FEASIBILITY STUDY ON SURFACE EXPRESSION OF ORGANOPHOSPHORUS HYDROLASE USING CYANOBACTERIAL SomA SYSTEM

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ABSTRACT

Display of heterologous proteins on the surface of microorganisms is becoming increasingly used in various applications in microbiology and biotechnology. The current knowledge concerning the surface display of cyanobacteria is still limited. Cyanobacteria are photosynthetic prokaryotes that have become hosts of interest for expression of heterologous genes with low cost production. The activated cyanobacteria-reactor has been recognized as an economical method for treating both domestic and industrial wastewater. Organophosphorus (OP) compounds are neurotoxic chemicals and used extensively as pesticides. Organophosphorus hydrolase (OPH) encoded from *opd* gene of *Flavobacterium* sp. ATCC27551 has been shown to detoxify OP effectively. Therefore, expression of OPH on the cyanobacterial surface could be an alternative for detoxification of OP. In this study, SomA (*Synechococcus* outer membrane), a porin in the outer membrane, was used as an anchoring motif for expression of OPH on the surface of unicellular cyanobacterium *Synechococcus* PCC7942. The *somA* gene of *Synechococcus* PCC7942 was cloned. The *somA-opd* gene cassette encoding the truncated SomA protein fused in frame with OPH was constructed. The shuttle plasmids pKT-Psom-opd and pKT-Tsom-opd containing *somA-opd* gene cassette under the control of *somA* promoter and $tRNA^{pro}$ promoter, respectively, were transformed into *Synechococcus* PCC7942. Results showed that the OPH activity of freeze-thaw whole cells harboring pKT-Psom-opd (6.26±0.34 μM/min/OD_{730}) was 5-fold higher than that of cells harboring pKT-Tsom-opd (1.09±0.14 μM/min/OD_{730}). Thus, the *somA* promoter is stronger than $tRNA^{pro}$ promoter. The surface location of OPH was investigated using proteinase K accessibility assay. The results were not conclusive.

KEY WORDS: CYANOBACTERIA/ *SYNECHOCOCCUS* PCC7942/ SURFACE EXPRESSION/ ORGANOPHOSPHORUS HYDROLASE/ *somA* GENE

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ศึกษาความเป็นไปได้ในการแสดงออกของเอ็นไซม์ออร์กาโนฟอสฟอรัสไฮโดรเลสบนผิวเซลล์โดยใช้ระบบ SomA ของไซยาโนแบคทีเรีย (FEASIBILITY STUDY ON SURFACE EXPRESSION OF ORGANOPHOSPHORUS HYDROLASE USING CYANOBACTERIAL SomA SYSTEM)

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บทคัดย่อ

การแสดง heterologous protein บนผิวเซลล์จุลชีพได้ถูกนำมาประยุกต์ใช้ทางด้านจุลชีววิทยาและเทคโนโลยีชีวภาพเพิ่มมากขึ้น ปัจจุบันความรู้เกี่ยวกับการแสดงออกที่ผิวเซลล์ของไซยาโนแบคทีเรียมีอย่างจำกัด ไซยาโนแบคทีเรียเป็น prokaryote ที่สามารถสังเคราะห์แสงซึ่งเป็นที่น่าสนใจนำมาใช้เป็นเจ้าบ้านในการแสดงออกของ heterologous gene ด้วยต้นทุนการผลิตต่ำ เป็นที่ยอมรับกันว่าการใช้ activated cyanobacteria-reactor ในการบำบัดน้ำเสียจากบ้านเรือนและอุตสาหกรรมเป็นวิธีที่ประหยัด Organophosphorus (OP) เป็นสารเคมีที่เป็นพิษต่อระบบประสาทและเป็นยาฆ่าแมลงที่ใช้กันอย่างกว้างขวาง เอ็นไซม์ organophosphorus hydrolase (OPH) ซึ่งสร้างจากยีน *opd* ของ *Flavobacterium* sp. ATCC27551 สามารถย่อยสลาย OP ได้อย่างมีประสิทธิภาพ ดังนั้นการแสดงออกของ OPH บนผิวเซลล์ไซยาโนแบคทีเรียอาจเป็นทางเลือกเพื่อใช้ขจัดความเป็นพิษของ OP ในการศึกษานี้ ได้ใช้ SomA (*Synechococcus* outer membrane) ซึ่งเป็น porin ใน outer membrane เป็น anchoring motif สำหรับแสดงออก OPH บนผิวเซลล์ของ *Synechococcus* PCC7942 ซึ่งเป็นไซยาโนแบคทีเรียเซลล์เดียว ได้โคลนยีน *somA* ของ *Synechococcus* PCC7942 และสร้าง *somA-opd* gene cassette ซึ่งถอดรหัสเป็น truncated SomA ซึ่ง fused in frame กับ OPH ได้สร้าง shuttle plasmid pKT-Psom-opd และ pKT-Tsom-opd ซึ่งมี *somA-opd* gene cassette ภายใต้การควบคุมของโปรโมเตอร์ของยีน *somA* และ $tRNA^{pro}$ ตามลำดับ แล้วนำไป transform เข้าสู่ *Synechococcus* PCC7942 ผลการทดลองพบว่าค่า OPH activity ของ freeze-thaw whole cells ที่มี pKT-Psom-opd (6.26±0.34 µM/min/ OD_{730}) มีค่าสูงกว่าที่ได้จากเซลล์ที่มี pKT-Tsom-opd (1.09±0.14 µM/min/OD_{730}) ถึง 5 เท่า ดังนั้นโปรโมเตอร์ของยีน *somA* จึงแข็งขันกว่าของยีน $tRNA^{pro}$ ได้ใช้ proteinase K accessibility assay เพื่อตรวจสอบ OPH ที่ผิวเซลล์ ผลการทดลองที่ได้ไม่สามารถสรุปผลที่แน่ชัด

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LIST OF ABBREVIATIONS

A	=	Absorbance
Amp^r	=	β-lactamase or ampicillin resistance gene
ATP	=	Adenosine triphosphate
ε	=	Absorption coefficient
bla (Ap^R)	=	β-lactamase or ampicillin resistance gene.
°C	=	Degrees Celsius, sometimes called centigrade
c	=	Molar concentration
CAT	=	Chloramphenicol acetyl transferase
CIAP	=	Calf intestinal alkaline phosphatase
CMC	=	Carboxymethylcellulose
CMCase	=	Carboxymethyl cellulase
Cp	=	Cyclopentadienyl anions ($\eta^5C_5H_5$)
d	=	Path length through the sample
Da	=	Dalton
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotidetriphosphate
EC	=	Enzyme classification number
EPA	=	The United States Environmental Protection Agency
EPN	=	Ethyl 4-nitrophenyl phenylphosphonothioate
Et	=	Ethyl Group (CH_3CH_2)
g	=	Gram (s), a unit of mass in the SI system
g	=	Relative centrifugal force (rcf)
GFP	=	Green fluorescent protein
GUS	=	β-glucuronidase gene
h	=	Hour (s)
iso	=	Isometric
IUPAC	=	International Union of Pure & Applied Chemistry
JPC	=	Junctional pore complex

LIST OF ABBREVIATIONS (CONT.)

kb	=	Kilobase pair (s)
K_{cat}	=	Overall enzymatic catalytic rate
Km	=	Kanamycin resistance gene
K_m	=	The Michaelis-Menten constant
lacZ	=	β-galactosidase gene
LamB	=	A trimeric protein located in the outer membrane of *E. coli* K 12
LevU	=	Levansucrase
M	=	Molar
MCS	=	Multiple cloning sites
Me	=	Methyl Group (-CH_3)
μg	=	Microgram (s)
μl	=	Microliter (s)
μM	=	Micromolar (s)
min	=	Minute (s)
ml	=	Milliliter (s)
mM	=	Millimolar (s)
mol	=	Mole (s), the base SI unit of amount of matter
ng	=	Nanogram (s)
nm	=	Nanometer (s)
no.	=	Number
Nos-ter	=	Nopaline synthase terminator
OD	=	Optical density
OmpA	=	*Escherichia coli* outer membrane protein A
OmpC	=	*Escherichia coli* outer membrane protein C
OP	=	Organophosphorus compound or organophosphate
OPA	=	Organophosphorus acid
opd	=	Organophosphate degradation gene

LIST OF ABBREVIATIONS (CONT.)

OPDA	=	Organophosphate-degrading enzyme from *Agrobacterium radiobacter* P230
OPH	=	Organophosphorus hydrolase or organophosphate hydrolase
OPs	=	Organophosphorus compounds or organophosphates
ori	=	Origin of replication
PCC	=	Pasteur Culture Collection
PCR	=	Polymerase chain reaction
P_{lac}	=	The *lac* promoter
pmol	=	Picomole (s)
P_{somA}	=	Promoter of *somA* of *Synechococcus* PCC7942
P_{tRNA}	=	$tRNA^{pro}$ promoter of *Synechococcus* PCC7942
η^5	=	Pentahapto
rep (pMB1)	=	A replicon from the pMB1 plasmid
RNase	=	Ribonuclease
rpm	=	Revolutions per minute
s	=	Second(s)
S-layer	=	Surface layer
sp.	=	Species
T	=	Threonine
ter	=	Transcription-translation terminator signal of phage T4
Tris-HCl	=	Tris-hydrochloric
tRNA	=	Transfer ribonucleic acid
U	=	Unit (s)
V_{max}	=	Velocity Maximum
VX	=	O-ethyl-S-[2(diisopropylamino)ethyl] methylphosphonothiolate ($C_{11}H_{26}NO_2PS$)
v/v	=	Volume/volume
w/v	=	Weight/volume

CHAPTER I
INTRODUCTION

Genetic engineering provides a lot of potential applications for displaying heterologous proteins on the surface of microorganisms. Cell surface display of several proteins or peptides was extensively studied in prokaryotes and eukaryotes, particularly in bacterial (Wernérus & Ståhl, 2004) and yeast cells (Kondo & Ueda, 2004). System of cell surface display can be classified into C-terminal fusion, N-terminal fusion and sandwich fusion (Xu & Lee, 1999). The C-terminal fusion type is used for anchoring the foreign peptide to the C-terminus of the native surface protein. The N-terminal fusion type is, on the contrary, anchoring the foreign peptide to the N-terminus of the native surface protein. Sandwich fusion is called for anchoring of the heterologous protein, which is inserted within surface-expose loops of the outer membrane protein on the host cell surface (Georgiou et al., 1993).

There are many uses of surface display of Gram-positive bacteria (Ståhl & Uhlén, 1997; Hansson et al., 2001; Chen & Georgiou, 2002; Samuelson et al., 2002; Wernérus et al., 2002; Wernérus & Ståhl, 2004) or Gram-negative bacteria (Georgiou et al., 1993; Ståhl & Uhlén, 1997; Chen & Georgiou, 2002; Samuelson et al., 2002; Wernérus & Ståhl, 2004). The advantages of surface display systems in Gram-positive bacteria above Gram-negative bacteria are: first, in Gram-positive bacteria, the recombinant protein associates with only a single membrane translocation; second, the more rigid of Gram-positive bacteria cell surface is potentially more suitable for field applications such as bioadsorption; third, the thick peptidoglycan layer of Gram-positive bacteria possesses the inherent metal-binding capacity (Wernérus et al., 2001). This introduction focused on cell-surface display applications of Gram-negative bacteria because the host cell that was used in this thesis were cyanobacteria, whose cell surface resembles the cell surface structure of Gram-negative bacteria (Hoiczyk & Hansel, 2000).

1. Surface display of heterologous proteins in Gram-negative bacteria

1.1 Development of live bacterial vaccines

Recombinant live bacterial vaccines benefit not only better recognition by the immune system since their surface-exposed antigens directly present on the cell surface. Outer-membrane lipopolysaccharides also induce a very strong immune response which, furthermore, promote as an adjuvant for surface-anchored polypeptides (Georgiou et al., 1993). Many microbial surface systems have been used to display antigens or epitopes as follows:

LamB protein, which is a trimeric protein located in the outer membrane of *E. coli* K 12, was used to present two distinct portions of the preS2 region of hepatitis B virus on the cell surface of live *Escherichia coli* by using sandwich fusion (Charbit et al., 1987; Charbit et al., 1988).

Salmonella flagellin was used to express *Vibrio cholera* toxin subunit B, which is the virulence factor responsible for the massive secretory diarrhea (Lencer, 2001), in a nonvirulent *Salmonella dublin* by using sandwich fusion (Newton et al., 1989).

The hypervariable region of a cloned *Salmonella* flagellin gene was used to express a hepatitis B virus surface antigen epitope in a nonvirulent *S. dublin* live vaccine strain by sandwich fusion (Wu et al., 1989). The recombinant *S. dublin* led to both anti-hepatitis B surface and anti-flagellin responses in immunized animals.

P-fimbriae were used to target foot and mouth disease virus (*Plasmodium falciparum*) epitopes on the cell surface of *E. coli* by using sandwich fusion (Van Die et al., 1990).

Foreign antigenic determinants were transported to the *E. coli* cell surface by using the phosphate-limitation-inducible outer membrane protein (PhoE) of *E. coli* K-12 as a carrier (Agterberg et al., 1990).

The major small surface antigen of hepatitis B virus was displayed on the *E. coli* cell surface by fusing with C-terminus of ice nucleation protein (Inp) (Kim & Yoo, 1999). Inp is an outer membrane protein of *Pseudomonas syringae* that can catalyze the ice crystal formation of supercooled water (Wolber et al., 1986; Gurian-Sherman & Lindow, 1993).

1.2 High-throughput screening of polypeptide

The promising advantages of surface expression in *E. coli* above phage display technology are as follows (Georgiou et al., 1993). Firstly, in bacteria, surface expression is more adaptable because the suitable expression vector presents the ability to adjust the number of surface-expressed polypeptides. Secondly, when considering correct folding, the polypeptide of interest can be fused at either terminus of the anchor protein but, conversely, it can be done to only N-terminus of coat proteins of phage.

A library of mutated carboxymethyl cellulase (CMCase) genes generated by DNA shuffling was fused to Inp (Kim et al., 2000). The resulting fusion proteins with bacterial surface display were selectively screened for improved variants of CMCase. This technique shows that bacterial surface display of enzyme libraries is a useful technology for directed evolution and high-throughput screening of industrial enzymes.

Surface-display OPH library in *E. coli* was used to isolate novel enzymes with improved substrate specificities among OPH variants by using Inp system (Cho et al., 2002).

1.3 The development of whole cell adsorbents

The use of microbial biomass for heavy metal (e.g. Cd^{2+}, Ni^{2+}) removal is more than chemical processes because it provides economic and friendly wastewater treatments (Gupta et al., 2000). One or two hexahistidine was inserted into an exposed protein loop of outer membrane LamB protein of *E. coli* to generate a recombinant *E. coli.* The recombinant *E. coli* accumulated Cd^{2+} eleven folds more than *E. coli* expressing LamB without the histidine insert. In addition, the recombinant *E. coli* cell can adhere reversibly to a Ni^{2+}-containing solid matrix (Sousa et al., 1996).

Two recombinant *E. coli* were constructed to express heterologous proteins by using LamB of *E. coli* as an outer membrane anchoring protein. The first recombinant *E. coli* expressed *Saccharomyces cerevisiae* metallothionein, which are responsible for copper tolerance (called CUP1) by inserting CUP1 into LamB. Another recombinant *E. coli* expressed a human metallothionein, which is predominant in the adipose tissue (called HMT-1A), by inserting HMT-1A into LamB. The two recombinant *E. coli* efficiently bind to Cd^{2+} 15- to 20-fold more than

natural ability of *E. coli* host cells (Sousa et al., 1998).

Different numbers of copies of hexahistidine linkers (polyhistidine peptides) were expressed on *E. coli* cell surface by insertion into the seventh exposed loop of *E. coli* outer membrane protein C (OmpC) (Xu & Lee, 1999). The recombinant *E. coli* successfully adsorbed up to 32 μmol of heavy metal Cd^{2+} per g (dry weight) of cells.

An antibody fragment (scFv) anti-pollutant antibody specific to atrazine herbicide was fused to N-terminus of peptidoglycan-associated lipoprotein of *E. coli* (PAL) (Dhillon et al., 1999). Recombinant *E. coli* that expressed scFv-PAL fusion showed the anti-atrazine antibody on the cell surface.

1.4 Surface expression of enzymes

The targeting of biologically active proteins to bacterial cell surface creates potential application for many biotechnological uses. This system provides direct reaction of active enzymes with their substrates. β-lactamase (EC 3.5.2.6), which is a soluble periplasmic protein, was tandemly fused to the N-terminal targeting sequence of *E. coli* lipoprotein (Lpp) and *E. coli* outer membrane protein A (OmpA). This fusion protein was transported through the outer membrane and then, anchored on the cell surface of *E. coli* (Francisco et al., 1992).

The *Bacillus subtilis* carboxymethylcellulase (CMCase) (an endo-1,4-*β*-glucanase), whose substrate is a high molecular weight polymer, carboxymethylcellulose (CMC), was expressed on the *E. coli* surface by using Inp of *Psuedomonas syringae* (Jung et al., 1998b).

Enzyme levansucrase (EC 2.4.1.10), which is the extracellular sucrase produced by *Zymomonas mobilis*, is responsible for sucrose hydrolysis, levan formation, and oligosaccharide production (Crittenden & Doelle, 1994). Levan is a polysaccharide of fructose, which is produced when *Z. mobilis* ferments sucrose (Tano & Buzato, 2003). Inp-LevU fusion protein was successfully constructed by fusing levansucrase (LevU) to the C-terminus of Inp of *P. syringe*. Recombinant *E. coli* expressing Inp-LevU fusion protein was found to retain both the ice nucleation and whole cell LevU enzyme activities (Jung et al., 1998a).

Recombinant *E. coli* cells coexpressing OPH and *Vitreoscilla* hemoglobin (VHb) has shown enhancement of whole cell OPH activity (Kang et al., 2002).

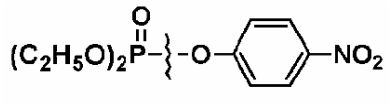
2. General background of organophosphate compound

Synthetic organophosphorus compound or organophosphate (OP) neurotoxins are used extensively as agricultural and domestic pesticides including insecticides, fungicides and herbicides. Examples of OP pesticides are paraoxon, fenamiphos, phorate, terbufos, ethoprophos, methylparathion (Singh et al., 2005; Reiner, 2007; Ortiz-Hernández et al., 2003) (figure 1). Furthermore, OPs can be used as chemical warfare agents such as sarin, tabun, VX and soman (Reiner, 2007) (figure 2).

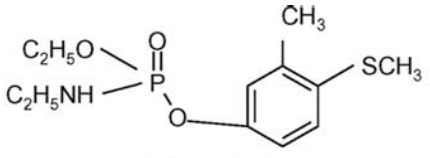
Two groups of esterases, which are serine esterases (e.g. acetylcholinesterase) and phosphoric triester hydrolases (EC 3.8.1) (e.g. organophosphorus hydrolase), can react with OPs (Reiner, 2007). OPs irreversibly inhibit acetylcholinesterase (AChE; EC 3.1.1.7) in the nervous system with subsequent accumulation of toxic levels of a neurotransmitter, acetylcholine (ACh) (EPA, 1999), by phosphorylating the serine hydroxy group of the active center of AChE (see the mechanism in Rosenfeld & Sultatos, 2006) as shown in three-dimensional structure of AChE with cholinesterase inhibitors (Silman et al., 1999; Shi et al., 2002). As a result of high toxicity of OPs to human health producing serious symptoms of muscle or vital organs and eventually death, the attentive decontamination of the waste and residual of OPs is necessary.

3. General background of organophosphorus hydrolase

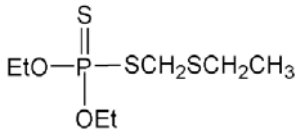
There have been reported that both chemicals and enzymes were used to efficiently detoxify OPs. Organomettallic compounds are the chemical compounds, whose structures contain both carbon (C) and a metal (Traeger, 2000). They are able to be used as catalysts for some of important industrial processes (Traeger, 2000). According to the definition by International Union of Pure & Applied Chemistry (IUPAC), a metallocene, which is a subset of a broader class of organometallic compounds, contains a transition metal and two cyclopentadienyl ligands coordinated in a sandwich structure, i.e. the two cyclopentadienyl anions are co-planar with equal bond lengths and strengths (*Metallocene*, 2006). Recently, the aquated form of the metallocene bis(η^5-cyclopentadienyl) molybdenum (IV) dichloride (Cp_2MoCl_2: Cp = cyclopentadienyl anions ($\eta^5C_5H_5$), η^5 pronounced "pentahapto" = the equivalent bonding of all five carbon atoms of each cyclopentadienyl ring) was shown to effectively hydrolyze the OP pesticides parathion and paraoxon via hydrolytic pathways in aqueous solution (Perera & Awana, 2006).



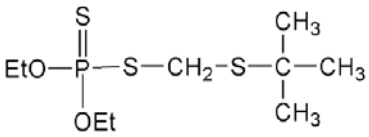
Paraoxon



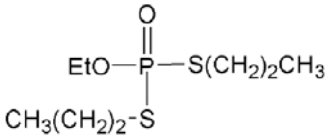
Fenamiphos



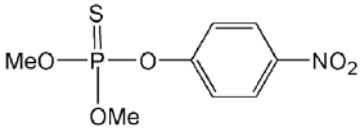
Phorate



Terbufos



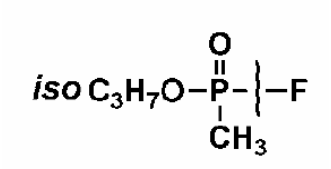
Ethoprophos



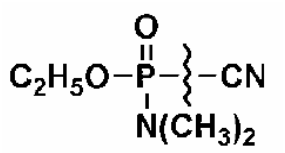
Methylparathion

Figure 1. Examples of organophosphorus pesticides

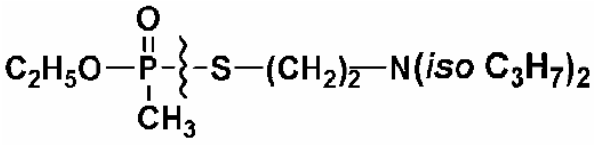
Examples of organophosphorus pesticides containing phosphotriester bonds are paraoxon (Reiner, 2007), fenamiphos (Singh et al., 2005), phorate, terbufos, ethoprophos and methylparathion (Ortiz-Hernández et al., 2003). Et = Ethyl group (CH_3CH_2). Me = Methyl group ($-CH_3$).



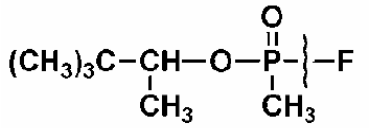
Sarin



Tabun



VX



Soman

Figure 2. Examples of organophosphorus compound (OP) nerve agents

Examples of OPs nerve agents are sarin, tabun, VX and soman (Reiner, 2007). *iso* = Isometric.

To date, by means of more studies than the chemical method, a diversity of enzymes has been shown to specifically hydrolyze organophosphorus esters with different phosphoryl bonds from the phosphotriester bonds of common insecticidal neurotoxins (e.g. paraoxon or coumaphos) to the phosphonate-fluoride bonds of chemical warfare agents (e.g. soman or sarin) (Dave et al., 1993). Examples of these enzymes are parathion hydrolases of an American isolate of *Pseudomonas diminuta* and a Philippine *Flavobacterium* sp. ATCC27551 (Mulbry et al., 1986), organophosphorus acid (OPA) anhydrolase enzymes found in a wide variety of prokaryotic organisms such as *Alteromonas undina* and *Alteromonas haloplanktis* C (Cheng et al., 1996) and an organophosphate-degrading enzyme from *Agrobacterium radiobacter* P230 (OPDA) (Yang et al., 2003). In addition, predicted aromatic hydrolase from *Flavobacterium* sp. ATCC27551 can degrade *p*-nitrophenol, a product from OPH hydrolysis (Siddavattam et al., 2003).

Nevertheless, most reports of OPs degradation by using enzymes produced from microorganisms referred to organophosphorus hydrolase or organophosphate hydrolase (OPH), which can detoxify OPs by hydrolyzing various phosphorus-ester bonds (P-O, P-F, P-CN, and P-S) between the phosphorus center and an electrophilic leaving group (Lai et al., 1995). OPH has been interesting for prevention, decontamination, treatment and remediation of OPs.

3.1 Characteristics of OPH

Organophosphorus or organophosphate hydrolase (OPH) also known as phosphotriesterase (PTE) was encoded from *opd* gene (Ang et al., 2005). It is a 35 kDa membrane-associated phosphoric triester hydrolase (EC 3.1.8.1), which contains a binuclear metal center with two metals (contains two Zn^{2+} ions per molecule) interactively involved in catalysis and/or structural functions (Lai et al., 1994). The *opd* gene from *P. diminuta* MG (McDaniel et al., 1988) and that from *Flavobacterium* sp. ATCC27551 (Mulbry & Karns, 1989) was sequenced. The *opd* gene encoded 35 kDa of the gene product, OPH.

Single crystal X-ray diffraction analysis of the apoenzyme showed the quaternary structure of dimeric enzyme OPH, whose overall fold consists of an alpha/beta barrel with eight strands of parallel beta-pleated sheet and two antiparallel beta-strands at the N-terminus (Benning et al., 1994; Grimsley et al., 1997). The

active site of OPH consists of a binuclear metal center, which contains up to two equivalents of zinc per a unit of the naturally isolated enzyme, and is located at the C-terminal portion of the beta barrel (Benning et al., 1995).

3.2 Hydrolysis of OPs by OPH

The examination of the mechanism and substrate specificity of OPH from *P. diminuta* with 30 analogues of OPs showed that paraoxon is the best substrate for OPH from *P. diminuta* and the rate limiting of the enzyme reaction is phosphorus-oxygen bond cleavage (Donarski et al., 1989). The catalytic mechanism for the enzymatic hydrolysis of paraoxon by OPH from *P. diminuta* showed that kinetic parameters V_{max} and V/K_m are 2100 s^{-1} and 4.8 X 10^7 $M^{-1}s^{-1}$ (Caldwell et al., 1991). The hydrolysis of OP pesticide by OPH of *Flavobacterium* sp. ATCC27551 is shown in figure 3 (Ortiz-Hernández et al., 2003). Specific activity of wild type OPH can be increased almost 2.5-fold by purifying or reconstituting the enzyme with Co^{2+} as shown in increase of turnover number (K_{cat}) of paraoxon from 2100 to over 5000 s^{-1} (Lai et al., 1994). Various detergents, including nonionic, anionic, cationic and amphoteric detergents, were used to study the compatibility with detergents of OPH from *Flavobacterium* sp. ATCC 27551 (Sode & Nakamura, 1997). They showed that the highest OPH activity using paraoxon (diethyl 4-nitrophenyl phosphate) as substrate in the presence of 0.01 % sodium carboxyl polyoxyethylene tridecylether was 1.7 fold higher than that without detergent. Furthermore, about 75 % of initial preparation of lyophilized OPH prepared in the presence of 60 mM trehalose, which is an excipient used in preparation of high active lyophilized biological materials, was stable after 25 days at 25 °C as 3 fold higher residual activity of the sample prepared in the absence of trehalose. These results lead to develop a detergent capable of both washing and degrading of OPs.

3.3 OPH mutants

OPH of *P. diminuta* and *Flavobacterium* sp. ATCC27551 have been thoroughly studied. Engineered mutants with enhanced activity and substrate specificity were constructed by substitutions of each of the histidine and cysteine residues in the catalytic domain of OPH to asparagine and serine residues respectively (Lai et al., 1994). In order to improve thermostability, catalytic properties and conformational stability of OPH, OPH mutants were constructed with substitution of

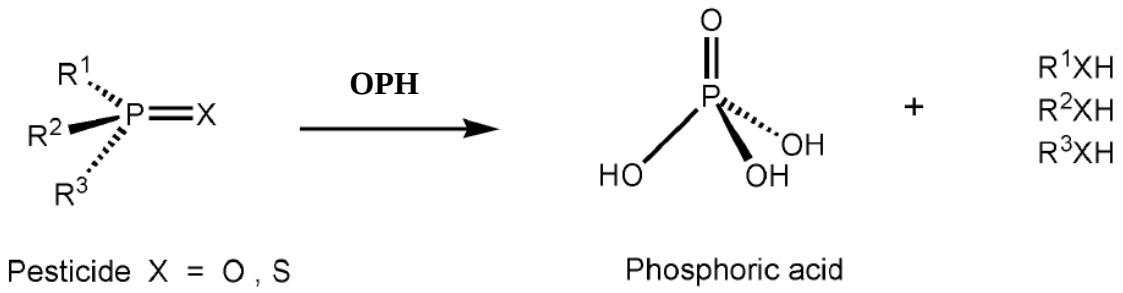


Figure 3. Pesticide hydrolysis by organophosphorus hydrolase from *Flavobacterium* sp.

