รายงานวิจัยฉบับสมบูรณ์

โครงการ

“การสังเคราะห์ไลบรารี่ regulatory-sequence เพื่อปรับปรุงการแสดงออกของยีน heterologous ในไซยาโนแบคทีเรีย”

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

โครงการวิจัยนี้ได้รับทุนสนับสนุนจาก ทุนวิจัยองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนา สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ในระหว่าง วันที่ 30 พฤษภาคม พ.ศ 2551 ถึงวันที่ 29 พฤษภาคม พ.ศ 2554 ทุนดังกล่าวมีส่วนสนันสนุนการทำวิทยานิพนธ์ของนักศึกษาปริญญาโทสาขาพันธุศาสตร์ระดับโมเลกุลและพันธุวิศวกรรมศาสตร์ ได้แก่ นายอรรถพล กำลังดี นายนวพล อุดพ้วย และนางสาวปาจรีย์ เลี้ยงหล่ำ นับว่าได้มีส่วนช่วยเสริมสร้างความเข้มแข็งแก่งานวิจัยอย่างยิ่ง คณะวิจัยขอขอบคุณ สกว. มา ณ. โอกาสนี้ด้วย

ขอขอบคุณ ศาสตราจารย์ ดร. โกวิท พัฒนาปัญญาสัตย์ สถานส่งเสริมการวิจัย คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล ที่เอื้อเฟื้อการใช้เครื่อง Fluorescence-activated cell sorter (FACS)

บทคัดย่อ

ไซยาโนแบคทีเรีย เป็นจุลชีพที่สังเคราะห์แสงให้ออกซิเจน สามารถเติบโตได้ในอาหารเลี้ยงเชื้อราคาถูก ได้ใช้เป็นเจ้าบ้านสำหรับการแสดงออกของยีน heterologous ต่างๆ อย่างไรก็ดี ระดับการแสดงออกของยีน heterologous ในไซยาโนแบคทีเรีย มีระดับต่ำกว่าใน *E. coli* โครงการวิจัยนี้มีเป้าหมายที่จะพัฒนา enhancer/ โปรโมเตอร์ (promoter) และบริเวณเริ่มถอดรหัส (translation initiation region) ให้สามารถทำงานแข็งขันในไซยาโนแบคทีเรีย *Synechococcus* PCC7942 ได้ทำการตรวจสอบการทำงานของโปรโมเตอร์ของ *Synechococcus* outer membrane protein A (SomA) ซึ่งเป็นโปรตีนที่พบมากที่สุดในที่หุ้มเซลล์ ผลการทดลองพบว่า การทำงานของโปรโมเตอร์ *somA* อยู่ระดับต่ำกว่าโปรโมเตอร์ $tRNA^{pro}$ (P_{tRNA}) ได้มีการประเมินการใช้ green fluorescent protein (GFP) เป็นโปรตีนรายงานใน *Synechococcus* ผลการทดลองแสดงให้เห็นว่า ระดับฟลูออเรสเซนต์ของเซลล์ที่มี cytoplasmic GFP สูงกว่า เซลล์ที่มี periplasmic GFP ถึง 6 เท่า ดังนั้นจึงใช้ cytoplasmic GFP เป็นตัวรายงาน สภาวะที่เหมาะสมที่สุดในการแสดงออกของ GFP คือการเลี้ยง *Synechococcus* หนึ่งวันภายใต้แสงที่มีความเข้ม 4000-4500 lux เพื่อตรวจหา enhancer/ โปรโมเตอร์ ที่ทำงานแข็งขันจึงได้ตรวจกรองหา GFP activity สูงจาก *Synechococcus* ที่มี synthetic libraries pLL และ pUL ซึ่งมี LysR binding sequences และ UP element/ LysR sequences (ตามลำดับ) อยู่ด้านหน้าของ P_{tRNA} พบว่าโคลนที่มี pUP-UP140, pLL-172, pLL-209 และ pUL-146 มีค่า GFP activity เป็น 1.56, 2.70, 2.37, 3.40 และ 3.15 เท่า (ตามลำดับ) สูงกว่าของ control (pKT-GFP) จากการวิเคราะห์ลำดับเบสพบว่า pUP-UP140 มี UP element จำนวน 3 ชุด ส่วน pLL-172, pLL-209 และ pUL-146 นั้นพบว่าส่วนของ transcription-translation terminator ที่อยู่ด้านหน้าของthe regulatory sequences หลุดหายไป ดังนั้น ค่า GFP activities ที่สูงขึ้นนั้นอาจเนื่องมาจาก read-through transcripts เพื่อตรวจหาบริเวณเริ่มถอดรหัสที่เหมาะสมที่สุด จึงได้ตรวจกรองหาค่า GFP activity สูงจาก *Synechococcus* ที่มี synthetic Shine-Dalgarno (SD)/ non-SD sequence libraries ซึ่งมีส่วนของ randomized decanucleotide อยู่ด้านหน้าของยีน *gfp* พร้อมทั้งได้ตรวจหาระดับของ *gfp* mRNA โดยวิธี real-time PCR แล้ววิเคราะห์ประสิทธิภาพการถอดรหัส (translation efficiency) ประสิทธิภาพการถอดรหัสของลำดับเบส SD-like AGGAGAAUGA, AAUGGAAAUA และ AAUUGGAUUU สูงกว่าของ control (GGUGGU) อยู่ 2.38, 3.96 และ 4.89 เท่าสูง (ตามลำดับ) แสดงให้เห็นว่าลำดับเบส SD-like มีประสิทธิภาพการถอดรหัสสูง อย่างไรก็ดี ประสิทธิภาพการถอดรหัสของลำดับเบส non-SD AUGUCAACUU ไม่แตกต่างอย่างมีนัยสำคัญจาก control ประสิทธิภาพการถอดรหัสของลำดับเบส non-SD UUCAAUAUUU, AUUUACCUCC และ CCAAUCUAC จาก *E. coli* ต่ำกว่า control อย่างชัดเจน ดังนั้นผลการทดลองบ่งชี้ว่า ลำดับเบส non-SD สามารถทำหน้าที่เริ่ม

ถอดรหัสใน *Synechococcus* แต่มีประสิทธิภาพต่ำกว่าลำดับเบส SD-like ประสิทธิภาพการถอดรหัสของลำดับเบส consensus-SD AAGGAGGU, AGGAGGU, AAAGGAGG และ AGGAGG สูงกว่าของ control อยู่ 12.24, 10.52, 6.36 และ 3.77 เท่า (ตามลำดับ) ดังนั้น ลำดับเบส consensus-SD มีประสิทธิภาพการถอดรหัสสูงกว่าลำดับเบส SD-like และลำดับเบส AAGGAGGU มีความเหมาะสมที่สุดสำหรับการเริ่มถอดรหัสใน *Synechococcus* สำหรับ translation enhancer นั้น การมี BoxA ของ *E. coli rrnB* และ pyrimidine-rich enhancer ที่อยู่ด้านหน้าของบริเวณเริ่มถอดรหัส ไม่สามารถเสริมแต่กลับลดประสิทธิภาพการถอดรหัส ดังนั้น translation enhancer จาก *E. coli* ไม่สามารถทำงานได้ใน *Synechococcus* ได้ตรวจสอบบริเวณที่ทำหน้าที่ของเบสควบคุม (regulatory sequence) ของ *rrnA* จาก*Synechococcus* โดยวิธี deletion analysis พบว่าเมื่อให้บริเวณที่ทำหน้าที่ของลำดับเบสควบคุมของ *rrnA* อยู่ด้านหน้าของลำดับเบส consensus-SD จะให้ GFP activity สูงกว่า control ถึง 23.97 เท่า ได้สำรวจการทำงานของโปรโมเตอร์ σ^{70} (P_{sig70}) ของ *E. col* ใน *Synechococcus* ซึ่งอยู่ด้านหน้าของลำดับเบส consensus-SD ผลการทดลองพบว่า GFP activity ของ P_{sig70} สูงกว่า control 6.17 เท่า เพื่อสร้างลำดับเบสควบคุมที่สามารถทำงานในระดับที่สูงเกินกว่าที่พบตามธรรมชาติในไซยาโนแบคทีเรีย จึงได้สร้างพลาสมิดที่มีการผสมของ tandem enhancer/ promoter และลำดับเบส consensus-SD ผลการทดลองพบว่า GFP activity ของ *Synechococcus* ที่มีพลาสมิด tandem promoters P_{rnnA} -P_{rnnA} (pRRSC), P_{sig70}- P_{rnnA} (pS70-RSC) และ P_{sig70}- P_{rnnA}- P_{rnnA} (pS70-2RSC) สูงกว่าของ control ถึง 32.33, 36.66 และ 35.88 เท่า(ตามลำดับ) เมื่อนำโปรตีนทั้งหมดที่สกัดจาก *Synechococcus* ที่มีพลาสมิด pS70-2RSC และ pS70-RSC มาวิเคราะห์โดยวิธี SDS-PAGE แล้วย้อมด้วย Comassie brilliant blue พบว่าสามารถมองเห็นแถบโปรตีน GFP เท่าที่คณะวิจัยโครงการนี้ทราบ รายงานนี้เป็นรายงานแรกที่แสดงให้เห็นว่าสามารถควบคุมให้การแสดงออกของโปรตีน heterologous ใน *Synechococcus* สูงมากพอที่จะตรวจสอบได้ด้วย SDS-PAGE ที่ย้อมด้วย Comassie brilliant blue ดังนั้น คณะวิจัยโครงการนี้ได้ประสบความสำเร็จในการสร้างลำดับเบสควบคุม ที่สามารถทำงานในระดับที่สูงเกินกว่าที่พบตามธรรมชาติในไซยาโนแบคทีเรีย

Abstract

Cyanobacteria, oxygenic photosynthetic prokaryotes, have simple growth requirements and inexpensive to maintain. They have been used as hosts to express several heterologous genes. However, the level of heterologous gene expression in cyanobacteria is low when compared with that in *E. coli*. This project is aimed to develop highly active enhancer/ promoter and translation initiation region including translation initiator and translation enhancer in cyanobacterium *Synechococcus* PCC7942. The promoter of *Synechococcus* outer membrane protein A (SomA), one of the most abundant proteins of the cyanobacterial total envelope, was investigated. Results indicated that the activity of *somA* promoter was dramatically lower than that of $tRNA^{pro}$ promoter (P_{tRNA}).The use of green fluorescent protein (GFP) as a reporter in *Synechococcus* was evaluated. Results showed that the fluorescence intensity of cells with cytoplasmic GFP was approximately 6-fold higher than that of cells with periplasmic GFP. Therefore, cytoplasmic GFP was used as a reporter. The optimal conditions for GFP expression in *Synechococcus* were 1-day cultures grown under light intensity of 4000-4500 lux. For highly active enhancer/ promoter, *Synechococcus* harboring synthetic libraries pLL and pUL containing LysR binding sequences and UP element/ LysR sequences upstream of P_{tRNA} , respectively, were screened for high GFP activities. The GFP activities of selected clones harboring pUP-UP140, pLL-172, pLL-209 and pUL-146 were 1.56-, 2.70-, 2.37-, 3.40- and 3.15- fold, respectively, higher than that of control (pKT-GFP). DNA sequence analysis revealed that there are three copies of UP element in pUP-UP140. In plasmids pLL-172, pLL-209 and pUL-146, the transcription-translation terminator upstream of the regulatory sequences was deleted. Therefore, the high GFP activities might be due to the read-through transcripts. For optimal translation initiators, *Synechococcus* harboring the synthetic Shine-Dalgarno (SD)/ non-SD sequence libraries containing randomized decanucleotide region upstream of the *gfp* gene were screened for high GFP activities. The level of *gfp* mRNA of the selected clones was determined using real-time PCR and the translation efficiency was analyzed. The translation efficiencies of SD-like sequences: AGGAGAAUGA, AAUGGAAAUA and AAUUGGAUUU were 2.38-, 3.96- and 4.89-fold, respectively, higher than that of control (GGUGGU), indicating that the SD-like sequences were highly efficient for translation initiation. However, the translation efficiency of non-SD sequence, AUGUCAACUU, was

