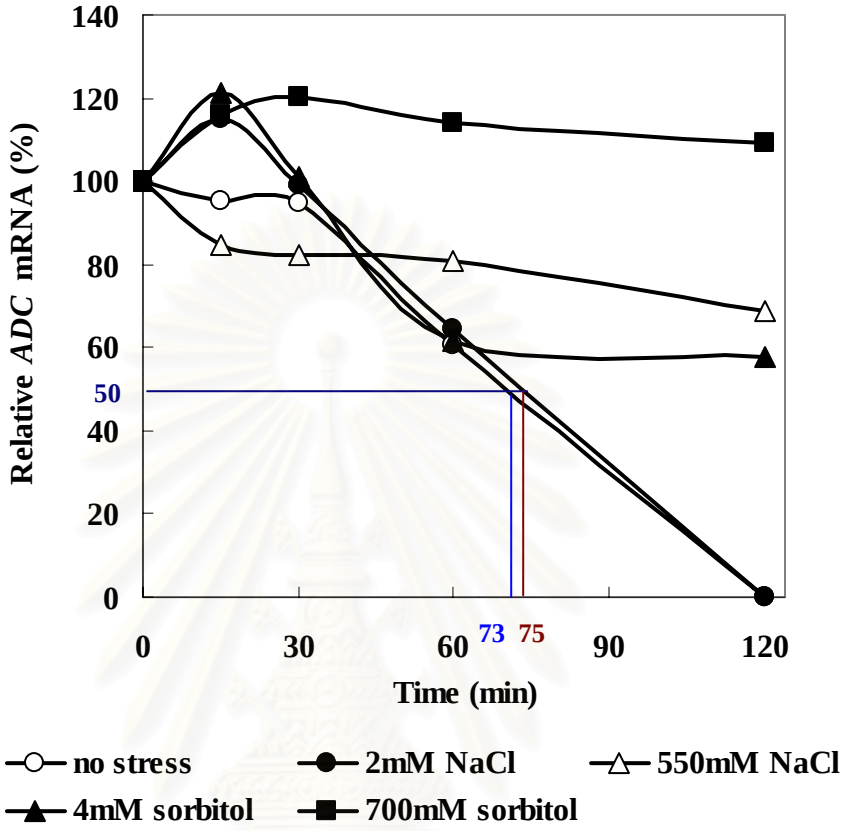

Figure 3.18 Decrease in the *ADC* mRNA amount as a function of time in *Synechocystis* sp. PCC 6803 after addition of rifampicin. The experiments were done as described in Figure 3.17.

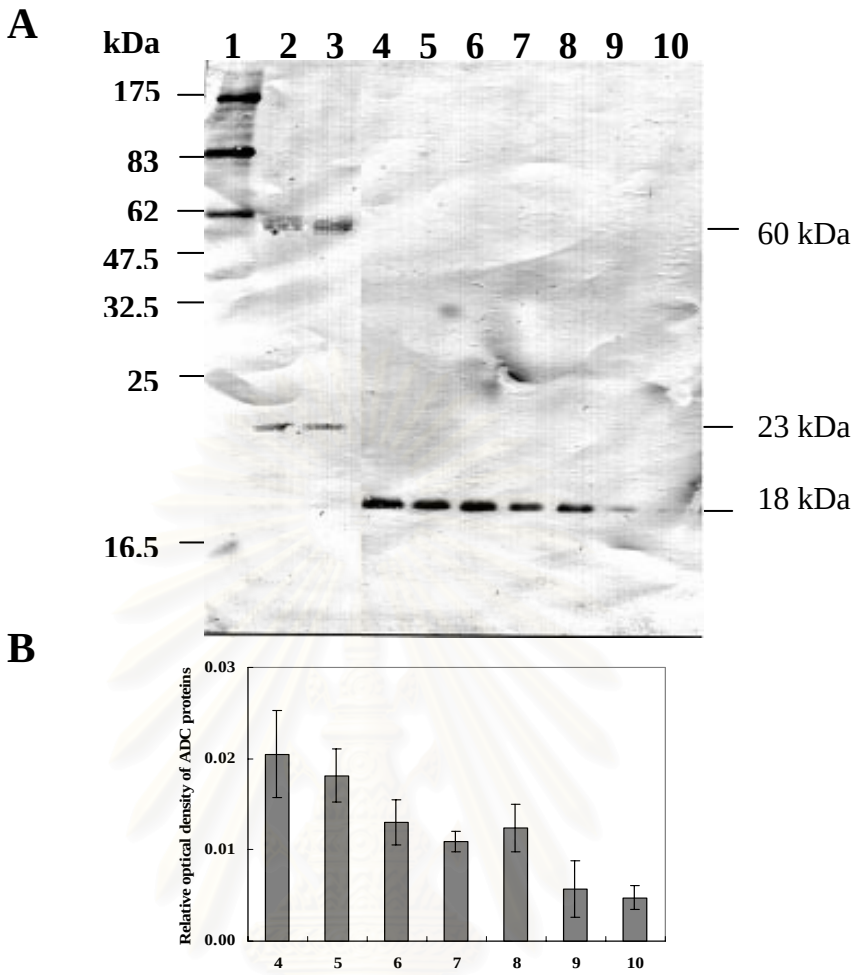

Figure 3.19 (A)Western blot analysis of ADC under salt and sorbitol stresses from *Synechocystis* cells. Ten-days cells grown in medium containing different concentrations of NaCl and sorbitol were used. Total protein extract (60 μg) was loaded onto the SDS-gel. After SDS-PAGE analysis, the gel was electrophoretically transferred onto nitrocellulose membrane and probed for 2 h at room temperature with a 1:2000 dilution of the antiserum against MBP-ADC. Lane 1, standard marker ; lane 2 and 3, 20 and 30 μg spinach (control) ; lane 4, no stress ; lane 5, 2 mM NaCl ; lane 6, 150 mM NaCl ; lane 7, 550 mM NaCl ; lane 8, 4 mM sorbitol ; lane 9, 300 mM sorbitol and lane 10, 700 mM sorbitol. Quantification was carried out using AIS^{TM} program. (B) ADC protein ± SE, n = 3.

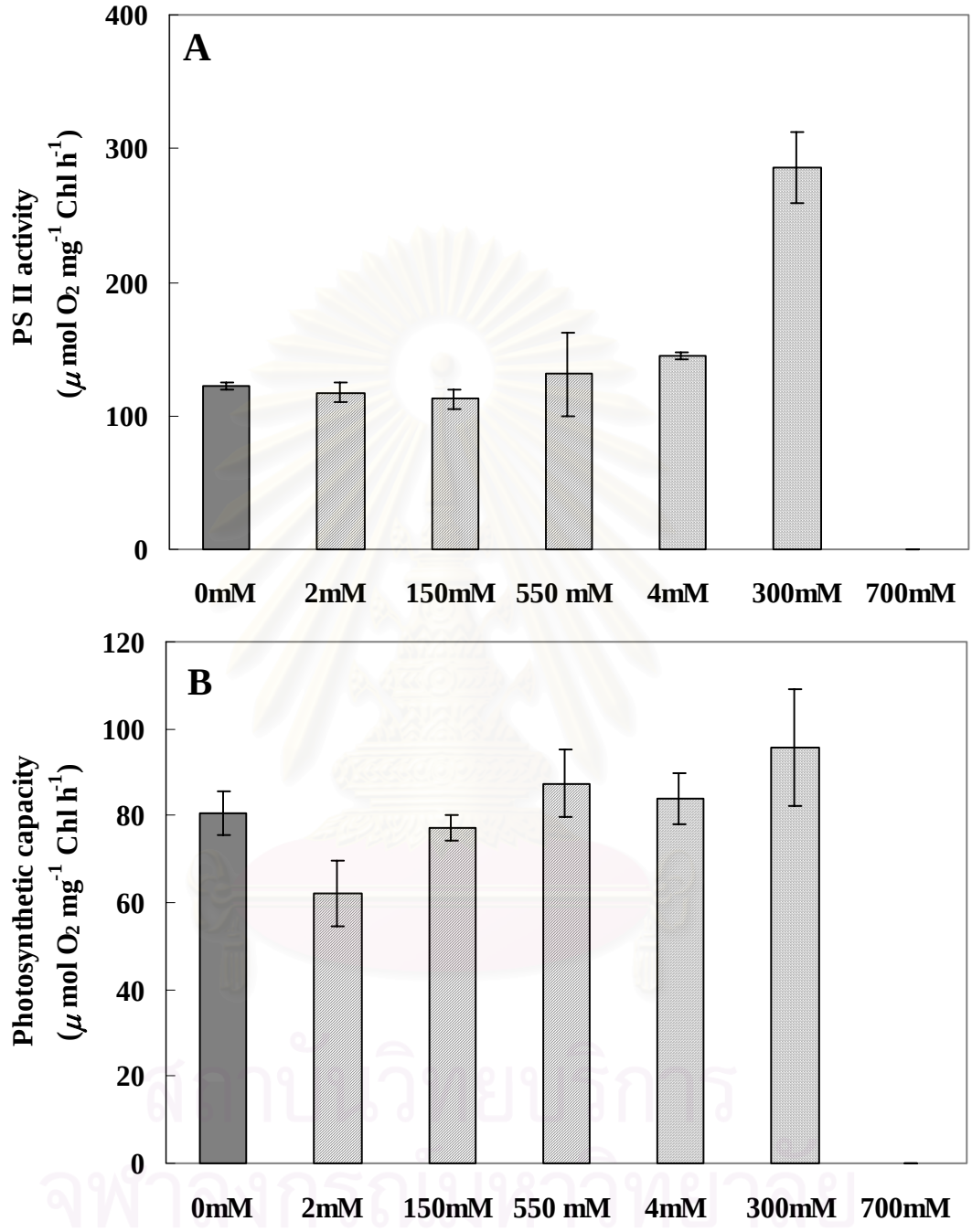

Figure 3.20 The light-saturated photosynthetic oxygen evolution of *Synechocystis* sp. PCC 6803 under salt and sorbitol stresses *in vivo*. The PSII activity (A) was measured in a Clark type oxygen electrode under saturating light intensity at 35°C using 0.5 mM DCBQ as electron acceptor and 0.5 mM ferricyanide. Photosynthetic capacity was measured using 0.6 mM bicarbonate (B). Solid bar, unstress ; hatched bar, [NaC] and dotted bar, [Sorbitol]. Means ± S.E., n=6.