X is either oxygen or sulfur. OPH is organophosphorus hydrolase. R^n is unspecified hydrocarbon chain. (Ortiz-Hernández et al., 2003)

the two zinc cations of the OPH active center by transition metal cations, Co^{2+} or Cd^{2+} (Rochu et al., 2004). The three isoforms had different pI (isoelectric point) values, pH-dependent activity profiles, catalytic properties and conformational stabilities, and showed non-superimposable electrophoretic titration curves. Optimum activity and maximum stability of OPH were displayed by Co^{2+}-OPH and Zn^{2+}-OPH, respectively. Three isoforms showed maximum activity at ≤ 35 ºC, an activation phase near 45 – 48 ºC and completely inactivated at 60 ºC. The optimum activities of three isoforms were between pH 8 and 9.4. The thermostability of the different OPHs at their optimum activities increased as the pH decreased, and the metal cation modulated stability.

3.4 OPH enzyme reactors

There were reports of enzyme reactors for OP degradation using both native and recombinant OPHs immobilized onto supports, such as cellulose matrixes and glass and nylon beads. The purification cost of OPH is high for enzyme reactor. Bifunctional fusion proteins consisting of OPH from *Flavobacterium* fused with C-terminus of cellulose binding domain (CBD) from *Clostridium cellulovorans* were immobilized onto a variety of cellulose matrixes (Richins et al., 2000). CBDs are structurally and functionally independent, non-catalytic modules that are found in many cellulose or hemicellulose degrading enzymes (Linder et al., 1998). They are used in fusion proteins as low-price tags for affinity purification or immobilization (Shpigel et al., 1999; Carrard et al., 2000). The immobilized enzyme reactor using this recombinant OPH was able to repeat hydrolysis with 100 % degradation efficiency over 45 days when paraoxon was used as substrate. The use of immobilized whole cells either growing or non-growing system in reactor for OP detoxification is more cost-effective without purification step of OPH and higher metabolic activity than the enzyme reactors (Mulchandani et al., 1999; Richins et al., 2000). Recombinant *Escherichia coli* have been used to express *opd* gene from *Pseudomonas* sp. and *Flavobacterium* sp. because it is easier to grow and to develop to large-scale detoxification processes (Chen & Mulchandani, 1998). The *opd* gene with deletion of signal peptide has intracellularly expressed in *E. coli* and caused greater hydrolase activity than intracellular expression of the cells that contained the entire *opd* gene (Mulbry & Karns, 1989).

3.5 OPH biosensors

The amperometric OPH electrodes generally rely on monitoring the oxidation of *p*-nitrophenol, a product from catalysis of OPs (Deo et al., 2005). An OPH biosensor using the enzyme electrode composed of OPH and albumin, which co-immobilized to a nylon net and directly contacted with a carbon paste electrode, was used to detect OPs including parathion, EPN, paraoxon and fenitrothion (Chough et al., 2002). The detection limit of this biosensor is 15 nM for parathion and 20 nM for paraoxon (about 100 times less than optical or electrochemical sensor methods). An amperometric OPH biosensor based on a carbon nanotube – modified transducer was developed by using a bilayer approach with the OPH layer atop of the carbon nanotube film (Deo et al., 2005). The carbon nanotube layer improved sensitivity and stability of anodic detection of p-nitrophenol.

The fiber-optic microbial biosensor is attractive for online monitoring of detoxification process of OPs in wastewaters. This biosensor was used to directly measure OPs by determination of *p*-nitrophenol, which is a product of OPH hydrolysis. Whole cells of *Flavobacterium* sp. expressing OPH was immobilized by trapping in glass fiber filter and was used as biocomponent along with optic fiber system (Kumar et al., 2006). The immobilized microbial biocomponent was disposable, commercial and showed high reproducibility and uniformity. The sample used only 75 µl. The detection limit is 0.3 µM methyl parathion.

3.6 Protein fusion of green fluorescent protein and OPH

As a consequence of fusion protein strategy, green fluorescent protein (GFP) was used as the quantitative marker of OPH (Cha et al., 2000) in *E. coli*. GFP tandemly fused with two copies of OPH (GFP-$OPH_{n=2}$) presented higher yield in comparison to one copy fusion (GFP-OPH) (Wu et al., 2001). It would seem that GFP-$OPH_{n=2}$ probably formed a structure enhancing stability and yield of OPH. In contrast, a fusion comprising two copies of OPH without GFP and an operon fusion of two OPHs with two independent ribosomal binding sites give lower yields than one OPH expressed alone (Wu et al., 2001).

4. OPH cell surface expression in bacteria

Pseudomonas putida KT2440, *Moraxella* sp and *E. coli* were used as hosts for OPH expression on the cell surface. *Pseudomonas putida* KT2440 expressed OPH on

the cell surface by using Inp from *P. putida* under the control of *tac* promoter (Shimazu, 2003). A recombinant *Moraxella* sp. expressed OPH on the surface by using Inp anchor can degrade *p*-nitrophenol, which is a product of OP hydrolysis (Shimazu et al., 2001b). The expression of OPH on the surface of *E. coli* using the ice nucleation protein InaV from *P. syringae* INA5 anchor system is 5-fold higher than either the surface expression of OPH using Lpp-OmpA or using the truncated ice nucleation protein InaK (Shimazu et al., 2001a).

4.1 OPH surface expression of *E. coli* immobilized cell

Whole cells of *E. coli* with surface display of OPH by using Lpp-OmpA fusion system was immobilized on highly porous sintered glass beads (Mansee et al., 2000). This provides subsequent application of these cells in a continuous-flow packed bed bioreactor for the biodetoxification of OPs.

E. coli with OPH expression on the cell surface was immobilized on nonwoven polypropylene fabric in order to detoxify OPs in wastewaters (Mulchandani et al., 1999). *E. coli* cell expressing both OPH and CBD on the cell surface enabled simultaneous hydrolysis of OPs and immobilization via specific adsorption to cellulose (Wang et al., 2002).

4.2 Surface expressed OPH in *E. coli* used as biosensors

Biosensors, which contain genetically engineered *E. coli* and different types of electrode, were used to determine OPs rapidly and cost-effectively. *E. coli* cells expressing OPH on the cell surface was used in an amperometric microbial biosensor for the direct measurement of OPs (Mulchandani et al., 2001). The sensor was based on a carbon paste electrode containing the recombinant *E. coli* cells. A biosensor was generated by using recombinant *p*-nitrophenol-degrading/oxidizing bacteria *Pseudomonas putida* JS444 with surface display of OPH as biological sensing element and a dissolved oxygen electrode as the transducer (Lei et al., 2006). Under the optimum conditions, detection limits of this biosensor were as 277 ppb and 1.6 ppm of OPs, fenitrothion and EPN, respectively.

5. Cyanobacteria

5.1 Characteristics of cyanobacteria

Cyanobacteria, which are also known as blue-green algae from their pigment color and their behavior like algae in nature, are prokaryotes that perform

oxygenic photosynthesis similar to photosynthesis of higher plant chloroplasts by using chlorophyll a and phycobilin pigments (Wilmotte, 1994; Brian, 1992).

Natural strains of cyanobacteria are robust and ubiquitous in a variety, wide range and extreme of environments (e.g. oceanic water, permanently frozen Antarctic lakes, ponds and hot spring) and terrestrial environments (e.g. deserts, soil and rocks). They also play an important role in symbiosis with plants (Brian, 1992). Many cyanobacteria can perform nitrogen fixation under either aerobic or anaerobic conditions (Wilmotte, 1994). Cyanobacteria can produce toxins causing serious health risks for the animal and human population when toxic cyanobacterial blooms occur in drinking water reservoirs. To remove cyanotoxins for cyanobacteria, *Planktothrix rubescens* and *Microcystis aeruginosa*, from raw water, an ozonation batch reactor coupled with several filtrations was developed (Hoeger et al., 2002).

Cyanobacteria are a very interesting resource with potential benefits in many different areas (e.g. health food, food and feed additive, soil conditioner, cosmetics and medicine). They can grow on a very simple culture medium (autotrophic) with the ability to produce high-value compounds using only light as their main energy source (phototrophic), carbon dioxide and water (Barbosa, 2003).

The cyanobacterium *Synechococcus* sp. PCC7942 responds to exposure of divalent heavy metal (copper, zinc and cadmium) ions by expressing messenger RNA for the stress protein GroEL and for the metal-binding protein metallothionein (Ybarra & Webb, 1999). These responses of cyanobacteria were used to protect the cell from damage, which caused by divalent metal ions.

5.2 Classification of cyanobacteria

Several systems have been used to classified cyanobacteria by considering either structural or ecological determination. For example, 22 genera of the collection culture deposited in the American Type Culture Collection were placed in 5 sections as follows (Rippka et al., 1979): Section I is composed of unicellular cyanobacteria with spherical, cylindrical or ovoid cells that reproduce either by binary transverse fission or by budding, such as member of genus *Synechococcus*. Section II is composed of unicellular cyanobacteria that reproduce by multiple fission, such as member of the genera *Dermocarpa* and *Xenococcus*. Section III is composed of non-heterocystous, filamentous cyanobacteria that divide in a plane, such as genera

Spirulina and *Oscillatoria*. Section IV is composed of heterocystous, filamentous cyanobacteria that divide in a plane, such as genera *Anabaena* and *Nostoc*. Section V is composed of heterocystous, filamentous cyanobacteria that divide in more than a plane, such as genera *Fischerella*.

5.3 Cyanobacterial cell surface

The overall structure of cyanobacteria (figure 4) is similar to that of Gram-negative bacteria, which consists of three distinct layers, the outer membrane, the peptidoglycan (murein) layer and the inner membrane (plasma membrane) (BenZ & Bauer, 1988), even though their peptidoglycan layer is markedly larger than that of most Gram-negative bacteria (Hoiczyk & Hansel, 2000). The cyanobacterial cell surface has the thick peptidoglycan layer like in Gram-positive bacteria and has the outer membrane like presence in Gram-negative features. The envelope structure of gliding cyanobacteria, *Phormidium uncinatum*, is shown in figure 5 (Hoiczyk & Baumeister, 1995).

S-layer (stand for surface layer) has been found in cyanobacterial envelope since 1972 as an external surface layer. S-layers are widely distributed in almost all phylogenetic branches of bacteria and *Archea* (Engelhardt & Peter, 1998). They are formed by two-dimentional, monomolecular crystalline arrays of identical units of protein or glycoprotein subunits. S-layer protein is the major protein of bacteria because S-layer protein is up to 25 % of the total protein of the bacterial cell (Šmarda et al., 2002). The cell walls of gliding filamentous cyanobacterium *Oscillatoriaceae* species are multilayered Gram-negative cell wall, which is covered with a complex external double layer (Hoiczyk & Baumeister, 1995). The first layer is a tetragonal crystalline S-layer anchored on the outer membrane. The second array is formed by parallel, helically arranged surface fibrils.

One of the major functions of cell envelope is to allow sufficient transport nutrients and metabolite into and out of the cell across membrane systems. The cyanobacterial cell membrane systems are divided into two systems (Mackle & Zilinskas, 1994; Hoiczyk & Hansel, 2000). First, the typical eubacterial cell envelope includes an inner membrane, periplasmic space and an outer membrane. The second system is the photosynthetic thylakoids. Examples of transport system of cyanobacteria are porins SomA (Umeda et al., 1996; Hansel et al., 1998),

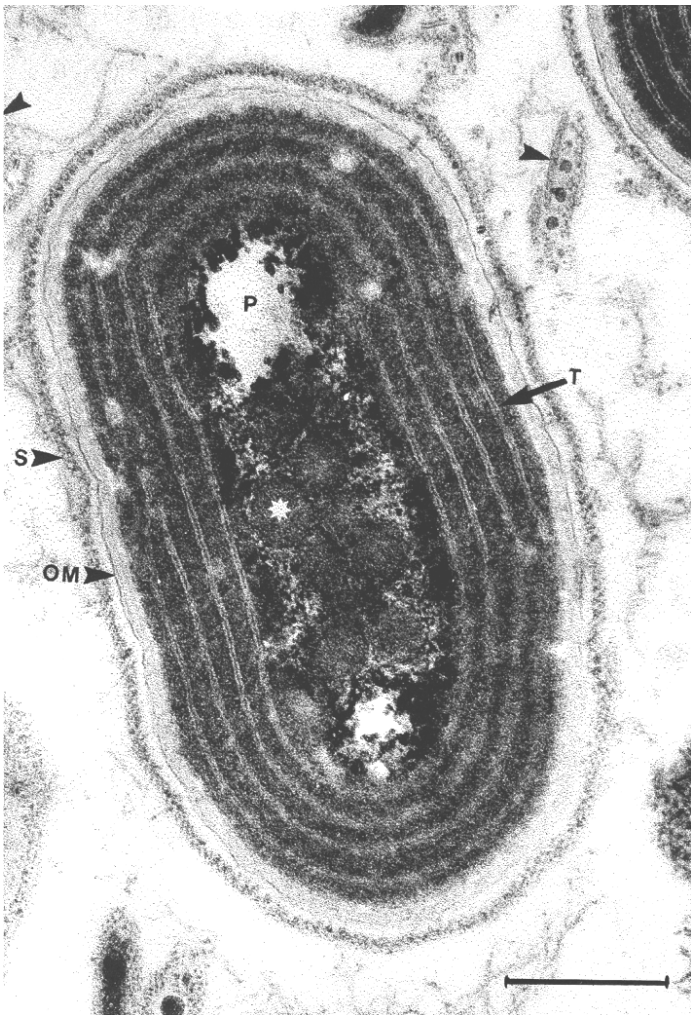


Figure 4. Electron micrograph of section of *Synechococcus* sp. strain GL24

Electron micrograph of longitudinal section of cell was covered by an S-layer (S). OM is outer membrane. Star is carboxysome. P is polyphosphate granule. T is thylakoid. Arrow-heads are S-layer fragments with associated outer membrane material. Bar is 500 nm long. (Hoiczyk & Hansel, 2000)

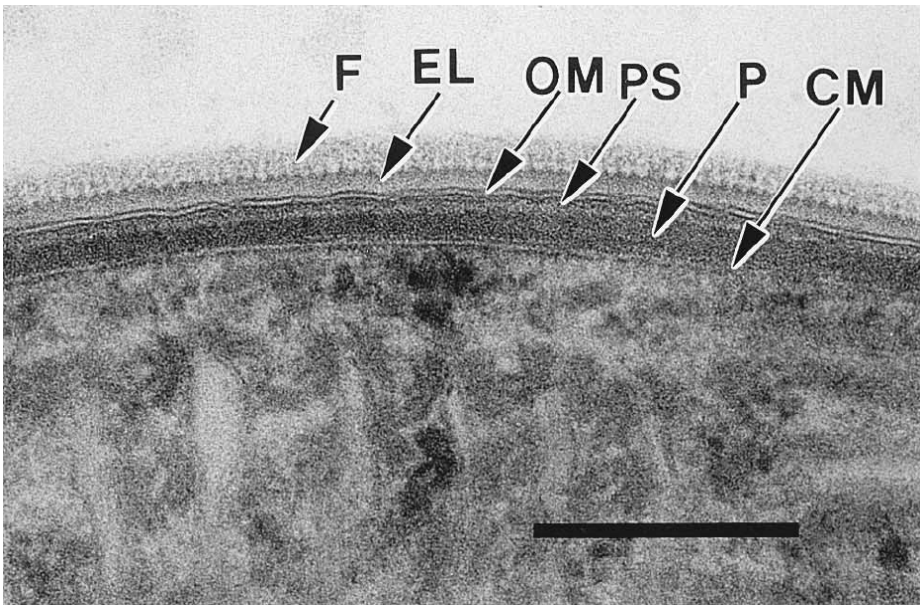


Figure 5. Electron micrograph of section of *Phormidium uncinatum*

Envelope profiles of freeze-substituted gliding cyanobacteria *P. uncinatum* (strain Tubingen) show the complex external layer with its serrated substructure. CM is cytoplasmic membrane. EL is serrated external layer. F is hair-like fibers. OM is outer membrane. P is peptidoglycan. PS is periplasmic space. A bar is 200 nm long. (Hoiczyk & Baumeister, 1995)

ATP-binding cassette (ABC) transporter and the junctional pore complex (JPC) (Hoiczyk & Hansel, 2000).

5.4 Cyanobacterial SomA system

The *somA* (*Synechococcus* outer membrane) gene is a gene that encodes aporin in the outer cell membrane protein of *Synechococcus* sp. PCC7942 (Umeda et al., 1996) (sequences information, figure 9 in materials and methods) and *Synechococcus* sp. PCC6301 (Hansel et al, 1998) (figure 13 in materials and methods). Based on the nucleotide sequence, the *somA* gene was predicted to comprise 531 amino acids with a calculated molecular mass of 57,136 Da. Parts of the deduced amino acid sequence of SomA protein share similarities with two bacterial cell-surface proteins, which are the S-layer protein of *Thermus thermophilus* (Faraldo et al., 1992) and the fagellin of *Campylobacter coli* (Guerry et al., 1991).

The *somA* gene contains a signal peptide-like sequence at its N–terminus which was capable of mediating protein translocation across the cytoplasmic membrane into the outer membrane of *E. coli* (the translocation of SomA::OmpF protein fusion) (Umeda et al., 1996). There was a report from Sauer et al. (2001) that nitrogen starvation can induce the expression of *somA* gene under the control of P_{somA} of *Synechococcus* PCC7942. The accumulation of SomA porin was detected under nitrogen starvation (Görl et al., 1998; Sauer et al., 2001).

5.5 Cyanobacterial molecular genetics

Molecular genetics were used to study various properties of cyanobacteria such as oxygenic photosynthesis, aerobic and anaerobic nitrogen fixation, light-regulated gene expression and cell differentiation (Tandeau de Marsac & Houmard, 1987). Examples of shuttle vectors that can replicate in both *Synechococcus* PCC7942 and *E. coli* are pUC104 (Kuhlemeier et al., 1981), pUC105 (Kuhlemeier et al., 1981), pUC303 (Kuhlemeier et al., 1983), pSG11 (Golden & Sherman, 1983), pUC105-31 (Dzelzkalns et al., 1984) and pKG (Chungjatupornchai et al., 1999).

Transfer of genetic material into cyanobacterial cells can occur by conjugation (Wolk et al., 1984; Flores & Wolk, 1985; Sode et al., 1992), electroporation (Thiel & Poo, 1989) or transformation (Devilly & Houghton, 1977; Stevens & Porter, 1980).

In addition, complete genome of a laboratorial used cyanobacterium

Synechococcus elongatus PCC7942 was sequenced (GenBank accession number NC_007604).

Several promoters were used to control the gene expression in cyanobacteria. The promoter of the *ps2B* gene of *Amaranthus hybridus* was used to control a reporter gene, chloramphenicol acetyl transferase (CAT) gene, in *Synechococcus* sp. PCC6301 (Dzelzkalns et al., 1984). Lamda promoter and the temperature-sensitive repressor gene *c*I857 of bacteriophage lambda were used to control the *cat* gene in *Synechococcus* PCC7942 (Friedberg & Seijffers, 1986). λP_R, *tac* and *recA* promoters were used to control the *cat* reporter gene in *Synechocystis* sp. PCC6803 (Ferino & Chauvat, 1989). The $tRNA^{pro}$ promoter of *Synechococcus* PCC7942 (P_{tRNA}) was isolated and characterized as a strong promoter (Chungjatupornchai et al., 1999; Chungjatupornchai et al., 2002).

Cyanobacteria have been used as hosts to express heterologous genes. For example, expression of the larvicidal gene of *Bacillus sphaericus* 1593M (Tandeau de Marsac et al., 1987) and expression of the gene for the ethylene-forming enzyme of *P. syringe* (Fukuda et al., 1994) in *Synechococcus* PCC7942. The gene coding mosquito-larvicidal protein of *Bacillus thuringiensis* was expressed in *Synechocystis* PCC6803 and in *Synechococcus* PCC7942 (Chungjatupornchai, 1990; Chungjatupornchai & Panyim, 1994).

5.6 OPH expression in cyanobacteria

In *Synechococcus* PCC7942, the *opd* gene from *Flavobacterium* sp. under the control of λP_R promoter was intracellularly expressed (Udtanut, 2005). The *opd* gene under the control of the P_{tRNA} was intracellularly expressed and surface expressed in *Synechococcus* PCC7942 by using Inp of *P. syringae* as anchoring protein (Chungjatupornchai, W., unpublished data).

CHAPTER II
OBJECTIVES

In this thesis, SomA (*Synechococcus* outer membrane) system of *Synechococcus* sp. PCC7942 (Umeda et al., 1996) was used as a surface-anchoring motif to express enzyme organophosphorus hydrolase (OPH), which is encoded from *opd* gene of *Flavobacterium* sp. ATCC27551. This approach was used for detoxification of organophosphorus pesticides. The *somA*-*opd*-[Nos-ter] gene cassette was expressed under the control of the promoter of *somA* gene of *Synechococcus* PCC7942 (P_{somA}), which is highly susceptible to nitrogen down-shift (Sauer et al., 2001), or $tRNA^{pro}$ promoter (P_{tRNA}), which is a strong promoter of *Synechococcus* PCC7942 (Chungjatupornchai et al., 2002).

The aims of this thesis are:

1. To study the expression of *opd* gene on the cell surface of *Synechococcus* PCC7942 using SomA system.
2. To compare the OPH activities of *somA*-*opd*-[Nos-ter] gene cassette under the control of *somA* promoter (P_{somA}) or $tRNA^{pro}$ promoter (P_{tRNA}) of *Synechococcus* PCC7942.
3. To investigate the expression of P_{somA}-*somA*-*opd*-[Nos-ter] gene cassette under nitrogen starvation condition.

CHAPTER III
MATERIALS AND METHODS

1. Materials

1.1 Bacterial strains

Synechococcus PCC7942 strain R2-SPc (Kuhlemeier et al., 1983) (hereafter referred to as *Synechococcus*) was used as a model system to study the cell surface display of OPH using SomA system. Genomic DNA of *Synechococcus* was used as template for the polymerase chain reaction (PCR) of the full-length *somA* gene. *Escherichia coli* strain MC1061 (Casadaban & Cohen, 1980) and strain DH5α were used as a host for construction and rescue of recombinant plasmids.

1.2 Plasmids

Plasmid pUC18 (figure 6) (Yanisch-Perron et al., 1985) was used to construct plasmid pUC18-SomA (figure 11).

Plasmid pUC18-PtOPH (figure 7) harbors the $tRNA^{pro}$ promoter (P_{tRNA}), the *opd* gene of *Flavobacterium* sp. ATCC27551 and the nopaline synthase terminator (Nos-ter) (Juntawong & Chungjatupornchai, unpublished data). The *opd*-[Nos-ter] gene fusion was used to construct gene cassette P_{somA}-*somA*-*opd*-[Nos-ter].

Plasmid pKGT-SalI (figure 8) was used to construct plasmid pKTB. pKGT-Sal has been modified from plasmid pKGT (Chungjatupornchai et al., 2002),which is able to replicate in both *Synechococcus* and *E. coli* and contains kanamycin resistance gene (Km).

1.3 Oligonucleotide primers

Synthetic oligonucleotides (table 1) were purchased from PROLIGO (Singapore). Locations and sequences of the primers targeting at the *somA* gene are shown in figure 9.

1.4 Chemicals and enzymes

Chemicals for BG-11 medium (William, 1988) were from Merck.

Table 1. Sequences of DNA primers

Primer	Sequences (5'→3') [a]	Target sequence
Lk2-somAF1	5'-**TCCCCGGGTGGTCAGTCCCTT**ATGAAAC-GCCTTTTCTCGGCGCTG-3'	P_{tRNA} (bold letters) and *somA*
Nos-rev2	5'-CGGGATCCATCTAGTAACATAGATGACA-CCG-3'	Nos-ter
OPH-fwd2	5'-ATGTCGATCGGCACAGGCGATCGG-3'	*opd*
OPH-rev2	5'-CGCCGCCACGATATGAACGTC-3'	*opd* [b]
somA-fwd1	5'-CGGGATCCGAGGGTGGGGTCCGCAAG-3'	Upstream of *somA*
somA-fwd2	5'-CGGGATCCTCTAGAGCTACGGCACTAG-3'	Upstream of *somA*
somA-rev1	5'-ACGCGTCGACAAAGCTCTCGACAGCGAG-3'	Downstream of *somA*
somA-rev2	5'-ACGCGTCGACTCGCCCATCCCTAACTAA-ACAG-3'	Downstream of *somA*
somA-rev3	5'-**CCGATCGCCTGTGCCGATCGACAT**GGT-AGCACCAAAAGGCTGGAAGGC-3'	*somA* (bold letters) and *opd*
T1	5'-CGGGATCCTTGCCCTCGCCTCCTAGTCC-3'	P_{tRNA} [c]

[a] Underline sequences are restriction sites, GGATCC for *Bam*HI and GTCGAC for *Sal*I. These sites were used for excision of inserts to construct recombinant plasmids.

[b] Udtanut, 2005

[c] Chungjatupornchai et al., 2002

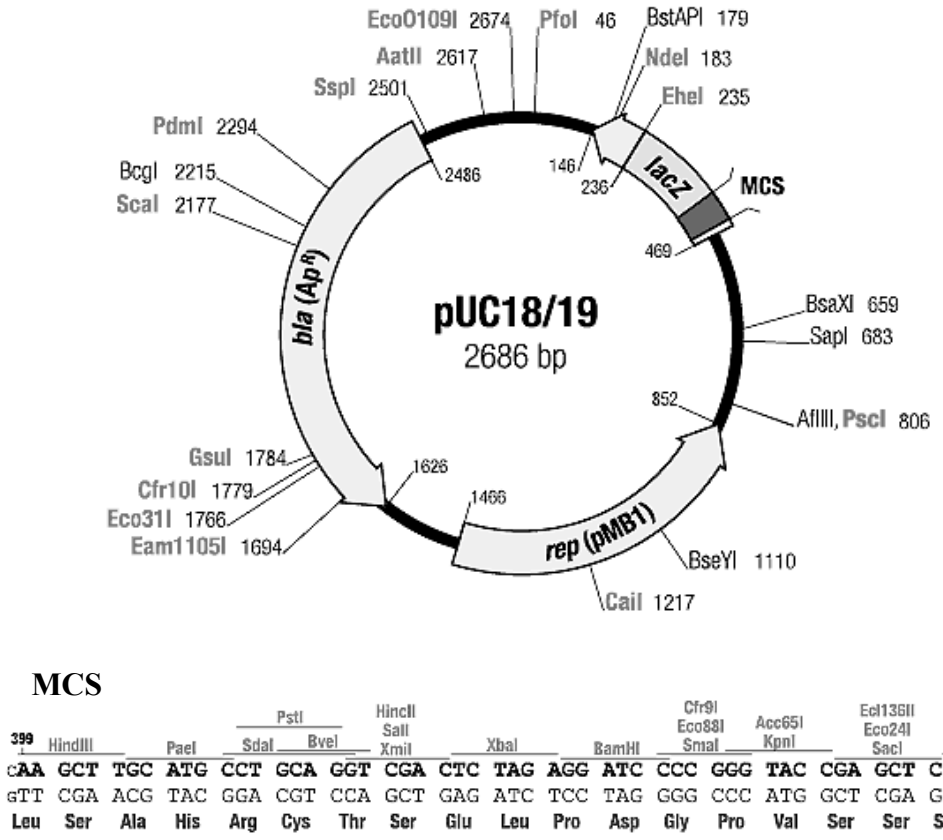


Figure 6. A description and partial restriction map of plasmid pUC18

This figure relates to GenBank/EMBL accession number L09136 (Fermentas Life Sciences, 2006). Plasmid pUC18 is a small, high copy number and generally used plasmid-cloning vector in *Escherichia coli*. This figure shows restriction sites of several restriction enzymes in multiple cloning sites (MCS). β-galactosidase gene (*lacZ*) is a reporter gene. *rep* (pMB1) is a replicon from the pMB1 plasmid. *bla* (Ap^R) is β-lactamase or ampicillin resistance gene.