not significantly different from that of control. The translation efficiencies of *E. coli* non-SD sequences UUCAAUAUUU, AUUUACCUCC and CCAAUCUAC were apparently lower than that of control. Therefore, the results indicated that the non-SD sequences could be translation initiator in *Synechococcus*, but were less efficient than SD-like sequences. The translation efficiencies of consensus-SD sequences AAGGAGGU, AGGAGGU, AAAGGAGG and AGGAGG were 12.24-, 10.52-, 6.36- and 3.77-fold, respectively, higher than that of control. Thus, the consensus-SD sequences were more efficient for translation initiation than the SD-like sequence and AAGGAGGU was the optimal translation initiator in *Synechococcus*. For translation enhancer, the present of BoxA of *E. coli rrnB* and pyrimidine-rich enhancer upstream of translation initiators did not enhance but decreased the translation efficiency. Therefore, the *E. coli* translation enhancer did not function in *Synechococcus*. The active region of *rrnA* regulatory sequence of *Synechococcus* was identified using deletion analysis. The GFP activity of active *rrnA* regulatory sequence upstream of the consensus-SD sequence was 23.97-fold higher than that of control. The activity of *E. coli* σ^{70} promoter (P_{sig70}) upstream of the consensus-SD sequence in *Synechococcus* was also investigated. Results showed that the GFP activity of the P_{sig70} was 6.17-fold higher than that of control. In order to construct regulatory sequences which are highly active exceeding the natural cyanobacterial regulatory sequences, plasmids harboring combination of tandem enhancer/ promoter and consensus-SD sequence were constructed. Results showed that the GFP activities of *Synechococcus* harboring plasmids with tandem promoters P_{rnnA} -P_{rnnA} (pRRSC), P_{sig70}- P_{rnnA} (pS70-RSC) and P_{sig70}- P_{rnnA}- P_{rnnA} (pS70-2RSC) were 32.33-, 36.66- and 35.88- fold, respectively, higher than that of control. When the total protein extracted from *Synechococcus* harboring plasmids pS70-2RSC and pS70-RSC were subjected to SDS-PAGE and stained with Comassie brilliant blue, the GFP band was visualized. To our knowledge, this is the first report that the expression of heterologous protein in *Synechococcus* is high enough to be detected by SDS-PAGE stained with Comassie brilliant blue. Thus, we have successfully constructed the synthetic regulatory sequences which are highly active exceeding the natural cyanobacterial regulatory sequences.

Executive Summary

Project title (Thai): การสังเคราะห์ไลบรารี่ regulatory-sequence เพื่อปรับปรุงการแสดงออกของยีน heterologous ในไซยาโนแบคทีเรีย

(English): Synthetic regulatory- sequence libraries for improvement of heterologous gene expression in cyanobacteria

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Duration of the project: 3 years (May 30, 2008- May 29, 2011)

Rationale

Since cyanobacteria are photoautotrophic prokaryotes and have simple growth requirements and inexpensive to maintain, they have been used as hosts to express several heterologous genes. However, the level of heterologous gene expression in cyanobacteria is low when compared with that in *E. coli*. Current knowledge of the relationship between nucleotide sequence structure and function of promoters and ribosome binding site for translation initiation recognized within cyanobacteria is still limited. In cyanobacterium *Synechocystic* PCC6803, only 48.6% of genes contain the Shine-Dalgarno (SD) sequence. Recently, large-scale analysis of distribution of SD sequences in completed microbial genomes (10 species) reveals that genes led by SD are as common as genes not led by SD.

A pool of various genes generated by random mutations introduced into a gene and selecting what is wished out of the pool can be a simulation of molecular evolution. Such a process is referred to as directed evolution. In this project, we will use this process to develop highly active regulatory sequences for heterologous gene expression in cyanobacteria by (i) generation of strong promoters from synthetic DNA library including the promoter and enhancer elements (ii) analysis of non-SD initiation of translation selected from synthetic DNA library. The knowledge obtained from (i) and (ii) can be used to construct regulatory sequences which are highly active exceeding the natural cyanobacterial regulatory sequence. There is no report of synthetic promoters and SD (or non-SD) sequence in cyanobacteria.

Objectives

This project is aimed to develop highly active enhancer/ promoter and translation initiation region including translation initiator and translation enhancer in cyanobacterium *Synechococcus* PCC7942.

1. To evaluate the use of green fluorescent protein (GFP) as a reporter in *Synechococcus.*
2. To evaluate the activity of *somA* promoter of *Synechococcus.*
3. To develop highly active enhancer/ promoter from synthetic libraries.
4. To develop highly active translation initiator (SD/ non-SD sequences) from synthetic libraries.
5. To develop highly active regulatory sequences using tandem enhancer/ promoter and optimal translation initiator.

Results and Discussion

The promoter of *Synechococcus* outer membrane protein A (SomA), one of the most abundant proteins of the cyanobacterial total envelope, was investigated.

Results indicated that the activity of *somA* promoter was dramatically lower than that of $tRNA^{pro}$ promoter. The use of green fluorescent protein (GFP) as a reporter in *Synechococcus* was evaluated. Results showed that the fluorescence intensity of cells with cytoplasmic GFP was approximately 6-fold higher than that of cells with periplasmic GFP. Therefore, cytoplasmic GFP was used as a reporter.

The optimal conditions for GFP expression in *Synechococcus* were 1-day cultures grown under light intensity of 4000-4500 lux. In order to develop highly active enhancer/ promoter, synthetic libraries containing the LysR binding sequence and UP element upstream of the $tRNA^{pro}$ promoter were constructed. A total of 830 and 930 clones from libraries pLL (LysR binding sequences) and pUL (UP/LysR sequences), respectively, were screened for high GFP activities. The GFP activities of selected *Synechococcus* harboring pUP-UP140, pLL-172, pLL-209 and pUL-146 were 1.56-, 2.70-, 2.37-, 3.40- and 3.15- fold, respectively, higher than that of control pKT-GFP. DNA sequence analysis revealed that there are three copies of UP in pUP-UP140. In plasmids pLL-172, pLL-209 and pUL-146, the transcription-translation terminator upstream of the regulatory sequences were deleted. Therefore, the high GFP activities might be due to the read-through transcripts.

To investigate the optimal translation initiators, the synthetic SD (Shine-Dalgarno)/ non-SD sequence libraries containing randomized decanucleotide regions, upstream of *gfp* gene were constructed. A total of 1,500, 650 and 710 clones from libraries pSD-TNG2, pSD-TNG3 and pNG-nPT, respectively, were screened for GFP activities. The level of *gfp* mRNA of the selected clones was determined using real-time PCR and the translation efficiency was analyzed. The translation efficiencies of SD-like sequences: AGGAGAAUGA, AAUGGAAAUA and AAUUGGAUUU were 2.38-, 3.96- and 4.89-fold, respectively, higher than that of control (GGUGGU), indicating that the SD-like sequences were highly efficient for translation initiation. However, the translation efficiency of non-SD sequence, AUGUCAACUU, was not significantly different from that of control.

It has been shown that in *E. coli*, the non-SD sequences UUCAAUAUUU, AUUUACCUCC and CCAAUCUAC are more efficient than the consensus-SD sequence. To investigate the previously reported non-SD sequences in *Synechococcus*, plasmids

pSDR-F1, pSDR-F2, pSDR-F3 and pAC6 harboring the non-SD sequences UUCAAUAUUU, AUUUACCUCC, CCAAUCUAC and ACCACCU, respectively, were constructed. The translation efficiencies of non-SD sequences UUCAAUAUUU, AUUUACCUCC, CCAAUCUAC and ACCACCU were apparently lower than that of control. Therefore, our results disagreed with the previous reports. The results indicated that the non-SD sequences could be translation initiator in *Synechococcus*, but was less efficient than SD-like sequences.

The activities of consensus-SD sequences in *Synechococcus* were also investigated. Results showed that the translation efficiencies of consensus-SD sequences AAGGAGGU (pSDC8), AGGAGGU (pSDC7), AAAGGAGG (pSDC-S8) and AGGAGG (pSDC-S6) were 12.24-, 10.52-, 6.36- and 3.77-fold, respectively, higher than that of control (GGUGGU). Thus, the consensus-SD sequences were more efficient for translation initiation than the SD-like sequence and the sequence AAGGAGGU was the optimal translation initiator in *Synechococcus*. The insertion of the BoxA (A/U-rich) of *E. coli rrnB* or pyrimidine-rich enhancer (CUUUCUCCCCAGCCUUUCCCCUUUCU CUUUUU) upstream of translation initiators did not enhance but decreased the translation efficiency. Therefore, the *E. coli* translational enhancer did not function in *Synechococcus*.

To investigate the active region of rRNA A operon (*rrnA*) of *Synechococcus*, deletion analysis of the *rrnA* regulatory sequence of *Synechococcus* was performed. The GFP activity of active *rrnA* regulatory sequence upstream of the consensus-SD sequence was 23.97-fold higher than that of control. The results indicated that the UP element of *rrnA* can function as enhancer in both *Synechococcus* and *E. coli*.

In order to investigate the activity of the *E. coli* σ^{70} promoter (P_{sig70}) in *Synechococcus*, plasmid pKT-Sig70 harboring the -35 (TTGACA) and -10 (TATAAT) region of P_{sig70} upstream of the consensus-SD sequence was constructed. Results showed that the GFP activities of P_{sig70} was 6.17-fold higher than that of control. Thus, the σ^{70} promoter is highly active in both organisms.

In order to construct regulatory sequences which are highly active exceeding the natural cyanobacterial regulatory sequence, plasmids harboring combination of tandem enhancer/ promoter and consensus-SD sequence were constructed. The GFP

activities of *Synechococcus* harboring plasmids with tandem promoters P_{rnnA} -P_{rnnA} (pRRSC), P_{sig70}- P_{rnnA} (pS70-RSC) and P_{sig70}- P_{rnnA}- P_{rnnA} (pS70-2RSC) were 32.33-, 36.66- and 35.88- fold, respectively, higher than that of control. To investigate the GFP expression, the total proteins extracts were subjected to SDS-PAGE and stained with Comassie brilliant blue. The results showed that the GFP band was detected in total proteins extracted from *Synechococcus* harboring plasmids pS70-2RSC and pS70-RSC. To our knowledge, this is the first report that the expression of heterologous protein in *Synechococcus* is high enough to be detected by SDS-PAGE stained with Comassie brilliant blue. Thus, we have successfully constructed the synthetic regulatory sequences which are highly active exceeding the natural cyanobacterial regulatory sequences.

Output

Presentation at conferences:

1. Chungjatupornchai W, Kitraksa P, Udpuay N and Fa-aroonsawat S. Improvement of heterologous gene expression in blue green alga using synthetic regulatory-sequence libraries. Abstract In: Proceeding of The 9th International Phycological Congress, August 2- 8, 2009, Tokyo, Japan.

2. Chungjatupornchai W, Kamlangdee A and Fa-aroonsawat S. Surface display of organophosphorus hydrolase on *Synechococcus* PCC7942. Abstract In: Proceeding of The 8th European Workshop on Molecular Biology of Cyanobacteria, August 28- September 1, 2011, Naatali, Finland.

Publication

@= corresponding author

1. Chungjatupornchai W@ and Fa-aroonsawat S (2009) Translocation of green fluorescent protein to cyanobacterial periplasm using ice nucleation protein. Journal of Microbiology, 47: 187-192.

2. Chungjatupornchai W[@], Kamlangdee A and Fa-aroonsawat S (2011) Display of organophosphorus hydrolase on the cyanobacterial cell surface using *Synechococcus* outer membrane protein A. Applied Biochemistry and Biotechnology, 164: 1048-1057.