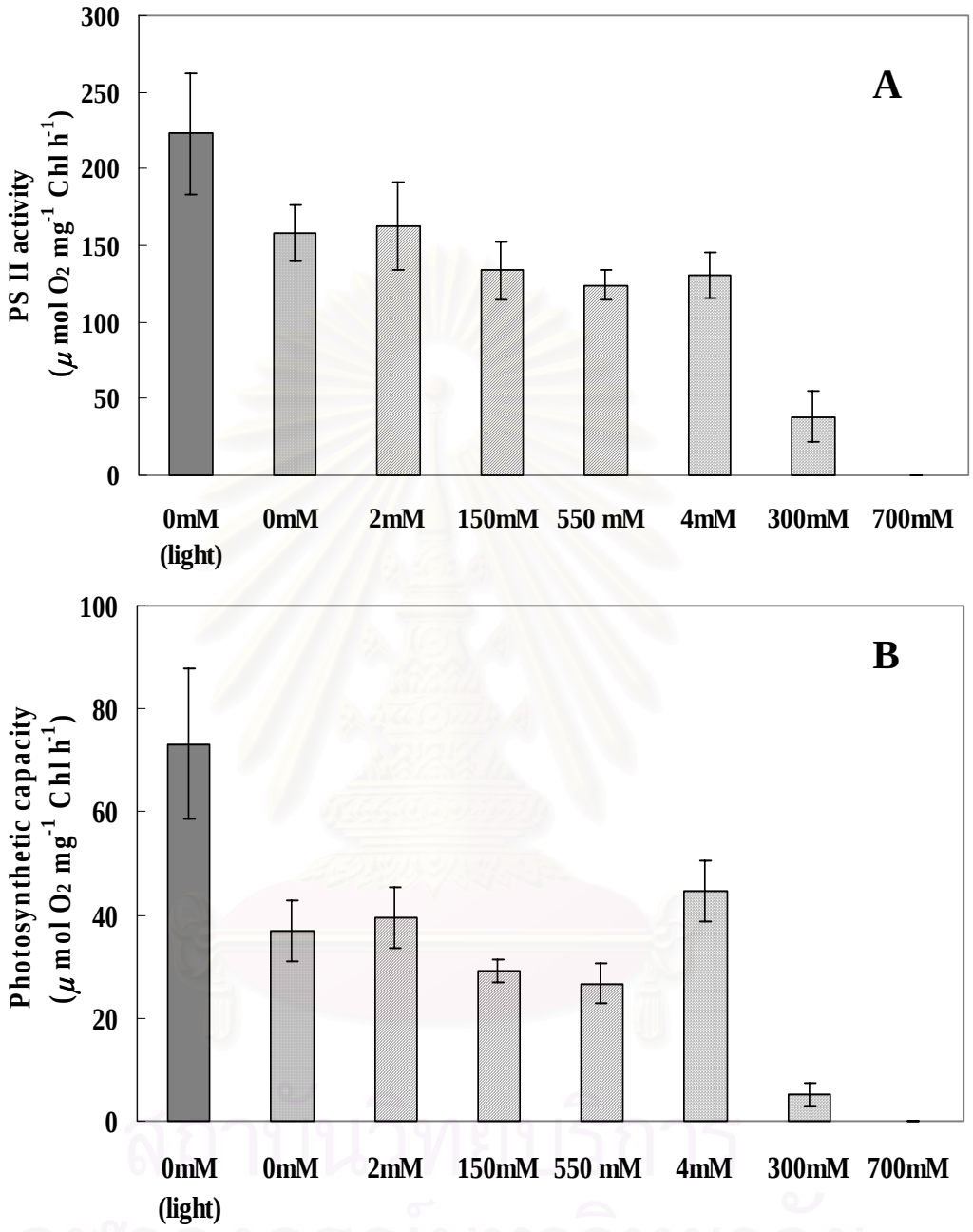

Figure 3.21 The photosynthetic oxygen evolution of *Synechocystis* sp. PCC 6803, incubated in darkness, under salt and sorbitol stresses. Before measurements, cells were incubated in darkness for 3 h. The PSII activity (A) was measured in a Clark type oxygen electrode under saturating light intensity at 35°C using 0.5 mM DCBQ as electron acceptor and 0.5 mM ferricyanide. Photosynthetic capacity was measured using 0.6 mM bicarbonate (B). Solid bar, unstress under light ; shaded bar, unstress under dark ; hatched bar, [NaCl] and dotted bar, [Sorbitol]. Means ± S.E., n=6.

3.8 Effects of salt and osmotic stresses on *psbA* and *psaA* transcripts

3.8.1 Effects of salt and sorbitol stresses on *psbA* transcripts

Total RNAs, extracted from *Synechocystis* cells grown for 10 days in various NaCl and sorbitol concentrations, were used for Northern blot analysis. The *psbA* encodes the reaction center protein D1 of PSII. The *psbA* transcript levels under salt and sorbitol stresses are shown in Figure 3.22. The abundance of *psbA* mRNA was slightly decreased under salt stress (Figure 3.22A and B). On the other hand, the *psbA* mRNA drastically decreased under 700 mM sorbitol condition. The results for rRNA represent an internal standard.

3.8.2 Effects of salt and osmotic stresses on the stability of *psbA* mRNA

To test whether the stability of *psbA* mRNA was affected by salt and osmotic stresses were the consequence of the stability of the transcript, the Northern blot analysis of the cells after treatment with rifampicin was performed. As shown in Fig. 3.23A, the half-life of *psbA* mRNA of unstressed cells was determined to be 47 min. The values of 44, 54 and 29 min were found for the half-life of *psbA* mRNA of cells under 2 mM NaCl, 550 mM NaCl and 4 mM sorbitol conditions, respectively (Figure 3.23B, C and D). In contrast, there was no *psbA* mRNA detected under 700 mM sorbitol-treated cells (Figure 3.23E). The pattern of the progressive decline in *psbA* mRNA is also shown in Figure 3.24 for cells under various salt and sorbitol treatments.

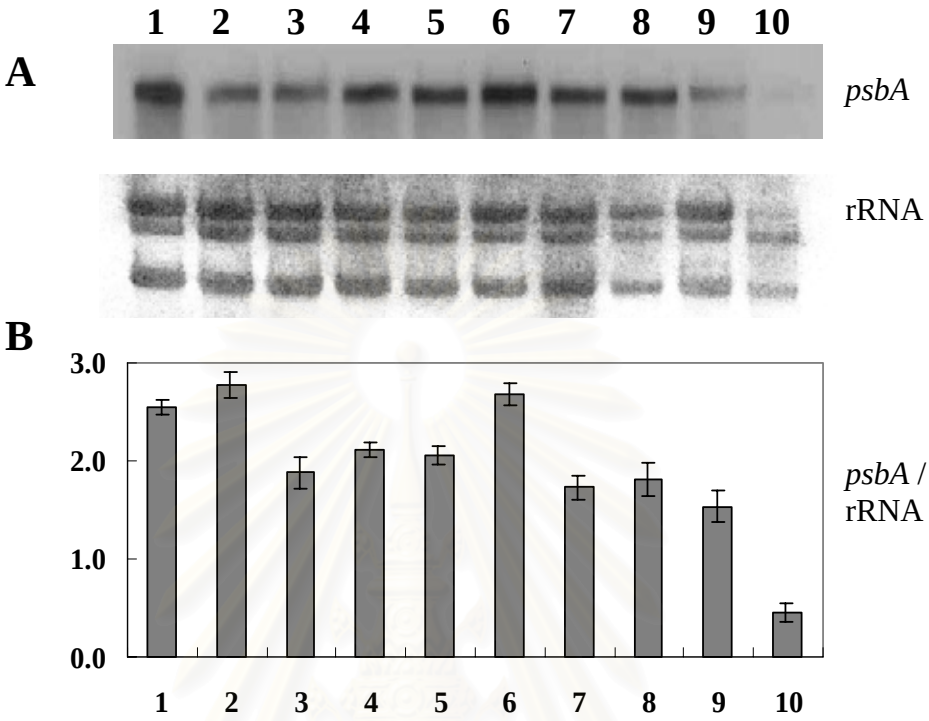

Figure 3.22 (A) Northern blot analysis of *psbA* mRNA levels in *Synechocystis* sp. PCC 6803 grown in different concentrations of NaCl and sorbitol. Total RNA was isolated from 10 d cells grown in medium containing no addition : lane 1, 2 mM NaCl : lane 2, 50 mM NaCl : lane 3, 150 mM NaCl : lane 4, 350 mM NaCl : lane 5, 550 mM NaCl : lane 6, 4 mM sorbitol : lane 7, 100 mM sorbitol : lane 8, 300 mM sorbitol : lane 9 and 700 mM sorbitol : lane 10. The relative abundance of rRNA is also shown. Quantification was carried out using AIS^{TM} program. (B) The ratio (*psbA* mRNA/rRNAs) ± SE, n = 3.

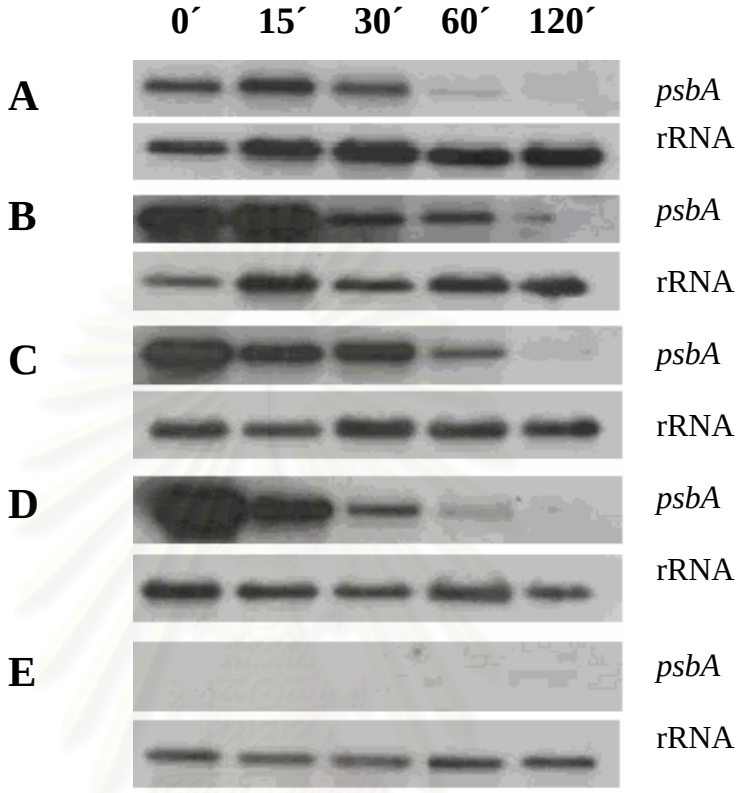

Figure 3.23 Stability of *psbA* mRNA in *Synechocystis* sp. PCC 6803 grown in high concentrations of NaCl and sorbitol. Northern blot analysis was done using 10 d cells grown in medium containing no addition (A), 2 mM NaCl (B), 550 mM NaCl (C), 4 mM sorbitol (D) and 700 mM sorbitol (E). Total RNA was isolated from cells after further incubation with rifampicin (500 μg ml^{-1}) at the times indicated (min). Quantification was carried out using AIS^{TM} program.

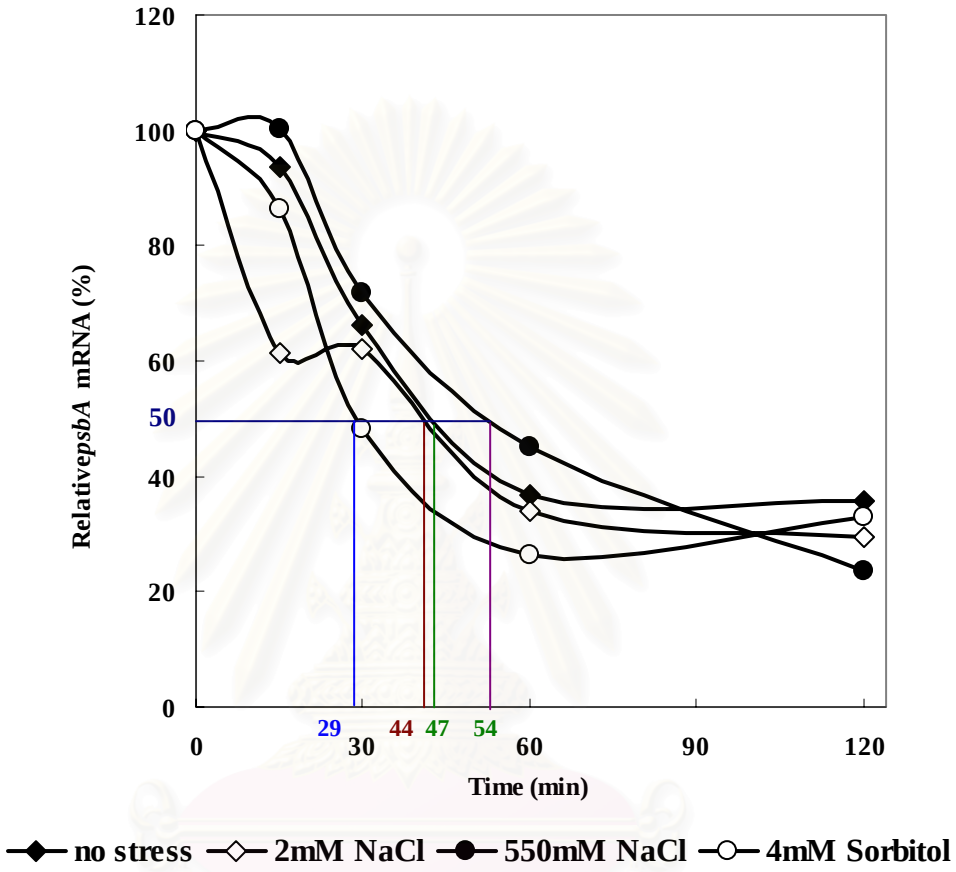

Figure 3.24 Decrease in the *psbA* mRNA amount as a function of time in *Synechocystis* sp. PCC 6803 after addition of rifampicin. The experiments were done as described in Figure 3.23.