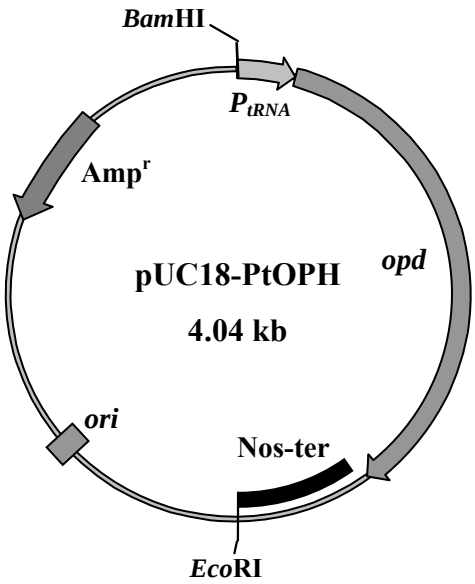


Figure 7. A description and partial restriction map of plasmid pUC18-PtOPH

The pUC18-PtOPH has been modified from plasmid pUC18 by inserting the gene cassette P_{tRNA}-opd-[Nos-ter] into *Bam*HI and *Eco*RI sites (Juntawong & Chungjatupornchai, unpublished data). *ori* is the origin of replication. Amp^r is β-lactamase or ampicillin resistance gene. The figure is not drawn to scale.

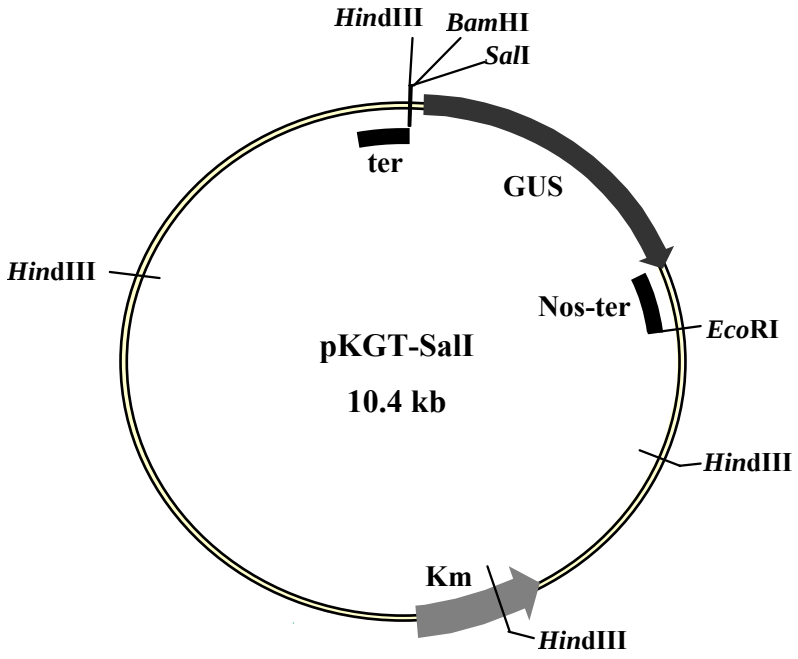
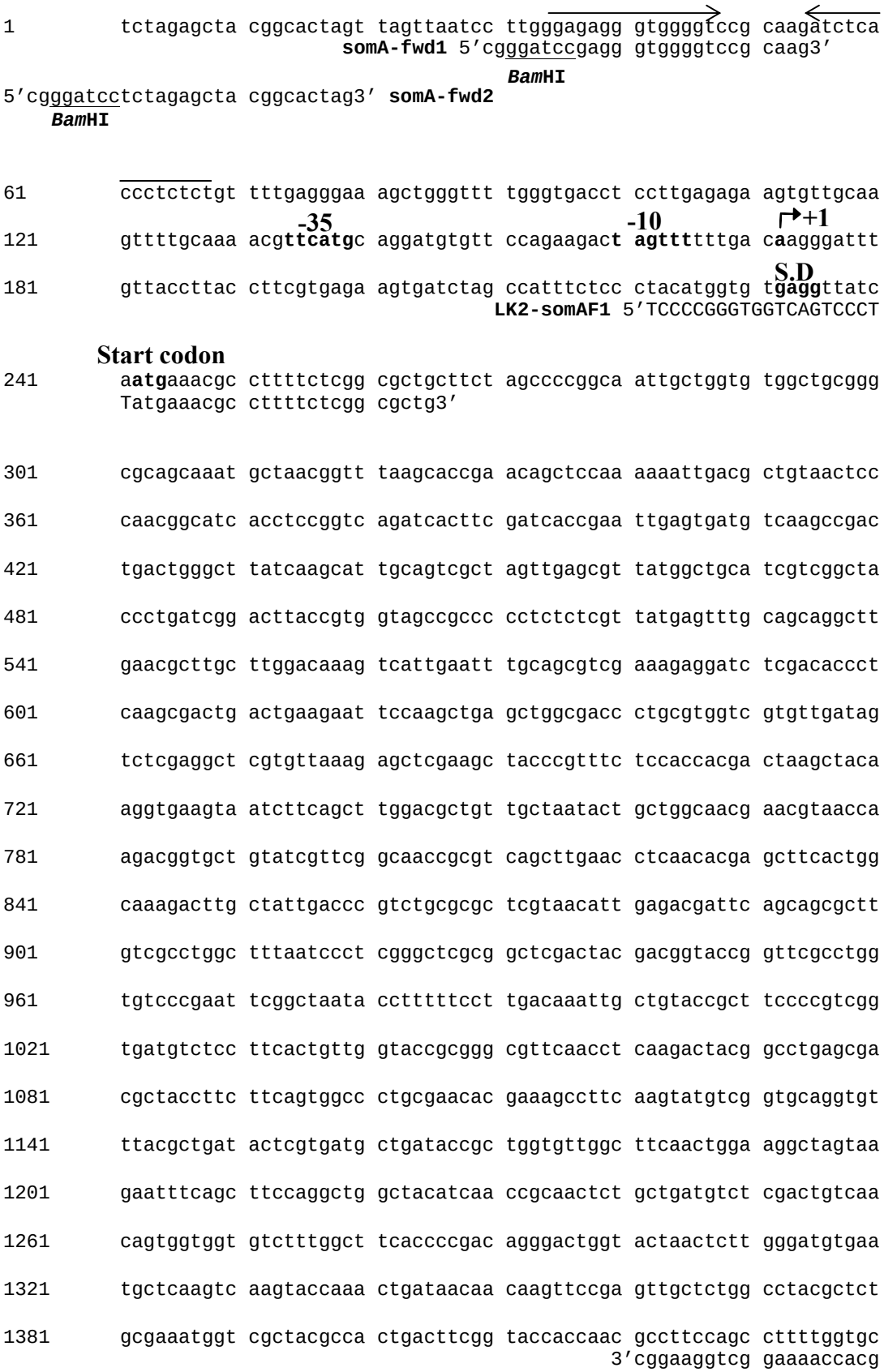


Figure 8. A description and partial restriction map of plasmid pKGT-SalI

The pKGT-SalI has been modified from plasmid pKGT (Chungjatupornchai *et al.*, 2002) by addition of *SalI* site (Chungjatupornchai, unpublished data). ter is the transcription-translation terminator signal of phage T4. GUS is β-glucuronidase gene. Km is kanamycin resistance gene. The figure is not drawn to scale.


somA-fwd1 5′cgggatccgagg gtggggtccg caag3′
BamHI
5′cgggatcctctagagcta cggcactag3′ somA-fwd2
BamHI
-35
-10
+1
S.D
LK2-somAF1 5′TCCCCGGGTGGTCAGTCCCT
Start codon
Tatgaaacgc cttttctcgg cgctg3′

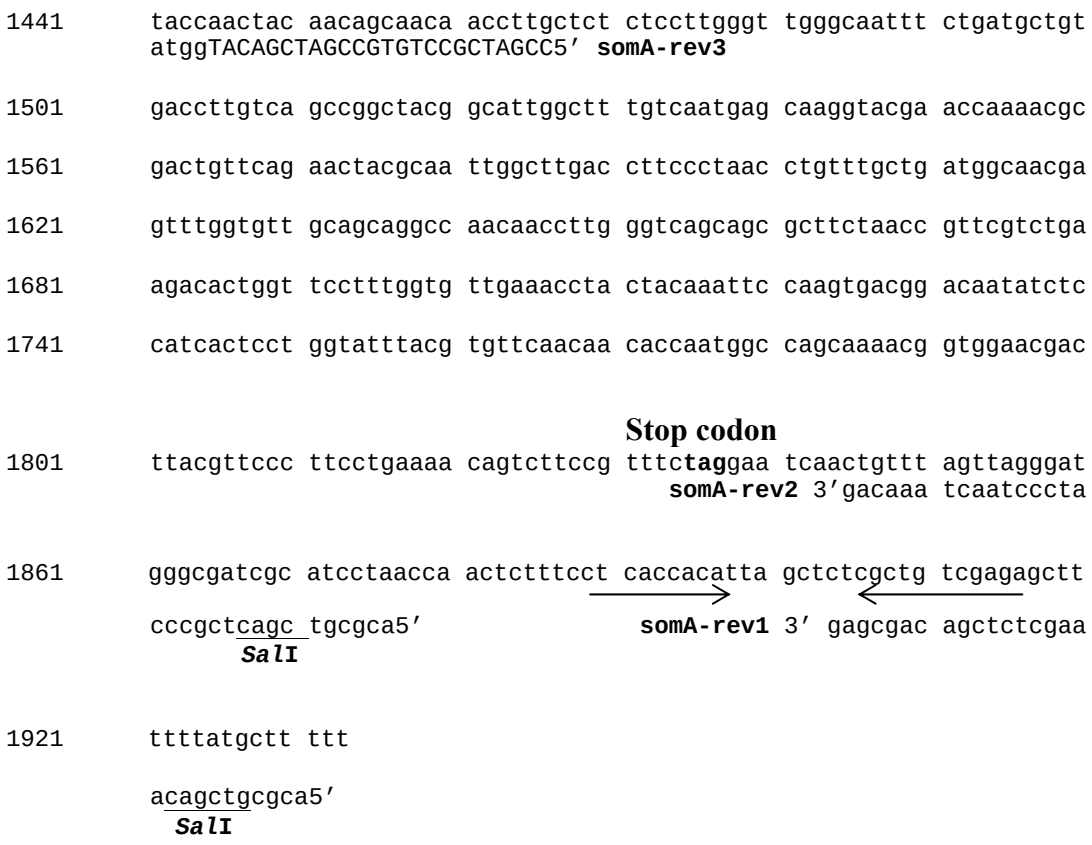


Figure 9. The nucleotide sequences of the *somA* gene of *Synechococcus* PCC7942

This figure shows the full-length *somA* gene from GenBank database accession number D64077. The putative transcription start site (+1), -10 and -35 promoter regions, possible Shine Dalgano sequences (S.D.), the start and stop codons of the *somA* gene are indicated. The *Bam*HI and *Sal*I sites in the specific primers are underlined. The uppercase letters in somA-rev3 primer are the sequences on a complementary strand of the *opd* gene of *Flavobacterium* ATCC27551 starting from the start codon (TAC). The uppercase letters in the sequences of LK2-somAF1 primer are the sequences of the $tRNA^{pro}$ promoter (P_{tRNA}). Inverted horizontal arrows indicate 2 regions of potential stem-loop formation presumably functioning as ρ-independent transcription terminators (Hansel et al., 1998).

Bacto-agar was from Difco Laboratories. Emzymes and chemicals for the polymerase chain reaction were from Promega. Restriction enzymes were from Promega and Biolabs. T4 DNA ligase, 5x T4 DNA ligase buffer and Large Fragment of DNA Polymerase I (Klenow Fragment) were from Invitrogen. Calf intestinal alkaline phosphatase (CIAP) and CIAP buffer were from Promega. GENECLEAN II® kit was from BIO 101 Inc. ABI PRISM™ Big Dye sequencing reaction kit was from Perkin Elmer. All antibiotics and others chemicals were from Sigma and Merck.

2. Growth conditions of microorganism

2.1 Growth conditions of *Synechococcus*

Synechococcus were cultured in liquid BG-11 medium with shaking or on solid BG-11 medium (1.5 % Difco Bactoagar). Recombinant *Synechococcus* were cultured in liquid BG-11 medium with shaking or on solid BG-11 medium (1.5 % Difco Bactoagar) with 25 μg/ml kanamycin in medium. The cells were grown at 28 °C or 30 °C, under continuous illumination of fluorescent bulbs (i.e. 4 daylight and 2 gro-lux bulbs) with constant light intensity 3,500 lux.

The selection of transformants used 10 μg/ml kanamycin in BG-11 medium.

2.2 Growth conditions of *E. coli*

E. coli was grown at 37 °C in LB broth with 250 rpm shaking or on LB agar plate (Sambrook & Russell, 2001).

The selection of transformants used 100 μg/ml ampicillin in medium.

3. Isolation of genomic DNA of *Synechococcus* (modified from method of Draper et al., 1988)

A pea sized globe of cells was scraped from a healthy stock plate (grown for 5 days) into a microtube and resuspended in 400 μl of TES buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA pH 7.5 and 0.1 % (w/v) SDS). Subsequently, 100 μl of 50 mg/ml lysozyme was mixed in the tube and incubated at 37 °C with shaking for 10 min. Then, 1/8 volume of 10 % sarkosyl was added before mixing by vortex. After that, 600 μl of phenol was added before the mixture was mixed and tortured on a rotating wheel for 15 min. The tube was centrifuged at 14,000 rpm (20,800 *g*) for 10 min. The aqueous layer was transferred to a new microtube. Next, 20 μl of 10 μg/μl RNase A was added into the new tube and incubated at 37 °C with shaking for 15 min. Then, ¼ volume of 5 M NaCl was added before mixing by vortex. After that, ¼ volume of

prewarmed at 65 °C CTAB-NaCl was added before mixing by vortex. Later, 600 µl of chloroform: isoamyl alcohol (24:1) was added into the mixture. After mixing by vortex, the mixture was centrifuged at 14,000 rpm (20,800 *g*) for 5 min. Next, equal volume of isopropanol was used to precipitate the DNA in aqueous layer. After centrifugation at 14,000 rpm (20,800 *g*) for 15 min, the genomic DNA pellet was dissolved in 50 µl in MilliQ water and its concentration and purity was estimated by 1 % agarose gel electrophoresis.

4. Agarose gel electrophoresis

The agarose gel was prepared by dissolving the gel powder in TBE buffer (89 mM Tris-HCl pH 8.0, 89 mM Boric acid and 2.5 mM EDTA) at 1-2 % (w/v) concentration and heated to complete dissolve. Then, ethidium bromide was added to a final concentration of 1.5 µg/ml. To analyze DNA patterns, DNA samples mixed with 1/5 volume of agarose gel loading dye (0.1 % Bromophenol blue, 40 % Ficoll and 5M EDTA) was loaded into gel slot under submarine condition. The electrophoresis was carried out at constant 100 volts. The DNA patterns were visualized under UV light and photographed by variable-intensity transilluminator Gel Doc 1000 (BIORAD) and UVP (Life science)**.**

5. Prediction of DNA secondary structure of locations of primers upstream and downstream of *somA* gene

The secondary structure formation of single stranded DNA of locations of primers on the coding strand (for somA-fwd1 and somA-fwd2 primers) or the complementary strand (for somA-rev1 and somA-rev2 primers) of nucleotide sequences upstream and downstream of the *somA* gene was predicted by using the computer program mfold version 3.1 by Zuker and Tuner (Mathews, 1999; Zuker et al., 1999; Zuker, 2003) at the default temperature, 37 °C.

6. DNA ligation

6.1 Restriction endonuclease digestion/analysis

Concentration of restriction endonucleases used for cloning and verifying plasmids (0.2-1 µg) and PCR products, reaction buffers, temperature and time for incubation were depending on suggestions of enzyme manufacturers.

6.2 Purification of desired insert by GENECLEAN II Kit

GENECLEAN II Kit (BIO 101 Inc.) was used to purify PCR products in

agarose gel. Under the UV light, the PCR band was excised from agarose gel and transferred to a microtube to determine the weight. Then, 3 volumes of NaI solution and 1/3 volume of TBE modifier was added into the tube. The mixture was incubated at 55 °C until the gel was completely melted. After that, 5 µl of GLASSMILK (50 % (v/v) acid-washed glass beads, 50 % (v/v) H_2O) was added into the tube. The mixture was incubated at room temperature for 15 min, centrifuged at 14,000 rpm (20,800 *g*) for 5 s. After removing supernatant, pellet was washed with 500 µl of NEW WASH solution (20 mM Tris, 1mM EDTA, 100 mM NaCl). Later, the mixture was centrifuged at 14,000 rpm (20,800 *g*) for 5 s and repeated washing 2 times. Then, 5 µl of TE buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.0) was used to resuspend the pellet after drying. The tube was incubated at 55 °C for 5 min. After centrifugation at 14,000 rpm (20,800 *g*) for 30 s, the supernatant containing the eluted DNA was transferred to a new microtube.

6.3 Purification of plasmid vector by phenol extraction

A pellet of a plasmid vector was dissolved in MilliQ water. A half of volume of phenol was added before mixing by vortex. Then, a half of volume of chloroform: isoamyl alcohol (24:1) was added. The mixture was mixed by vortex and centrifuged at 14,000 rpm (20,800 *g*) for 5 min. A volume of chloroform: isoamyl alcohol (24:1) was added. The reaction was mixed and centrifuged at 14,000 rpm (20,800 *g*) for 5 min before 1/10 volume of sodium perchlorate ($NaClO_4$) was added. After mixing by vortex, a volume of isopropanol was added. The mixture was mixed and centrifuged at 14,000 rpm (20,800 *g*) for 5 min to obtain the pellet. Finally, the pellet was dissolved by MilliQ water.

6.4 DNA ligation condition (modified from Sambrook & Russell, 2001)

A digested plasmid and a desired insert DNA fragment were ligated together using T4 DNA ligase. The 10 µl ligation reaction was included 100 ng of plasmid DNA (50 ng of plasmid pKTB after end-filling by using large fragment of DNA polymerase I (Klenow fragment)), 400-800 ng of inserted DNA, 5 U of T4 DNA ligase (1 U for ligation of pKTB after end-filling), 1X bacteriophage T4 DNA ligase buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT and 5 % (w/v) Polyethylene Glycol-8000) and 1 µl of 10 mM ATP, adjusted with MilliQ

water and slowly cooled down in water from 25 °C to 4 °C for 16 – 20 h.

7. Construction of plasmid pKT-Psom-opd and pKT-Tsom-opd

7.1 The DNA end-filling of *Sal*I/*Eco*RI digested plasmid pKGT-SalI using Klenow fragment

To generate plasmid pKTB for construction of plasmid pKT-Psom-opd and pKT-Tsom-opd, large fragment of DNA polymerase I (Klenow fragment) was used for filling the *Sal*I and *Eco*RI termini of digested plasmid pKGT-SalI to be blunt ends (figure 10). The reaction was performed in 30 µl by adding 1 µg of DNA, 1X buffer (50mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl), 0.017 mM of each dNTP, 0.017 U/µl of Klenow fragment diluted with Klenow dilution buffer (50 mM potassium phosphate (pH 7.0), 100 mM KCl, 1mM Dithiothreitol, 50 % (v/v) glycerol) and milliQ water to 30 µl of final volume. After gently mixing and briefly centrifuging, the reaction was incubated on ice for 20 min. Fill-in reaction was terminated by phenol extraction.

7.2 Dephosphorylation at 5' end of digested plasmid pKGT-SalI fragment by using CIAP

pKTB was digested with *Bam*HI and then, generated two sticky ends. The digested pKTB was dephosphorylated in 50 µl reaction including 1 µg of plasmid pKTB, 5 µl of 10X CIAP buffer, 1 U of calf intestinal alkaline phosphatase (CIAP) (Promega) and adjusted with MilliQ water. Then, the reaction was incubated in water bath at 37 °C for 30 min and immediately purified by phenol.

7.3 Amplification of the *somA* gene using the polymerase chain reaction (PCR)

Synechococcus genomic DNA was used as the template for amplification of the *somA* gene. The somA-fwd1 and somA-rev2 primer were designed to amplify the *somA* gene. The 25 µl of PCR reaction mixture contained 1x reaction buffer (75 mM tris-HCl pH 9.0, 50 mM KCl and 20 mM $(NH_4)_2SO_4$), 200 µM of each dNTPs, 62 ng of genomic DNA template, 0.4 pmol of each primer and 1.5 U of *Pfu* DNA polymerase. The volume of the reaction was adjusted with miliQ water to 25 µl. 35 cycles of PCR was carried out after an initial denaturation step at 94 °C for 5 min. Each cycle consisted of 3 holding temperature, at 94 °C for 1 min for denaturation, at

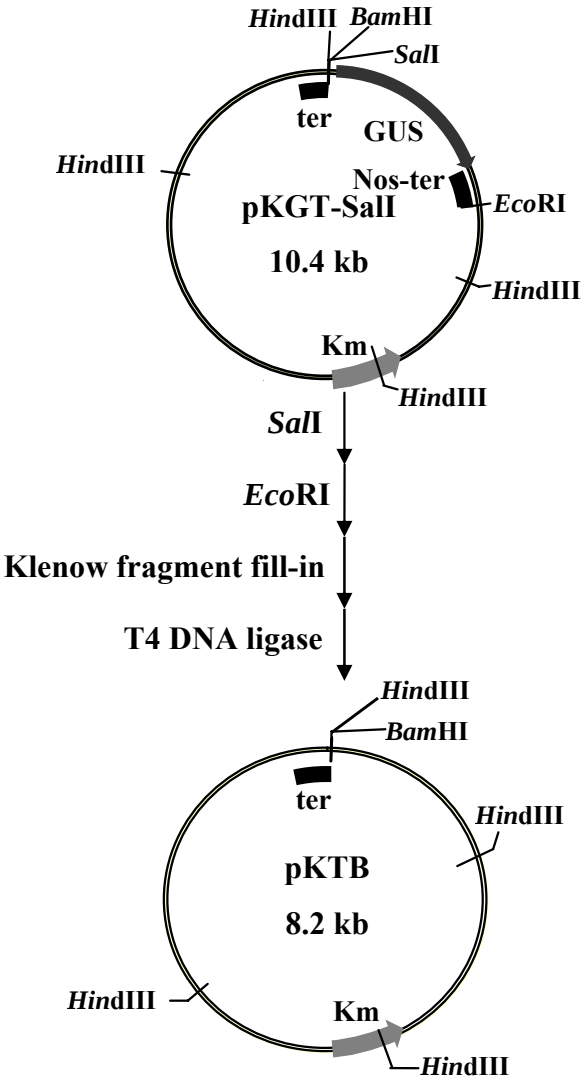


Figure 10. Schematic diagram for construction of plasmid pKTB

Description and partial restriction maps of plasmid pKGT-SalI and pKTB were shown. β-glucuronidase gene (GUS) was eliminated from pKGT-SalI by using *Sal*I and *Eco*RI digestions respectively. The *Sal*I and *Eco*RI termini were filled in by Klenow fragment to make blunt ends. Then, the end filled plasmid was self ligated by using T4 DNA ligase. ter is the transcription-translation terminator signal of phage T4. Km is kanamycin resistance gene. The figure is not drawn to scale.

55 °C for 30 s for annealing and at 72 °C for 4 min for extension, followed by final extension at 72 °C for 7 min.

7.4 Construction of plasmid pUC18-SomA

The PCR product was digested with *Bam*HI and *Sal*I to generate sticky ends for ligation. The digested PCR product was purified using GENECLEAN Kit II. 1.8 kb of *somA* PCR product from 6.3 (materials and methods) was cloned into the *Bam*HI and *Sal*I sites of plasmid pUC18 to generate 4.5 kb of recombinant plasmid pUC18-SomA (figure 11).

7.5 Overlap extension PCR

Reactions were the same as those of 6.3 (materials and methods) except using 15 pmol of each primer and about 100 ng of each dissimilar DNA template containing complementary sequences with each other at the 3' ends.

7.6 Construction of plasmid pKT-Psom-opd

With the same compositions as PCR reaction of 6.3 (materials and methods), PCR of the *somA* and the *opd* genes were performed. 50 ng of plasmid pUC18-SomA was used as the template for the amplification of the *somA* gene by using somA-fwd1 and somA-rev3 primers. 50 ng of plasmid pUC18-PtOPH was used as the template to amplify the *opd*-[Nos-ter] by using OPH-fwd2 and Nos-rev2 primers. 35 cycles of both PCR were carried out after an initial denaturation step at 94 °C for 5 min. Each cycle consisted of 3 holding temperatures: 94 °C for 1 min for denaturation, 55 °C for 30 s for annealing and 72 °C for 3 min for extension, and followed by final extension at 72 °C for 7 min.

The PCR products of the *somA* and the *opd* genes from the above amplifications were used as templates for overlap extension PCR using somA-fwd1 and Nos-rev2 primers to obtain gene cassette P_{somA}-*somA*-*opd*-[Nos-ter] (figure 12). The *opd* gene was fused in frame with the threonine (T) of the SomA protein (figure 13). The 25 cycles of PCR was carried out after an initial denaturation step at 94 °C for 5 min. Each cycle consists of 3 holding temperature: 94 °C for 1 min, 60 °C for 1 min, 72 °C for 6 min following by a final extension at 72 °C for 7 min. The PCR product of overlap extension PCR was cloned into *Bam*HI site of dephosphorylated pKTB fragment (6.2 in materials and methods) to obtain plasmid pKT-Psom-opd (figure 14).

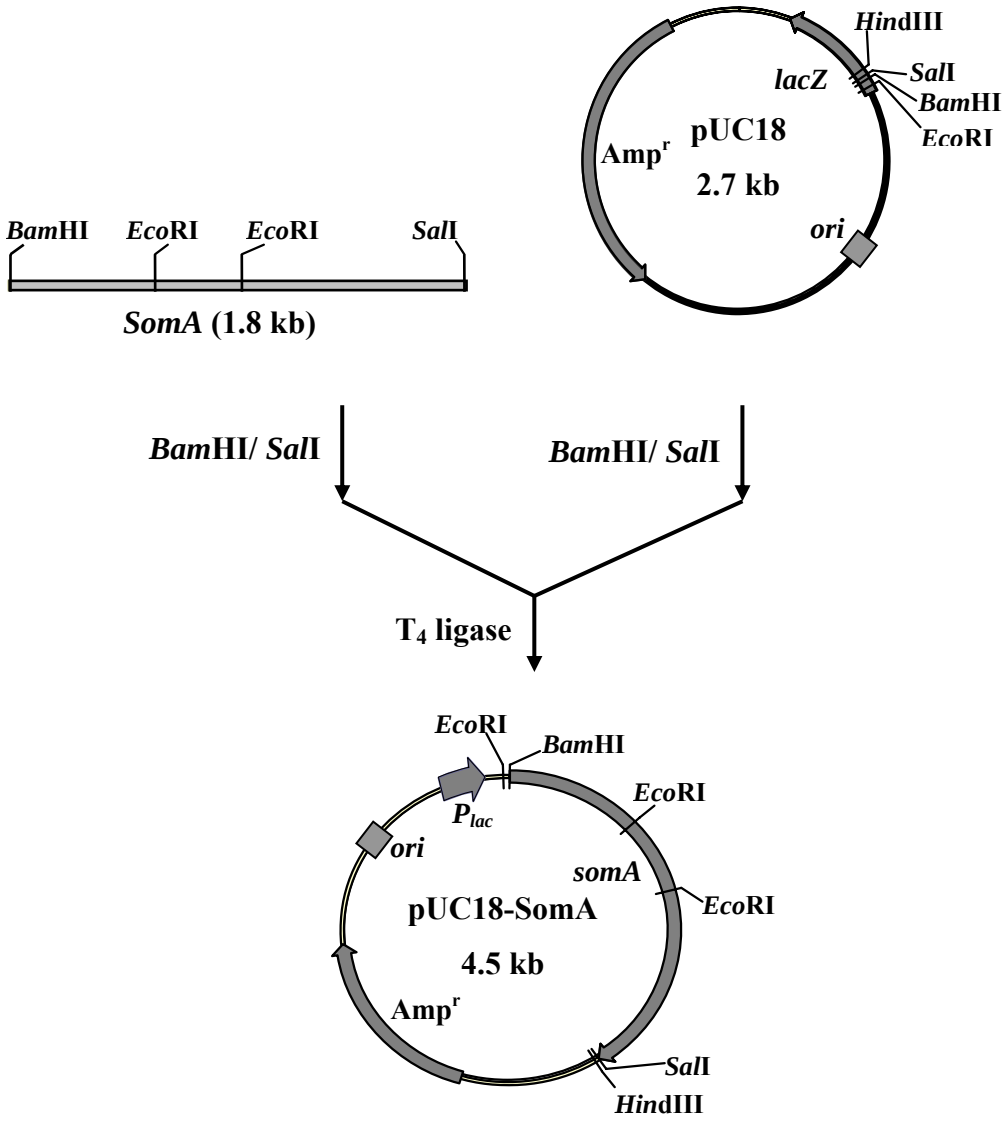


Figure 11. Schematic diagram for construction of plasmid pUC18-SomA

The 1.8 kb of the *somA* gene from PCR product was cloned into the *Bam*HI and *Sal*I sites of plasmid pUC18. The resulting plasmid, pUC18-SomA, is 4.5 kb. *lacZ* is β-galactosidase gene. *ori* is the origin of replication. P_{lac} is the *lac* promoter. Amp^r is β-lactamase or ampicillin resistance gene.