3. Chungjatupornchai W[@] and Fa-aroonsawat S. (2011) Characterization of the regulatory sequence of *rrnA* operon of Synechococcus PCC7942. (In preparation)

4. Chungjatupornchai W[@] and Fa-aroonsawat S. (2011) Enhance constitutive expression of heterologous gene in cyanobacteria using tandem promoters. (In preparation)

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Rationale

Cyanobacteria are oxygenic photosynthetic prokaryotes that are ultrastructurally and biochemically similar to both bacteria and chloroplasts of higher plants. As cyanobacteria have simple growth requirements and inexpensive to maintain, they have been used as hosts to express several heterologous genes. For examples: mosquitocidal protein genes (Chungjatupornchai, 1990); salmon growth hormone gene (Kawata *et al*., 1991); ethylene-forming enzyme gene (Fukuda *et al*., 1994); and organophosphorus hydrolase (Chungjatupornchai and Fa-aroonsawat, 2009). However, the level of heterologous gene expression in cyanobacteria is low when compared with that in *E. coli*. The possible ways to improve the gene expression are (i) to use endogenous strong promoter for transcription and (ii) to use the optimal ribosome binding site for translation initiation. However, current knowledge of the relationship between nucleotide sequence structure and function of promoters and ribosome binding site for translation initiation recognized within cyanobacteria is still limited.

Although cyanobacteria are classified as eubacteria, their RNA polymerase holoenzyme is unique in that it contains a subunit γ, in addition to the $\alpha_2\beta\beta'\sigma$ structure common to the RNA polymerase of other eubacteria (Schneider and Haselkorn 1988). Many cyanobacterial promoters contain the conserved motif that conforms in both sequence and position to the -10 hexamer consensus of *E. coli* σ^{70} promoters, but do not contain, or contain relatively weak, -35 sequence of *E. coli* σ^{70} promoters. Previously, we isolated and analyzed several endogenous strong promoters of cyanobacterium *Synechococcus* PCC7942 (Chungjatupornchai *et al.*, 1999; Chungjatupornchai *et al*., 2002; Plansangkate *et al*., 2004). These endogenous cyanobacterial strong promoters either become weak or do not function in *E. coli.* However, the level of heterologous gene expression in *Synechococcus* PCC7942 is low when compared with that in *E. coli.*

Many bacterial ribosome binding sites located in the 5’ untranslated region of mRNA have the consensus sequence known as the Shine-Dalgarno (SD) sequence. However, the consensus SD sequences were not detected in the cyanobacterial genes

isolated previously (Chungjatupornchai *et al.*, 1999; Chungjatupornchai *et al*., 2002; Plansangkate *et al*., 2004). In cyanobacterium *Synechocystic* PCC6803, only 48.6% of genes contain the SD sequence (Sazuka and Ohara, 1996). Recently, large-scale analysis of distribution of SD sequences in completed microbial genomes (10 species) reveals that genes led by SD are as common as genes not led by SD (Chang *et al.,* 2006). Whether the 5' UTRs of genes not led by SD contain any type of reserved sequence, remains to be investigated.

When a pool of various genes is generated by random mutations introduced into a gene, and then the preferred genes are selected out of the pool, it can be a simulation of molecular evolution. This process is referred to as directed evolution (Albelson 1990; Shao and Arnold, 1996). In this project, we will use this process to develop highly active regulatory sequences for heterologous gene expression in cyanobacteria by (i) generation of strong promoters from synthetic DNA library including the promoter and enhancer elements, for examples: UP sequence (Asato, 2005) and LysR binding sequence (Maeda *et al.*, 1998); (ii) analysis of non-SD initiation of translation selected from synthetic DNA library. The knowledge obtained from (i) and (ii) can be used to construct regulatory sequences which are highly active exceeding the natural cyanobacterial regulatory sequence. There is no report of synthetic promoters and SD (or non-SD) sequence in cyanobacteria.

Objectives

This project is aimed to develop highly active enhancer/ promoter and translation initiation region (TIR) including translation initiator and translational enhancer in cyanobacterium *Synechococcus* PCC7942.

1. To evaluate the use of green fluorescent protein (GFP) as a reporter in *Synechococcus.*
2. To evaluate the activity of *somA* promoter of *Synechococcus.*
3. To develop highly active enhancer/ promoter from synthetic libraries.
4. To develop highly active translation initiator (SD/ non-SD sequences) from synthetic libraries.
5. To develop highly active regulatory sequences using tandem enhancer/ promoter and optimal translation initiator.

Results and Discussion

1. GFP as a reporter in *Synechococcus*

GFP is a popular reporter for gene expression and protein localization studies. GFP does not require the addition of any substrate or co-factor, it promises real time visualization of gene expression. The GFPmut2, a GFP variant with a triple amino acid substitution (S65A/V68L/S72A), has been shown to express in *E. coli* as a soluble protein with improved fluorescence (Cormack *et al.,* 1996). In this study, we have used the GFPmut2 as a reporter to investigate the protein translocation in cyanobacterium *Synechococcus* PCC 7942. The GFP was fused in frame to the carboxyl-terminus of ice nucleation protein (InpNC). We demonstrated that the InpNC was able to direct translocation across the intracellular membranes and that the transported GFP accumulated almost entirely in the periplasm. The fluorescence intensity of cells with periplasmic GFP was approximately 6-fold lower than that of cells with cytoplasmic GFP. [ดูภาคผนวก, Chungjatupornchai W and Fa-aroonsawat S (2009) Display of organophosphorus hydrolase on the cyanobacterial cell surface using *Synechococcus* outer membrane protein A as an anchoring motif. Journal of Microbiology, 47: 187-192]. Therefore, cytoplasmic GFP can be used as a reporter in *Synechococcus.*

2. The activity of *somA* promoter

Synechococcus outer membrane protein A (SomA), a porin, is one of the most abundant proteins of the total envelope proteins of *Synechococcus* PCC 7942 (Umeda *et al*., 1996). SomA of *Synechococcus* PCC 7942 showed an overall homology of 97% to that of the closely related strain *Synechococcus* PCC 6301 (Hansel *et al.,*1998). The N-terminus of SomA contains a typical signal peptide and a highly conserved surface layer homology (SLH) domain; therefore, it may have a dual function: the formation of diffusion pores as well as act as linkers connecting the outer membrane with the peptidoglycan layer (Hansel *et al.,*1998). In this study, we have used the organophosphorus hydrolase (OPH) as a reporter to investigate the ability of SomA to act as an anchoring motif for cyanobacterial surface display system. The OPH was fused in frame to the carboxyl-terminus of different cell-surface exposed loops of SomA.

We demonstrated that OPH was successfully translocated across the membrane and completely displayed onto the outermost cyanobacterial cell surface. It has been reported that under the condition of nitrogen deprivation, *somA* promoter is induced, and SomA increases slightly (Sauer *et al.,* 2001). However, our results indicated that nitrogen deprivation did not affect the expression of OPH. Since the size of *somA* promoter fragment in the gene cassettes is 204 bp (upstream of *somA* start codon), this fragment might not contain the regulatory sequence responding to nitrogen deprivation. The activity of *somA* promoter is dramatically lower than that of tRNA promoter. [ดูภาคผนวก, Chungjatupornchai W, Kamlangdee A and Fa-aroonsawat S (2011) Display of organophosphorus hydrolase on the cyanobacterial cell surface using *Synechococcus* outer membrane protein A as an anchoring motif. Appl Biochem Biotechnol 164: 1048-1057]

3. Optimal conditions for expression of GFP in *Synechococcus*

In this study, the GFP was used as a reporter. The *Synechococcus* harboring pKT-GFP [Chungjatupornchai and Fa-aroonsawat, 2009] was used as control (Fig. 1). In order to optimize the GFP- activity determination using spectrofluorometer, the culture time and light intensities used to grow cells were investigated. The results in Fig. 2 showed that GFP activity of cells grown under constant light at 4,000 lux was not significantly different from that at 4,500 lux but was higher than that of cells grown at 3,200 lux. The GFP activity of 3-day culture was significantly higher than those of 1-, 2- and 4-day cultures (Fig. 3). Thus, the optimal conditions for GFP expression in *Synechococcus* were 1-day cultures grown under light intensity of 4000-4500 lux.

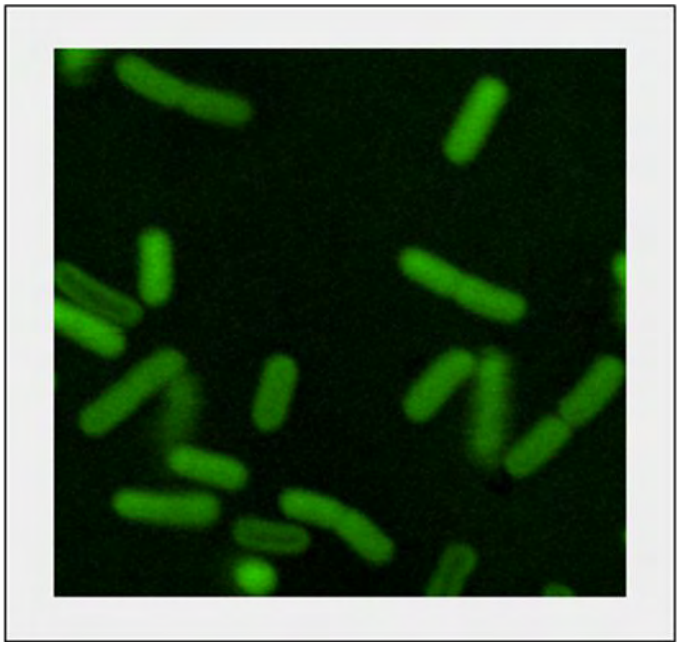


Fig. 1. Confocal laser scanning microscopy images of *Synechococcus* cells expressing GFP.

The cytoplasmic GFP in *Synechococcus* cells harboring pKT-GFP were visualized under confocal laser scanning microscope with excitation at 488 nm and emission measured at 520 nm.

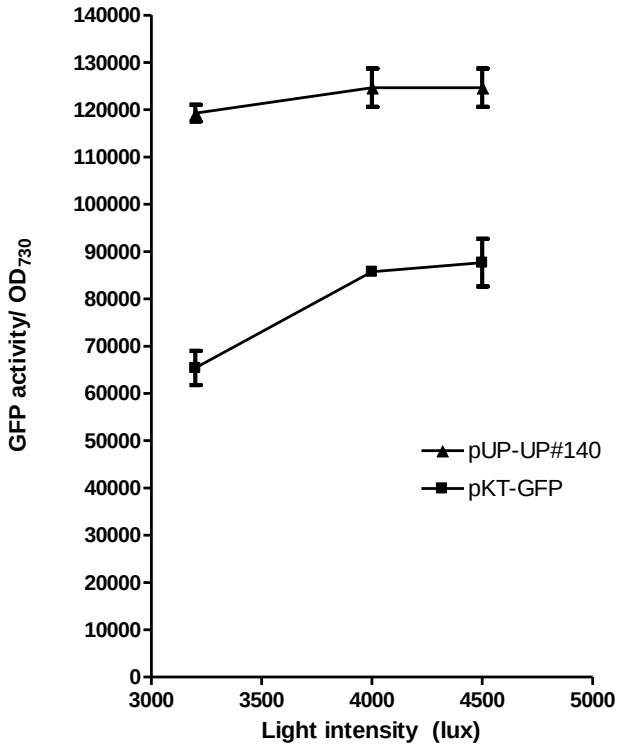


Fig. 2. GFP activities of *Synechococcus* grown at various light intensities.

Cell cultures, harboring the recombinant plasmids and grown under constant light at various intensities, were harvested and the fluorescence intensity of whole cells was measured.