3.8.3 Effects of salt and sorbitol stresses on *psaA* transcripts

Northern blot analysis was performed using total RNAs extracted from *Synechocystis* cells grown for 10 days in various NaCl and sorbitol concentrations. The *psaA* encodes the reaction center protein of PSI. The *psaA* transcript levels under salt and sorbitol stresses are shown in Figure 3.25. The abundance of *psaA* mRNA decreased under salt stress, except for 550 mM NaCl-treated cells (Figure 3.25A and B). Similarly, *psaA* mRNA decreased in sorbitol-treated cells comparing with unstressed treatment. The results for rRNA in samples derived from the same amount of total RNAs represent an internal standard.

3.8.4 Effects of salt and osmotic stresses on the stability of *psaA* mRNA

To test whether the decrease of *psaA* mRNA level by salt and osmotic stresses was the consequence of decreased transcriptional activity or decreased stability of the transcript, the Northern blot analysis of the cells after treatment with rifampicin was performed. The half-life of *psaA* transcripts under stresses is demonstrated in Figure 3.26.

The half-life of *psaA* mRNA of unstressed cells was determined to be 33 min and 24 min of 2 mM NaCl-treated cells (Figure 3.26A and B). The half life of *psaA* mRNA for cells under 550 mM NaCl condition was 8.3 min (Figure 3.26C). Similar short half-life of 9 min was found for cells under 4 mM sorbitol condition (Figure 3.26D). In contrast, there was hardly any *psaA* mRNA detected for cells under 700 mM sorbitol treatment (Figure 3.26E). The pattern of *psaA* mRNA levels at various times for cells under salt and sorbitol treatments is also shown in Figure 3.27.

3.9 Effects of salt and osmotic stresses on photosynthetic protein

3.9.1 Effects of salt and osmotic stresses on D1 and D2 proteins

The D1 and D2 proteins are the reaction center cores of PSII and form heterodimer containing all redox active components of PSII. Western blot analysis was performed using D1 and D2 antibody probes. The amount of D1 protein was unaffected under NaCl stress condition while down regulation was observed under 700 mM sorbitol stress condition (Figure 3.28A and B). Similar results were obtained for D2 protein (Figure 3.28A and C).

3.9.2 Effects of salt and osmotic stresses on PSI and ndhF3 protein complexes

The abundance of PSI and ndhF3 protein complexes are demonstrated in Figure 3.29. The unchanged amount of PSI protein complex was observed under 550 mM NaCl- and 700 mM sorbitol-treated cells (Figure 3.29A). In contrast, the ndhF3 protein appeared to slightly decrease under high concentrations of NaCl and moderately decrease under high sorbitol treatment (Figure 3.29B).

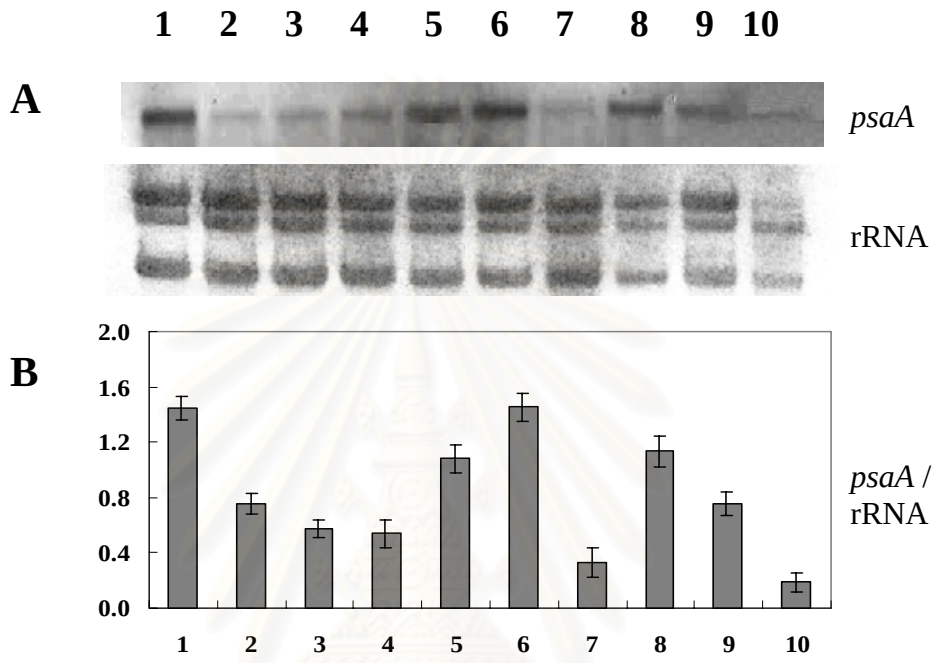

Figure 3.25 (A) Northern blot analysis of *psaA* mRNA levels in *Synechocystis* sp. PCC 6803 grown in different concentrations of NaCl and sorbitol. Total RNA was isolated from 10 d cells grown in medium containing no addition : lane 1, 2 mM NaCl : lane 2, 50 mM NaCl : lane 3, 150 mM NaCl : lane 4, 350 mM NaCl : lane 5, 550 mM NaCl : lane 6, 4 mM sorbitol : lane 7, 100 mM sorbitol : lane 8, 300 mM sorbitol : lane 9 and 700 mM sorbitol : lane 10. The relative abundance of rRNA is also shown. Quantification was carried out using AIS^{TM} program. (B) The ratio (*psaA* mRNA/rRNAs) ± SE, n = 3.

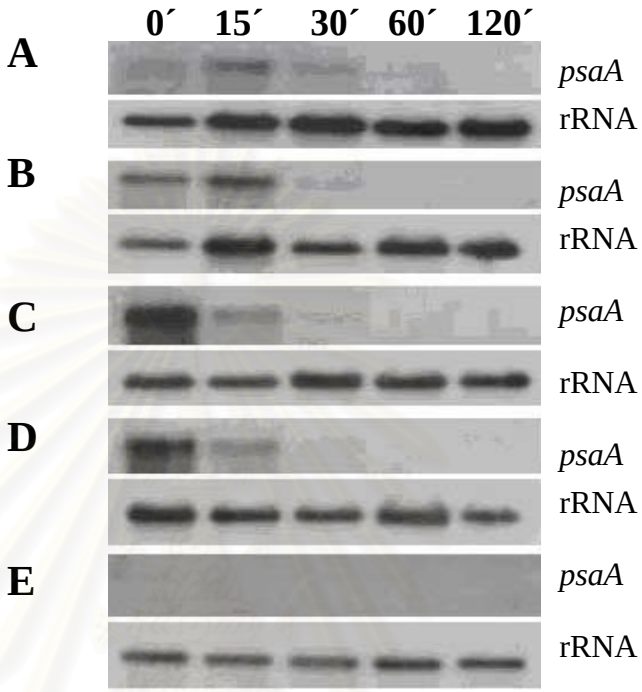

Figure 3.26 Stability of *psaA* mRNA in *Synechocystis* sp. PCC 6803 grown in high concentrations of NaCl and sorbitol. Northern blot analysis was done using 10 d cells grown in medium containing no addition (A), 2 mM NaCl (B), 550 mM NaCl (C), 4 mM sorbitol (D) and 700 mM sorbitol (E). Total RNA was isolated from cells after further incubation with rifampicin (500 μg ml^{-1}) at the times indicated (min). Quantification was carried out using AIS^{TM} program.

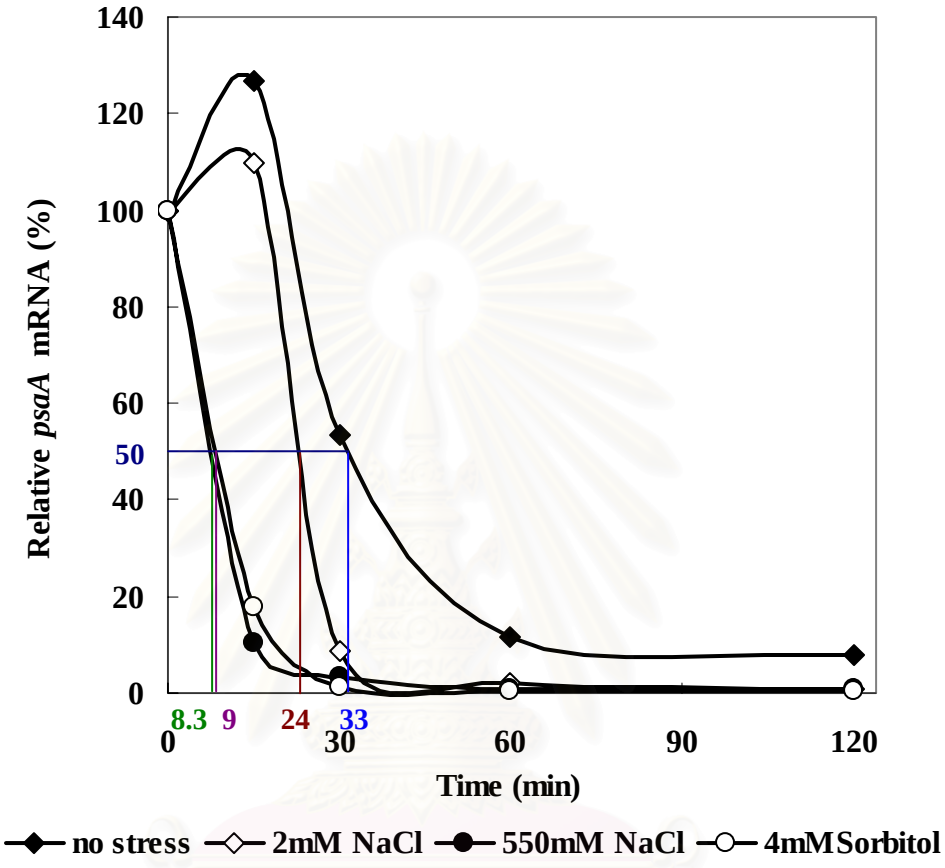

Figure 3.27 Decrease in the *psaA* mRNA amount as a function of time in *Synechocystis* sp. PCC 6803 after addition of rifampicin. The experiments were done as described in Figure 3.26.