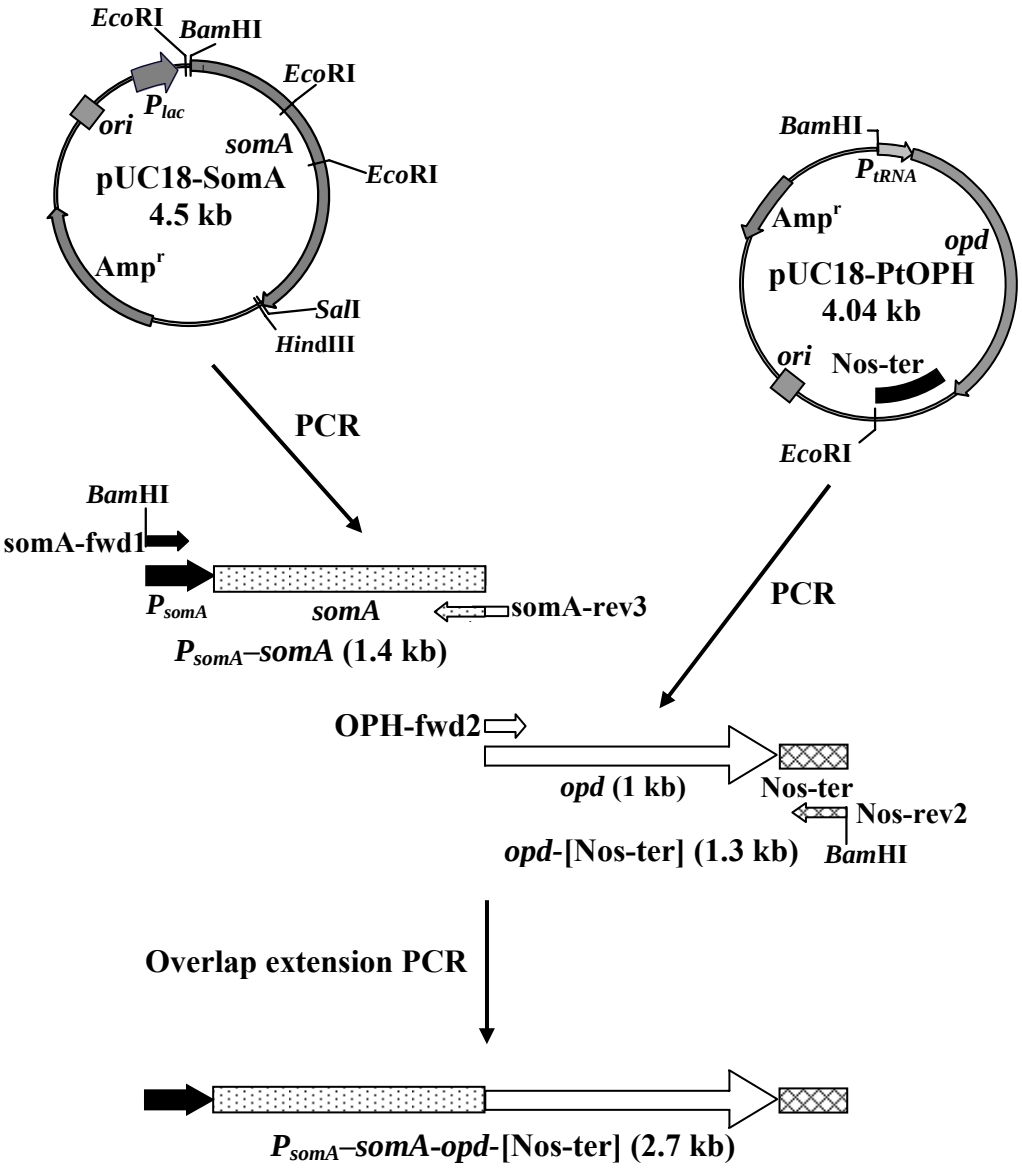


Figure 12. Schematic diagram for construction of gene cassette P_{somA}–*somA-opd*-[Nos-ter]

The 1.4 kb of the *somA* PCR product including the promoter of the *somA* gene was fused in frame with the 1.3 kb of PCR product of *opd*-[Nos-ter] gene fusion by using overlap extension PCR. The resulting gene fusion is 2.7 kb of gene cassette P_{somA}–*somA-opd*-[Nos-ter]. Amp^r is β-lactamase or ampicillin resistance gene. *ori* is the origin of replication. P_{lac} is the *lac* promoter. The figure is not drawn to scale.

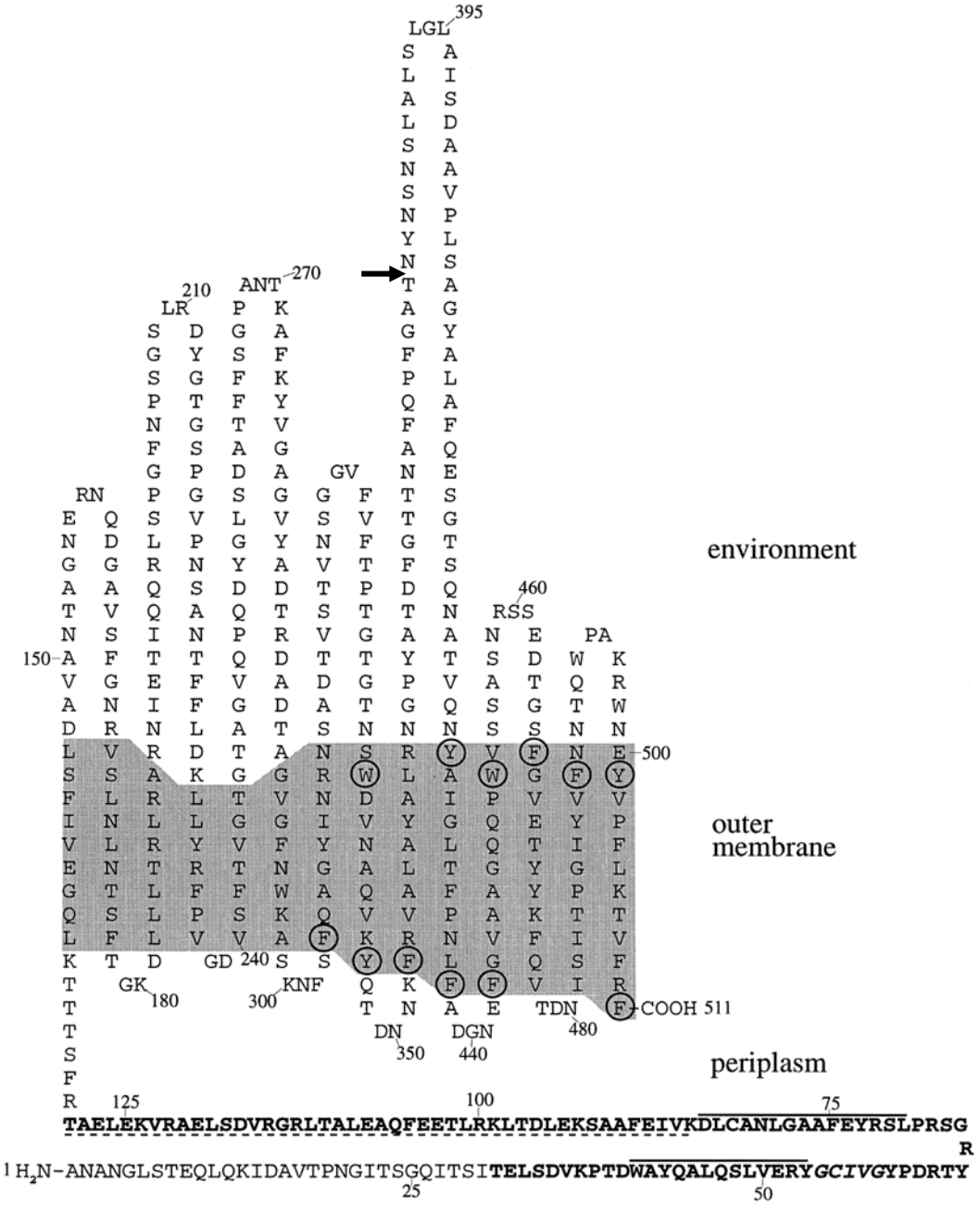


Figure 13. A putative topology of SomA protein in *Synechococcus* PCC6301

The putative topology of SomA protein encoded from the *somA* (*S*ynechococcus outer membrane) gene of *Synechococcus* PCC6301 (Hansel et al., 1998). The nucleotide sequences of the *opd* gene that start from its start codon (ATG) were fused in frame with the *somA* gene next to threonine (T) of the *somA* gene indicated by an arrow (figure 22).

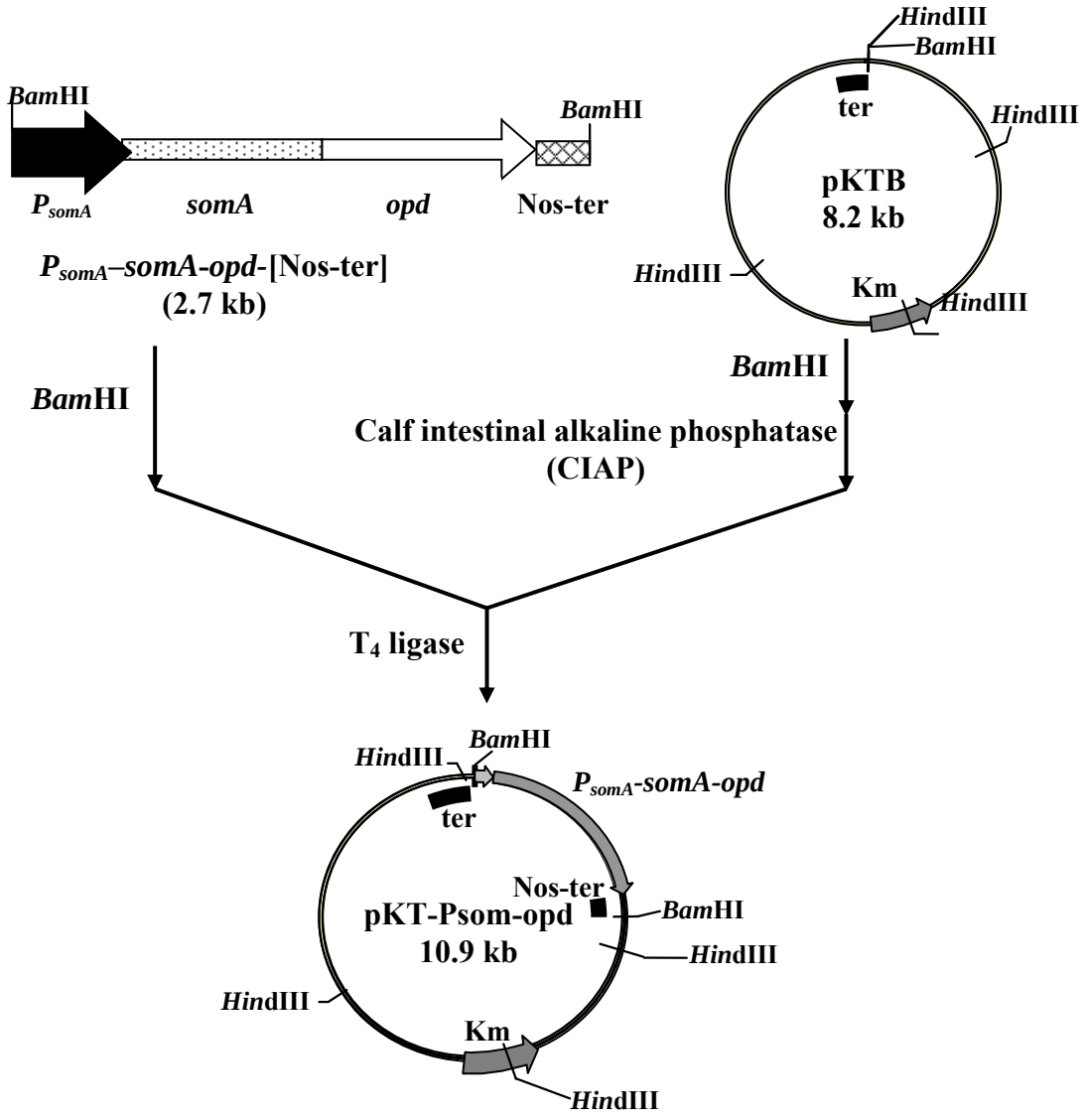


Figure 14. Schematic diagram for construction of plasmid pKT-Psom-opd-[Nos-ter]

The 2.7 kb of gene cassette P_{somA}-*somA*-*opd*-[Nos-ter] from overlap extension PCR was cloned into the *Bam*HI site of plasmid pKTB. The resulting 10.9 kb of plasmid pKT-Psom-opd contained the promoter of the *somA* gene, the *somA* gene, the *opd* gene and the nopaline synthase terminator (Nos-ter). ter is the transcription-translation terminator signal of phage T4. GUS is β-glucuronidase gene. Km is kanamycin resistance gene. The figure is not drawn to scale.

7.7 Construction of plasmid pKT-Tsom-opd

In order to generate gene cassette P_{tRNA}-*somA*-*opd*-[Nos-ter], the PCR reaction using plasmid pKT-Psom-opd as a template was performed (figure 15) with the same compositions as that of 6.3 (materials and methods), except the amount of templates. The 10 ng of plasmid pKT-Psom-opd was the template for amplification by using Lk2-somAF1 and Nos-rev2 primers. 35 cycles of these PCR were carried out after an initial denaturation step at 94 °C for 5 min. Each cycle consisted of 3 holding temperature, at 94 °C for 1 min, at 55 °C for 30 s and at 72 °C for 5:30 min, and followed by final extension at 72 °C for 7 min.

The P_{tRNA} and the *somA*-*opd*-[Nos-ter] gene fusion were used as template for the overlap extension PCR using T1 and Nos-rev2 primers to obtain gene cassette P_{tRNA}-*somA*-*opd*-[Nos-ter] (figure 15). The 35 cycles of PCR was carried out after an initial denaturation step at 94 °C for 5 min. Each cycle consisted of 3 holding temperature: 94 °C for 1 min, 55 °C for 30 s, 72 °C for 5:30 min following by a final extension at 72 °C for 7 min. The PCR product of overlap extension PCR was cloned into the dephosphorylated *Bam*HI site of pKTB (from 6.2 in materials and methods) to obtain plasmid pKT-Tsom-opd (figure 16).

8. Transformation of *E. coli* and *Synechococcus* PCC7942

8.1 Transformation of plasmids into *E. coli*

8.1.1 Preparation of *E. coli* competent cells

Single colony of *E. coli* MC1061 was inoculated in 3 ml LB broth and then grown for 16-20 h at 37 °C with shaking. The 250 µl of stock culture was transferred into 25 ml LB broth in 200 ml flask, incubated at 37 °C until OD_{600} reached 0.2, and then cell was collected by centrifugation at 4,500 rpm for 6 min at 4 °C. The cell pellet was then resuspened in 20 ml of ice-cold 0.1 M $MgCl_2$. This cell suspension was subsequently centrifuged at 4,500 rpm for 6 min at 4 °C. After that the cell pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and recovered by centrifugation. The cell pellet was added with 2 ml of ice-cold 0.2 M $CaCl_2$ and 15 % glycerol, then aliquoted 100 µl of each in 1.5 ml tube and kept at -80 °C.

8.1.2 Transformation of plasmids into *E. coli* (Sambrook & Russell, 2001)

The 2 µl of ligation mixture was added into 10 µl pre-warmed 65 ˚C

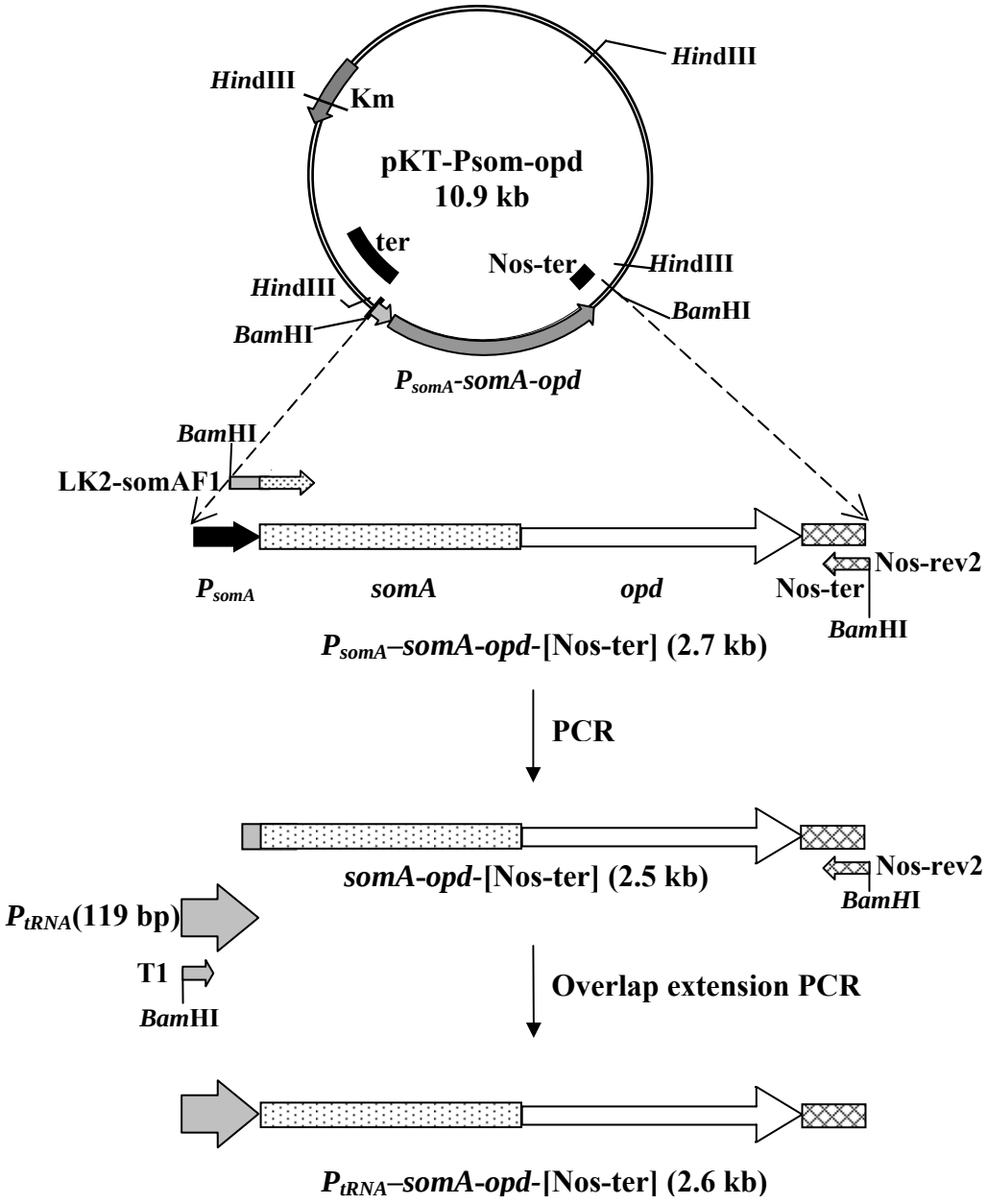


Figure 15. Schematic diagram for construction of gene cassette P_{tRNA}–*somA-opd*-[Nos-ter]

The 2.5 kb of *somA-opd*-[Nos-ter] gene cassette was amplified by using LK2-somAF1 and Nos-rev2 primers. The result is 2.6 kb of gene cassette P_{tRNA}–*somA-opd*-[Nos-ter] obtained from overlap extension PCR by using the $tRNA^{pro}$ promoter (P_{tRNA}) and *somA-opd*-[Nos-ter] as templates and T1 and Nos-rev2 primers. Km is kanamycin resistance gene. ter is the transcription-translation terminator signal of phage T4 The figure is not drawn to scale.

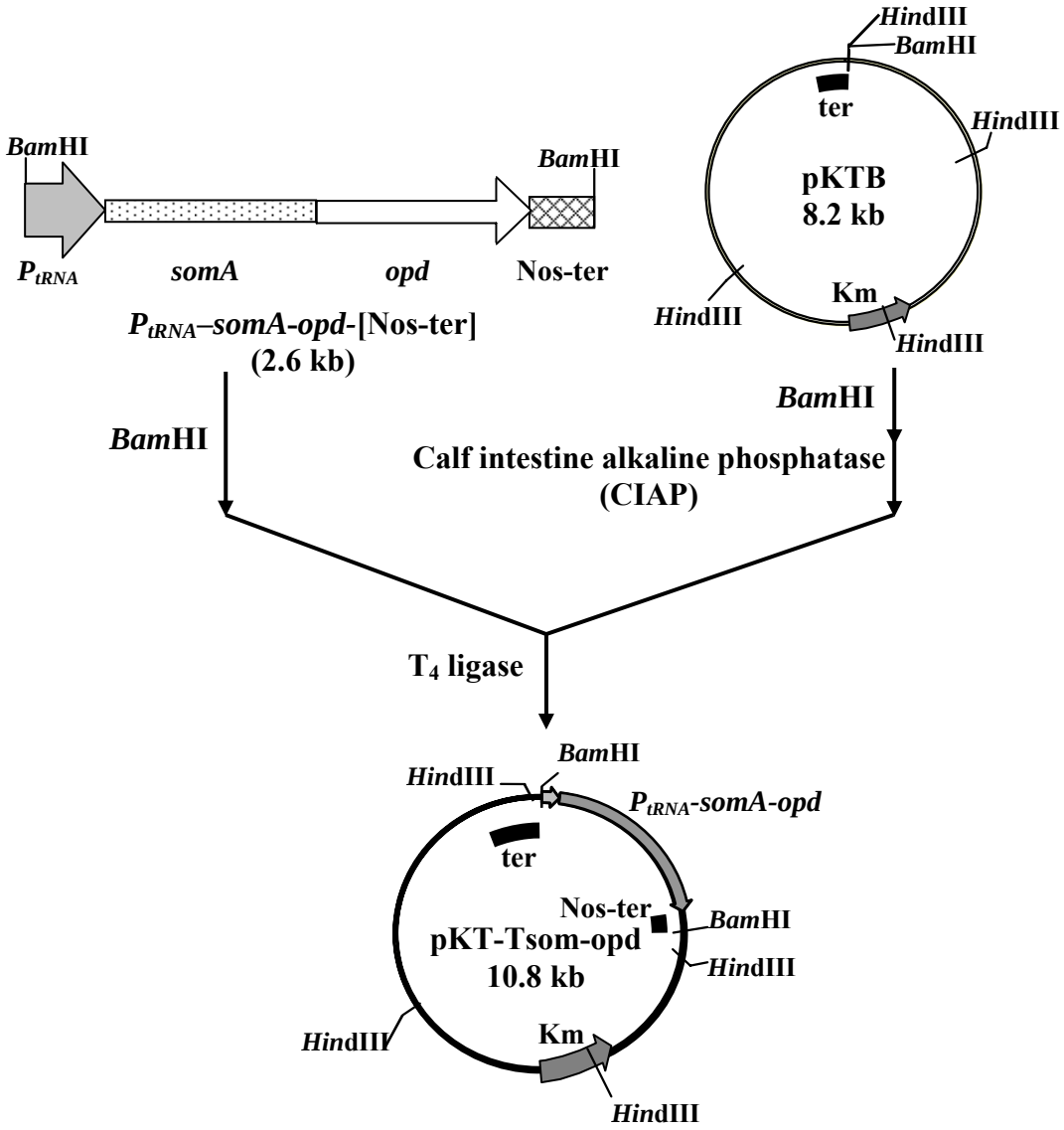


Figure 16. A description and partial restriction map of plasmid pKT-Tsom-opd

The 2.6 kb of P_{tRNA}-*somA*-*opd*-[Nos-ter] gene cassette from overlap extension PCR was cloned into the *Bam*HI site of plasmid pKTB. The resulting plasmid is 10.8 kb of pKT-Tsom-opd containing the $tRNA^{pro}$ promoter (P_{tRNA}), *somA*, *opd* and the nopaline synthase terminator (Nos-ter). Km is kanamycin resistance gene. Ter is the transcription-translation terminator signal of phage T4. The figure is not drawn to scale.

MilliQ water, mixed and incubated at 65 °C for 20 min. Then 200 µl of competent cells on ice was added with 12 µl pre-warmed ligation mixture, mixed and incubated on ice for 30 min. The plasmids were transformed into the competent cells by heat-shock at 42 °C for 50 s and put on ice immediately for 2 min. 800 µl of LB broth was added into the cell mixture. After incubation at 37 °C for 1 h, the transformed cells were spread on LB plates which contain antibiotics as selectable marker and incubated overnight at 37 °C.

8.2 Transformation of plasmids into *Synechococcus* (Kuhlemeier et al., 1983)

Synechococcus were grown on BG-11 medium liquid at the growth condition (2.1 in materials and methods) to log phase (OD_{730} = 0.4-0.6) and then was diluted to OD_{730} = 0.2. After that, the cells were grown further until OD_{730} reached 0.4. Then, cells were harvested by centrifugation at room temperature. The cell pellet was resuspended in BG-11 broth to be an approximate density of 2×10^8 cells/ml (OD_{730} = 1.0 corresponds to 1.2×10^8 cell/ml). The *Synechococcus* cell suspension (2×10^8 cells/ml) was used further for transformation.

For transformation, 300 µl of the *Synechococcus* cell suspension (2×10^8 cells/ml) was mixed with 10 µg of plasmid DNA. The mixture was incubated at 28°C for 2-4 h with occasionally shaking in the dark. The 100 µl of the mixture was spread on 40 ml of BG-11 agar plate and incubated for 24 – 48 h at 28 °C under continuous illumination with constant light intensity 3,500 lux. Then, kanamycin was added beneath the agar. The cells were incubated for 7-10 days to select the kanamycin-resistant transformants.

9. Small scale plasmid extraction from *E. coli* using CTAB method (Del-Sal et al., 1988)

The pellet of *E. coli* harboring recombinant plasmid was collected from 3 ml of overnight culture. The cell pellet was resuspended in 200 µl of STET buffer (8 % sucrose, 0.1 % Triton X-100, 50 mM Tris-HCl pH 8.0) and 10 µl of 50 mg/ml lysozyme, mixed and incubated for 10 min at 37 °C. The suspension was boiled for 45 s and centrifuged at 14,000 rpm (20,800 *g*) for 15 min at room temperature immediately. The pellet was removed from the suspension by using a toothpick. The supernatant was added with 1/10 volume of 5 % CTAB (cetyl trimethyl ammonium

bromide), centrifuged at 14,000 rpm (20,800 *g*) for 5 min, resuspended in 300 µl of 1.2 M NaCl, vortexed, added with 5 µl of 10 mg/ml RNase and incubated at 37 °C. The mixture was added with equal volume of chloroform: isoamyl alcohol (24:1), vortexed and centrifuged at 14,000 rpm (20,800 *g*) for 5 min. The upper phase was taken to a new tube, added with 1/10 volume of 5M $NaClO_4$ and mixed. Then, the mixture was added with equal volume of isopropanol and mixed. After centrifugation at 14,000 rpm (20,800 *g*) for 15 min, the pellet of DNA was resuspended with 30 µl of MilliQ water.