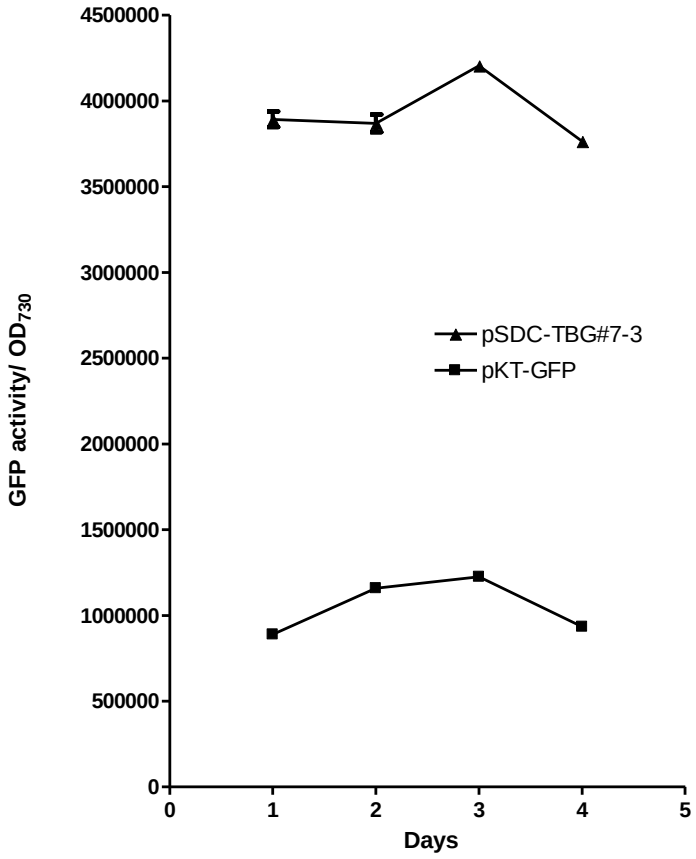


Fig. 3. GFP activities of *Synechococcus* cultures grown at various time courses.

Cell cultures, harboring the recombinant plasmids and grown under constant light intensity of 4,000 lux, were harvested each day. The fluorescence intensity of whole cells was measured

4. The synthetic enhancer/ promoter libraries in *Synechococcus*

4.1 Investigation of the optimal insertion site for synthetic UP element.

In order to investigate the activity of single copy of synthetic UP element (AAAAAATTTTTCTAAAA) in cyanobacteria, plasmids pUP1-GFP and pUP2-GFP were constructed (Fig. 4). Plasmids pUP1-GFP and pUP2-GFP contained a single copy of UP element located at position -47 and -85, respectively, upstream of the tRNA promoter of *Synechococcus* PCC7942 (see Fig. 5). Results showed that the GFP activity of *Synechococcus* harboring pUP1-GFP was approximately 1.29-fold higher than that of control pKT-GFP (containing tRNA promoter without UP element). The GFP activity of pUP2-GFP was not significantly different from that of control. Therefore, pUP1-GFP was used as a template for construction of the synthetic UP element library.

4.2 Investigation of the optimal sequences of synthetic LysR binding site.

In order to investigate the optimal nucleotide sequences of LysR binding element ($TGCAN_5TGCA$) in cyanobacteria, synthetic LysR libraries were constructed (Fig. 4). The single copy of LysR element was located at position -64 and -85, upstream of the tRNA promoter of *Synechococcus* PCC7942 (see Fig. 6). The resulting plasmid libraries pLR1-GFP (at -64), pLR2-GFP (at -85) and pLR3-GFP (at -85) in *E. coli* clones were extracted and transformed into *Synechococcus* PCC7942. The transformants were sorted for high GFP activities using fluorescence-activated cell sorter (FACS). The transformants were sorted for high GFP activities using fluorescence-activated cell sorter (FACS). The sorted cells with high GFP activities were isolated as single colonies. The GFP of each clone was measured using spectrofluorometer. The plasmids of selected *Synechococcus* were rescued in *E. coli* and the DNA sequences were determined. Results showed that the GFP activity of *Synechococcus* harboring pLR3 (TGCAAAGCATGCA) were not significantly difference from that of control pKT-GFP, whereas, the GFP activity of pLR2-742

(TGCAAAGAATGCA) was 1.3-fold higher than that of pKT-GFP. Therefore, pLR2-742 was used as a template for further construction of the synthetic LysR element library.

4.3 Construction of the synthetic enhancer/ promoter libraries

To investigate the activities of synthetic UP element (AAAAAATTTTTCTAAAA) and LysR- binding sequences ($TGCAN_5TGCA$) in cyanobacteria, the PCR products were amplified using primer sets: TUP-F2 and Nos-ter; LysR-F2 and Nos-ter, with gene cassette P_{tRNA}-gfp-[Nos-ter] as template (see Fig.4). The BamHI/EcoRI digested PCR products or the double stranded oligos (UP-F1 and UP-R1; LysR-F4 and LysR-R1) were cloned into the corresponding sites of the shuttle vector pKGT. The locations of UP and LysR insertion are shown in Fig. 5 and 6, respectively.

The recombinant plasmids were transformed into *E. coli*. The total plasmids extracted from *E. coli* transformants were transformed into cyanobacterium *Synechococcus* PCC7942. The transformants were sorted for high GFP activities using FACS. Flow cytometric analysis of GFP of *Synechococcus* harboring the synthetic libraries was shown in Fig. 7. The sorted cells with high GFP activities were further isolated as single colonies. The GFP activity of each clone was measured using spectrofluorometer. The plasmids of selected *Synechococcus* were rescued in *E. coli* and the DNA sequences were determined.

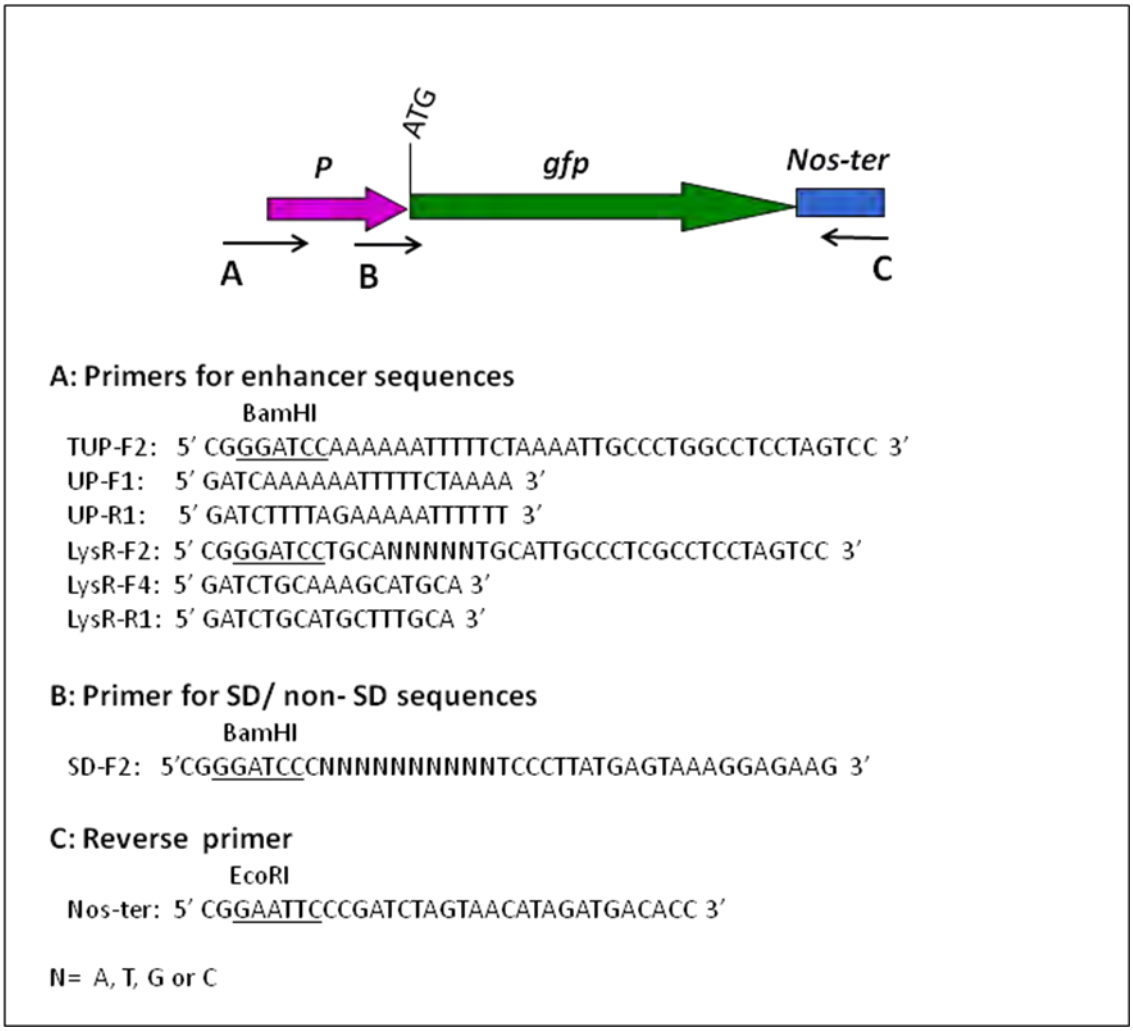


Fig. 4. PCR products for construction of synthetic regulatory-sequence libraries.

The gene cassette containing: P, tRNA promoter; *gfp*, green fluorescent protein; Nos-ter, nopaline synthase terminator, was used as template. Locations and sequences of primers are indicated. The figure is not drawn to scale.

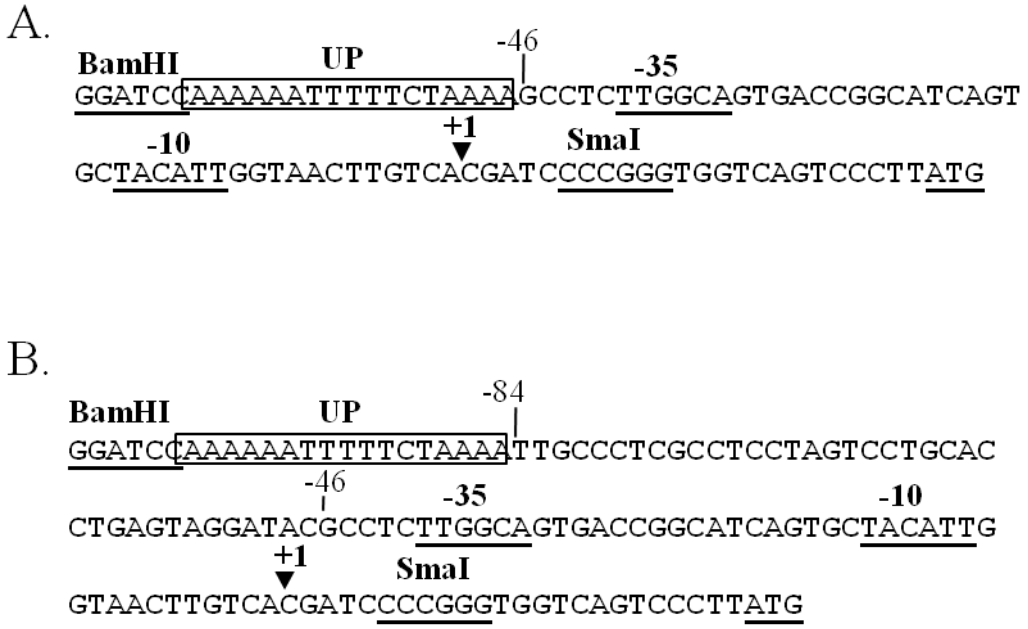


Fig. 5. Insertion of UP element upstrem of tRNA promoter.

The regulatory sequences of *gfp* gene in plasmids pUP1-GFP (A) and pUP2-GFP (B) are shown. The UP element was inserted upstream of the cyanobacterial tRNA promoter, using PCR with primer containing the UP element. The tRNA promoter sequence including the -35 and -10 regions, starting site of mature transcript (+1), and start codon (ATG) of *gfp* gene are indicated.