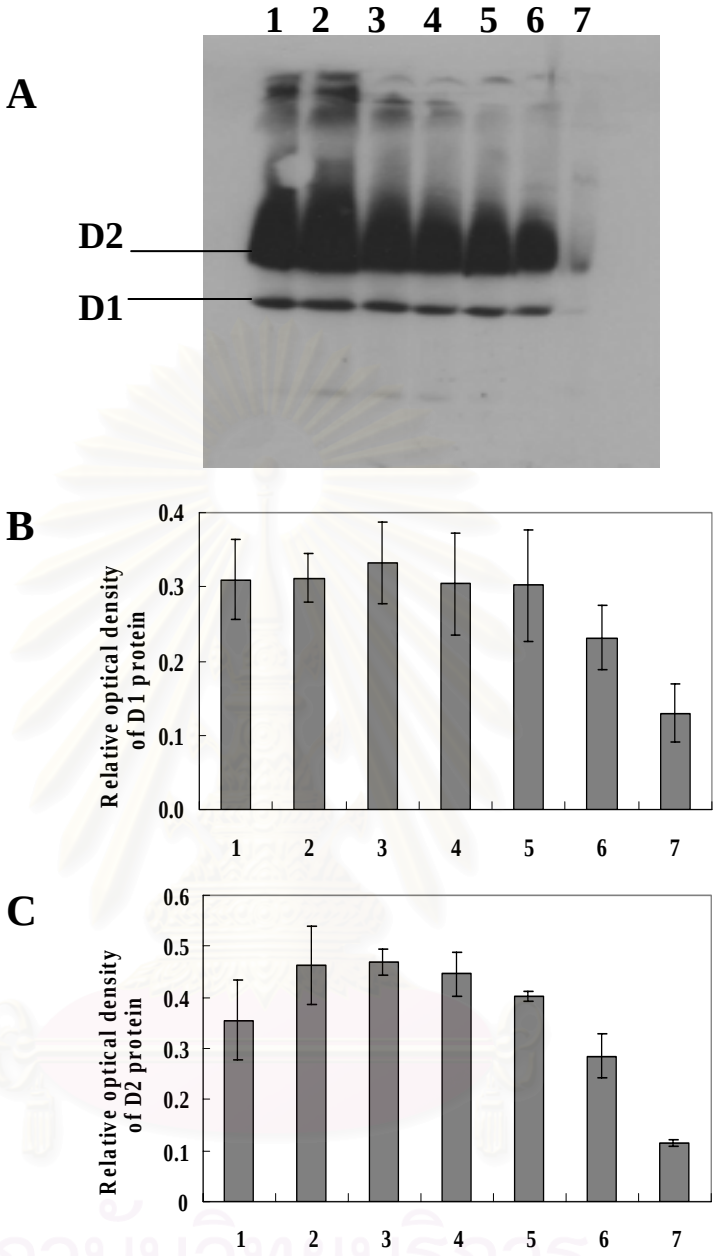

Figure 3.28 (A) Western blot analysis of D1 and D2 protein in *Synechocystis* sp. PCC 6803 grown under NaCl and sorbitol stresses. Thylakoid extraction was done using 10 d cells grown in medium containing no addition (lane 1), 2 mM NaCl (lane 2), 150 mM NaCl (lane 3), 550 mM NaCl (lane 4), 4 mM sorbitol (lane 5), 300 mM sorbitol (lane 6) and 700 mM sorbitol (lane 7). 1 μg Chl of each extracted sample was loaded onto the SDS-gel followed by transferring to nitrocellulose membrane probed with D1 and D2 antibody. B and C show the relative D1 and D2 level, respectively. The data represent Mean± S.E., n=3.

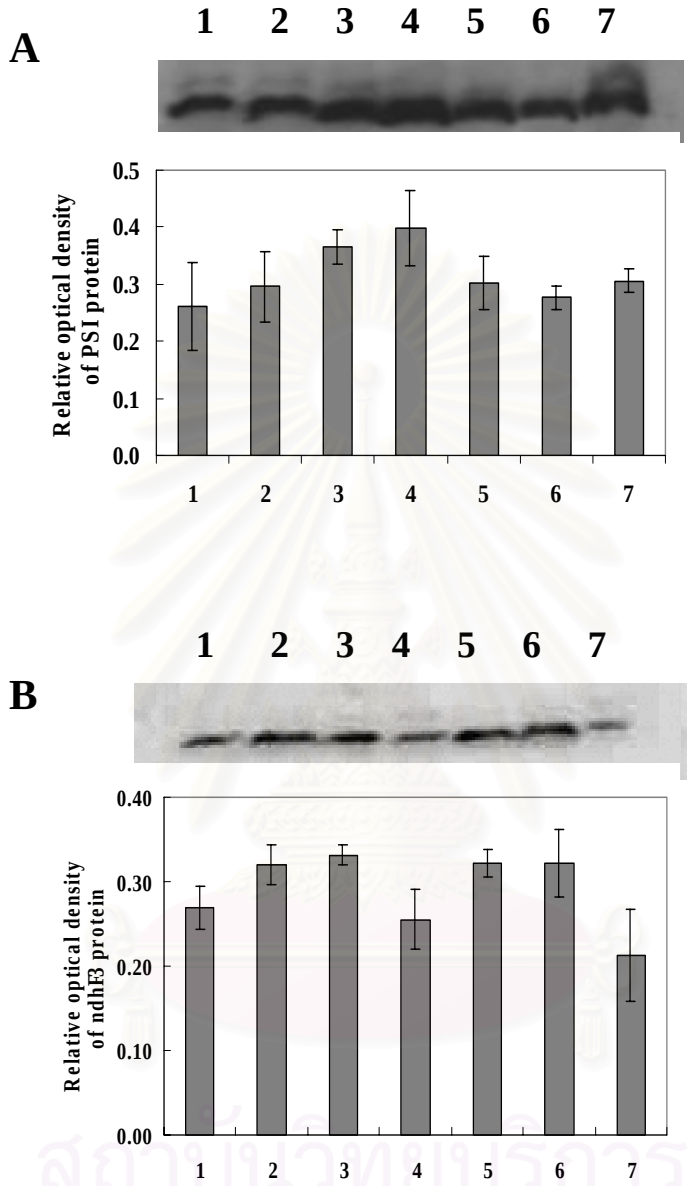

Figure 3.29 Western blot analysis of PSI (A) and ndhF3 (B) protein complex in *Synechocystis* sp. PCC 6803 grown under NaCl and sorbitol stresses. Thylakoid extraction was done using 10 d cells grown in medium containing no addition (lane 1), 2 mM NaCl (lane 2), 150 mM NaCl (lane 3), 550 mM NaCl (lane 4), 4 mM sorbitol (lane 5), 300 mM sorbitol (lane 6) and 700 mM sorbitol (lane 7). 1 μg Chl of each extracted sample was loaded onto SDS-gel followed by transferring to nitrocellulose membrane probed with PSII and ndhF3 antibody. Quantification was carried out using AIS^{TM} program. The data represent Mean ± S.E., n=3.

CHAPTER IV

DISCUSSION

4.1 Effects of salt and sorbitol on polyamine biosynthesis

We have demonstrated that 10-days old culture of *Synechocystis* sp. PCC 6803 under unstressed and stressed treatments was at mid-logarithmic phase and this represented a long term stress acclimation of the cells (Figure 3.1). A previous study of cellular polyamine contents under pH and osmotic stress in soybean rhizobia *Rhizobium fredii* P220 and *Bradyrhizobium japonica* A1017 was also carried out using cells at the stationary phase of growth (Fujihara and Yoneyama, 1993). Several studies aiming at analyzing the short term effect of stress on polyamine accumulation in plants use simplified experimental systems such as tomato leaf discs (Santa-Cruz *et al*., 1997b, Aziz *et al*., 1998). Nevertheless, most previous studies of the long term adaptation under stress were conducted in plants such as tomato (Botella *et al*., 2000), maize and maize callus (Szalai *et al*., 1997, Willadino *et al*., 1996).

We have also shown that the increase in the cellular level of polyamine in *Synechocystis* cells was induced by long term osmotic stress, and to a less extent by salt stress (Figures 3.2 and 3.7). The cellular polyamines in this study include soluble and insoluble polyamine fractions. The supernatant phase after cells extraction in 5% cold $HClO_4$ contained the 'free' polyamine fraction (Flores and Galston, 1982). In nature, polyamines often occur as free molecular bases (Bouchereau *et al*., 1999) which are translocated (Antognoni *et al*., 1998) and that conjugated polyamines have no effect on cell division process (Bagni *et al*., 1994). Many studies of polyamine accumulation under stress were focused on soluble or free polyamine fraction (Kaur-

Sawhney *et al*., 1982b, Santa-Cruz *et al*., 1997b, Motyl *et al*., 1995) or on both separated polyamine fractions as free and bound polyamines (Marián *et al*., 2000, Mo and Pua, 2002). Different classes of polyamine including Put, Spd and Spm could be induced under stress leading to change in the titer of soluble and insoluble forms. Free Put content increased obviously under 550mM NaCl while the insoluble Put form showed no response under salt stress (Figure 3.2) as well as under sorbitol stress (Figure 3.7). The high abundance of insoluble polyamine contents occurred under high osmotic stresses (700 and 1100 mM sorbitol) while lower soluble polyamine contents could be observed under the same conditions. Therefore, the insoluble polyamine represented the major form of total cellular polyamine content under high sorbitol treatment. These results indicated that sorbitol induced high level of insoluble conjugated or bound polyamine forms like membrane bound or macro-biomolecule in the *Synechocystis* cells. In maize, polyamine bound to membrane proteins has been found to be an intermediate in a number of cellular events (Tassoni *et al*., 2002). Moreover, growth under high stress could affect several features of membranes, mostly the cytoplasmic membranes, resulting in increased fluidity and decreased permeability to H^+ and Na^+ (Joset *et al*., 1996). We have followed the changes in cellular polyamine levels under salt and sorbitol stresses during growth for 20 days (Figures 3.3-3.6 and Figures 3.8-3.11) and found that the changes of cellular polyamine pools occurred mostly in early 0-8 days cultivation under salt and sorbitol stress treatments. Subsequently, the polyamine accumulation showed clearly stable level in long term acclimation.

Furthermore, the main class of total cellular polyamine induced by osmotic stress and salt stress was Spd and Spm, respectively. Put appeared to be unaffected by osmotic stress despite the observed increase in ADC activity under osmotic stress

(Figures 3.7 and 3.13). It is likely that cellular Put level would rise initially in *Synechocystis* cells aided by the increased activity of ADC and would subsequently be converted to a more preferably accumulated Spd. The polyamine pool does not only depend on its synthesis, but also on several other processes including degradation, conjugation and transport (Bouchereau *et al*., 1999). Previously, *Escherichia coli* cells grown in high concentration of sucrose have been shown to contain reduced cellular Put (Munro *et al*., 1972). However, Spd content in *E. coli* was unaffected by sucrose-induced osmotic stress. Another study in *Rhizobium fredii* P220 reported the unaffected Put content and the reduced level of homospermidine, an analog of Spd, when external osmolarity increased (Fujihara and Yoneyama, 1993). The effect of osmotic stress on polyamine content was different in higher plants. Osmotic treatments using sorbitol as well as water stress imposed by withholding water caused a rise in Put but a decline in Spd and Spm levels in detached oat leaves (Flores and Galston, 1984). However, the response of the plant cells to accumulate different classes of polyamine is very complex and is partly determined by the plant species concerned. For instance, cell cultures of alfalfa subjected to water-deficit caused by polyethylene glycol showed a trend toward increased accumulation of Spd and Spm and a loss of Put (Kuehn *et al*., 1990).