10. Automated DNA sequencing

For automated DNA sequencing, the PCR-based sequencing reaction was carried out using ABI PRISM^{TM} Dye Terminator Cycle Sequencing Kit (Perkin Elmer). The 20 µl of the PCR reaction mixture contained 2 µl of 5X reaction buffer, 4 µl of Big Dye, 400 ng of plasmid DNA, 20 pmol of a primer and MilliQ water to adjust volume. 25 cycles of PCR was carried out after an initial denaturation step at 96 °C for 5 min. Each cycle consisted of 3 holding temperature, at 96 °C for 10 s, at 50 °C for 5 s and at 60 °C for 4 min, followed by final extension at 60 °C for 5 min. The DNA sequences were analyzed on the ABI Prism 3100 DNA sequencer (Perkin Elmer) by service of BSU (bioservice unit), National Science and Technology Development Agency (NSTDA) building, Rama VI Road, Bangkok 10400, Thailand.

11. Expression of the *opd* gene under the control of the P_{somA} or the P_{tRNA}

Three different media, which are BG-11 (William, 1988), BG-11^{N} (Görl et al., 1998) and BG-11^{0} (Görl et al., 1998), were used to grow cyanobacterial cells. BG-11 and BG-11^{N} media were media that contained nitrogen. The different between BG-11 and BG-11^{N} media was BG-11^{N} medium contained ferric citrate instead of ferric ammonium citrate in BG-11 medium when $NaNO_3$ was used as nitrogen source in both BG-11 and BG-11^{N} media. Components of nitrogen-free medium, BG-11^{0}, were identical to components of BG-11^{N} medium except $NaNO_3$ in BG-11^{N} medium was replaced by the same molarity of NaCl.

The recombinant *Synechococcus* was streaked on BG-11 agar and grown at 28 °C with luminescence 3,500 lux for 6 days as stock culture. The OD_{730} = 4 of the cell culture was spread on BG-11 agar plates containing kanamycin at 30 °C with with constant light intensity 3,500 lux for 3 days. Then, OPH enzyme activity assay was

performed to select the clones that expressed high level of OPH activities.

11.1 Comparison between whole cell OPH activities of *Synechococcus* harboring plasmid pKT-Psom-opd and that harboring plasmid pKT-Tsom-opd

Three loops from stock culture of the selected recombinant *Synechococcus* grown on BG-11 agar containing kanamycin plate were collected and resuspended with 1 ml of BG-11 broth. The OD_{730} = 10 of the cell culture was spread on a BG-11 agar plate containing kanamycin and grown at 30 °C with constant light intensity 3,500 lux for 5 days. The OD_{730} = 1 of 1-day to 5-day cell culture was collected and stored at -80 °C before being used in whole cell OPH enzyme activity assay.

11.2 Nitrogen starvation in *Synechococcus* (modified from Görl et al., 1998)

The recombinant *Synechococcus* was grown in 150 ml of BG-11^N medium at 30 °C with constant light intensity 3,500 lux overnight as stock culture. Subsequently, 300 ml of the stock culture in BG-11^N medium was incubated in 2 L Erlenmeyer flask at 30 °C with luminescence 3,500 lux with 250 rpm of shaking until OD_{730} reached 0.5. Then, the exponentially growing cells were harvested by 10,000 rpm (10,600 *g*) of centrifugation for 5 min. Next, the harvested cells were washed twice with 5 ml BG-11^0 broth and resuspended in 100 ml BG-11^0 broth to give an OD_{730} = 0.5. In another flask, the harvested cells were washed twice with 5 ml BG-11^N broth and resuspended in 100 ml BG-11^N broth to give an OD_{730} = 0.5. The incubation was continued under constant light intensity 3,500 lux. The cell culture was taken from nitrogen-deprived or nitrogen-containing cultures to immediately perform OPH enzyme activity assay from the first hour until 48 h after incubation.

12. Whole cell OPH enzyme activity assay of *Synechococcus* (modified from Udtanut, 2005)

OPH activity was measured by following the increase in absorbancy of *p*-nitrophenol which is a product from the hydrolysis of substrate paraoxon at 400 nm. Pellets of cell culture were collected by centrifugation at 12,000 rpm (15,300 *g*) for 5 min and then, soaked with buffer of proteinase K (15 % sucrose, 15 mM Tris-HCl, 0.1 mM EDTA) for 1 h. In experiment with proteinase K treatment, the cells were treated with 200 μg/ml of final concentration of proteinase K in the buffer of proteinase K for 1 h. The cell pellets were collected by centrifugation, washed once with 400 μl of CHES buffer (250 mM CHES (2-(*N*-cyclohexylamino) ethanesulfonic acid), 50 μM

$CoCl_2$) and collected. The reaction was performed in 1 ml volume by adding 900 µl of CHES buffer and 100 µl of 20 mM paraoxon (dissolved in 10 % methanol in CHES buffer) into the cell pellet. The reaction was mixed and incubated at 37 °C for 5 min. The cell pellets was removed by centrifugation. The supernatant was measured OPH activity by using UV spectrophotometer (Hitachi U-200 spectrophotometer). The negative control was *Synechococcus* harboring plasmid pKGT (Chungjatupornchai et al., 2002), which does not contain *opd* gene.

Whole cell OPH activities were expressed in unit of µM of paraoxon hydrolysed/min/OD_{730}. Absorbance of *p*-nitrophenol obeyed the Beer-Lambert law (i.e. A = εdc). The absorbance of wave length at 400 nm measured by spectrophotometer (A) is directly proportional with the absorption coefficient (ε = 17000 $M^{-1}cm^{-1}$ for *p*-nitrophenol), the path length through the sample (d = 1 cm) and molar concentration (c).

CHAPTER IV
RESULTS

Gene cassette P_{somA}-*somA*-*opd*-[Nos-ter] and P_{tRNA}-*somA*-*opd*-[Nos-ter], in which the *somA* gene was under the control of its own promoter or the $tRNA^{pro}$ promoter respectively, were cloned into shuttle plasmid pKTB with the purpose of generating recombinant plasmid pKT-Psom-opd and pKT-Tsom-opd for cell surface expression of OPH.

1. Construction of plasmid pKTB

The *gus* gene was deleted from plasmid pKGT-SalI (see figure 8) by using *Sal*I and *Eco*RI digestions (see figure 10). The resulting recombinant plasmids from eighteen *E. coli* clones (no. 2 – 19) were digested with *Eco*RI (figure 17). The recombinant plasmid obtained from clone no. 19 was not cleaved in *Eco*RI digestion (figure 17, lane 18). This result was different from digested recombinant plasmids obtained from other clones bearing *Eco*RI sites (figure 17, lanes 1 – 17).

The recombinant plasmid from clone no. 19 was verified by digestion with *Hin*dIII generating fragments of 1.4, 1.6, 1.8 and 3.5 kb and by digestion with *Bam*HI generating a fragment of 8.2 kb (figure 18). All the DNA band patterns are as expected when they are compared with fragments of plasmid pKGT digested with *Hin*dIII and *Bam*HI (figure 18).

The plasmid obtained from clone no. 19 still contained *Sal*I site (figure 19) despite the fact that the original plasmid pKGT-SalI has been already digested with *Sal*I and *Eco*RI and then fill-in for blunt end ligation. Recombinant plasmid from clone no. 19 was designated pKTB (see figure 10).

2. Construction of plasmids pKT-Psom-opd and pKT-Tsom-opd

2.1 Cloning of the *somA* gene

Genomic DNA of *Synechococcus* was isolated (figure 20). No *somA* PCR product was observed using primer sets: somA-fwd1 and somA-rev1; or somA- fwd2

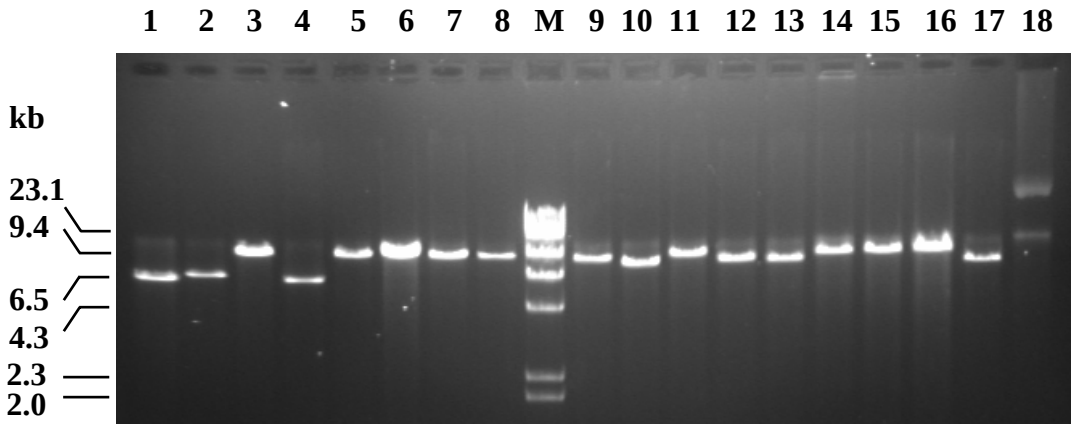


Figure 17. Screening of plasmid pKTB by *Eco*RI digestion

Gel electrophoresis was performed on 1 % agarose gel containing ethidium bromide. Lane M is 200 ng of the λ/*Hin*dIII DNA marker. Lanes 1 – 18 are *Eco*RI digestion of recombinant plasmids obtained from clones no. 2 – 19 respectively.

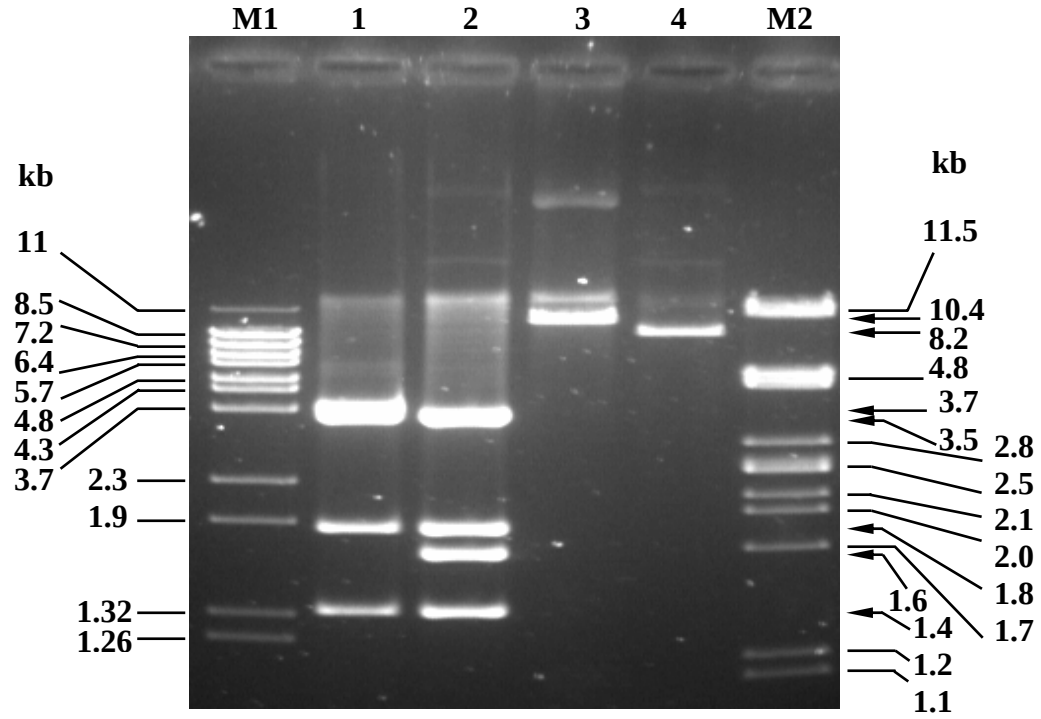


Figure 18. Verification of plasmid pKTB

Gel electrophoresis was performed on 1 % agarose gel containing ethidium bromide. Lane M1 is 200 ng of the λ/*Bst*EII DNA marker. Lane M2 is 200 ng of the λ/*Pst*I DNA marker. Lane 1 is plasmid pKGT digested with *Hin*dIII. Lane 2 is plasmid obtained from clone no. 19 digested with *Hin*dIII. Lane 3 is plasmid pKGT digested with *Bam*HI. Lane 4 is plasmid obtained from clone no. 19 digested with *Bam*HI.

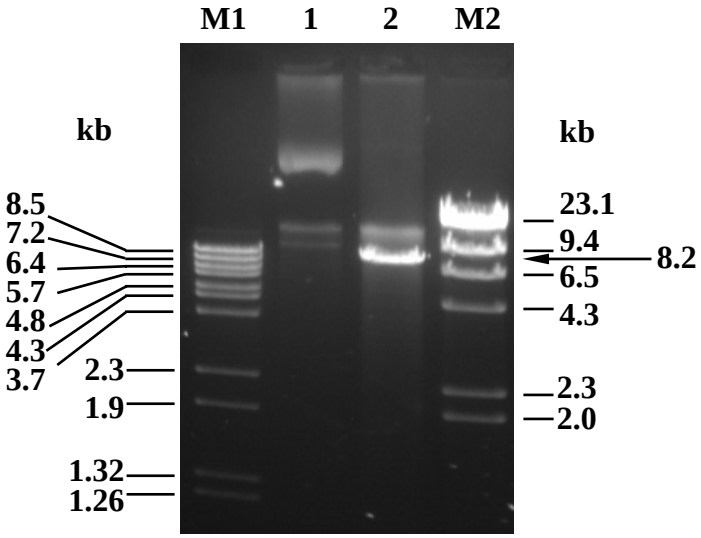


Figure 19. Plasmid from clone no. 9 digested with *Sal*I

Gel electrophoresis was performed on 1 % agarose gel containing ethidium bromide. Lane M1 is 200 ng of the λ/*Bst*EII DNA marker. Lane M2 is 200 ng of the λ/*Hin*dIII DNA marker. Lane 1 is plasmid pKGT digested with *Sal*I. Lane 2 is plasmid obtained from clone no. 19 digested with *Sal*I.

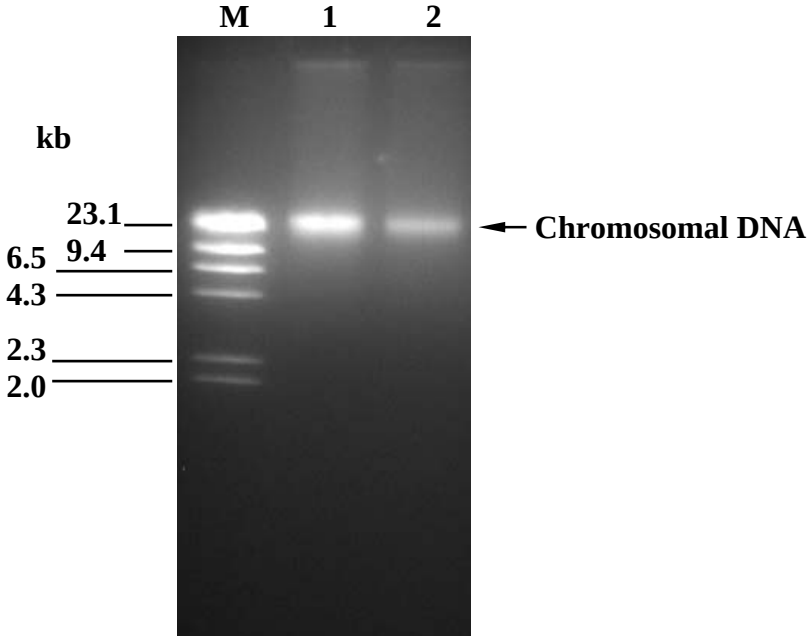


Figure 20. Chromosomal DNA of *Synechococcus*

Gel electrophoresis was performed on 1 % agarose gel containing ethidium bromide. Lane M is 200 ng of the λ/*Hin*dIII DNA marker. Lanes 1 and 2 are 1 µl of chromosomal DNA of *Synechococcus* from different preparations.

and somA-rev2. The primers somA-fwd1, somA-fwd2, somA-rev1 and somA-rev2 were located on the regions that form stem-loop structure of *somA* gene (see figure 9). The free energies of the predicted stem-loop structure of the primers on the *somA* gene were calculated using the computer program mfold version 3.1 by Zuker and Tuner (Mathews, 1999; Zuker et al., 1999; Zuker, 2003) (figure 21). Thus, these primers may not be efficient in PCR resulted in no PCR product. Amplification of the 1.8 kb of *somA* gene was successful by using primers somA-fwd1 and somA-rev2 (figure 22, lane 1). The 1.8 kb of *somA* PCR product was digested with *Eco*RI generating fragments of 0.35, 0.6 and 0.9 kb (figure 22, lane 2). The DNA bands are as expected.

Plasmid pUC18 (see figure 6) and the 1.8 kb of *somA* PCR product were digested with *Bam*HI and *Sal*I (figure 23). Subsequently, the digested *somA* PCR product was cloned into *Bam*HI/*Sal*I site of plasmid pUC18 to generate the resulting plasmid pUC18-SomA (see figure 11). Plasmids from one *E. coli* MC1061 clone and ten *E. coli* DH5α clones were extracted and characterized with *Bam*HI/*Sal*I digestion. The results showed that digested plasmid obtained from *E. coli* MC1061 clone no. 9 produced a band of the 1.8 kb of *somA* PCR product (figure 24).

The recombinant plasmid from clone no. 9 was verified by digestions with *Bam*HI, *Eco*RI, *Xmn*I and *Sac*I (figure 25). The *Bam*HI digestion generated a fragment of 4.5 kb. The *Eco*RI digestion generated fragments of 0.35, 0.6 and 3.6 kb. The *Xmn*I digestion generated fragments of 1.6 and 3.0 kb. The *Sac*I digestion generated fragments of 0.7 and 3.9 kb. All the DNA band patterns are as expected. Therefore, the plasmid obtained from clone no. 9 was the correct plasmid pUC18-SomA (see figure 11).

The nucleotide sequences of the *somA* gene in plasmid pUC18-SomA obtained from clone no. 9 was investigated by using Automated DNA sequencing. The results showed that the nucleotide sequences on the coding strand of the *somA* gene covering upstream of the *somA* gene (figure 26A) and on the complementary strand of the *somA* gene covering downstream of the *somA* gene (figure 26B) are as expected by comparison with GenBank database accession no. D64077 (see figure 9). Therefore, plasmid pUC18-SomA obtained from clone no. 9 harbored the correct *somA* gene.

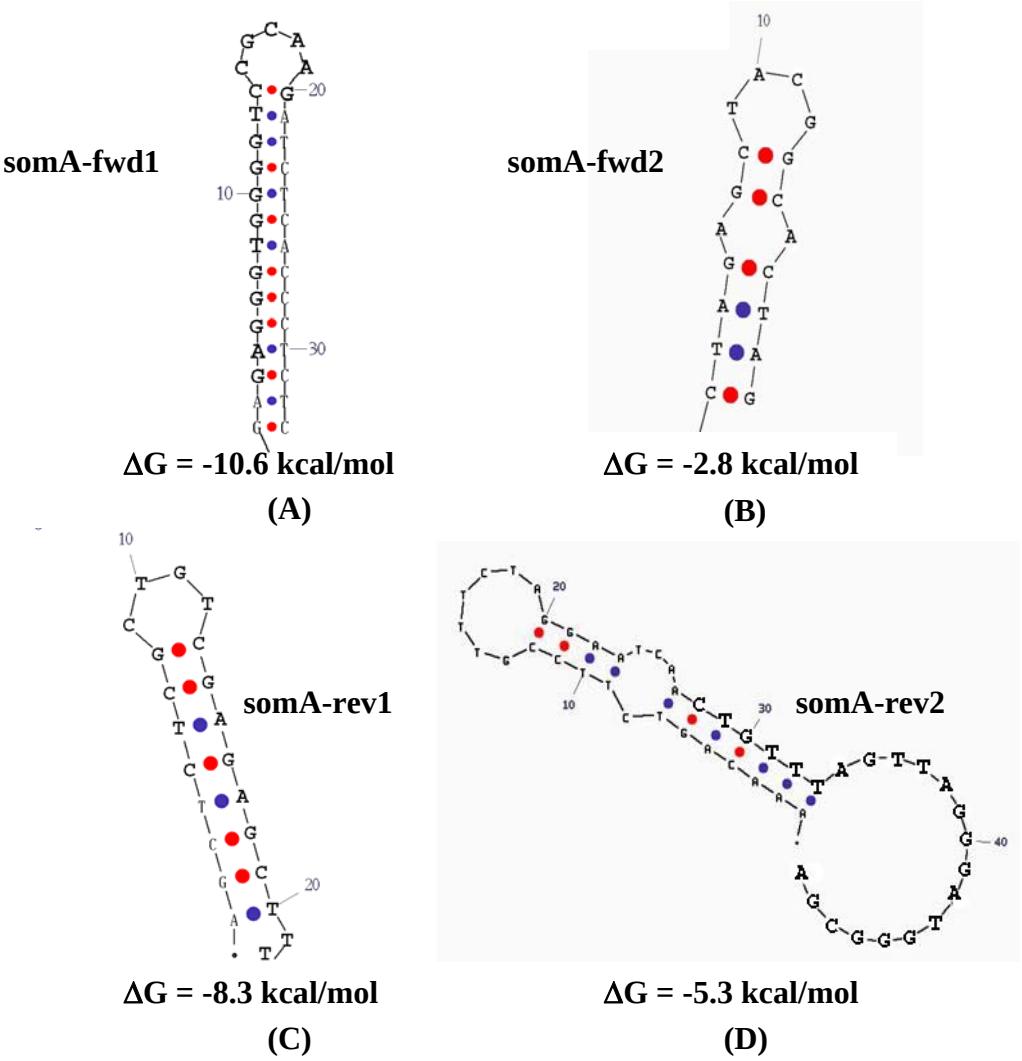


Figure 21. Predicted secondary structure of nucleotide sequences at the locations of somA-fwd1, somA-fwd2, somA-rev1 and somA-rev2 primers

The secondary structure formations of single stranded DNA upstream and downstream of the coding strand of *somA* gene were analyzed by using computer program mfold version 3.1 by Zuker and Tuner (Mathews, 1999; Zuker et al., 1999; Zuker, 2003) with default prediction at 37 °C. Predicted loop forming at the locations of primers somA-fwd1 (A), somA-fwd2 (B), somA-rev1 (C) and somA-rev2 (D) were shown.

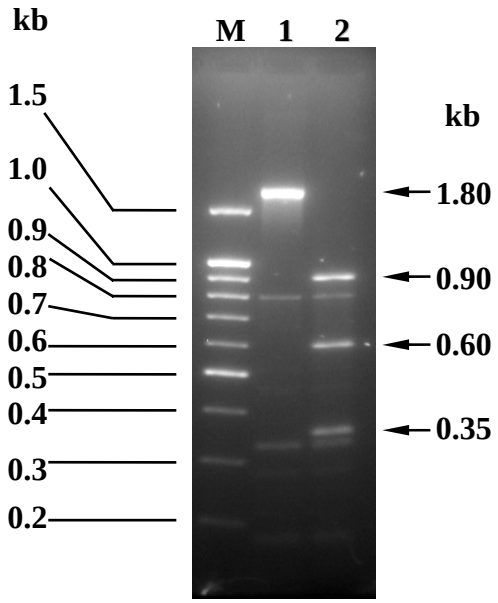


Figure 22. Verification of the 1.8 kb of *somA* PCR product

Gel electrophoresis was performed on 2 % agarose gel containing ethidium bromide. Lane M is 200 ng of the 100 bp DNA ladder marker. Lane 1 is the *somA* PCR product. Lane 2 is the *somA* PCR product digested with *Eco*RI.

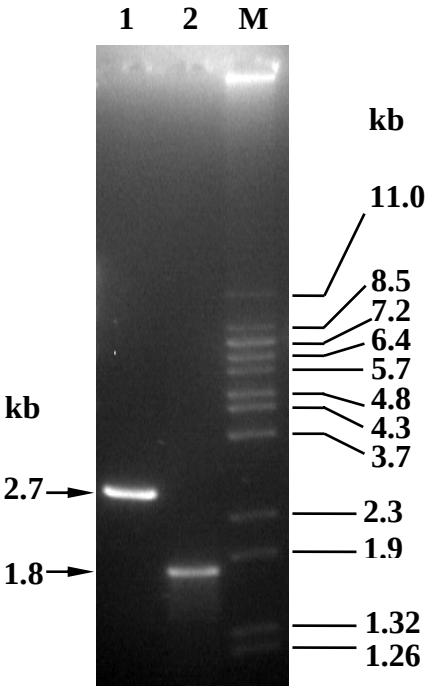


Figure 23. *Bam*HI and *Sal*I digestions of the 1.8 kb of *somA* PCR product and plasmid pUC18

Gel electrophoresis was performed on 1 % agarose gel containing ethidium bromide. Lane M is 200 ng of the λ/*Bst*EII DNA marker. Lane 1 is plasmid pUC18 digested with *Bam*HI and *Sal*I. Lane 2 is the *somA* PCR product digested with *Bam*HI and *Sal*I.

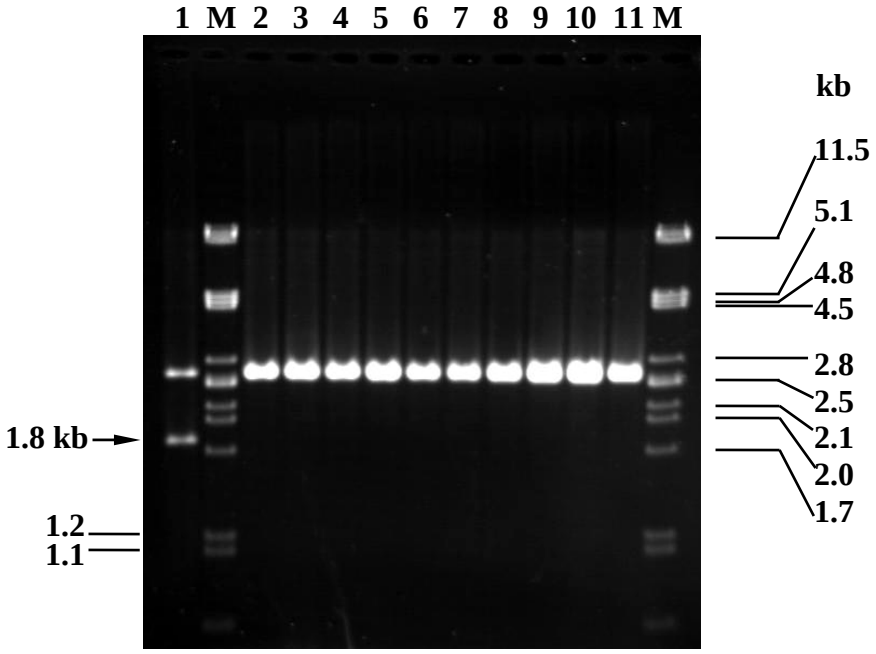


Figure 24. Screening of plasmid pUC18-SomA with *Bam*HI/*Sal*I digestion

Gel electrophoresis was performed on 1 % agarose gel containing ethidium bromide. Lane M is 200 ng of the λ/*Pst*I DNA marker. Lane 1 is recombinant plasmids obtained from *E. coli* MC1061 clone no. 9 digested with *Bam*HI/*Sal*I. Lanes 2 – 11 are *Bam*HI/*Sal*I digestions of recombinant plasmids from *E. coli* DH5α clones no. 1 – 10, respectively.