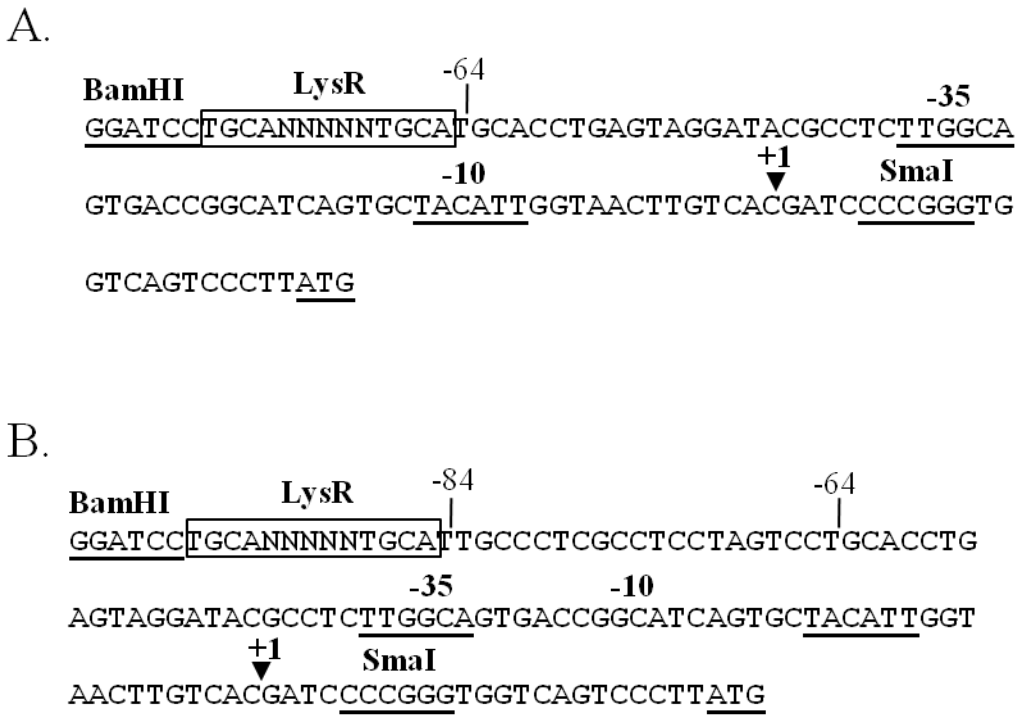


Fig. 6. Insertion of LysR binding sequence upstrem of tRNA promoter

The regulatory sequences of gfp gene in plasmids pLR1-GFP (A) and pLR2-GFP (B) are shown. The LysR binding sequence was inserted upstream of the cyanobacterial tRNA promoter, using PCR with primer containing the LysR binding sequence. The random nucleotides A, T, G, or C are marked by N. The tRNA promoter sequence including the -35 and -10 regions, starting site of mature transcript (+1), and start codon (ATG) of *gfp* gene are indicated.

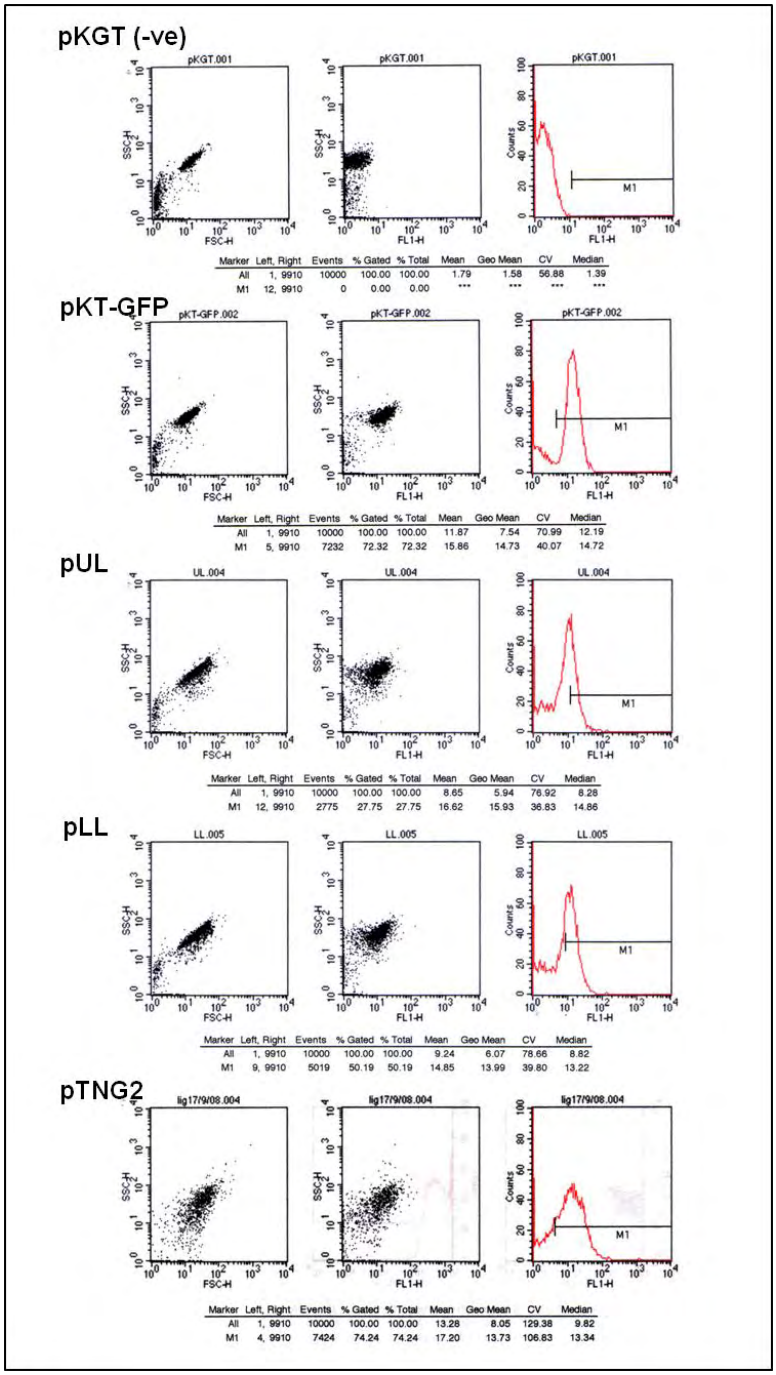


Fig. 7. Flow cytometric analysis of GFP of *Synechococcus* harboring the synthetic libraries. FSC, forward scatter; SSC, sideward scatter; M1, range of gated events; FL1-H, arbitary logarithmic values of GFP.

4.4 Analysis of the relationship between the synthetic enhancer/ promoter sequences and their activities in *Synechococcus*

A total of 830 and 930 clones from libraries pLL (LysR binding sequences) and pUL (UP/LysR sequences), respectively, were screened for high GFP activities. The GFP activities of selected *Synechococcus* harboring pUP-UP140, pLL-172, pLL-209 and pUL-146 were 1.56-, 2.70-, 2.37-, 3.40- and 3.15- fold higher than that of control (Fig. 8). The plasmids of the selected clones were rescued in *E. coli* and the DNA sequences were determined. DNA sequence analysis revealed that there are three copies of UP in pUP-UP140. In plasmids pLL-172, pLL-209 and pUL-146, the transcription-translation terminator (ter) upstream of the regulatory sequences were deleted (Fig. 8). Therefore, the high GFP activities might be due to the read-through transcripts.

To investigate whether inversion of the gene cassette would prevent the deletion of transcription-translation terminator, plasmid vector pNG containing restriction sites for cloning at opposite orientation of pKT-GFP was constructed. Libraries pNG-LL and pNG-UL were generated. The gene cassette (ter-X-Pt-GFP-[Nos-ter]) was cloned into the same location of vector but in the opposite orientation. A total of 742 and 556 clones from libraries pNG-LL and pNG-UL, respectively, were screened for high GFP activities using spectofluorometer either before or after sorting with FACS. Results in Table 1 showed that the GFP activities of selected *Synechococcus* harboring pNG-LL#131 pNG-LL#131, pNG-LL#35, pNG-UL#190 and pNG-LL172#10 were approximately 2- fold higher than that of control.

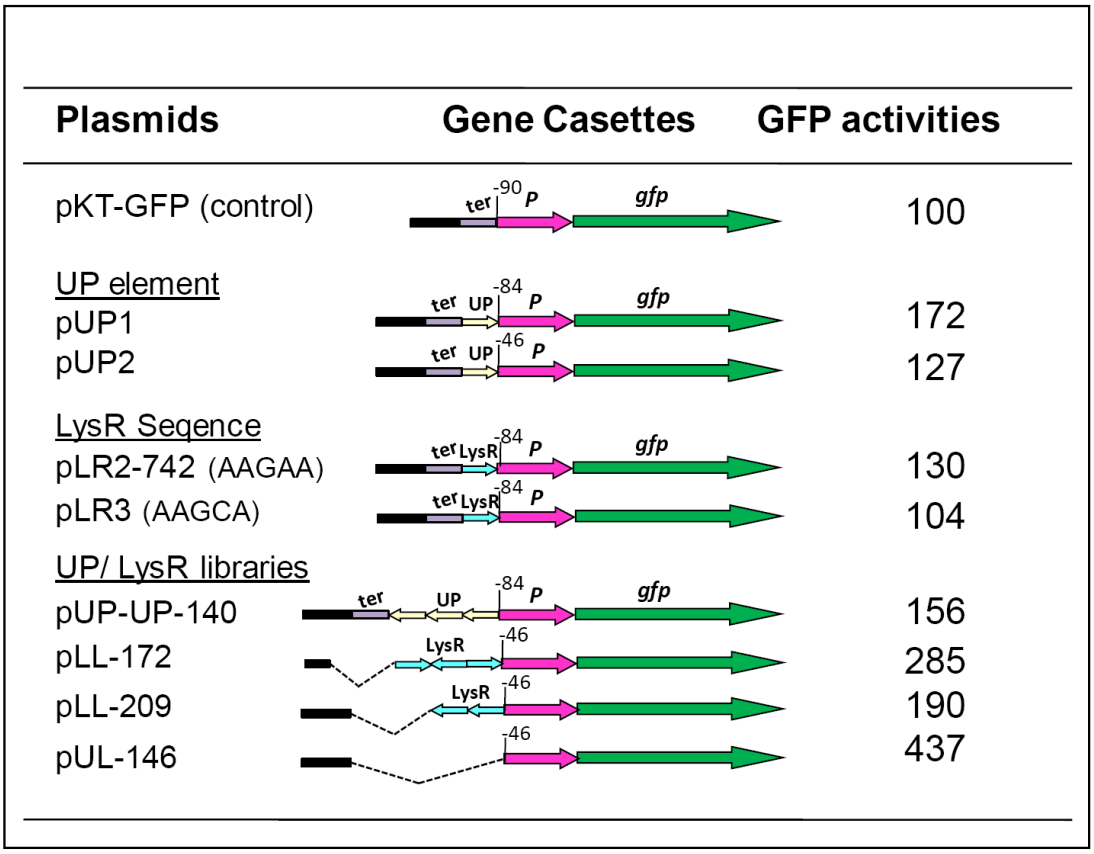


Fig. 8. The GFP activities of *Synechococcus* harboring plasmids with *gfp* under the control of synthetic enhancers/promoters

The GFP activity is defined as GFP fluorescence units per OD_{730}. Ter, transcription-translation terminater; UP, UP element; LysR, LysR binding sequence; *P*, tRNA promoter; *gfp,* green fluorescent protein gene.

Table 1. GFP activities of *Synechococcus* harboring plasmids from libraries pNG-LL and pNG-UL.

Plasmids[a]	GFP activities (Fluorescence intensities/OD_{730})	
pKT-GFP#2 (A, control)	100	
pNGPtd#2-4 (B, control)	80	100
pNG-LL#112 (B)	137	170
pNG-LL#131 (B)	171	210
pNG-LL#4 (B) (FACS)	128	160
pNG-LL#16 (B) (FACS)	124	160
pNG-LL#20 (B) (FACS)	133	170
pNG-LL#35 (B) (FACS)	169	210
pNG-UL#190 (B)	178	220
pLL-172 (A)	331	-
pNG-LL172#10 (B)	168	210
pLL-209 (A)	220	-
pKP-LL209#5 (A)	86	-

[a]: The gene cassette (ter-X-Pt-GFP-[Nos-ter]) in the same orientation as that in pKT-GFP was indicated as (A), the gene cassette in the opposite orientation was indicated as (B). Cells obtained after sorting with FACS were indicated.