The levels of cellular polyamines accumulated in *Synechocystis* sp. PCC 6803 after salt and osmotic stress treatments were too low to account for any osmotic importance (Figures 3.2 and 3.7). Indeed, glucosylglycerol has been identified as a major osmolyte for *Synechocystis* sp. PCC 6803 (Hageman and Erdmann, 1994). The increase of various classes of polyamines observed in this study represents the steady state level reflecting the acclimatization of the cells toward the stress rather than the response mechanism. Previously, it has been suggested that the main role of

polyamines is to maintain a cation-anion balance in a long term salt treatment of tomato leaves (Santa-Cruz *et al*., 1997a). In *Synechocystis* sp. PCC 6803, a similar role for polyamines is also likely in view of the fact that intracellular Na^+ would be maintained at a low level by the functional Na^+/H^+ antiporter of *Synechocystis* sp. PCC 6803 under salt stress (Hamada *et al*., 2001), thereby making anions especially Cl^- in excess.

It was found that the activities of ADC and SAMDC in 10-day cells were unaffected under stress conditions (Figures 3.12A - 3.15A). However, the changes in enzyme activities occurred during the stage of cell growth under salt stress. For example, ADC activity showed a response to low NaCl treatment in early 4 days acclimation while it was much induced later under 550 mM NaCl condition. On the other hand, ADC activity was largely induced in 4 days old culture under sorbitol stress (Figure 3.13B). These results suggested different regulation in different stress conditions. The SAMDC activity showed high activity under high sorbitol stress and was regulated in the same way by both salt and osmotic factors during the growth stage (Figure 3.15).

The results showing the increase of *ADC* mRNA by 550 mM NaCl-stress (Figure 3.16) did not correlate with those showing unchanged ADC activity subjected to the same stress treatment (Figure 3.12). This absence of correlation can be partly explained by the inhibitory effect of Spm on the processing of ADC. It is clearly evident that a 2-fold increase in Spm was observed in 550 mM NaCl-treated *Synechocystis* cells (Figure 3.2C). In experiments with osmotically-stressed oat leaves, Spm was shown to inhibit the processing of ADC proenzyme resulting in an increased level of inactive ADC form (Borrell *et al*., 1996). The notion that high cellular level of Spm can bring about inactive ADC is further substantiated by results

in Figure 3.13 showing low ADC activity at 300 mM sorbitol but high ADC activity at 700 mM sorbitol. At 300 mM sorbitol, the observed Spm content was 3-fold of that at 700 mM sorbitol (Figure 3.7C). The phenomenon where *ADC* mRNA levels do not correlate with ADC activity has previously been reported in *Arabidopsis thaliana* upon potassium deficiency stress (Watson and Malmberg, 1996). However, the phenomenon in *A. thaliana* is somewhat opposite to what observed in the present study, i.e. *A. thaliana* showed an increase in ADC activity with no change in *ADC* mRNA suggesting translational or posttranslational regulation of *ADC* due to potassium deficiency stress. On the other hand, the 700 mM sorbitol stressed cells showed a slight increase of *ADC* mRNA (Figure 3.16) but with high activity of ADC protein (Figure 3.13A) indicating that both transcriptional and post-transcriptional regulation are involved in osmotic response with regard to ADC protein. Taken together, it seems that salt and osmotically stressed *Synechocystis* cells upregulate polyamine levels by completely different regulatory machanisms at transcriptional, post-transcriptional, as well as protein level. It should be noted that the results of *ADC* mRNA in the present study represent the transcripts from both slr1312 (*adc1*) and slr0662 (*adc2*) genes. Further investigation on the response of each gene to salt stress and osmotic stress together with single and double mutation analysis is now under way. Such study would help identify the gene(s) which is specifically involved in the stress tolerance imposed by either salt or osmotic stress. A recent novel study by Murata's group using DNA microarray analysis has shown that salt stress and osmotic stress regulate different sets of genes in *Synechocystis* sp. PCC 6803 (Kanesaki *et al*., 2002). Although slr1312 (*adc1*) and slr0662 (*adc2*) are not identified as genes induced by salt stress and osmotic stress, there remains a possibility that these two

genes might be involved in the components of the signaling pathways for salt and osmotic stresses.

The changes in cellular polyamine contents in response to salt stress were less profound compared to what observed under osmotic stress (Figures 3.2C and 3.7C). It is therefore likely that an osmotic effect rather than an ionic effect is the main signal triggering the response. In fact, the influx of Na^+ and Cl^- can occur at a certain rate in cells thriving in hypersaline environments, thereby resulting in a lowered osmotic potential.

The present study has also shown that *Synechocystis* sp. PCC 6803 contains Spm, albeit at a low level. Hitherto, the tetramine Spm has hardly been detected in prokaryotic cells (Kashiwagi *et al*, 1988, Nakabachi *et al*., 2000). The content of cellular Spm in *Synechocystis* cells appeared to be mediated by salt stress and a moderately imposed osmotic stress by 300 mM sorbitol (Figures 3.2C and 3.7C). At present it is unclear how the high osmotic stress by 700 mM sorbitol resulted in a low level of cellular Spm (Figure 3.7C). However, since cellular Spd level was increased at 700 mM sorbitol, it can be speculated that there might exist two distinct isoforms of SAMDC which can provide a substrate, decarboxylated S-adenosylmethionine, for Spd and Spm synthesis. One isoform may be specific for Spd and the other for Spm. Under high osmotic stress the Spd-specific enzyme is activated whereas the Spm-specific enzyme is repressed. In fact, two distinct enzymes, Spd and Spm synthases which are both substrate and product-specific were found in mammalian cells (Pegg, 1983). Alternatively, the gene(s) for Spd and Spm synthases of *Synechocystis* cells may have been switched on and off respectively by high osmotic stress. This avenue of research is of great interest for the elucidation of the possible roles of SAMDC in stress-tolerance. Indeed, the *SAMDC* gene (s) in *Synechocystis* sp. PCC 6803 appear

to be unique since by comparing with the known sequences in other organisms, the putative *SAMDC* gene (s) could not be found in *Synechocystis* sp. PCC 6803.

The imposition on *Synechocystis* cells of salt and osmotic stresses by NaCl and sorbitol respectively could bring about the stabilization of *ADC* transcript (Figure 3.17). This stabilization might arise as a result of the formation of the polyamine-*ADC* mRNA complex. The higher contents of Spd or Spm in the stressed cells of *Synechocystis* would facilitate the formation of such complex. In *E. coli*, 90% of the total Spd was complexed with RNA, whereas 85% of the total Spm bound to RNA in rat liver (Igarashi and Kashiwagi, 2000). It was coincident with a reported work that the *ADC* mRNA levels from leaves, shoots and roots of grapevine remained constant throughout the 72-h experiment period under stress (Primikirios and Roubelakis-Angelakis, 1999). Recently, it has been reported that Spd binds to a GC-rich double-stranded region close to the Shine-Dalgarno sequence of mRNA for oligopeptide-binding protein A (Yoshida *et al*., 1999). Such binding leads to the stabilization of the RNA structure. Moreover, the nuclear degradation which is accompanied by apoptosis-like DNA fragmentation was observed when the barley roots were exposed to more than 300 mM NaCl for 24 h. This DNA degradation leads to sequential nuclear degradation, cell death and inhibition of root growth (Katsuhara and Kawasaki, 1996). On the other hand, the stable *ADC* mRNA observed under salt or osmotic stress in *Synechocystis* cells might be due to its interaction with glucosylglycerol, a compatible solute accumulated in the salt-stressed cells of *Synechocystis* (Hageman and Erdmann, 1994). Nevertheless, we cannot rule out the possibility that the polyamine-*ADC* mRNA complex mentioned above is facilitated or mediated by glycosylglycerol. Very recently, it has been reported that *psbA* transcripts could also be stabilized in *Synechocystis* sp. PCC 6803 in the presence of either 0.5 or

1.0 M NaCl (Allakhverdiev *et al*., 2002). However, the effect of salt stress was shown to suppress the transcription of *psbA* genes which was in contrast to our study where salt stress stimulated the transcription of *adc* genes (Figure 3.16).

In conclusion, both long term salt and osmotic stresses could induce an increase in polyamine contents in *Synechocystis* sp. PCC 6803, particularly with the increase in Spm and Spd by salt stress and osmotic stress respectively. The increased accumulation of *ADC* mRNA under both stress conditions is accounted for by the stabilization of the transcript.

In this study we have also shown that low levels of ADC proteins were observed under high salt and osmotic stresses (Figure 3.19). These results did not correlate with those showing unchanged ADC activity subjected to the same stress treatments (Figure 3.12). The size of the identified protein band is indeed very small. However, we cannot exclude the possibility that the ADC protein in *Synechocystis* is the observed one of 18 kDa. The molecular mass of ADC polypeptide, observed after isolation of the native enzyme and SDS-PAGE, could be completely different from that deduced from the open reading frames of the respective clone. For example, although two ADC cDNA clones isolated from *Arabidopsis* bear open reading frames which encode a 76 (Watson and Malmberg, 1996) and a 77 kDa protein (Watson *et al*., 1997), a monoclonal antibody produced against the 76 kDa product recognized only a 42 kDa protein in Western blot analysis of plant protein extracts, suggesting that there is post-translational processing of the ADC protein. This ADC pre-protein may be processed by a general protease rather than a specific cleavage factor (Hanfrey *et al*., 2001, Hanfrey *et al*., 2002). ADC differs in that several other amino-acid decarboxylases such as SAMDC which are found to be autocatalytically processing. The proenzyme of the carnation SAMDC was auto catalytically converted

into two subunits by processing during in vitro transcription/translation (Lee *et al*., 1997, Franceschetti *et al*., 2001). It was hypothesized however that the grapevine ADC precursor molecule of 80 kDa undergoes post-translational autocatalytic processing and its product is the active form of the ADC enzyme (Primikirios and Roubelakis-Angelakis, 2001). Likewise, the ADC enzyme of *Brassica campestris* (a species in the same family as *Arabidopsis*) was found to be homotetramerous, with a subunit molecular mass of 60 kDa (Das *et al*., 1996), and the open reading frame of an ADC cDNA clone isolated from *Brassica juncea* encodes for a 76 kDa protein (Mo and Pua, 1998).

The two spinach bands of about 60 and 23 kDa were not shown on Western blot membrane. Although no nucleotide or protein ADC spinach sequences have been characterized yet, the ADC spinach polypeptide could also be post-translationally processed. The reaction mechanism of ADC is poorly understood, but the pioneering work of Malmberg and colleagues in isolating the oat ADC cDNA (Bell and Malmberg, 1990) indicated that the oat ADC pro-enzyme was synthesized as a 66 kDa preprotein and subsequently cleaved into 42 kDa N-terminal and 24 kDa C-terminal domain polypeptides (Malmberg *et al*., 1992), after post-translational proteolytic cleavage by a specific protease. It was suggested that the two processed domains of the peptide were held together in the enzyme by a disulphide bridge. Furthermore, Western blot analysis with a polyclonal antibody developed against a protein from carnation ADC cDNA (Chang *et al*., 2000) showed that a soluble ADC enzyme consisted of 45 and 33 kDa polypeptides, derived from a 78 kDa protein.