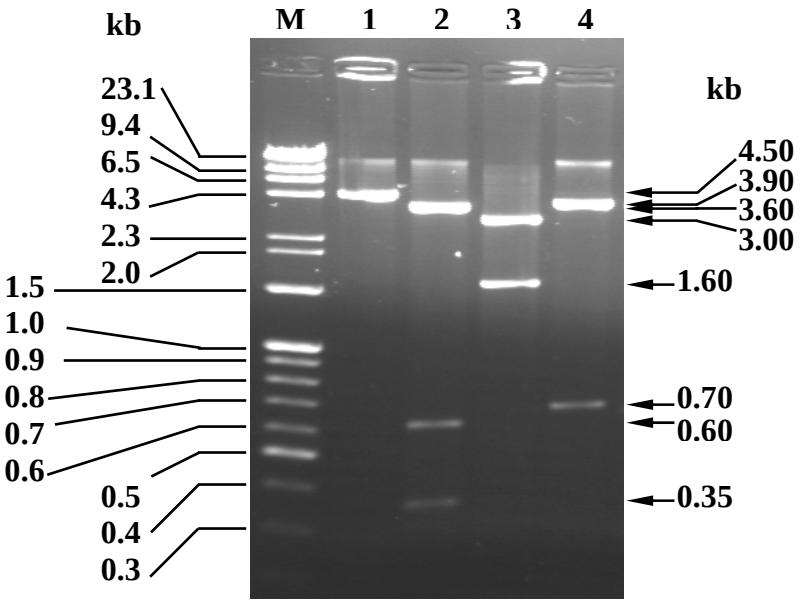
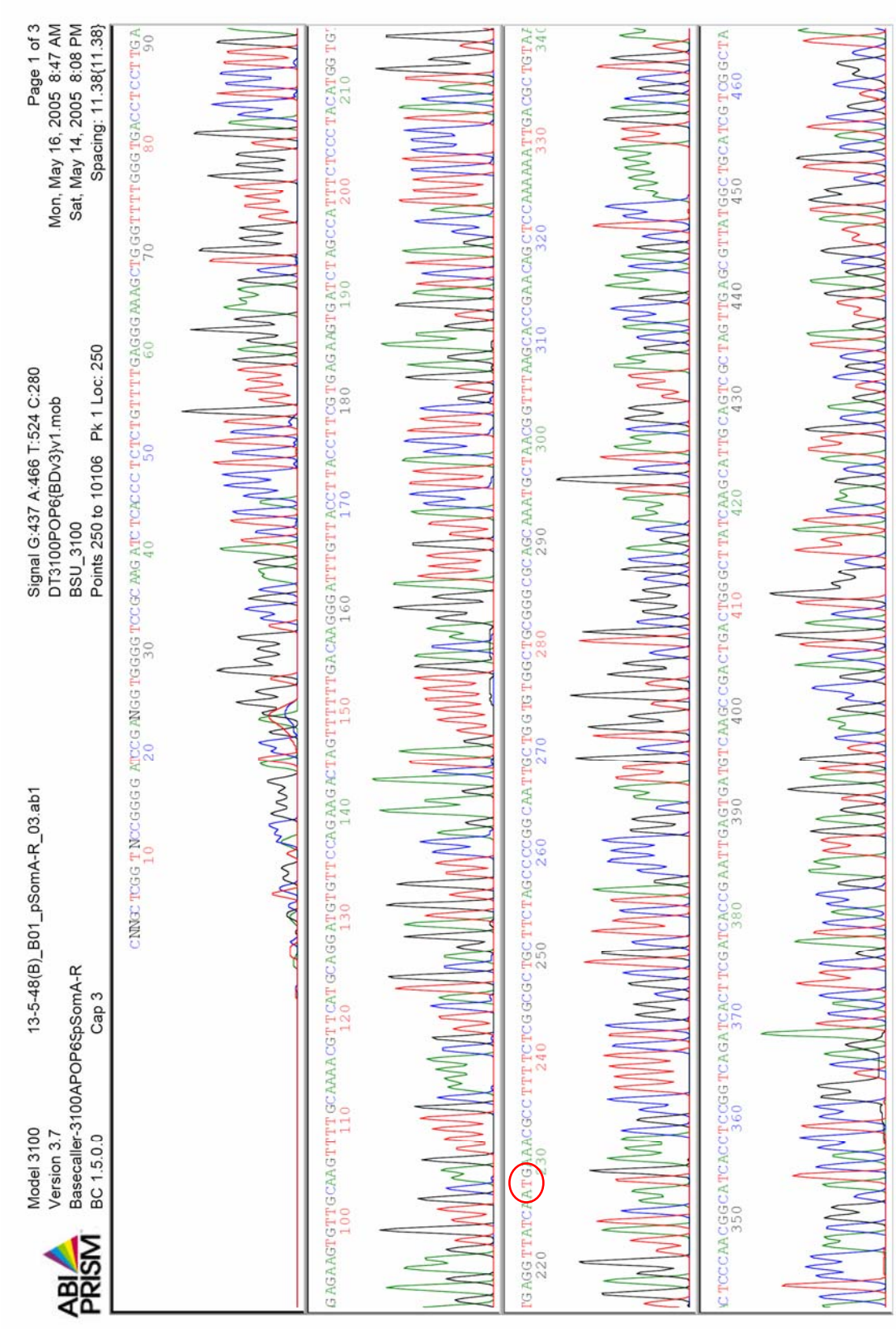


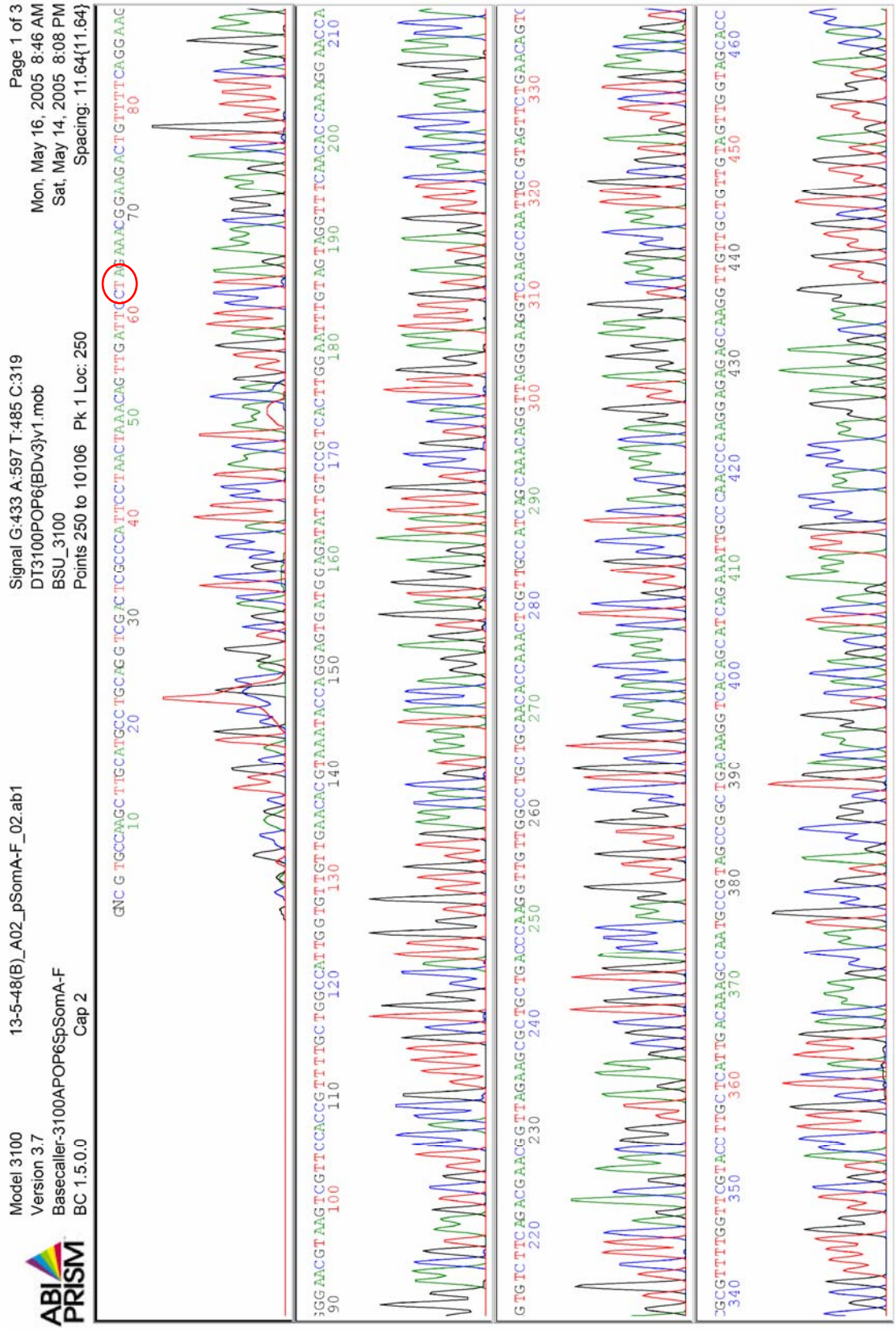
Figure 25. Verification of plasmid pUC18-SomA

Gel electrophoresis was performed on 2 % agarose gel containing ethidium bromide. Lane M is 200 ng of the λ/*Hin*dIII DNA marker mixed with 200 ng of the 100 bp DNA ladder marker. Lanes 1 – 4 are plasmids from clone no. 9 digested with *Bam*HI, *Eco*RI, *Xmn*I and *Sac*I, respectively.

(A)


ABI PRISM
Model 3100
Version 3.7
Basecaller-3100APOP6SpSomA-R
BC 1.5.0.0
13-5-48(B)_B01_pSomA-R_03.ab1
Cap 3
Signal G:437 A:466 T:524 C:280
DT3100POP6{BDv3}v1.mob
BSU_3100
Points 250 to 10106 Pk 1 Loc: 250
Page 1 of 3
Mon, May 16, 2005 8:47 AM
Sat, May 14, 2005 8:08 PM
Spacing: 11.38{11.38}

(B)


ABI PRISM
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BC 1.5.0.0
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Cap 2
Signal G:433 A:597 T:485 C:319
DT3100POP6{BDv3}v1.mob
BSU_3100
Points 250 to 10106 Pk 1 Loc: 250
Page 1 of 3
Mon, May 16, 2005 8:46 AM
Sat, May 14, 2005 8:08 PM
Spacing: 11.64{11.64}

Figure 26. Partial nucleotide sequences of the *somA* gene in plasmid pUC18-SomA

(A) Partial nucleotide sequences on the coding strand of the *somA* gene. The start codon was indicated by a red circle. (B) Partial nucleotide sequences on the complementary strand of the *somA* gene. The complementary sequences of the stop codon were indicated by a red circle.

2.2 Construction of gene cassette P_{somA}-*somA*-*opd*-[Nos-ter] using overlap extension PCR

The 1.4 kb of *somA* PCR product with its own promoter was amplified by PCR using primers somA-fwd1 and somA-rev3, and using plasmid pUC18-SomA from clone no. 9 as the template (see figure 12). The 1.3 kb of the *opd* gene was amplified by PCR using primers OPH-fwd2 and Nos-rev2, and using plasmid pUC18-PtOPH (see figure 7) as the template (see figure 12). Then, PCR products of *somA* and *opd* genes were fused in-frame by using overlap extension PCR (see figure 12). The resulting 2.7 kb overlap-extension PCR product (figure 27) contained gene cassette P_{somA}-*somA*-*opd*-[Nos-ter]. The 2.7 kb overlap-extension PCR product was digested with *Eco*RI and *Eco*RV (figure 28). The *Eco*RI digestion generated fragments of 0.35, 0.6 and 1.8 kb. The *Eco*RV generated fragments of 1.05 and 1.6 kb. All the DNA band patterns are as expected. Therefore, the 2.7 kb overlap-extension PCR product was digested with *Bam*HI and cloned into *Bam*HI site of dephosphorylated pKTB fragment to obtain recombinant plasmid pKT-Psom-opd (see figure 14), which contains gene cassette P_{somA}-*somA*-*opd*-[Nos-ter] (figure 29). The resulting recombinant plasmids from thirteen *E. coli* clones (no. 8 – 10, 26 – 30 and 66 – 70) were digested with *Bam*HI. The recombinant plasmids obtained from clones no. 8, 30, 67 and 70 produced 2.7 kb of gene cassette P_{somA}-*somA*-*opd*-[Nos-ter] (figure 30). Plasmids obtained from clones no. 8 and 70 were verified by digestion with *Hin*dIII, *Sal*I and *Sac*I (figure 31). The *Hin*dIII digestion generated fragments of 1.35, 1.8, 3.5 and 4.3 kb. The *Sal*I digestion generated fragments of 1.6 and 9.8 kb. The *Sac*I digestion generated fragments of 1.8, 3 and 6 kb. The entire DNA band patterns are as expected. Therefore, the plasmids obtained from clones no. 8 and 70 were the correct plasmid pKT-Psom-opd.

The nucleotide sequences of P_{somA}-*somA*-*opd*-[Nos-ter] in plasmids from clones no. 8 and 70 were investigated by using Automated DNA sequencing (figure 32). OPH-rev2 primer was used in the sequencing reaction. The results showed that the nucleotide sequences of clones no. 8 and 70 are as expected (clone no. 8, data not shown). Therefore, plasmids pKT-Psom-opd from clones no. 8 and 70 contained correct *somA*-*opd* gene fusion.

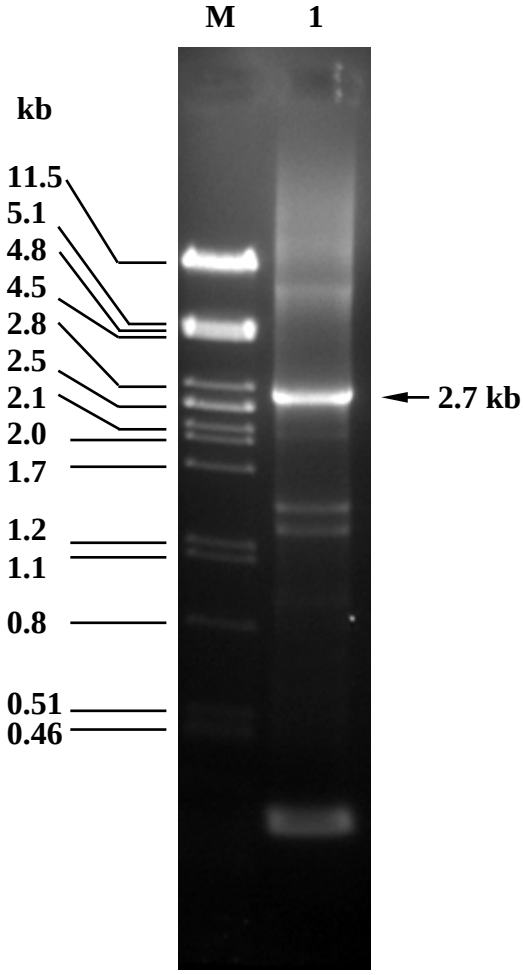


Figure 27. Overlap-extension PCR product containing P_{somA}-*somA*-*opd*-[Nos-ter]

Gel electrophoresis was performed on 1 % agarose gel containing ethidium bromide. Lane M is 200 ng of the λ/*Pst*I DNA marker. Lane 1 is the overlap extension PCR of the *somA* and the *opd* genes using primers somA-fwd1 and Nos-rev2.

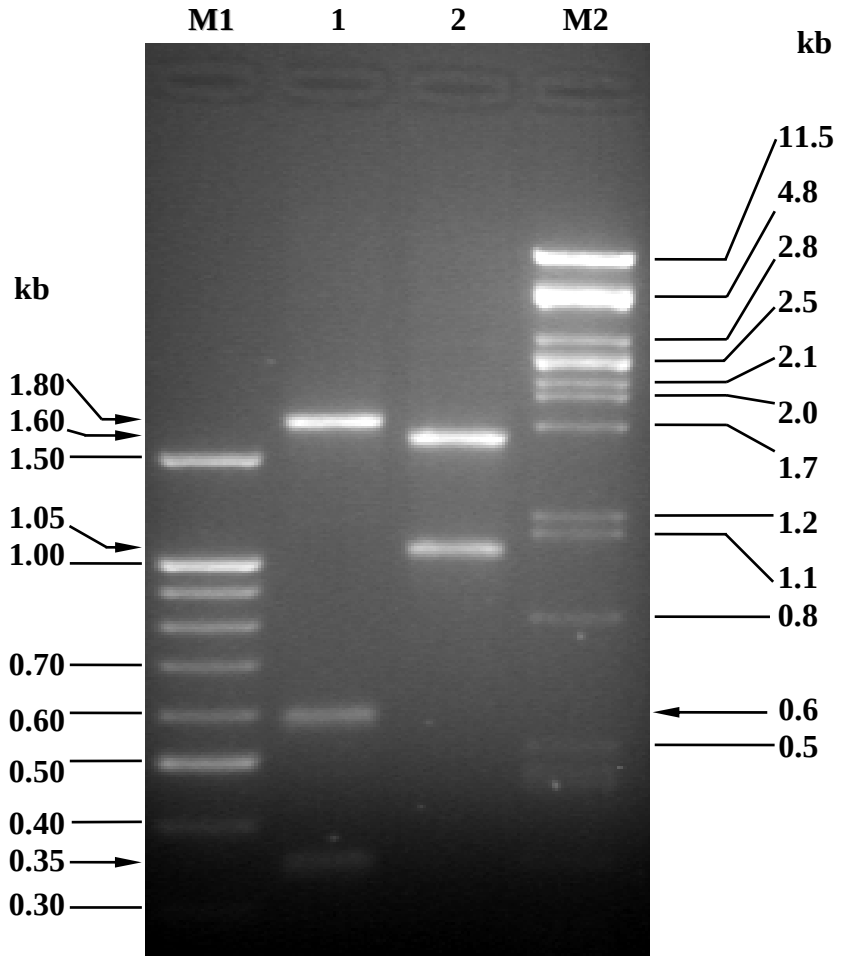


Figure 28. Verification of the 2.7 kb overlap-extension PCR product containing *P_{somA}-somA-opd*-[Nos-ter]

Gel electrophoresis was performed on 1.5 % agarose gel containing ethidium bromide. Lane M1 is 200 ng of the 100 bp DNA ladder marker. Lane M2 is 200 ng of the λ/*Pst*I DNA marker. Lanes 1 and 2 are the 2.7 kb overlap-extension PCR product digested with *Eco*RI and *Eco*RV, respectively.

```
          M   K   R   L   F   S   A   L   L   L   A   P   A   I   A   G   V   A   A   G
    1     ATG AAA CGC CTT TTC TCG GCG CTG CTT CTA GCC CCG GCA ATT GCT GGT GTG GCT GCG GGC

          A   A   N   A   N   G   L   S   T   E   Q   L   Q   K   I   D   A   V   T   P
   61     GCA GCA AAT GCT AAC GGT TTA AGC ACC GAA CAG CTC CAA AAA ATT GAC GCT GTA ACT CCC

          N   G   I   T   S   G   Q   I   T   S   I   T   E   L   S   D   V   K   P   T
  121     AAC GGC ATC ACC TCC GGT CAG ATC ACT TCG ATC ACC GAA TTG AGT GAT GTC AAG CCG ACT

          D   W   A   Y   Q   A   L   Q   S   L   V   E   R   Y   G   C   I   V   G   Y
  181     GAC TGG GCT TAT CAA GCA TTG CAG TCG CTA GTT GAG CGT TAT GGC TGC ATC GTC GGC TAC

          P   D   R   T   Y   R   G   S   R   P   L   S   R   Y   E   F   A   A   G   L
  241     CCT GAT CGG ACT TAC CGT GGT AGC CGC CCC CTC TCT CGT TAT GAG TTT GCA GCA GGC TTG

          N   A   C   L   D   K   V   I   E   F   A   A   S   K   E   D   L   D   T   L
  301     AAC GCT TGC TTG GAC AAA GTC ATT GAA TTT GCA GCG TCG AAA GAG GAT CTC GAC ACC CTC

          K   R   L   T   E   E   F   Q   A   E   L   A   T   L   R   G   R   V   D   S
  361     AAG CGA CTG ACT GAA GAA TTC CAA GCT GAG CTG GCG ACC CTG CGT GGT CGT GTT GAT AGT

          L   E   A   R   V   K   E   L   E   A   T   R   F   S   T   T   T   K   L   Q
  421     CTC GAG GCT CGT GTT AAA GAG CTC GAA GCT ACC CGT TTC TCC ACC ACG ACT AAG CTA CAA

          G   E   V   I   F   S   L   D   A   V   A   N   T   A   G   N   E   R   N   Q
  481     GGT GAA GTA ATC TTC AGC TTG GAC GCT GTT GCT AAT ACT GCT GGC AAC GAA CGT AAC CAA

          D   G   A   V   S   F   G   N   R   V   S   L   N   L   N   T   S   F   T   G
  541     GAC GGT GCT GTA TCG TTC GGC AAC CGC GTC AGC TTG AAC CTC AAC ACG AGC TTC ACT GGC

          K   D   L   L   L   T   R   L   R   A   R   N   I   E   T   I   Q   Q   R   L
  601     AAA GAC TTG CTA TTG ACC CGT CTG CGC GCT CGT AAC ATT GAG ACG ATT CAG CAG CGC TTG

          S   P   G   F   N   P   S   G   S   R   L   D   Y   D   G   T   G   S   P   G
  661     TCG CCT GGC TTT AAT CCC TCG GGC TCG CGG CTC GAC TAC GAC GGT ACC GGT TCG CCT GGT

          V   P   N   S   A   N   T   F   F   L   D   K   L   L   Y   R   F   P   V   G
  721     GTC CCG AAT TCG GCT AAT ACC TTT TTC CTT GAC AAA TTG CTG TAC CGC TTC CCC GTC GGT

          D   V   S   F   T   V   G   T   A   G   V   Q   P   Q   D   Y   G   L   S   D
  781     GAT GTC TCC TTC ACT GTT GGT ACC GCG GGC GTT CAA CCT CAA GAC TAC GGC CTG AGC GAC

          A   T   F   F   S   G   P   A   N   T   K   A   F   K   Y   V   G   A   G   V
  841     GCT ACC TTC TTC AGT GGC CCT GCG AAC ACG AAA GCC TTC AAG TAT GTC GGT GCA GGT GTT

          Y   A   D   T   R   D   A   D   T   A   G   V   G   F   N   W   K   A   S   K
  901     TAC GCT GAT ACT CGT GAT GCT GAT ACC GCT GGT GTT GGC TTC AAC TGG AAG GCT AGT AAG

          N   F   S   F   Q   A   G   Y   I   N   R   N   S   A   D   V   S   T   V   N
  961     AAT TTC AGC TTC CAG GCT GGC TAC ATC AAC CGC AAC TCT GCT GAT GTC TCG ACT GTC AAC

          S   G   G   V   F   G   F   T   P   T   G   T   G   T   N   S   W   D   V   N
 1021     AGT GGT GGT GTC TTT GGC TTC ACC CCG ACA GGG ACT GGT ACT AAC TCT TGG GAT GTG AAT

          A   Q   V   K   Y   Q   T   D   N   N   K   F   R   V   A   L   A   Y   A   L
 1081     GCT CAA GTC AAG TAC CAA ACT GAT AAC AAC AAG TTC CGA GTT GCT CTG GCC TAC GCT CTG

          R   N   G   R   Y   A   T   D   F   G   T   T   N   A   F   Q   P   F   G   A
 1141     CGA AAT GGT CGC TAC GCC ACT GAC TTC GGT ACC ACC AAC GCC TTC CAG CCT TTT GGT GCT

          T   M   S   I   G   T   G   D   R   I   N   T   V   R   G   P   I   T   I   S
 1201     ACC ATG TCG ATC GGC ACA GGC GAT CGG ATC AAT ACC GTG CGC GGT CCT ATC ACA ATC TCT

          E   A   G   F   T   L   T   H   E   H   I   C   G   S   S   A   G   F   L   R
 1261     GAA GCG GGT TTC ACA CTG ACT CAC GAG CAC ATC TGC GGC AGC TCG GCA GGA TTC TTG CGT

          A   W   P   E   F   F   G   S   R   K   A   L   A   E   K   A   V   R   G   L
 1321     GCT TGG CCA GAG TTC TTC GGT AGC CGC AAA GCT CTA GCG GAA AAG GCT GTG AGA GGA TTG

          R   R   A   R   A   A   G   V   R   T   I   V   D   V   S   T   F   D   I   G
 1381     CGC CGC GCC AGA GCG GCT GGC GTG CGA ACG ATT GTC GAT GTG TCG ACT TTC GAT ATC GGT

          R   D   V   S   L   L   A   E   V   S   R   A   A   D   V   H   I   V   A   A
 1441     CGC GAC GTC AGT TTA TTG GCC GAG GTT TCG CGG GCT GCC GAC GTT CAT ATC GTG GCG GCG

          T   G   L   W   F   D   P   P   L   S   M   R   L   R   S   V   E   E   L   T
 1501     ACC GGC TTG TGG TTC GAC CCG CCA CTT TCG ATG CGA TTG AGG AGT GTA GAG GAA CTC ACA

          Q   F   F   L   R   E   I   Q   Y   G   I   E   D   T   G   I   R   A   G   I
 1561     CAG TTC TTC CTG CGT GAG ATT CAA TAT GGC ATC GAA GAC ACC GGA ATT AGG GCG GGC ATT
```

```
          I   K   V   A   T   T   G   K   A   T   P   F   Q   E   L   V   L   K   A   A
1621      ATC AAG GTC GCG ACC ACA GGC AAG GCG ACC CCC TTT CAG GAG TTA GTG TTA AAG GCG GCC

          A   R   A   S   L   A   T   G   V   P   V   T   T   H   T   A   A   S   Q   R
1681      GCC CGG GCC AGC TTG GCC ACC GGT GTT CCG GTA ACC ACT CAC ACG GCA GCA AGT CAG CGC

          D   G   E   Q   Q   A   A   I   F   E   S   E   G   L   S   P   S   R   V   C
1741      GAT GGT GAG CAG CAG GCC GCC ATT TTT GAG TCC GAA GGC TTG AGC CCC TCA CGG GTT TGT

          I   G   H   S   D   D   T   D   D   L   S   Y   L   T   A   L   A   A   R   G
1801      ATT GGT CAC AGC GAT GAT ACT GAC GAT TTG AGC TAT CTC ACC GCC CTC GCT GCG CGC GGA

          Y   L   I   G   L   D   H   I   P   H   S   A   I   G   L   E   D   N   A   S
1861      TAC CTC ATC GGT CTA GAC CAC ATC CCG CAC AGT GCG ATT GGT CTA GAA GAT AAT GCG AGT

          A   S   A   L   L   G   I   R   S   W   Q   T   R   A   L   L   I   K   A   L
1921      GCA TCA GCC CTC CTG GGC ATC CGT TCG TGG CAA ACA CGG GCT CTC TTG ATC AAG GCG CTC

          I   D   Q   G   Y   M   K   Q   I   L   V   S   N   D   W   L   F   G   F   S
1981      ATC GAC CAA GGC TAC ATG AAA CAA ATC CTC GTT TCG AAT GAC TGG CTG TTC GGG TTT TCG

          S   Y   V   T   N   I   M   D   V   M   D   R   V   N   P   D   G   M   A   F
2041      AGC TAT GTC ACC AAC ATC ATG GAC GTG ATG GAT CGC GTG AAC CCC GAC GGG ATG GCC TTC

          I   P   L   R   V   I   P   F   L   R   E   K   G   V   P   Q   E   T   L   A
2101      ATT CCA CTG AGA GTG ATC CCA TTC CTA CGA GAG AAG GGC GTC CCA CAG GAA ACG CTG GCA

          G   I   T   V   T   N   P   A   R   F   L   S   P   T   L   R   A   S
2161      GGC ATC ACT GTG ACT AAC CCG GCG CGG TTC TTG TCA CCG ACC TTG CGG GCG TCA
```

Figure 29. Nucleotide and deduced amino acid sequences of gene cassette *somA-opd*

Nucleotide sequences on the coding strand of the *somA* gene were fused in frame with that of the *opd* gene. The *somA-opd*-[Nos-ter] gene fusion was under the control of either P_{somA} or P_{tRNA}. The start codons of the *somA* and the *opd* genes are underlined. Nucleotide sequences of the *opd* gene and deduced amino acid sequences of OPH are in the box.