5. The synthetic SD/ non SD sequence libraries in *Synechococcus*

5.1 Construction of the SD/ non-SD sequence libraries.

Plasmids harboring *gfp* gene used in this study were shown in Fig. 9. The *gfp* gene in all constructs was under the control of cyanobacterial $tRNA^{pro}$ promoter (Chungjatupornchai *et al*, 1999; Chungjatupornchai *et al*, 2002) enabling constitutive expression of GFP.

The *Bam*HI/*Eco*RI-digested or *Bgl*II/*Eco*RI-digested PCR product containing gene cassette *X-gfp*-(*nos-ter*) was cloned into the *Bam*HI/*Eco*RI sites of shuttle vector pKTN-T to obtain plasmid libraries pSD-TNG2 and pSD-TNG3, respectively (Fig. 9 and 10). Therefore, the *Bam*HI site was retained in plasmid library pSD-TNG2 but lost in pSD-TNG3. The plasmid library pSD-TNG3 extracted from *E. coli* was digested with *Bam*HI in order to get rid of background vector prior to be transformed into *Synechococcus*. The gene cassette *X-gfp*-(*nos-ter*) in plasmid libraries pSD-TNG2 and pSD-TNG3 contained ten randomized nucleotides located at the 7th nucleotide upstream of initiation codon of *gfp* gene which was used as a reporter (Fig. 9 and 10). The gene cassettes of plasmids pNG-PT and pNG-nPT were in the opposite orientation to those of plasmids pSD-TBG3 and pSD-TNG3, respectively (Fig. 9, 10 and 11).

The plasmid mixtures extracted from the pooled transformants of *E. coli* were transformed into *Synechococcus*. The *Synechococcus* cells harboring plasmid libraries pSD-TNG2 and pSD-TNG3 were sorted by FACS. The sorted cells with high GFP activities were further isolated as single colonies. The GFP activity of each clone was measured using spectrofluorometer. The plasmids of selected *Synechococcus* were rescued in *E. coli* and the DNA sequences were determined.

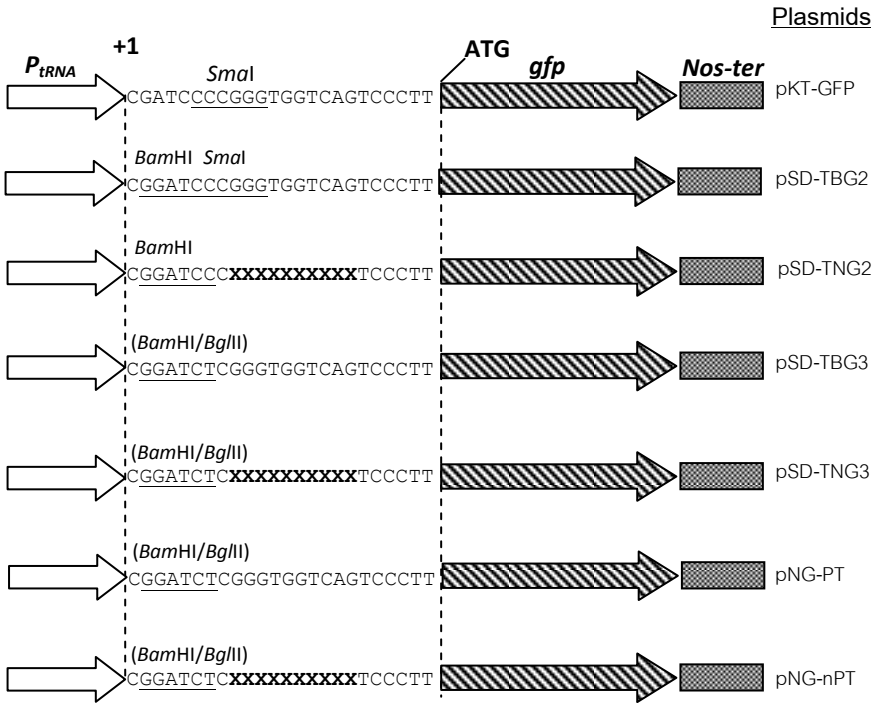


Fig. 9. Plasmids used for construction of SD/ non-SD sequence libraries.

Plasmids pKT-GFP, pSD-TBG2, pSD-TBG3 and pNG-PT were used as controls. The randomized nucleotides (A, T, C and G) of plasmid libraries pSD-TNG2, pSD-TNG3 and pNG-nPT are marked by X. The G content of randomized nucleotides of library pNG-nPT was restricted to 3%. The gene cassettes of plasmids pNG-PT and pNG-nPT were in the opposite orientation to those of plasmids pSD-TBG3 and pSD-TNG3, respectively. The start codon (ATG) of *gfp* mRNA are indicated. P_{tRNA}, $tRNA^{pro}$ promoter; *gfp*, green fluorescence protein gene; *Nos-ter*, nopaline synthase terminator.

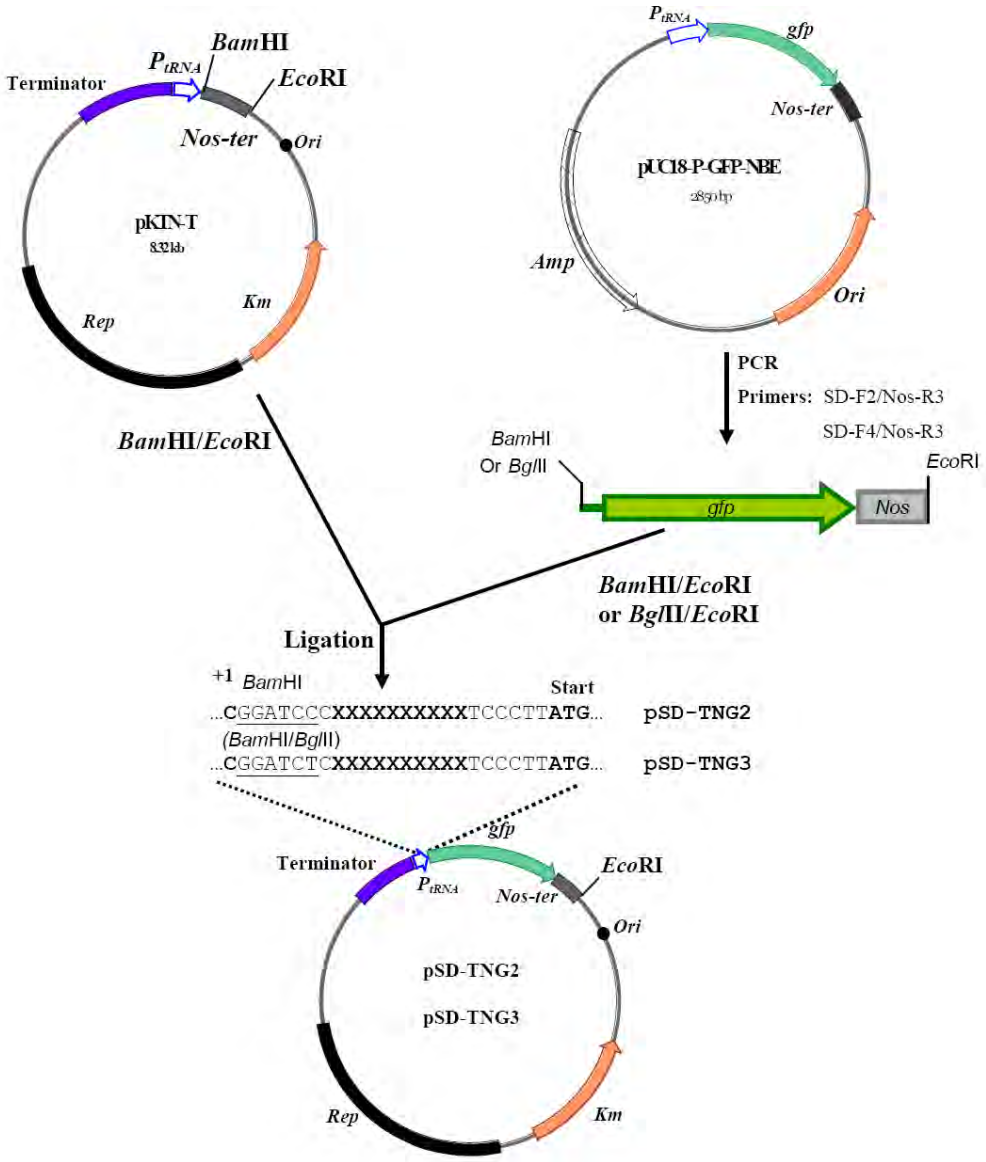


Fig. 10. Schematic diagram for construction of pSD-TNG2 and pSD-TNG3 libraries

The 1,017 bp of PCR products containing gene cassette x-gfp-(Nos-ter) were amplified from pUC18-P-GFP-NBE with primer sets: SD-F2 and Nos-R3; SD-F4 and Nos-R3. The PCR products were cloned into the cloning vector pKTN-T to obtain the resulting plasmids library pSD-TNG2 and pSD-TNG3. The random nucleotides containing equal contents of A, T, C and G are marked by X. The transcription start site (+1) and start codon (ATG) of *gfp* mRNA are indicated.

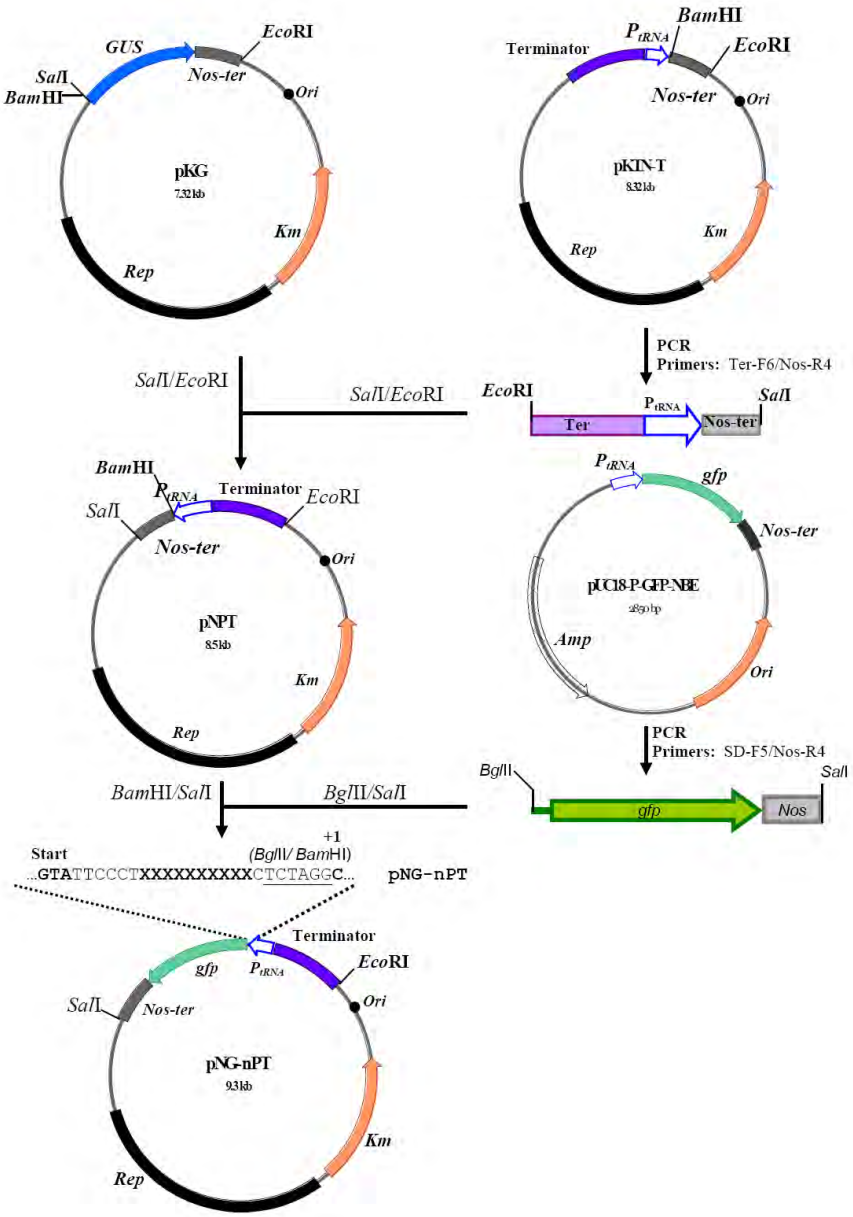


Fig. 11. Schematic diagram for construction of pNG-nPT library

The 1,179 bp of cassette Ter-P_{tRNA}-(*Nos-ter*) was amplified from plasmid pKTN-T with primers Ter-F6 and Nos-R4. The PCR product was digested with *Eco*RI/*Sal*I and inserted into corresponding sites of plasmid pKG to obtain plasmid pNPT. The 1,017 bp of gene cassette *x-gfp*-(*Nos*-ter) was amplified from pUC18-P-GFP-NBE with primers SD-F5 and Nos-R4. The PCR product was digested with *Bgl*II/*Sal*I and inserted into *Sal*I/*Bam*HI site of the cloning vector pNPT. The resulting plasmid was pNG-nPT. The randomized nucleotides containing equal contents of A, T and C and 3% of G content are marked by X. The transcription start site (+1) and start codon (ATG) of *gfp* mRNA are indicated.