4.2 Effects of salt and sorbitol on photosynthetic systems

We have shown that the long term ionic stress had no effect on PSII activity and photosynthetic capacity in *Synechocystis* cells whereas the PSII activity was affected by the long term osmotic stress (Figure 3.20A and B). The results suggested that PSII is unaffected under long term salt and low osmotic stresses. It was reported previously that in *Synechocystis* PCC 6803, PSII is resistant to salt stress alone (Allakhverdiev *et al*., 1999). In contrast, *Spirulina platensis* cells exposed to medium containing 0.8 M NaCl for 12 h showed a decrease in photosynthetic oxygen evolution activity, which correlated with the decrease in the quantum yield of PSII electron transport (Lu and Vonshak, 2002). In this study, we have also demonstrated the dark inactivation of photosynthetic oxygen evolution activities (Figure 3.21A and B). These results suggested that the decrease of PSII activity was induced by darkness, not from salt or sorbitol stress, but sorbitol together with darkness enhanced the inhibition. The previous work of Tyystjärvi *et al*. (1996) could support this dark-inactivation. When *Synechocystis* cells were cultivated in a 12 h light-dark rhythm in a strictly autotrophic growth medium, the oxygen evolution capacity of the cells was almost totally inactivated during the dark period. Light has been suggested to regulate both synthesis and degradation of the PSII reaction center polypeptide D1, encoded by the *psbA* gene (Tyystjärvi *et al*, 1996). In our study, high-osmotic stress seemed to regulate the photosynthetic oxygen evolution activity. In natural habitats, photosynthetic organisms are often exposed to light stress and, in many instances, salt stress is combined with light stress. Thus, the combined effects of salt and light stress are of considerable importance in nature and agriculture.

We have also found that the steady-state amount of *psbA* mRNA encoding D1 protein was slightly decreased under salt stress and more clearly down-regulated under high sorbitol stress (Figure 3.22). The half-lives of *psbA* mRNA under unstressed and 2 mM NaCl condition were similar (47 and 44 min, respectively) whereas only a slightly longer half-life (54 min) was observed under 550 mM NaCl condition. These results suggested that salinity had no marked effect on *psbA* transcripts. It was reported previously that the *psbA* gene transcription is not solely under light control (Tyystjärvi *et al*, 1996). The transcripts of *psbA2* gene are short-lived in light but stable in darkness. However, no dark stabilization was observed when a fragment of open reading frame located downstream of *psbA2* was deleted (Mulo *et al*., 1998). This fragment was identified to encode part of the ADC gene. The Spd content of the mutant was lower than that of the wild type suggesting the role of Spd in the stability of the *psbA2* mRNA (Mulo *et al*., 1998). In our study, the stabilization of *psbA* mRNA was unaffected by salinity (Figure 3.23B and C) and by the changes in the polyamine titers caused by salinity (Figure 3.2). High osmotic stress (700 mM sorbitol) induced the decreases in *psbA* mRNA and its stabilization (Figures 3.22 and 3.23), which correlated with the photosynthetic oxygen evolution activity results in Figures 3.20 and 3.21. The *psaA* transcripts, which encode the reaction center subunit of PSI, decreased obviously under salt stress as well as under sorbitol stress comparing with unstressed treatment (Figure 3.25). Furthermore, their stability decreased more than that of the *psbA* transcripts (Figure 3.26). Sorbitol stresses decreased the stability of *psbA* mRNA. This was in agreement with the previous work showing that the osmotic stress reversibly inactivates photosynthetic electron transport via shrinkage of the intracellular space (Allakhverdiev *et al*., 2000).

Salt stress showed no effect on the amounts of the D1 and D2 proteins (Figure 3.28) which correlated with constant PSII activities (Figure 3.20). Previously it has been reported that the light intensity-dependent damage results in an accelerated turnover of the D1 protein with increasing irradiance. The D2 reaction center protein has also been shown to undergo light-dependent degradation to some extent, whereas the other PSII proteins remain stable even under high-light conditions (reviewed by Aro *et al*., 1993). We have shown the unchanged amount of PSI protein under salt and osmotic stresses (Figure 3.29A). The results showed the decrease of the *psaA* mRNA but no effect on PSI protein under salt stress. Similarly, 700 mM sorbitol stress caused the decrease of *psaA* transcripts whereas no apparent changes of PSI protein were observed. These results suggested that the regulation occurred at the transcriptional level. We have also shown that the slight decrease also occurred with ndhF3 protein under high salt (550 mM NaCl) and sorbitol (700 mM sorbitol) stresses (Figure 3.29B). Effects of salt stress on light-induced gene expression have also been investigated previously. After *Synechocystis* cells had been grown at high light for 10 min in 0.5 M NaCl, the sll1732 ORF of *ndhF3* gene encoding NADH dehydrogenase I, chain L was diminished (Allakhverdiev *et al*, 2002). Interestingly, Tanaka *et al*. (1997) reported that the electron flow from the cytosol to PSI via NAD(P)H-dehydrogenase is essential for the adaptation of cyanobacteria to salt shock.

In conclusion, we can classify three groups of protein under regulation by ionic and osmotic stresses: 1) PSII is down regulated only under osmotic stress. 2) PSI is not down regulated under any stresses and 3) NDH is down regulated under both ionic and osmotic stresses. All these results seem to suggest the complicated regulation mechanism for photosynthetic processes in *Synechocystis* sp. PCC 6803.

CHAPTER V

CONCLUSION

Based on the results, the following specific conclusions were drawn :

1) *Synechocystis* sp. PCC 6803 cells showed no difference in the growth rate when the concentration of NaCl was raised up to 550 mM. The growth rate of the cells decreased at 300 mM sorbitol, whereas the inhibition of growth occurred at or higher than 700 mM sorbitol.

2) For the first time, three polyamine classes are detected in *Synechocystis*, importantly spermine has never been detected in *Synechocystis*.

3) Salt and sorbitol stresses affected the soluble and insoluble polyamine titers in the *Synechocystis* cells at 10 days cultivation;

3.1 Soluble Put content increased under 550 mM NaCl and 300 mM sorbitol conditions whereas insoluble Put content was unchanged under these conditions. On the other hand, soluble Put content was unchanged under 700 and 1000 mM sorbitol stressed conditions whereas insoluble Put increased under the same conditions.

3.2 Both soluble and insoluble Spd contents moderately decreased under 50, 150 and 350 mM NaCl conditions, except for 550 mM NaCl condition. The unchanged content of soluble Spd was observed under sorbitol stress. The insoluble Spd content showed no apparent changes under 0-300 mM sorbitol but markedly increased under 700 and 1100 mM sorbitol stressed conditions.

3.3 Both soluble and insoluble Spm contents increased moderately under salt stressed condition. On the other hand, soluble Spm markedly increased under 300 mM sorbitol and decreased at or higher than 700 mM sorbitol stressed condition while insoluble Spm was no changes under sorbitol stressed condition.

4) Salt stress induced a moderate increase in the total cellular polyamine content, spermine in particular.

5) Osmotic stress caused an apparent increase in the total cellular polyamine content with a marked increase of spermidine induced by 700 mM sorbitol.

6) Three polyamine classes are specifically regulated under the stress conditions studied. These refer to their specific role *in vivo* under different conditions.

7) The cellular polyamine contents rapidly increased at early stage of cultivation up to 4 days and decreased afterwards during growth for 20 days.

8) ADC, a key enzyme for putrescine synthesis, was unaffected by salt stress but showed a 6-fold increase of enzyme activity upon osmotic stress imposed by 700 mM sorbitol.

9) SAMDC, another important enzyme for spermidine and spermine synthesis, responded to salt and osmotic stresses similarly to that observed for ADC.

10) An analysis by RT-PCR revealed an increase of *ADC* mRNA level in cells under salt and osmotic stresses.

11) The high amount of *ADC* transcripts observed under high osmotic and ionic stress conditions are due to stabilization of the *ADC* mRNA.

12) High salt stress was unaffected on the photosynthetic oxygen evolution whereas high sorbitol stress (700 mM sorbitol) inhibited photosynthetic oxygen evolution completely.

13) Darkness inactivated the photosynthetic oxygen evolution and the dark-inactivation was accelerated by a concomitant sorbitol stress.

14) The abundance of *psbA* transcripts was slightly decreased under salt stress and more under sorbitol stress condition, especially under 700 mM sorbitol condition.

15) The abundance of *psaA* transcripts decreased under salt stress, except for 550 mM NaCl treated cells, as well as under sorbitol stress condition.

16) The stability of *psbA* mRNA was unaffected by salt stress whereas the decrease of *psaA* mRNA stability was observed. On the other hand, sorbitol stress decreased the stability of both *psbA* and *psaA* transcripts.

17) Salt stress did not affect the amounts of D1 and D2 and PSI proteins whereas the ndhF3 protein was slightly decreased by salinity.

18) High sorbitol stress induced the decreases of D1, D2 and ndhF3 proteins while the unchanged amount of PSI protein was observed under the same condition.

19) There are three groups of proteins distinguished according to their steady-state amounts under the stress conditions studied:

19.1 PSII proteins are down regulated only under osmotic stress.

19.2 PSI protein is not affected under any stresses.

19.3 ndhF3 protein is down regulated under both ionic and osmotic stresses.

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APPENDICES

APPENDIX 1

BG-11 medium (1,000 ml)

	Solid medium	Liquid medium
H_2O	947 ml	967 ml
Bacto-agar	15 g	-
100 × 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 Ferric ammonium citrate	1 ml	1 ml
1 M TES	10 ml	-
30% $Na_2S_2O_3 \times 5H_2O$	10 ml	-
1 M Hepes-NaOH, pH 7.5	20 ml	20 ml

100 × BG-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
0.5 M Na-EDTA	55.4 μl

*After autoclaved, add 10 ml of '1000 × Trace metal mix'

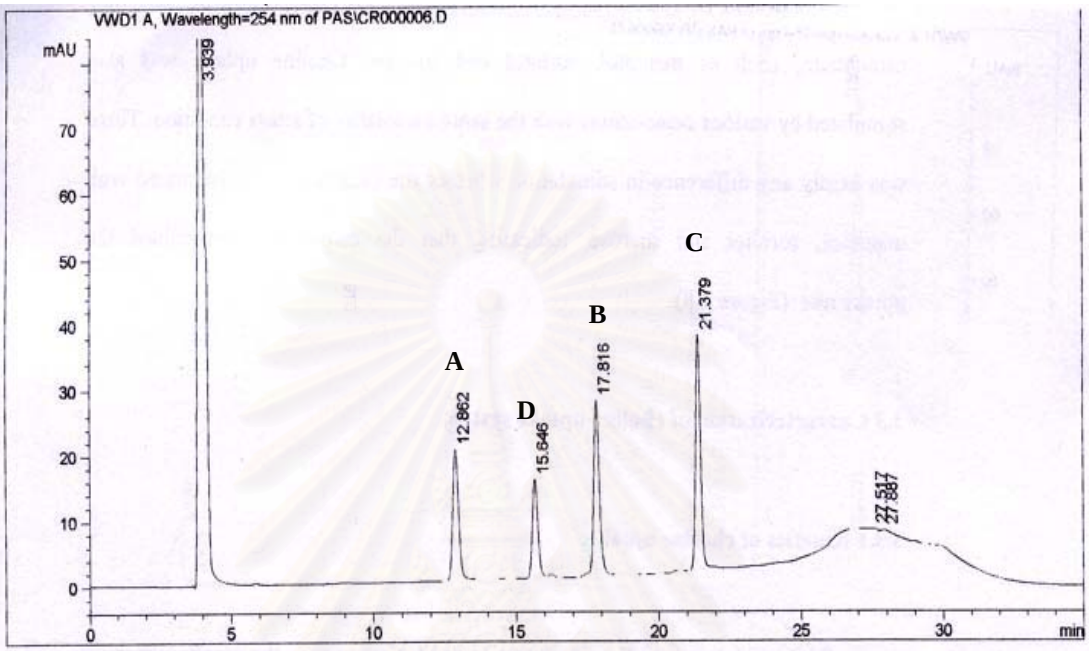
1000 × Trace metal mix**

	1,000 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 2

Chromatogram and standard curve of polyamines



Area Percent Report

Sorted by Signal
Multiplier : 1.000000
Dilution : 1.000000

Signal 1: VWD1 A, Wavelength=254 nm

Peak #	RT [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.939	PB	0.228	1842.12769	119.06691	49.6759
2	12.862	BB	0.230	291.45950	19.98807	7.8597
3	15.646	BV	0.212	204.35982	14.95904	5.5109
4	17.816	BB	0.219	385.13538	26.86992	10.3858
5	21.379	BB	0.191	438.18250	35.64256	11.8163
6	27.517	BV	1.472	412.42618	3.37727	11.1217
7	27.887	VB	0.603	134.60002	2.93439	3.6297
Totals :				3708.29102	222.83815	

Figure A.1 Chromatogram of standard polyamines (A, Put ; B, Spd and C, Spm) at final concentration of 70 μM and internal standard (D, 1,6-Hexanediamine) at 7 μM.