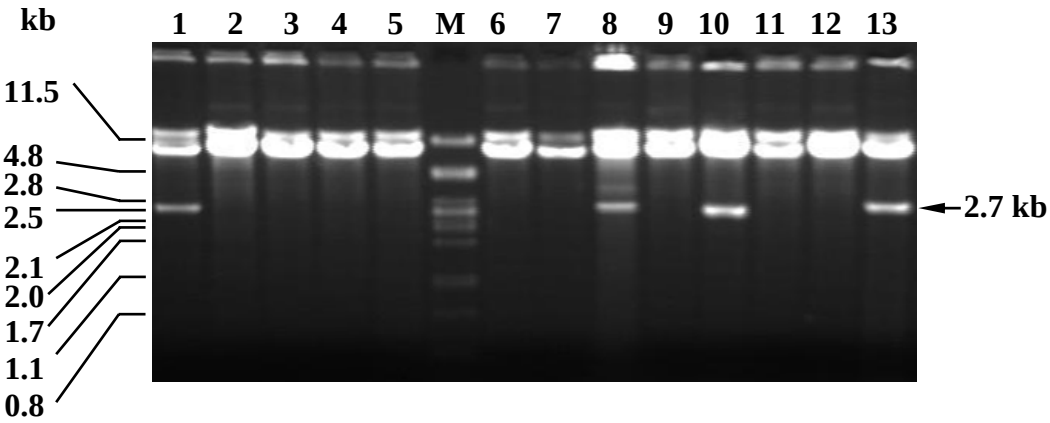


Figure 30. Screening of plasmid pKT-Psom-opd by *Bam*HI digestion

Gel electrophoresis was performed on 1 % agarose gel containing ethidium bromide. Lane M is the λ/*Pst*I DNA marker. Lanes 1 – 13 are *Bam*HI digestions of plasmids from *E. coli* clones no. 8 – 10, 26 – 30 and 66 – 70, respectively. The 2.7 kb of gene cassette P_{somA}–*somA*-*opd*-[Nos-ter] were indicated with an arrow.

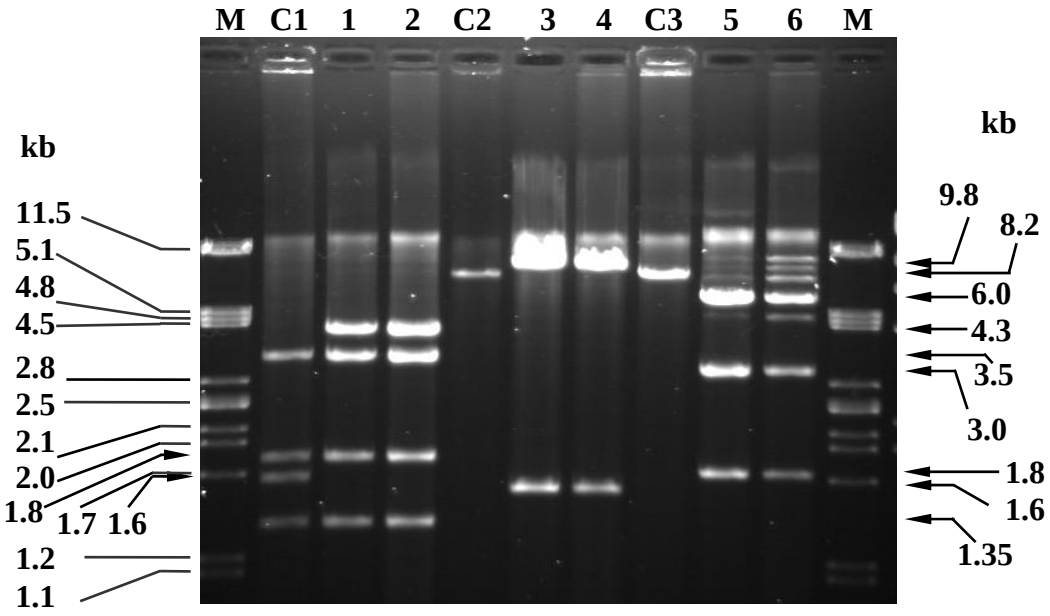
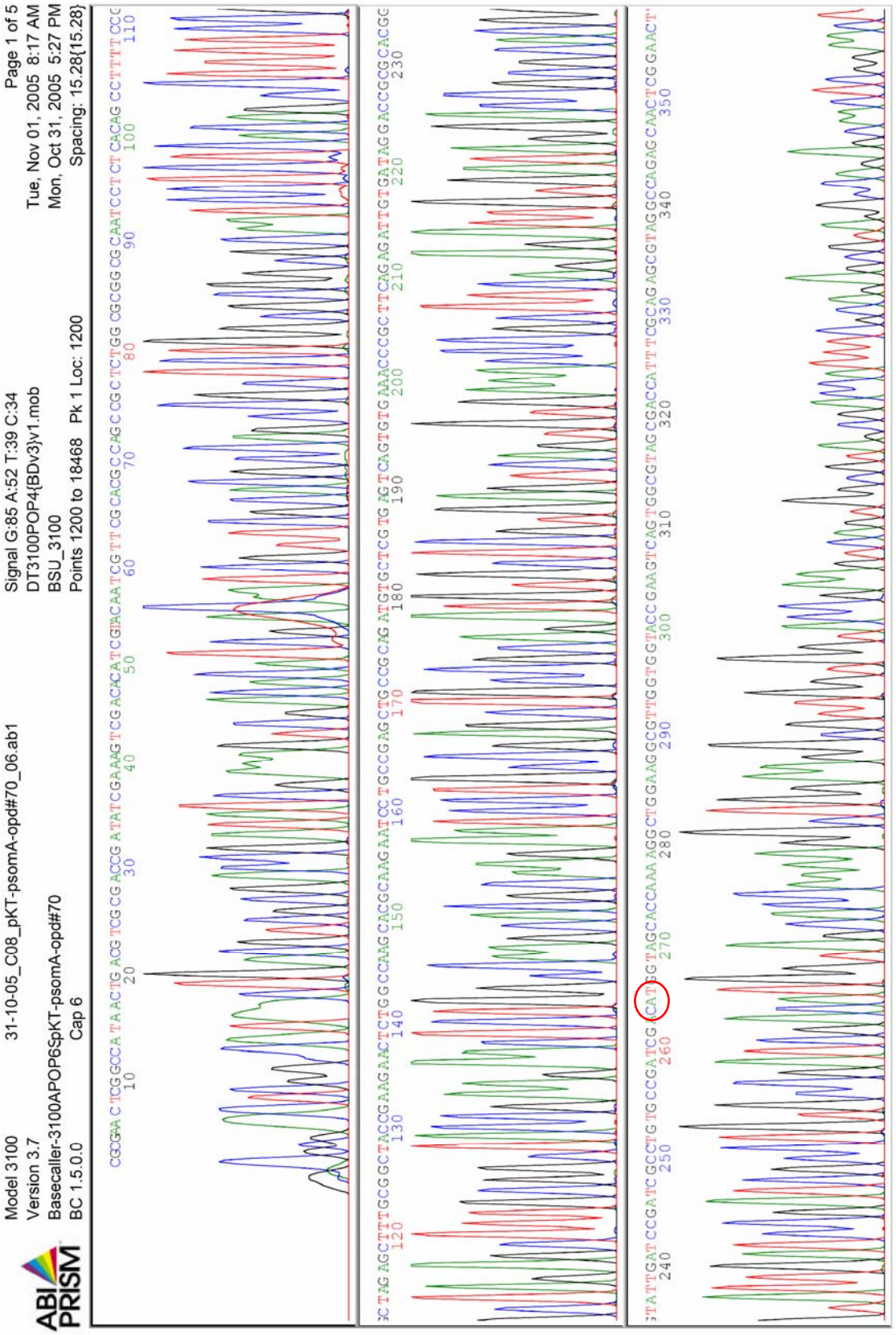


Figure 31. Verification of plasmid pKT-Psom-opd

Gel electrophoresis was performed on 1 % agarose gel containing ethidium bromide. Lane M is 200 ng of the *λ/Pst*I DNA marker. Lane C1 is plasmid pKTB digested with *Hin*dIII. Lanes 1 and 2 are *Hin*dIII digestions of plasmids from clones no. 8 and 70, respectively. Lane C2 is plasmid pKTB digested with *Sal*I. Lanes 3 and 4 are *Sal*I digestions of plasmids from clones no. 8 and 70, respectively. Lane C3 is plasmid pKTB digested with *Sac*I. Lanes 5 and 6 are *Sac*I digestions of plasmids from clones no. 8 and 70, respectively.

(A)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Cap 6
Signal G:85 A:52 T:39 C:34
DT3100POP4{BDv3}v1.mob
BSU_3100
Points 1200 to 18468 Pk 1 Loc: 1200
Page 1 of 5
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Mon, Oct 31, 2005 5:27 PM
Spacing: 15.28{15.28}

(B)

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1351  CTGCGAAATG GTCGCTACGC CACTGACTTC GGTACCACCA ACGCCTTCCA
1401  GCCTTTTGGT GCTACCATGT CGATCGGCAC AGGCGATCGG ATCAATACCG
1451  TGCGCGGTCC TATCACAATC TCTGAAGCGG GTTTCACACT GACTCACGAG
1501  CACATCTGCG GCAGCTCGGC AGGATTCTTG CGTGCTTGGC CAGAGTTCTT
```

Figure 32. Partial nucleotide sequences of the *somA-opd*-[Nos-ter] in plasmid pKT-Psom-opd from clone no. 70

(A) The chromatogram of partial nucleotide sequences on the complementary strand shows the *somA-opd*-[Nos-ter] fusion region in plasmid pKT-Psom-opd from clone no. 70. The start codon of the *opd* gene is indicated by a red circle. (B) Nucleotide sequences of the *somA-opd*-[Nos-ter] fusion region. Partial sequences of the *somA* and *opd* genes are indicated as blue and black letters, respectively. The start codon of the *opd* gene is underlined. The somA-rev3 primer is indicated as bold letters.

2.3 Construction of plasmid pKT-Tsom-opd

The 2.5 kb of PCR product, which contains partial sequences of the P_{tRNA} fused with *somA-opd*-[Nos-ter], was amplified by PCR using primers LK2-somAF1 and Nos-rev2 and using plasmid pKT-Psom-opd from clone no. 70 as the template (see figure 15). In previous study, the 0.1 kb of the P_{tRNA} PCR product was amplified by PCR using primers T1 (Chungjatupornchai, 2002) and tRNA-rev4 (Chungjatupornchai, W., unpublished data) and using plasmid pKGT-T1R1 (Chungjatupornchai, 2002). The P_{tRNA} was fused with the 2.5 kb of PCR product by using overlap extension PCR to achieve gene cassette P_{tRNA}-*somA-opd*-[Nos-ter] construction (see figure 15).

After that, the 2.6 kb of the resulting overlap extension PCR product (figure 33) was verified by digestions with *Dde*I, *Sma*I and *Sal*I (figures 34 and 35). The *Dde*I digestion generated fragments of 0.36, 0.39, 0.4, 0.47 and 1.3 kb. The *Sma*I digestion generated fragments of 0.1, 0.8 and 1.7 kb. The *Sal*I digestion generated fragments of 1 and 1.5 kb. All the DNA band patterns are as expected. The 2.6 kb overlap-extension PCR product was cloned into *Bam*HI site of dephosphorylated pKTB fragment to obtain recombinant plasmid pKT-Tsom-opd (figure 16), which contains gene cassette P_{tRNA}-*somA-opd*-[Nos-ter] (figure 29). The resulting recombinant plasmids from five *E. coli* clones (no. 16 – 20) were digested with *Bam*HI (figure 36). The 2.6 kb of gene cassette P_{tRNA}-*somA-opd*-[Nos-ter] was amplified from plasmids obtained from clones no. 1, 17, 28 and 54 (clones no. 1, 28 and 54, data not shown). Plasmids obtained from clones no. 1, 17, 28 and 54 were verified by *Xho*I digestion to generate fragments of 5.39 and 5.42 kb (figure 37A). Then, plasmids obtained from clones no. 17 and 28 were confirmed by two PCRs. In the first PCR using primers T1 and Nos-rev2, 2.6 kb of P_{tRNA}–*somA-opd*-[Nos-ter] was amplified (figure 37B). In another PCR using primers T1 and somA-rev3, 1.3 kb of P_{tRNA}–*somA* PCR product was amplified (figure 37C). The entire DNA band patterns are as expected therefore the plasmids obtained from clones no. 17 and 28 were the correct plasmid pKT-Tsom-opd.

The nucleotide sequences of P_{tRNA}–*somA-opd*-[Nos-ter] of plasmids from clones no. 17 and 28 were investigated by using Automated DNA sequencing (figure 38, clone no. 28, data not shown). T1 primer was used in the sequencing reaction.

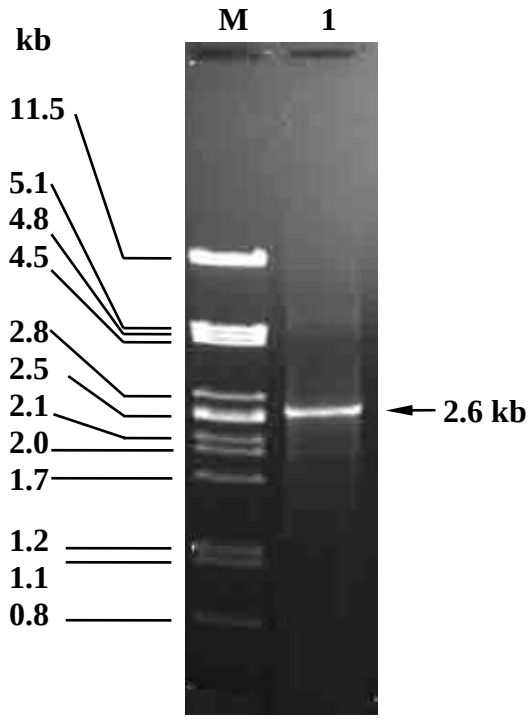


Figure 33. Overlap-extension PCR product containing P_{tRNA}-*somA*-*opd*-[Nos-ter]

Gel electrophoresis was performed on 1 % agarose gel containing ethidium bromide. Lane M is 200 ng of the *λ/Pst*I DNA marker. Lane 1 is the overlap-extension PCR product using a primer set of T1 and Nos-rev2.

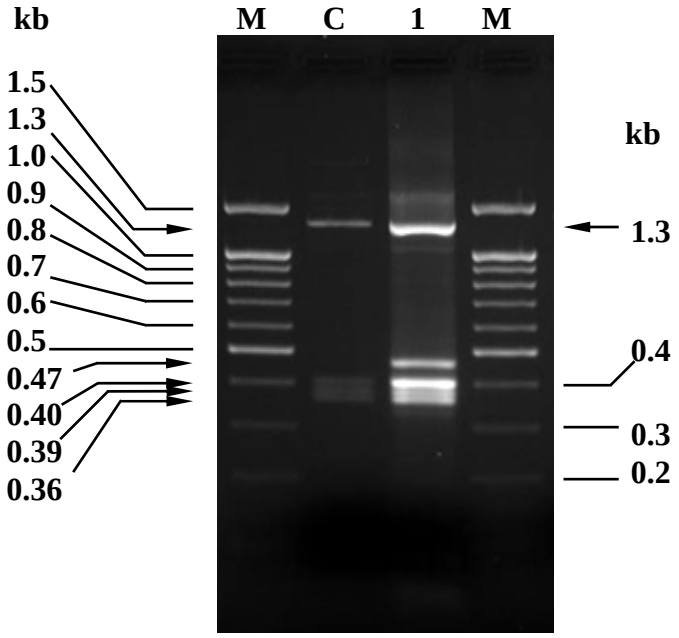


Figure 34. Verification of 2.6 kb overlap-extension PCR product by *Dde*I digestion

Gel electrophoresis was performed on 1.5 % agarose gel containing ethidium bromide. Lane M is 200 ng of the 100 bp DNA ladder marker. Lane C is gene cassette P_{somA}–*somA*-*opd*-[Nos-ter] digested with *Dde*I. Lane 1 is *Dde*I digestion of 2.6 kb overlap-extension PCR product.

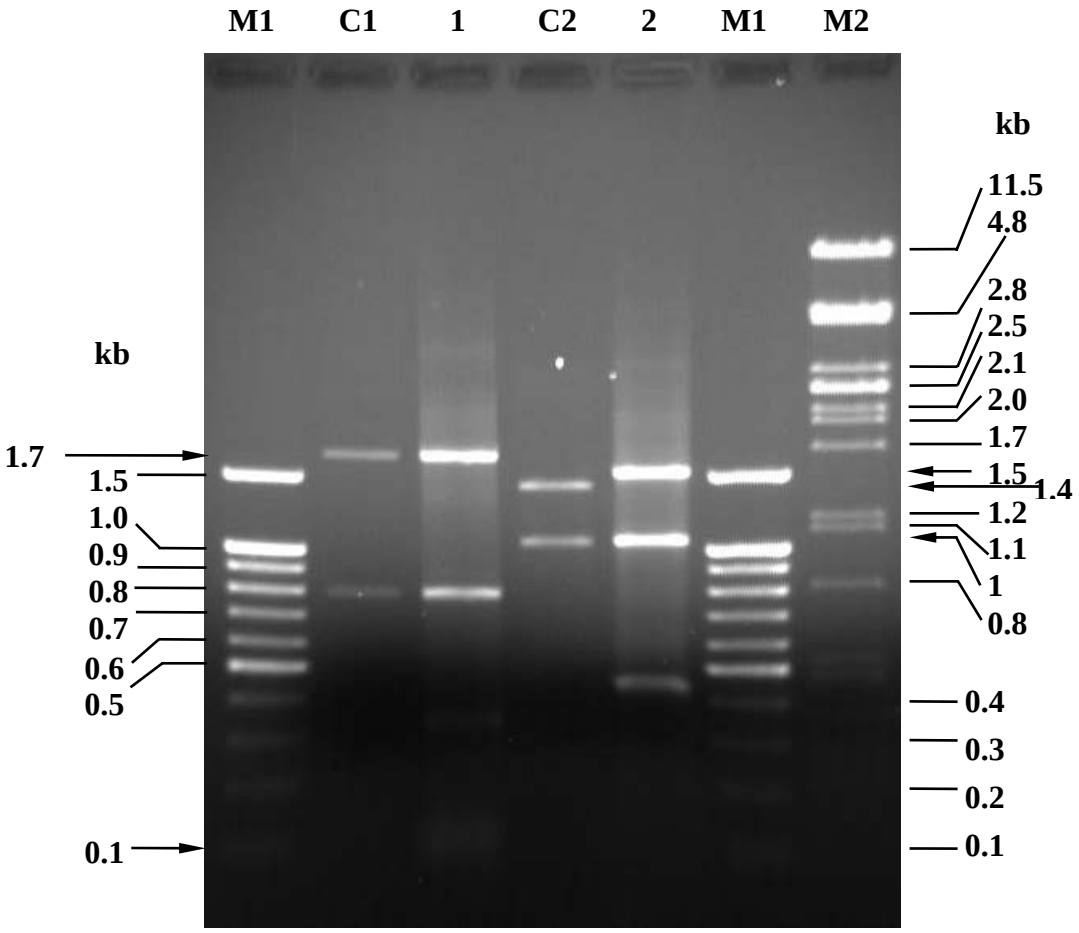


Figure 35. Verification of the 2.6 kb overlap-extension PCR product by *Sma*I and *Sal*I digestions

Gel electrophoresis was performed on 1.5 % agarose gel containing ethidium bromide. Lane M1 is 200 ng of the 100 bp DNA ladder marker. Lane M2 is 200 ng of the λ/*Pst*I DNA marker. Lane C1 is gene cassette P_{somA}-*somA*-*opd*-[Nos-ter] digested with *Sma*I. Lane 1 is *Sma*I digestion of 2.6 kb overlap-extension PCR product of P_{tRNA}-*somA*-*opd*-[Nos-ter]. Lane C2 is gene cassette P_{somA}-*somA*-*opd*-[Nos-ter] digested with *Sal*I. Lane 2 is *Sal*I digestion of 2.6 kb overlap-extension PCR product containing P_{tRNA}-*somA*-*opd*-[Nos-ter].

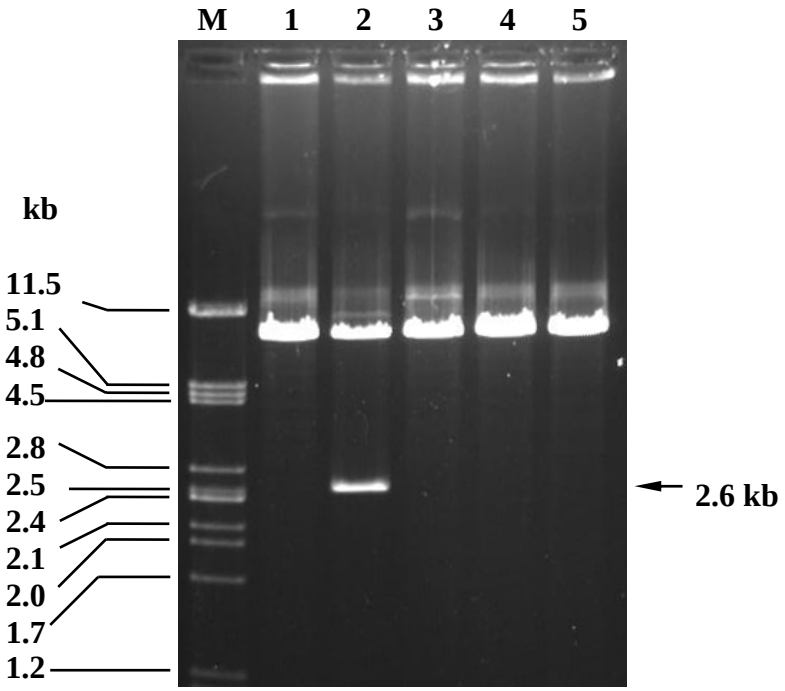
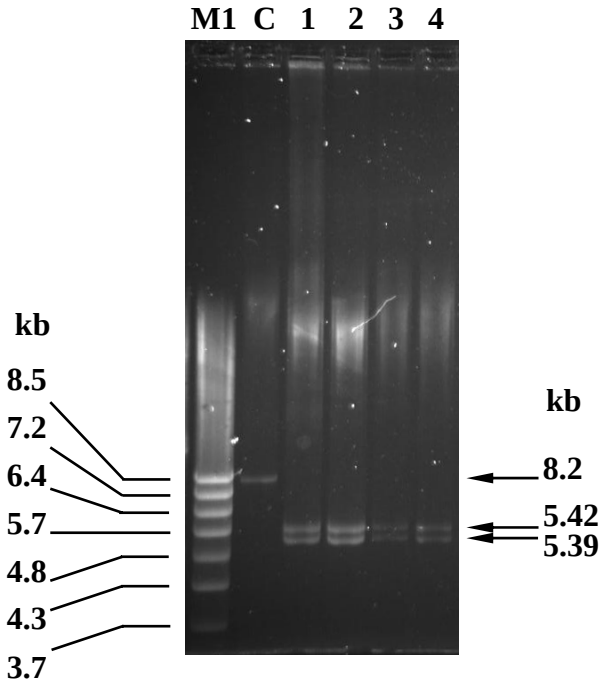


Figure 36. Screening of plasmid pKT-Tsom-opd

Gel electrophoresis was performed on 1 % agarose gel containing ethidium bromide. Lane M is 200 ng of the λ/*Pst*I DNA marker. Lanes 1 – 5 are *Bam*HI digestions of plasmids obtained from *E. coli* clones no. 16-20, respectively.

(A)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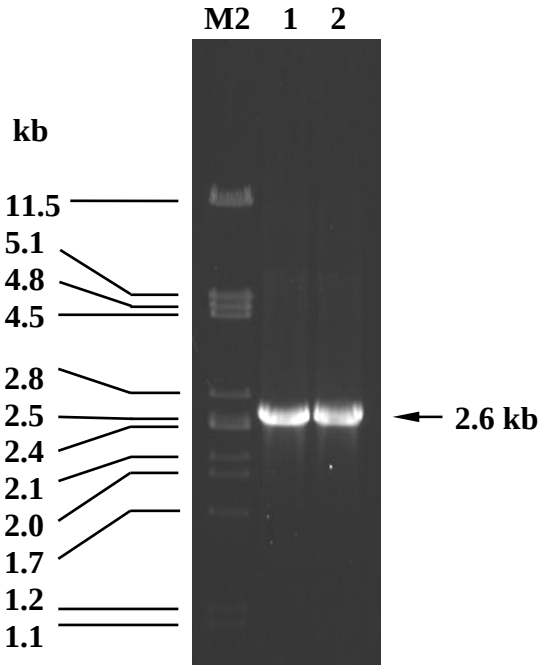
kb
8.5
7.2
6.4
5.7
4.8
4.3
3.7
kb
8.2
5.42
5.39

(B)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kb
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5.1
4.8
4.5
2.8
2.5
2.4
2.1
2.0
1.7
1.2
1.1
2.6 kb

(C)

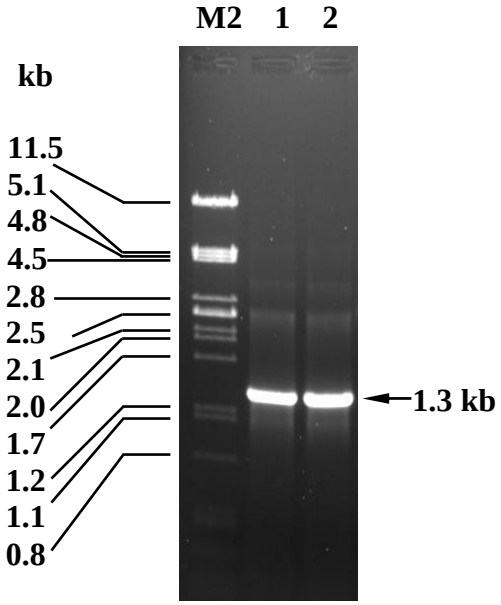


Figure 37. Verification of the plasmid pKT-Tsom-opd

All gel electrophoresises were performed on 1 % agarose gel containing ethidium bromide. Lane M1 is 200 ng of the λ/*Bst*EII DNA marker. Lane M2 is 200 ng of the λ/*Pst*I DNA marker. (A) Lane C is plasmid pKTB digested with *Xho*I. Lanes 1 and 2 are *Xho*I digestions of plasmids from clones no. 1, 17, 28 and 54, respectively. (B) Lanes 1 and 2 are 2.6 kb of P_{tRNA}–*somA-opd*-[Nos-ter] PCR product amplified from plasmids obtained from clones no. 17 and 28, respectively. (C) Lanes 1 and 2 are 1.3 kb of P_{tRNA}–*somA* PCR product amplified from plasmids obtained from clones no. 17 and 28, respectively.

The results showed that the nucleotide sequences of clones no. 17 and 28 are as expected. Therefore, plasmid pKT-Tsom-opd from clones no. 17 and 28 contained the correct P_{tRNA}-*somA* (figure 38).

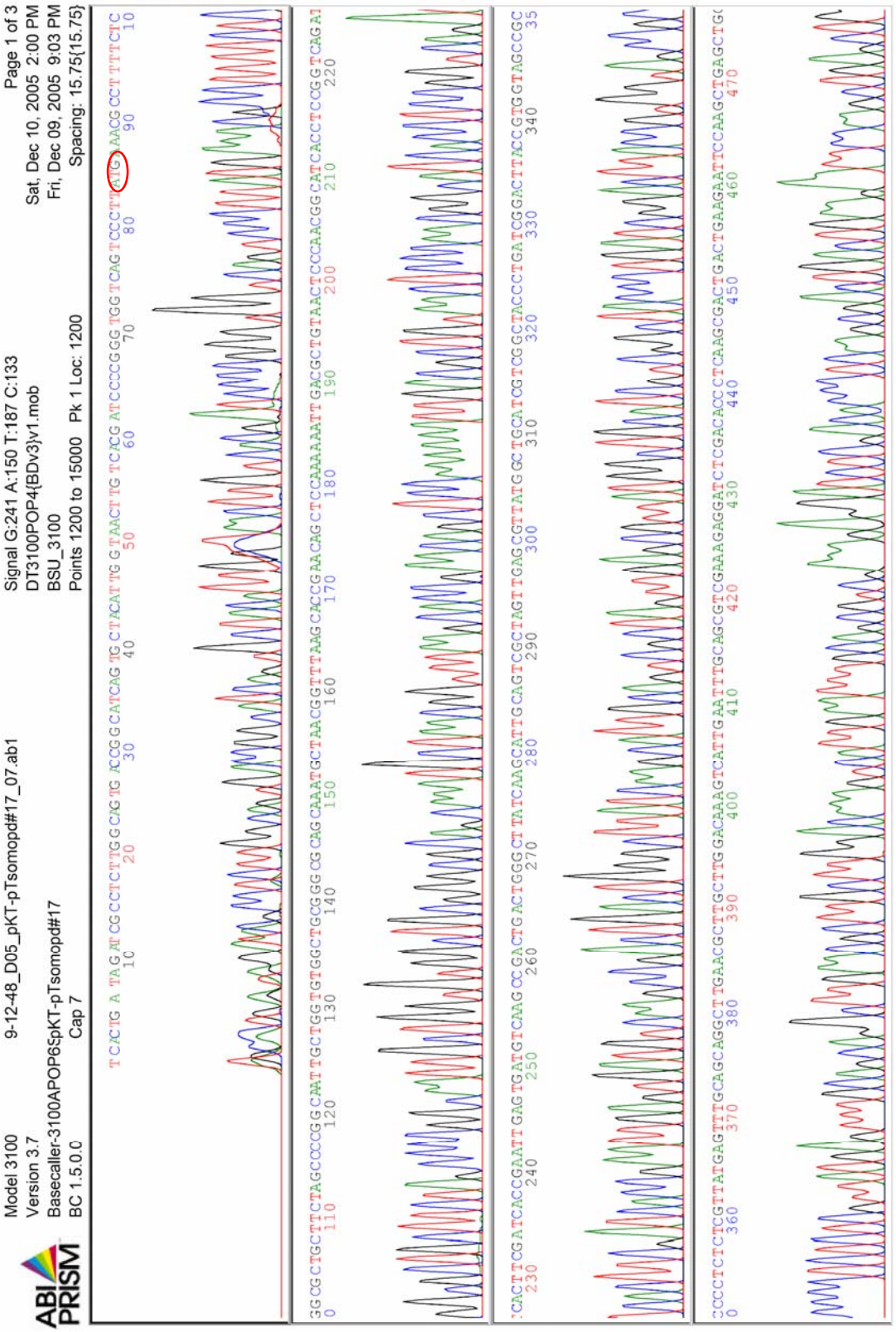
3. Selection of *Synechococcus* harboring pKT-Psom-opd and pKT-Tsom-opd

Plasmid pKT-Psom-opd (see figure 14) from *E. coli* clones no. 8 and 70 weretransformed into *Synechococcus* to generate cyanobacterial clones no. 8-1 to 8-11 and 70-1 to 70-12, respectively. Plasmid pKT-Tsom-opd (see figure 16) from *E. coli* clones no. 17 and 28 were transformed into *Synechococcus* to generate cyanobacterial clones no. 17-1 to 17-31 and 28-1 to 28-31, respectively. Then, cyanobacterial transformants were harvested and were screened by using OPH enzyme activity assay. Clones no. 70-1 and 17-31 were selected for further analysis because they expressed OPH activities that were higher than other cyanobacterial clones. Plasmid pKT-Psom-opd in clone no. 70-1 was verified by PCR using primers somA-fwd1 and Nos-rev2 and using genomic DNA of clone 70-1 as the template (figure 39). Plasmid pKT-Tsom-opd in clone no. 17-31 was verified by PCR using primers T1 and Nos-rev2 and using genomic DNA of clone no. 17-31 as the template (figure 39). All the DNA band patterns are as expected. Therefore, plasmid from clone no. 70-1 and plasmid from clone no. 17-31 were the correct plasmid pKT-Psom-opd and pKT-Tsom-opd, respectively.

4. Whole cell OPH activities of *Synechococcus* harboring plasmid pKT-Psom-opd and pKT-Tsom-opd

Freeze-thaw cells were cells that were frozen at -80 °C and were thawed before performing OPH reaction. Fresh cells were cells that were harvested and then, were immediately performed OPH reaction. Clone pKT-Psom-opd no. 70-1 and clone pKT-Tsom-opd no. 17-31 from stock cultures were streaked on BG-11 agar containing kanamycin and were grown at 30 °C under continuous illumination for 5 days. Freeze-thaw whole cells of both clones were used in whole cell OPH enzyme activity assay. Clone no. 70-1 expressed the maximum value of OPH activity (6.26±0.34 μM/min/OD_{730}) at day 3 (table 2 and figure 40). Whereas clone no. 17-31 expressed the maximum value of OPH activity (1.18±0.04 μM/min/OD_{730}) at day 2 (table 2 and figure 40). The maximum value of OPH activity of cell harboring pKT-Psom-opd (6.26±0.34 μM/min/OD_{730}) was approximately five-fold higher than that of cell

(A)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Cap 7
Signal G:241 A:150 T:187 C:133
DT3100POP4{BDv3}v1.mob
BSU_3100
Points 1200 to 15000 Pk 1 Loc: 1200
Page 1 of 3
Sat, Dec 10, 2005 2:00 PM
Fri, Dec 09, 2005 9:03 PM
Spacing: 15.75{15.75}

(B)

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151   CGGCAATTGC TGGTGTGGCT GCGGGCGCAG CAAATGCTAA CGGTTTAAGC
201   ACCGAACAGC TCCAAAAAAT TGACGCTGTA ACTCCCAACG GCATCACCTC
```