5.2 GFP activities of *Synechococcus* harboring SD/ non-SD sequence libraries.

A total of 1,500, 650 and 710 clones from libraries pSD-TNG2, pSD-TNG3 and pNG-nPT, respectively, were screened for high GFP activities. The results in Fig. 12 showed that the GFP activities of clones harboring pSD-TNG2-357, pSD-TNG2-831, pNG-nPT-496 and pNG-nPT-622 were higher than that of corresponding controls; (ii) the GFP activities of clone harboring pSD-TNG3-423 was not significantly different from that of pSD-TBG3; (iii) the GFP activities of clones harboring pSD-TNG2-1459, pSD-TNG3-462, pSD-TNG3-493, pSD-TNG3-552, pSD-TNG3-556, pNG-nPT-284 and pNG-nPT-422 were lower than that of corresponding controls.

5.3 Analysis of the relationship between synthetic SD/ non-SD sequences and GFP activities in *Synechococcus.*

Plasmids of the selected *Synechococcus* were rescued in *E. coli* and the DNA sequences were determined. The DNA sequences of the SD/ non-SD sequences of the selected clones were shown in Table 2. The GFP activities of clones pSD-TNG2-357, pSD-TNG2-831, pNG-nPT-496 and pNG-nPT-662 harboring the SD-like sequences AAGGAACATA, AGGAGAATGA, AATGGAAATA and AATTGGATTT were 1.19-, 1.94-, 1.41- and 1.38-fold, respectively, higher than that of the corresponding controls. In addition, the GFP activity of clone pSD-TNG3-423 harboring SD-like sequence AACAGGAAAAA (1.01-fold) was not significantly different from that of pSD-TBG3. However, the GFP activities of other selected clones harboring the SD-like sequences AAGAACAATG (pSD-TNG2-1459), AAGGCCCAAA (pSD-TNG3-552), GGGAAAAGCA (pSD-TNG3-556) and AAATCAAGGT (pNG-nPT-422) were 0.45-, 0.47-, 0.44- and 0.74-fold, respectively lower than that of the corresponding controls. For the selected non-SD sequences, the GFP activities of clones harboring pSD-TNG3-462 (TAACAAAAAA), pSD-TNG3-493 (AAAAAACAAA) and pNG-nPT-284 (ATGTCAACTT) were 0.31-, 0.26- and 0.46-fold, respectively, lower than that of the corresponding controls. The results indicated that the GFP activities of SD-like sequences are higher than those of non-SD sequences. In addition, the GFP activity of pSD-TBG3 was 1.27-fold higher than that of pSD-TBG2, however, both plasmids contained almost identical 5'UTR, except the nucleotide at position +7 (Table 2). The result indicated that nucleotides upstream of the translational initiator might affect the expression of *gfp* gene in *Synechococcus.* The GFP activity of pNG-PT was 1.76-fold higher than that of pSD-TBG3, both plasmids contained

identical gene cassette P_{tRNA}-*gfp*-(*Nos-ter*) but in the opposite orientation. The result indicated that the orientation of gene cassette P_{tRNA}-*gfp*-(*Nos-ter*) might affect the expression of *gfp* gene in *Synechococcus.*

5.4 Determination of *gfp* mRNA levels in the selected clones by real time PCR.

Total RNA were extracted from selected clones and used as template for the first cDNA synthesis. The cDNAs of *16S* rRNA and *gfp* mRNA were co-synthesized using primers 16S-rev6 and gfp-rev3. The *16S* rRNA was used as an internal control. The *gfp* mRNA levels of the selected clones are shown in Table 3. The results showed that the *gfp* mRNA levels of the selected clones were not significantly different when compared with each other. However, the *gfp* mRNA levels of the selected clones and controls (pSD-TBG2, pSD-TBG3 and pNG-PT) were lower than that of clone harboring plasmid pKT-GFP, although these plasmids contain the $tRNA^{pro}$ promoter similar to pKT-GFP. Therefore, the results indicated that 5' UTR of *gfp* mRNA might affect the levels of *gfp* mRNA.

5.5 Translation efficiencies of the selected SD/ non-SD sequences in *Synechococcus.*

The translation efficiencies of synthetic SD/ non-SD sequences in the selected *Synechococcus* clones were evaluated. The translation efficiency was defined as % of GFP activities/ *gfp* mRNA and normalized to that of the corresponding controls. The results in Table 4 showed that the efficiencies of SD-like sequences AACAGGAAAA (pSD-TNG3-423), AAGGAACATA (pSD-TNG2-357), AGGAGAATGA (pSD-TNG2-831), AAATCAAGGT (pNG-nPT-422), AATGGAAATA (pNG-nPT-496) and AATTGGATTT (pNG-nPT-662) were 1.39-, 1.91-, 2.38-, 2.27-, 3.96- and 4.89-fold, respectively, higher than that of control (GGGTGGTCAG). The results indicated that the SD-like sequences were highly efficient for translation. The translation efficiencies of the non-SD sequences TAACAAAAAA (pSD-TNG3-462) and AAAAAACAAA (pSD-TNG3-493) were lower than that of control. However, the translation efficiency of non-SD sequence ATGTCAACTT (pNG-nPT-284) was not significantly different from that of control. Thus, the results indicated that the non-SD sequence could be the translational initiator.

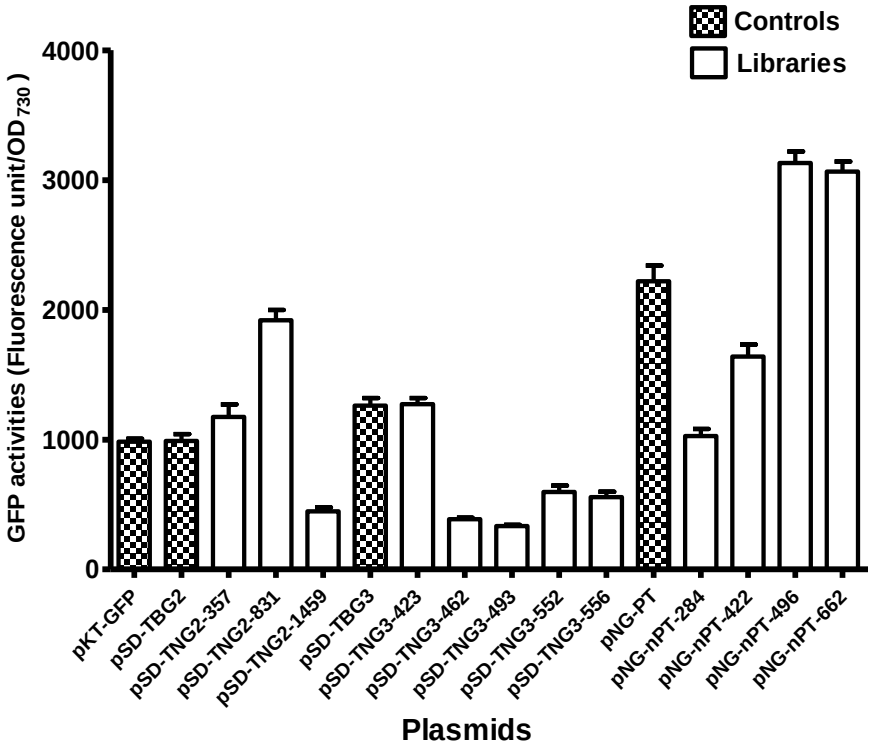


Fig. 12. GFP activities of the selected *Synechococcus* clones from SD/ non-SD libraries.

Cell cultures of selected *Synechococcus* clones were harvested at day 1. The GFP activities of *Synechococcus* cells were measured using spectrofluorometer (Multimode DTX880). Each value and error bar represents the mean of three independent experiments and its standard deviation.

Table 2. GFP activities of selected *Synechococcus* clones and the SD/ non-SD sequences upstream of *gfp*.

Plasmids [a]	Sequences (5'-3 ') [b]	GFP activities (%) [c]			
SD consensus	A**AGGAGG**TTT				
pKT-GFP	cgatccccG**GG**T**GG**TCAGtcccttATG	100			
pSD-TBG2	cggatcccG**GG**T**GG**TCAGtcccttATG	101	100		
pSD-TBG3	cggatctcG**GG**T**GG**TCAGtcccttATG	128	127	100	
pNG-PT	cggatctcG**GG**T**GG**TCAGtcccttATG	226	224	176	100
pSD-TNG2-357	cggatcccA**AGGA**ACATAtcccttATG	119	119	93	53
pSD-TNG2-831	cggatccc**AGGAG**AATGAtcccttATG	195	194	152	86
pSD-TNG2-1459	cggatcccA**AG**A**A**CAATGtcccttATG	45	45	35	20
pSD-TNG3-423	cggatctcAAC**AGGA**AAAtcccttATG	129	128	101	57
pSD-TNG3-462	cggatctcTAACAAAAAAtcccttATG	39	39	31	17
pSD-TNG3-493	cggatctcAAAAAACAAAtcccttATG	34	34	26	15
pSD-TNG3-552	cggatctcA**AGG**CCCAAAtcccttATG	61	60	47	27
pSD-TNG3-556	cggatctcG**GGA**AAAGCAtcccttATG	57	56	44	25
pNG-nPT-284	cggatctcATGTCAACTTtcccttATG	105	104	81	46
pNG-nPT-422	cggatctcAAATCA**AGG**Ttcccttatg	167	166	130	74
pNG-nPT-496	cggatctcAAT**GGA**AATAtcccttATG	318	316	248	141
pNG-nPT-662	cggatctcAATT**GGA**TTTtcccttATG	312	310	243	138

[a] Plasmids pSD-TBG2, pSD-TBG3 and pNG-PT are control of libraries pSD-TNG2, pSD-TNG3 and pNG-PT, respectively.

[b] The SD sequence A**AGGAGG**TTT was referred from Shine and Dalgarno, 1974. The first nucleotide is the transcription start site (+1).

The randomized decanucleotides identical to consensus SD sequence are in bold. The start codon (ATG) of *gfp* gene is indicated. The *Bam*HI site is underlined.

[c] The GFP activities were GFP fluorescence unit per OD_{730} and normalized to that of control (pKT-GFP, pSD-TBG2, pSD-TBG3 and pNG-PT)

Table 3. The levels of *gfp* mRNA of the selected *Synechococcus* clones.