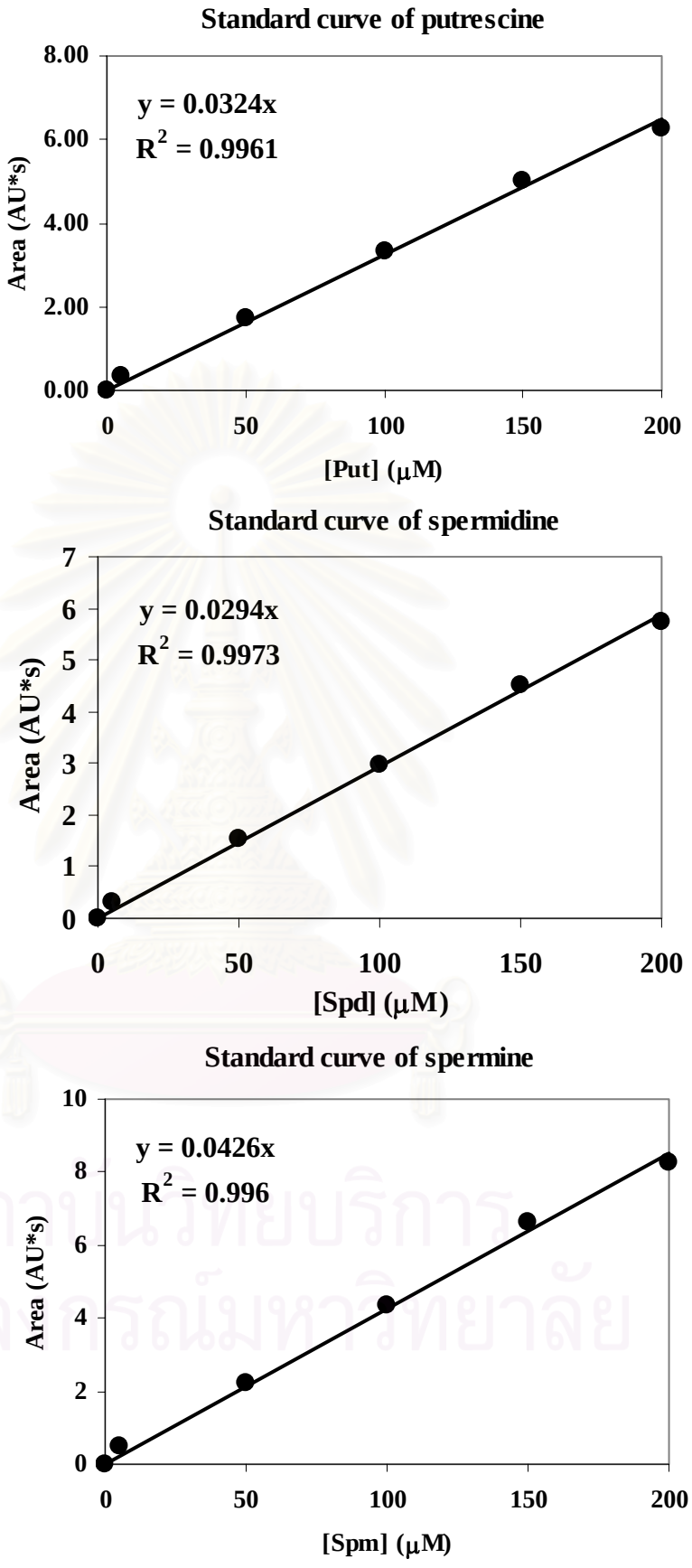


Figure A.2 Standard curve of polyamines.

APPENDIX 3

Standard curve of ^{14}C

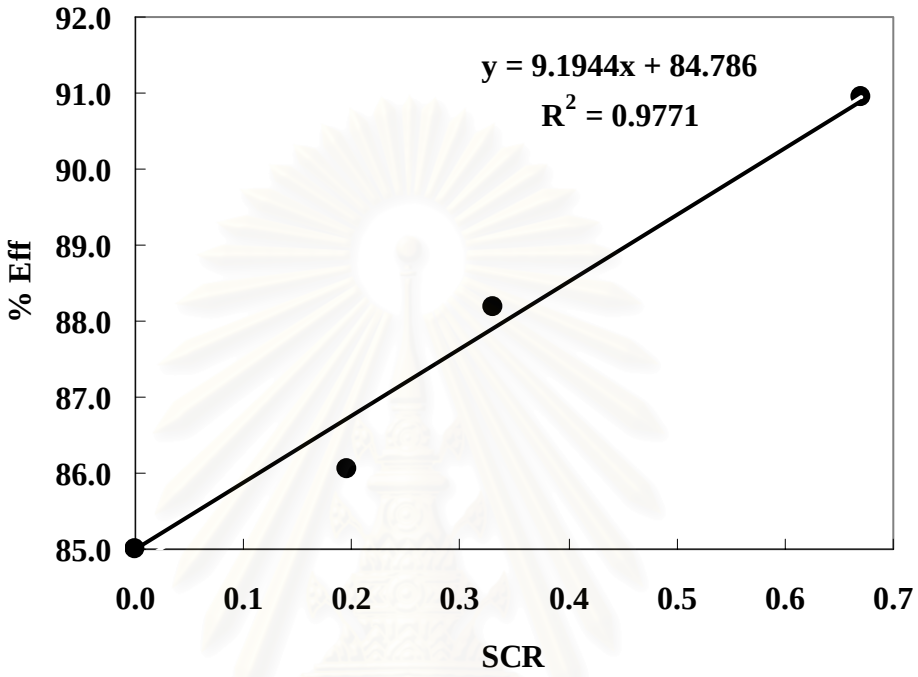


Figure A.3 Standard curve of ^{14}C.

APPENDIX 4

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 trough Whatman No.1 paper, Store at room temperature in brown glass bottle. Usable for several week, but may need to be refiltered.

Assay

1. Pipet protein solution 100 μl into tube
2. Add 1 ml Bradford working buffer and vortex.
3. Read OD_{595} after 2 min but before 1 h.

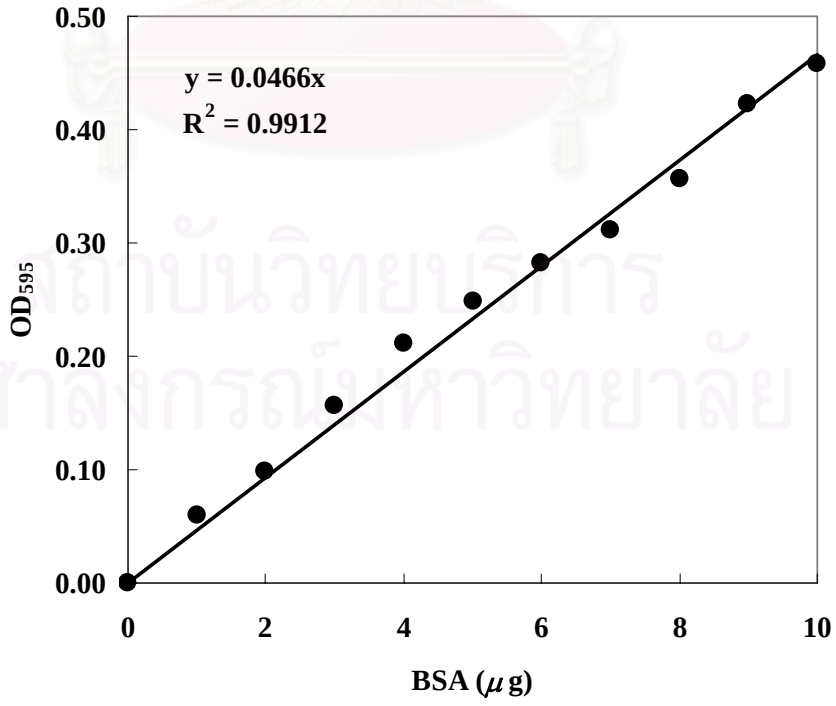


Figure A.4 Standard curve of BSA.

APPENDIX 5

RNA extraction buffer

1. Resuspension buffer

    0.3 M sucrose

    10 mM sodium acetate, pH 4.5

2. Lysis buffer

    2 % SDS

    10 mM sodium acetate, pH 4.5

3. RNA storage buffer

    20 mM Na-phosphate buffer pH 6.5

APPENDIX 6

Preparation and examination of agarose gels

1. Sealed the edges of a clean, dry, glass plate and set the mold on a horizontal section of the bench.
2. Prepared electrophoresis buffer (100 ml).
3. Weighed 0.6 g of agarose and put into buffer.
4. Boiled for 2-3 minutes in microwave.
5. Added ethidium bromide from a stock solution of 1 mg/ml (final concentration of 0.5 μg/ml and mixed.
6. Put the comb in its position.
7. Poured warm agarose solution into the tray.
8. After the gel was completely set (30-45 min at room temperature), carefully removed the comb.
9. Moved the gel in the electrophoresis tank.
10. Added 6×load dye into each samples and loaded samples into the slot.
11. Closed the lid of the gel tank and attached the electrical leads (80 volts).
12. Turned off the electric current and removed the gel into UV-light machine to see their bands.

APPENDIX 7

TBE buffer

Working solution

0.5 × : 0.045 M Tris-borate

0.001 M EDTA

Concentrated stock solution (per liter)

5 × : 54 g Tris base

27.5 g boric acid

20 ml 0.5 M EDTA (pH 8.0)

APPENDIX 8

TAE buffer

Working solution

1 × : 0.04 M Tris-acetate

0.001 M EDTA

Concentrated stock solution (per liter)

50 × : 242 g Tris base

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA (pH 8.0)

APPENDIX 9

PCR amplification mixture

10× PCR buffer	5.0	μl
5 mM deoxy NTPmix	2.5	μl
Primer 5´ (5 *p*mol/μl)	5.0	μl
Primer 3´ (5 *p*mol/μl)	5.0	μl
cDNA or DNA	X	μl
Milli-Q water	Y	μl
*DyNAzyme^{TM}II DNA Polymerase enzyme	0.5	μl
Mineral Oil	50.0	μl

$Y = 50 – [5.0 + 2.5 + 5.0 + 5.0 + X]$

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

10× PCR buffer

100 mM Tris-HCl, pH 8.8 at 25 ºC

15 mM $MgCl_2$

500 mM KCl

1 % Triton X-100

APPENDIX 10

Crude protein extraction

1. Pre-cooled glass beads for 1 hour.
2. The pellet was diluted with extraction buffer (Primikirios and Roubelakis-Angelakis, 1999) in ratio of 1:2 (cells:buffer, w/v) in tube.