Figure 38. Partial nucleotide sequences of the P_{tRNA}–*somA*-*opd*-[Nos-ter]

(A) The chromatogram of partial nucleotide sequences on the coding strand shows the P_{tRNA}–*somA* fusion region in plasmid pKT-Tsom-opd from clone no. 17. The start codon of the *somA* gene is indicated by a red circle. (B) Nucleotide sequences of the P_{tRNA}–*somA* fusion region. The start codon of the *somA* gene is underlined. The T1 primer is indicated as bold letters. Partial sequences of the P_{tRNA} and the *somA* gene are indicated as black and blue letters, respectively.

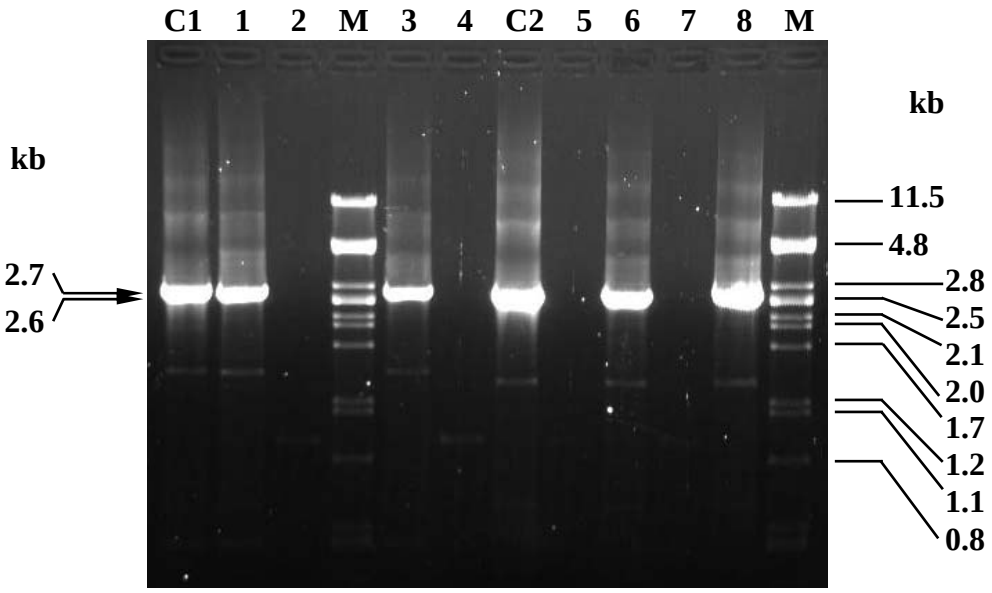

Figure 39. Verification of plasmid pKT-Psom-opd and pKT-Tsom-opd from recombinant *Synechococcus*

Gel electrophoresis was performed on 1 % agarose gel containing ethidium bromide. Lane M is 200 ng of the λ/*Pst*I DNA marker. Lane C1 is PCR product using plasmid isolated from *E. coli* clone no. 70 as template and primers somA-fwd1 and Nos-rev2. Lanes 1, 3 and 2, 4 are PCR products using plasmids isolated from *Synechococcus* clones no. 70-1 and 17-31, respectively, as templates and primers somA-fwd1 and Nos-rev2. Lane C2 is PCR product using plasmid isolated from *E. coli* clone no. 17 as template and primers T1 and Nos-rev2. Lanes 5, 7 and 6, 8 are PCR products using plasmids isolated from *Synechococcus* clones no. 70-1 and 17-31, respectively, as templates and primers T1 and Nos-rev2.

Table 2. OPH activities of freeze-thaw whole cells of *Synechococcus* harboring the *opd* gene

The *Synechococcus* clones were grown on BG-11 agar at 30 °C with continuous illumination. OD_{730} = 1 of the cells was collected to perform OPH enzyme activity assay. The data relate with figure 40.

Day	OPH activity (µM of paraoxon hydrolysed/min/OD_{730})[a]	
	pKT-Psom-opd	pKT-Tsom-opd
1	0.85 (±0.04)	0.82 (±0.07)
2	4.73 (±0.20)	1.18 (±0.04)
3	6.26 (±0.34)	1.09 (±0.14)
4	3.97 (±0.43)	1.00 (±0.11)
5	3.81 (±0.33)	0.95 (±0.12)

[a] The OPH activity is the mean of at least three independent experiments (±standard error mean).

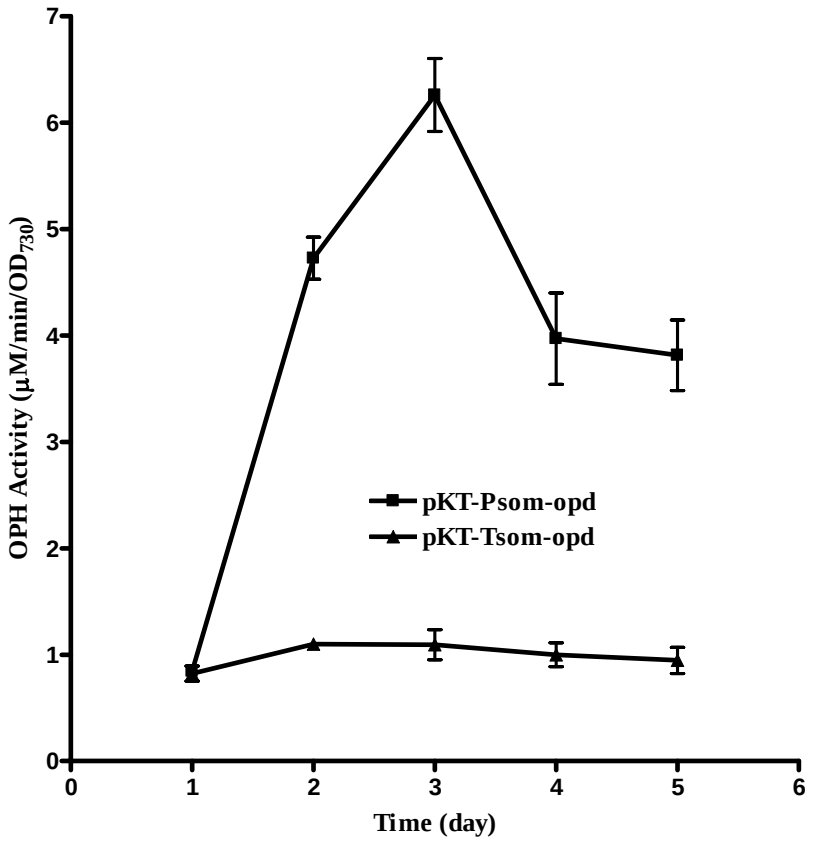

Figure 40. OPH activities of freeze-thaw whole cells of *Synechococcus* harboring plasmid pKT-Psom-opd and pKT-Tsom-opd

Synechococcus were grown on BG-11 agar containing kanamycin at 30 °C under 3,500 lux continuous illumination. OD_{730} = 1 of the cells was collected on days 1 – 5 to perform whole cell OPH enzyme activity assay with a final concentration of 2 mM paraoxon as substrate. Means with standard error means (SEM) are shown. This figure was drawn from data in table 2.

harboring pKT-Tsom-opd (1.18±0.04 μM/min/OD_{730}). Thus, the P_{somA} is stronger than the P_{tRNA}.

5. Proteinase K treatment of *Synechococcus* harboring pKT-Psom-opd and pKT-Tsom-opd

Proteinase accessibility experiments were performed to ascertain the presence of OPH on the cell surface of recombinant *Synechococcus*, since proteinase K cannot readily diffuse across the cell membrane and degrades only proteins exposed on the cell surface (Shimazu et al., 2001b). Clone pKT-Psom-opd no. 70-1 was grown in BG-11^0 and in BG-11^N broth. Fresh cells of clone no. 70-1 were harvested and incubated with proteinase K for 1 h before performing whole cell OPH enzyme activity assay. The results from more than three independent experiments were not consistent (tables 3 and 4 and figure 41). Most results showed that the OPH activities of treated cells slightly increased when compared with those of untreated cells (the results of nitrogen-replete cells collected at 0, 12, 36 and 48 h and nitrogen-deprived cells collected at 0 and 12 and 48 h). Furthermore, the OPH activities of treated cells were significantly higher than those of untreated cells when nitrogen-deprived cells were collected at 6 and 24 h. In contrast, the OPH activities of treated cells were significantly lower than those of untreated cells when nitrogen-replete cells were collected at 6 and 24 h and nitrogen-deprived cells were collected at 36 h. Therefore, it was not able to conclude that the OPH was successfully targeted onto the surface of *Synechococcus* by using the SomA system.

6. OPH enzyme activities of fresh whole cells of *Synechococcus* harboring pKT-Psom-opd in nitrogen starvation condition

Nitrogen starvation of *Synechococcus* was performed to improve the OPH activity of clone pKT-Psom-opd no. 70-1 harboring P_{somA}-*somA-opd*-[Nos-ter] since it has been shown that P_{somA} is susceptible to nitrogen down-shift (Sauer et al., 2001). Clone no. 70-1 was grown in BG-11^0 medium for nitrogen starvation condition and in BG-11^N medium for nitrogen repletion condition.

When clone no. 70-1 was grown on BG-11^0 agar, the cells died within 3 days. Clone no. 70-1 was grown in BG-11^0 and in BG-11^N broth at 30 °C with continuous illumination for 48 h. OD_{730} = 1 of the cells was harvested at 0, 6, 12, 24, 36 and 48 h to perform whole cell OPH enzyme activity assay. The result showed inconsistent

Table 3. OPH activities of fresh whole cells of *Synechococcus* harboring pKT-Psom-opd under nitrogen starvation without proteinase K treatment

Synechococcus clone no. 70-1 harboring plasmid pKT-Psom-opd was grown in $BG\text{-}11^0$ and in $BG\text{-}11^N$ broth at 30 °C with continuous illumination for 48 h. OD_{730} = 1 of the cells was collected at 0, 6, 12, 24, 36 and 48 h. Then, whole cell OPH enzyme activity assay was performed. The data relate with figure 41.

Hour	OPH activity (µM of paraoxon hydrolysed/min/OD_{730})[a]	
	Clone no. 70-1 in $BG\text{-}11^N$	Clone no. 70-1 in $BG\text{-}11^0$
0	0.89 (±0.17)	0.83 (±0.11)
6	0.86 (±0.23)	0.32 (±0.06)
12	0.92 (±0.11)	0.84 (±0.12)
24	0.92 (±0.17)	0.36 (±0.04)
36	0.64 (±0.10)	0.77 (±0.08)
48	0.73 (±0.16)	0.80 (± 0.11)

[a] The OPH activity is the mean of at least three independent experiments (±standard error mean).

Table 4. OPH activities of fresh whole cells of *Synechococcus* harboring pKT-Psom-opd under nitrogen starvation with proteinase K treatment

Synechococcus clone no. 70-1 harboring plasmid pKT-Psom-opd was grown in BG-11^0 and in BG-11^N broth at 30 °C with continuous illumination for 48 h. OD_{730} = 1 of the cells was collected at 0, 6, 12, 24, 36 and 48 h. Then, the cells were treated with proteinase K for 1 h before whole cell OPH enzyme activity assay was performed. The data relate with figure 41.

Hour	OPH activity (μM of paraoxon hydrolysed/min/OD_{730})[a]	
	Clone no. 70-1 in BG-11^N with proteinase K treatment	Clone no. 70-1 in BG-11^0 with proteinase K treatment
0	0.96 (±0.11)	0.89 (±0.17)
6	0.60 (±0.12)	0.66 (±0.08)
12	0.99 (±0.17)	0.93 (±0.11)
24	0.64 (±0.09)	0.62 (±0.07)
36	0.68 (±0.11)	0.61 (±0.12)
48	0.83 (±0.13)	0.93 (±0.10)

[a] The OPH activity is the mean of at least three independent experiments (±standard error mean).

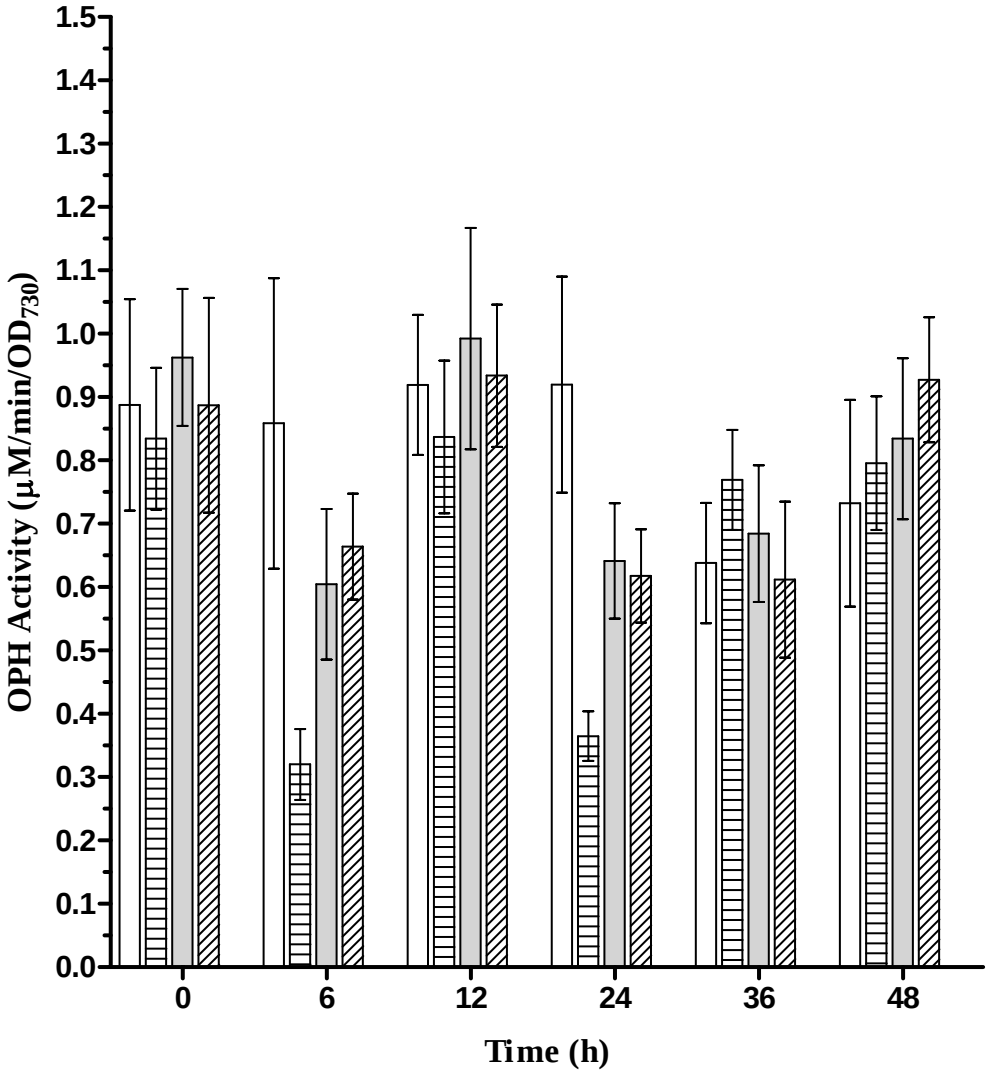

Figure 41. OPH activities of fresh whole cells of *Synechococcus* harboring pKT-Psom-opd under nitrogen starvation condition with and without proteinase K treatment

OPH activities of fresh whole cells of clone no. 70-1 that was grown in BG-11^N (□) and in BG-11^0 (▤) broth without proteinase K treatment and was grown in BG-11^N (■) and in BG-11^0 (▨) broth with proteinase K treatment for 1 h are shown as means with standard error means (SEM). OD_{730} = 1 of the cells was collected at 0, 6, 12, 24, 36 and 48 h to perform whole cell OPH enzyme activity assay. This figure was drawn from data in tables 3 and 4.

data (table 3 and figure 41) as follows: The OPH activities of nitrogen-deprived fresh cells, which collected at 0 and 12 h (0.83±0.11 and 0.84±0.12 μM/min/OD_{730}, respectively), slightly decreased when compared with those of nitrogen-replete fresh cells, which collected at 0 and 12 h (0.89±0.17 and 0.92±0.11 μM/min/OD_{730}, respectively). The OPH activities of nitrogen-deprived fresh cells, which collected at 6 and 24 h (0.32±0.06 and 0.36±0.04 μM/min/OD_{730}, respectively), were significantly lower than those of nitrogen-replete fresh cells, which collected at 6 and 24 h (0.86±0.22 and 0.92±0.17 μM/min/OD_{730}, respectively). In contrast, the OPH activity of nitrogen-deprived fresh cells, which collected at 36 and 48 h (0.77±0.08 and 0.80±0.11 μM/min/OD_{730}), slightly increased when compared with those of nitrogen-replete fresh cells, which collected at 36 and 48 h (0.64±0.10 and 0.73±0.16 μM/min/OD_{730}). Therefore, the results were not conclusive.

CHAPTER V
DISCUSSION

Surface expression of OPH, rather than intracellular, offers improved biodegradation of organophosphorus compounds (Richins et al., 1997) and becomes more attractive when water insoluble substrates such paraoxon are used. Although recombinant cyanobacterium *Synechococcus* PCC7942 that can degrade paraoxon have been developed by using Inp and OmpA cell surface display systems (Chungjatupornchai, W., unpublished data), in this study, the cyanobacterial SomA system was used as a challenge to develop an efficient system to surface-express functional OPH under the control of the P_{som} or the P_{tRNA}.

In proteinase K accessibility assay of fresh whole cells of *Synechococcus* harboring pKT-Psom-opd, more than three independent experiments were performed but the results were not conclusive (figure 41). The variable data were found both in different experiments and in the same experiment (within duplicate or triplicate of each sample). The possible variable factors, which affected these results, are as follows: The first factor is the solubility of substrate. Poor solubility of substrate caused more variable results. Since paraoxon is a water-insoluble substrate, its solubility is important in whole cell OPH enzyme activity assay. In order to increase solubility of the substrate, paraoxon was diluted in 10 % methanol in citrate-phosphate buffer (Shimazu et al., 2001a; Shimazu et al., 2001b; Shimazu et al., 2003). Therefore, in this study, paraoxon was diluted in 10 % methanol in CHES buffer and was prewarmed at 37 °C about 1 h and mixed well before performing the OPH reaction. The second factor is inconsistent cell growth in each experiment. Batch-to-batch variability depended on environmental conditions, especially temperature and light. The third factor is method to stop the OPH reaction. Instead of adding inhibitor, the OPH reaction was stopped by incubation on ice for about 5 min and centrifugation. Then, supernatant was separated to measure OPH activity by using

spectrophotometer. It was noted that fresh cells were more difficult to be separated from the supernatant than freeze-thaw cells. After centrifugation, residual fresh whole cells in the supernatant may have the OPH reaction, which causes the inaccuracy of OPH activity measured by using spectrophotometer. The forth factor is pipette error during the dilution step before OPH activity measurement by spectrophotometer. Therefore, it was not able to conclude that the OPH was successfully targeted onto the surface of *Synechococcus* by using the SomA system. In Shimazu et al. (2001a), the percentage of decrease in OPH activity of proteinase-treated recombinant *Escherichia coli* cells was used to ascertain the surface localization of OPH. The OPH activity of the *E. coli* cells surface-expressing Inp-OPH fusion protein decreased 64 % after 1 h of incubation with proteinase K at room temperature. In addition to estimation of the percentage of decrease in OPH activity of proteinase-treated cells, the following methods have been reported for detection of surface localization of OPH; (i) measurement of OPH activity in the membrane fraction of disrupted cells (Shimazu et al., 2001a; Wang et al., 2002; Shimazu et al., 2003), (ii) immunoblotting of membrane fraction of disrupted cells by using antiserum against OPH as the first antibody (Shimazu et al., 2001b, Wang et al., 2002; Shimazu et al., 2003), (iii) immunofluorescence of microscopy by probing cells with rabbit anti-OPH serum as the first antibody and then fluorescently staining with fluorescein isothiocyanate (FITC) – labled goat anti-rabbit IgG as the second antibody (Shimazu et al., 2001a; Shimazu et al., 2001b; Wang et al., 2002) and (iv) whole cell ELISA by probing cells with rabbit antiserum against the anchor protein as the primary antibody and with goat anti-rabbit antibody conjugated with alkaline phosphate as the second antibody and using *p*-nitrophenyl phosphate as a substrate (Shimazu et al., 2003).

Since the outer-membrane porins SomA and SomB accumulated in *Synechococcus* PCC7942 under nitrogen starvation condition (Sauer et al, 2001), it was expected that SomA-OPH fusion protein in this study might increase when *Synechococcus* harboring plasmid pKT-Psom-opd was grown in nitrogen-free liquid medium (BG-11^0). However, the results were not inconsistent. The SomA porin is slightly increased under the nitrogen starvation condition (Sauer et al, 2001).

In addition to paraoxon, it is possible to use the recombinant *Synechococcus* in this thesis for degradation of other organophosphorus compounds such as coumaphos,

diazinon, methyl parathion and parathion, as shown in *Psuedomonas putida* KT2440 (Shimazu et al., 2003), *Moraxella* sp. (Shimazu et al., 2001b; Mulchandani et al., 2001) and *E. coli* (Mulchandani et al., 1998; Mulchandani et al., 1999) with OPH on the surface.

The newly explored SomA-based fusion system reported in this thesis offers an attractive alternative to display a wide range of proteins on the surface of *Synechococcus* PCC7942.

CHAPTER VI
CONCLUSIONS

1. Gene cassettes P_{som}-*somA*-*opd* and P_{tRNA}-*somA*-*opd* were successfully constructed by using overlap extension PCR. OPH was fused to C-terminus of outer membrane SomA porin in order to generate the SomA-OPH fusion protein.

2. Gene cassettes P_{som}-*somA*-*opd* and P_{tRNA}-*somA*-*opd* were successfully cloned into *E. coli*–cyanobacteria shuttle plasmid pKTB in order to generate plasmids pKT-Psom-opd and pKT-Tsom-opd, respectively. The resulting plasmids were transformed into *Synechococcus* PCC7942.

3. The OPH activity of cells harboring pKT-Psom-opd (6.26±0.34 μM/min/OD_{730}) was five times higher than that of cells harboring pKT-Tsom-opd (1.09±0.14 μM/min/OD_{730}). Thus, the P_{som} is stronger than the P_{tRNA}.

4. The results from nitrogen starvation of fresh whole cells harboring pKT-Psom-opd were not conclusive.

5. In proteinase K accessibility assay of fresh whole cells of *Synechococcus* harboring pKT-Psom-opd, the results were not conclusive. Thus, it was not able to conclude that the OPH was targeted onto the surface of *Synechococcus* by using the SomA system.

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