   Extraction buffer : 50 mM Tris-HCl pH 8.0
   1 mM EDTA
   50 μM Pyridoxal phosphate
   5 mM DTT
   0.5 mM PMSF
   10 μM Leupeptin
   10 % (v/v) Glycerol
   0.2 % Triton X-100
3. Weighed glass beads (ratio of 1:1, glass beads: solution, w/v) and added into the tube.
4. Vortexed each sample for 20 sec per time and repeated 7-8 times (kept on ice after vortexing).
5. Crude protein sample solutions were transferred to new tubes (~1.5 ml).
6. Added 5 ml of extraction buffer into the tube and repeated steps 4 and 5 for 3 times. Collected the supernatant in new tube (~15 ml).
7. Centrifuged at 20,000 rpm at 4 ºC for 30 min.
8. The supernatant was taken and kept in –20 ºC.

APPENDIX 11

Preparation for polyacrylamide gel electrophoresis

1) Working 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

Added distilled water to make 100 ml and stirred until completely dissolved.

1.5 M Tris-HCl, pH 8.8, 100 ml

Tris (hydroxymetyl)-aminomethane	18.17 g

Adjusted pH to 8.8 with concentrated HCl slowly and added distilled water to a total volume of 100 ml.

0.5 M Tris-HCl, pH 6.8, 100 ml

Tris (hydroxymetyl)-aminomethane	6.06 g

Adjusted pH to 6.8 with concentrated HCl slowly and added distilled water to a total volume of 100 ml.

20 % SDS, 100 ml, stored at room temperature

SDS	20 g

Added distilled water to a total volume of 100 ml.

10 % Ammoniumpersulfate (APS), 1 ml

APS	0.1 g

Added distilled water to a total volume of 1 ml.

22.2 % Glycerol, 100 ml

100 % Glycerol	22.2 ml

Added distilled water to a total volume of 100 ml.

2) SDS-PAGE

15 % Separating gel (for 1 gel)

1.5 M Tris-HCl, pH 8.8	12.7 ml
30 % Acrylamide solution	16.6 ml
Urea	19.86 g
Distilled water	9.7 ml
20 % SDS	1.15 ml
10 % APS	150 μl
TEMED	15 μl

12 % Separating gel (for 1 gel)

1.5 M Tris-HCl, pH 8.8	11.0 ml
30 % Acrylamide solution	11.0 ml
Urea	15.89 g
Distilled water	10.43 ml
20 % SDS	880 μl
10 % APS	100 μl
TEMED	15 μl

6 % Stacking gel (for 1 gel)

0.5 M Tris-HCl, pH 6.8	5.0 ml
30 % Acrylamide solution	2.4 ml
Urea	7.21 g
Distilled water	6.9 ml
20 % SDS	400 μl
10 % APS	150 μl
TEMED	15 μl

Laemmli solution, 45 ml

0.5 M Tris-HCl, pH 6.8	12.5 ml
Urea	16.22 g
22.2 % Glycerol	11.5 ml
20 % SDS	9.75 ml
Distilled water	11.25 ml

Solubilizing buffer

Laemmli solution	900 μl
2-Mercaptoethanol	100 μl
Dye (bromophenol blue)	5 μl

The mixture was 1:1 (sample : solubilizing buffer) and incubated at 65 ºC for 5 min. After centrifugation at 9,000 rpm for 3 min, samples were loaded into the gel.

Electrophoresis buffer

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

Adjust volume to 1 litre with distilled water (pH should be approximately 8.3).

APPENDIX 12

Staining the SDS-gel

Step	Reagent	Duration
1. Fixing	40 % Methanol / 10 % Acetic acid	> 1 h
2. Wash	30 % Ethanol	3 ×10 min
3. Reduction	Thiosulfate reagent	1 min
4.Wash	Deionized water	3 × 20 sec
5. Incubation	Silver nitrate reagent	20 min
6. Wash	Deionized water	3 × 20 sec
7. Development	Developer	3 – 5 min
8. Stop	Stop reagent (0.5 % Glycine)	5 min
9. Wash	Deionized water	2 × 30 min

Working solutions

1) Thiosulfate reagent, 1 litre

$Na_2S_2O_3.5H_2O$	250	mg

2) Silver nitrate reagent, 150 ml (make fresh)

Silver nitrate	0.3	g
Formaldehyde (37%)	30	μl

3) Developer, 150 ml (make fresh)

Sodium carbonate (anhydrous)	4.5	g
Formaldehyde (37%)	75	μl
0.02 % Sodium thiosulfate	2.5	ml

APPENDIX 13

The electron acceptors used in oxygen evolution measurement

Stock solutions

1) 250 mM DCBQ, 1 ml

DCBQ	0.045	g

Dissolved in absolute ethanol 1 ml

2) 250 mM Ferricyanide, 5 ml

Ferricyanide	0.412	g

Dissolved in Milli-Q water 5 ml

3) 0.6 M Bicarbonate, 1 ml

Sodium bicarnobate	63	mg

Dissolved in Milli-Q water 1 ml

APPENDIX 14

Oxygen content of air-saturated water

Temp (°C)	O_2 Concentration (μmol/ml)
0	**0.442**
5	**0.386**
10	**0.341**
15	**0.305**
20	**0.276**
25	**0.253**
30	**0.230**
35	**0.210**

- Changes in solubility of oxygen in water with temperature.

APPENDIX 15

Northern blotting reagents

1) 50 × Phosphate buffer pH 6.8

250 ml 0.5 M Na_2HPO_4 (44.5 g)

250 ml 0.5 M NaH_2PO_4 (34.5 g)

2) Glyoxal denaturation mix

DMSO	500	µl
30 % Glyoxal	220	µl
50 × Phosphate buffer pH 6.8	20	µl

3) Glyoxal dye mix

Xylene cyanol	2.5	mg
Bromphenol blue	2.5	mg
50 × Phosphate buffer pH 6.8	1.0	ml

4) 20 × SSC, 500 ml

Sodium citrate (300 mM)	44.12	g
NaCl (3 M)	87.66	g

Adjusted with 2 N NaOH to pH 7.0

5) RNA-membrane dye

0.5 M Sodium acetate

0.04 % Methylene blue

6) 10 % SDS

SDS	10	g

Adjusted with Milli-Q water to final volume 100 ml

7) 50 × Denhardt, 100 ml

Ficoll	1	g
PVP	1	g
BSA	1	g

Adjusted with Milli-Q water to total volume 100 ml

8) Prehybridizing solution

20 × SSC	30	ml
50 × Denhardt	10	ml
10 % SDS	5	ml

Adjusted with Milli-Q water to total volume 100 ml

APPENDIX 16

Upward capillary transfer in Northern blotting

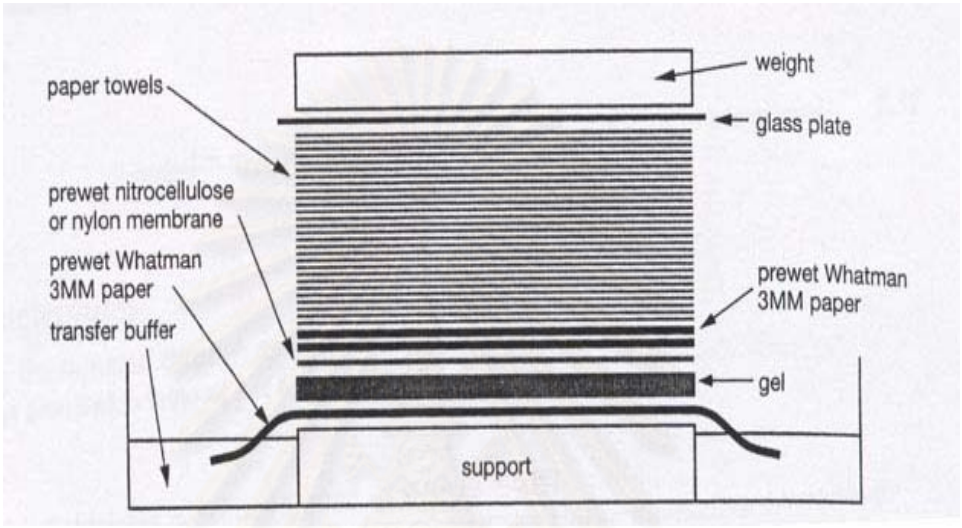


Figure A.5 Upward capillary transfer from an agarose gel to nitrocellulose membrane.

APPENDIX 17

Making the probe for Northern hybridization

		Add	Final Conc.
A	Nuclease-Free Water to achieve final volume of 50 μl		
B	Denatured DNA template	X	500 ηg/ml
C	Labelling 5 × Buffer	10 μl	1 ×
D	Mixture of the unlabeled dNTPs (dTTP, dATP, dGTP)	2 μl	20 μM each
E	Nuclease-Free BSA	2 μl	400 μg/ml
F	[α-^{32}P] dCTP, 50 μCi, 3000 Ci/mmol	5 μl	333 nM
G	DNA polymerase I, Large (Klenow) Fragment -5 Units	1 μl	100 U/ml
	Final volume	**50 μl**	

1) DNA sample was diluted with "**A**" to final concentration of 500 ηg/ml.

2) Denatured by heating at 95-100 °C for 2 min followed by rapidly chilling the tube in an ice bath.

3) Added "**C, D, E, F and G**" into the denatured DNA-tube

4) The reaction mixture was mixed gently and incubated at room temperature for 1 h.

5) Terminated the reaction by heating at 95-100 °C, 2 min and rapidly chilled in an ice bath.

6) Added 2 μl of 0.5 M EDTA, pH 8.0.

7) The labeled DNA probe was ready for hybridization.

APPENDIX 18

Isolation of Thylakoids

1) Twenty ml of cell culture was harvested and centrifuged at 12000 × *g* for 1 min, 4 °C. The pellet was taken and froze in liquid nitrogen.
2) Thawed the sample and added 200 μl STNE buffer.
3) The glass beads were added for 3 spoonfuls into the tube.
4) Vortexed 1 min, 6 times.
5) Added 1 ml STNE buffer into the tube followed by centrifuging at 650 × *g* for 4 min at 4 °C
6) Took the supernatant to a new tube and centrifuged at 18000 × *g* for 15 min at 4 °C.
7) The pellet (thylakoid fraction) was resuspended in 30 μl STNE buffer.

APPENDIX 19

Chlorophyll content determination

1) The 795 μl of 80 % acetone was added into the eppendorf.
2) Added 5 μl extracted thylakoid sample.
3) Vortexed for 2 – 3 min.
4) Centrifuged at 12000 × *g* for 5 min at 4 °C.
5) Measured the OD at 663 nm.
6) Calculated the chlorophyll content :

$$[A663 \times 12.7 \times (800/5)] / 1000 = \ldots\ldots. \ \mu g \text{ Chl} / \mu l$$

Coefficient constant: 12.7

APPENDIX 20

Buffer for western blotting

10 × Blotting solution

100 mM Tris-HCl, pH 9.5

100 mM NaCl

10 mM $MgCl_2$

(used time = 1×)

10 × TBS (Tris-buffer-saline)

200 mM Tris-HCl, pH 7.5

5 M NaCl

TTBS

1 × TBS

0.05 % Tween-20

Blocking solution

5 % Skim milk in TBS

Antibody buffer

1 % Skim milk in TTBS

Chemiluminescence kit

Milli-Q water	24	ml
CDP-Star	1	ml
1:6000 Chemiluminescence	8.3	μl

BIOGRAPHY

Miss Saowarath Jantaro was born on August 31, 1974 in Chiang-Rai, Thailand. She has graduated with a Bachelor of Science degree in Biotechnology and Master of Science degree in Biotechnology from Prince of Songkla University in 1997 and 2000 respectively. She has further studied for the Doctor of Philosophy (Ph.D.) degree in Biotechnology program, Department of Science, Chulalongkorn University since 2000.