Plasmids	Fold difference of *gfp* mRNA levels [a]
pKT-GFP	1.00
pSD-TBG2	0.52
pSD-TNG2-357	0.33
pSD-TNG2-831	0.43
pSD-TNG2-1459	0.40
pSD-TBG3	0.51
pSD-TNG3-423	0.37
pSD-TNG3-462	0.29
pSD-TNG3-493	0.28
pSD-TNG3-552	0.36
pSD-TNG3-556	0.32
pNG-PT	0.82
pNG-nPT-284	0.39
pNG-nPT-422	0.27
pNG-nPT-496	0.29
pNG-nPT-662	0.23

[a] The fold differences of *gfp* mRNA levels were calculated from $2^{-\Delta\Delta C_T}$ using the Ct values of *gfp* gene and *16S* rRNA (internal control). The plasmid pKT-GFP was used as control.

Table. 4. Translation efficiencies of selected synthetic SD/ non-SD sequences.

Plasmids [a]	Sequences (5'-3 ') [b]	GFP activities (%) [c]	mRNA levels (%) [d]	Translation efficiencies (% GFP/mRNA [e])
SD consensus	A**AGGAGG**TTT			
pKT-GFP	cgatccccG**GG**T**GG**TCAGtcccttATG	100	100	100
pSD-TBG2	cggatcccG**GG**T**GG**TCAGtcccttATG	100	100	100
pSD-TNG2-357	cggatcccA**AGGA**ACATAtcccttATG	119	62	191
pSD-TNG2-831	cggatccc**AGGAG**AATGAtcccttATG	194	82	238
pSD-TNG2-1459	cggatcccA**AG**A**A**CAATGtcccttATG	45	77	59
pSD-TBG3	cggatctcG**GG**T**GG**TCAGtcccttATG	100	100	100
pSD-TNG3-423	cggatctcAAC**AGGA**AAAtcccttATG	101	73	139
pSD-TNG3-462	cggatctcTAACAAAAAAtcccttATG	31	56	56
pSD-TNG3-493	cggatctcAAAAAACAAAtcccttATG	26	54	48
pSD-TNG3-552	cggatctcA**AGG**CCCAAAtcccttATG	47	71	66
pSD-TNG3-556	cggatctcG**GGA**AAAGCAtcccttATG	44	63	70
pNG-PT	cggatctcG**GG**T**GG**TCAGtcccttATG	100	100	100
pNG-nPT-284	cggatctcATGTCAACTTtcccttATG	46	48	97
pNG-nPT-422	cggatctcAAATCA**AGG**Ttcccttatg	74	33	227
pNG-nPT-496	cggatctcAAT**GGA**AATAtcccttATG	141	36	396
pNG-nPT-662	cggatctcAATT**GGA**TTTtcccttATG	138	28	489

[a] Plasmids pSD-TBG2, pSD-TBG3 and pNG-PT are a control of libraries pSD-TNG2, pSD-TNG3 and pNG-PT, respectively.

[b] The SD sequence A**AGGAGG**TTT was referred from Shine and Dalgarno, 1974. The first nucleotide is the transcription start site (+1).The randomized decanucleotides identical to consensus SD sequence are in bold. The start codon (ATG) of *gfp* gene is indicated. The *Bam*HI site is underlined.

[c] GFP activities were GFP fluorescence unit per OD_{730} and normalized to that of corresponding controls .

[d] The *gfp* mRNA levels (Fold different x 100) were normalized to that of corresponding controls.

[e] The translation efficiencies were determined by dividing the GFP activities by the levels of *gfp* mRNA.

6. The synthetic non-SD sequence library in *Synechococcus.*

To investigate the activities of the non-SD sequence in *Synechococcus,* the plasmid library pNSD harboring non-SD sequences upstream of *gfp* gene were constructed (Fig. 13 and 14). The plasmid library pNSD extracted from *E. coli* were transformed into *Synechococcus*. A total of 1,500 *Synechococcus* clones were screened for GFP activities using spectrofluorometer. The activities of the screened clones were lower than that of corresponding control (data not shown). The plasmids in the selected clones pNSD#24 and pNSD#88 were rescued in *E. coli* and the DNA sequences were determined. Results showed that the GFP activities of non-SD sequences UAAUUUUUUU (pNSD#24) and UACCUAUUUU (pNSD#88) were 0.27-and 0.36-fold, respectively, lower than that of control (pNG-PT) (Fig. 21).

The *gfp* mRNA levels of *Synechococcus* harboring plasmids pNSD#24 and pNSD#88 were determined using real-time PCR. (see Table 5). The translation efficiency was analyzed. Results in Table 6 showed that the translation efficiencies of non-SD sequences, UAAUUUUUUU and UACCUAUUUU were apparently lower than those of consensus-SD and SD-like sequences. Therefore, the results indicated that the non-SD sequences could be translation initiator in *Synechococcus*, but was less efficient than SD-like sequences.

7. Translation efficiency of the previously reported non-SD sequences in *Synechococcus.*

It has been shown that in *E. coli*, the non-SD sequences UUCAAUAUUU, AUUUACCUCC and CCAAUCUAC are more efficient than the consensus-SD sequence (Kolev *et al.,* 2003). In cyanobacterial *in vitro* translation system, the mutation of SD-like sequence AGGAU to ACCAU results in 200-250% increase in translation (Mutsuda and Sugiura 2006). To investigate the previously reported non-SD sequences in *Synechococcus*, plasmids pSDR-F1, pSDR-F2, pSDR-F3 and pAC6 harboring the non-SD sequences UUCAAUAUUU, AUUUACCUCC, CCAAUCUAC and ACCACCT, respectively, were constructed (Fig. 13, 15 and 16). The GFP activities of *Synechococcus* harboring pSDR-F1, pSDR-F2, pSDR-F3 and pAC6 were apparently lower than that of control pSD-TBG3 (Fig. 21).

The *gfp* mRNA levels of *Synechococcus* harboring plasmids pSDR-F1, pSDR-F2 pSDR-F3, and pAC6 were determined using real-time PCR (Table 5). The translation efficiency was analyzed. Results in Table 6 showed that the translation efficiencies of non-SD sequences UUCAAUAUUU, AUUUACCUCC, CCAAUCUAC and ACCACCT were apparently lower than that of SD-like sequence GGUGGU (pSD-TBG3) and consensus-SD sequence AAGGAGGU (pSDC8). Therefore, our results disagreed with the previous reports.

8. The consensus-SD sequences in *Synechococcus.*

8.1 Construction of plasmids harboring various length of consensus-SD sequences.

The SD sequence generally found 8 nucleotides (UAAGGAGG) upstream from the start codon and interacts with the complementary region at the 3'-end of 16S rRNA. This mRNA-rRNA base pairing plays an important role in the initiation of protein biosynthesis. It has been predicted that the extent to which a SD sequence is conserved relates to its translation efficiency (Ma *et al.,* 2003). In *E. coli*, a six-nucleotide SD (AGGAGG) is more efficient than shorter or longer sequences (Vimberg *et al*., 2007).

In order to investigate the activities of consensus-SD sequence and its length in *Synechococcus*, plasmids pSDC8, pSDC7, pSDC-S8 and pSDC-S6 were constructed (Fig. 13 and 17). Plasmids pSDC8 and pSDC7 harbored 8 nt. (AAGGAGGU) and 7 nt. (AGGAGGU), respectively, of consensus-SD sequence which is complementary to 3'end of the *E. coli 16S* rRNA. Plasmids pSDC-S8 and pSDC-S6 harbored 8 nt. (AAAGGAGG) and 6 nt. (AGGAGG), respectively, of consensus-SD sequence which is complementary to 3'end of the*16S* rRNA of *Synechococcus* PCC7942. .

8.2 The GFP activities of plasmids harboring various lengths of consensus-SD sequences.

The GFP activities of *Synechococcus* harboring pSDC8, pSDC7, pSDC-S8, and pSDC-S6 were 3.06-, 3.26-, 2.80-, and 2.83-fold, respectively, higher than that of control pSD-TBG2 (Fig. 21). The GFP activity of pSDC8 was not significantly different from that of pSDC7. The GFP activity of pSDC-S8 was not significantly different from that of pSDC-S6,

however both were significant lower than those of pSDC7 and pSDC8. Plasmid pNG-SDC8 harboring the gene cassette TER- P_{tRNA}–*gfp*-(*Nos*-ter) of in the opposite orientation of pSDC8 (Fig. 13). The GFP activity of *Synechococcus* harboring pNG-SDC8 was significantly higher than that of pSDC8 (Fig.21). Therefore, the results indicated that the inverted gene cassette may affect the GFP expression in *Synechococcus*. The results agreed well with those of pSD-TBG3 and PNG-PT.

8.3 The *gfp* mRNA levels of various lengths of consensus-SD sequences.

The *gfp* mRNA levels of plasmids harboring various lengths of consensus-SD sequences were determined by using real-time PCR. The results in (Table 5) showed that the *gfp* mRNA levels of *Synechococcus* harboring consensus-SD sequences (pSDC8, pSDC7, pSDC-S8, pSDC-S6 and pNG-SDC8) were significantly lower than that of SD-like sequences (pKT-GFP, pSD-TBG2, pSD-TBG3 and pNG-PT).

8.4 The translation efficiency of various length of consensus-SD sequences.

Results in Table 6 showed that the translation efficiency of *Synechococcus* harboring pSDC8 was significantly higher than that of pSDC7. The translation efficiencies of pSDC-S8 was significantly higher than that of pSDC-S6, however, both were significant lower than pSDC7 and pSDC8 (Table 6). Therefore, the results indicated that the consensus-SD sequences of 8 nt. Length were more efficient for translation than 7 nt. The translation efficiencies of consensus-SD sequences (pSDC8, pSDC7, pSDC-S8 and pSDC-S6) were apparently more efficient for translation initiation than those of SD-like sequences (Table 6).

9. The activities of translational enhancers in *Synechococcus.*

9.1 Construction of plasmids harboring translational enhancers

The boxA (A/U-rich enhancer) of *E. coli rrnB* is the binding target of the S1 protein of 30S ribosomal subunit (Komarova *et al.,* 2002; Vimberg *et al.,* 2007). The increased 30S ribosomal complexes on 5'-UTR enhance protein synthesis. In order to investigate the effect

of boxA on translation in *Synechococcus*, plasmid pSDC7-BoxA harboring the boxA sequence upstream of consensus-SD sequence was constructed (see Fig. 13 and 18).

It has been shown that using a cyanobacterial *in vitro* translation system, the pyrimidine-rich sequence (C/U-rich enhancer : CUUUCUCCCCAGCCUUUCCCCUUUCUCU UUUU) at the 5'UTR of *rbcS* mRNA is necessary for efficient translation. The pyrimidine-rich sequence might be the binding target of S1 protein of 30S ribosomal subunit (Mutsuda and Sugiura 2006). In order to investigate the effect of pyrimidine-rich sequence on translation, plasmid pSDC7-46Y10 and pAC6-46Y10 harboring the pyrimidine-rich sequence upstream of consensus-SD and non-SD sequence, respectively, were constructed (see Fig. 13, 19 and 20).

9.2 The GFP activities of plasmids harboring SD/ non-SD sequences with translational enhancers

The GFP activities of *Synechococcus* harboring pSDC7-BoxA and pSDC7-46Y10 were significantly lower than that without translational enhancer (pSDC7) (Fig. 21). The results indicated that the boxA sequence (pSDC7-BoxA) and the pyrimidine-rich sequence (pSDC7-46Y10) did not enhance but decreased the translation in *Synechococus*.

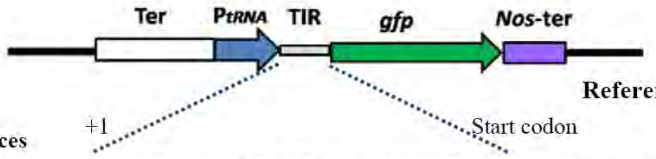
9.3 The *gfp* mRNA levels of plasmids harboring SD/ non-SD sequences with translational enhancers

The *gfp* mRNA levels of plasmids harboring SD/ non-SD sequences with translational enhancers were determined by using real-time PCR. The results in Table 5 showed that the *gfp* mRNA levels of *Synechooccus* harboring pSDC7-BoxA and pSDC7-46Y10 were significantly higher than that of consensus-SD sequences without the previously reported enhancers (pSDC7).

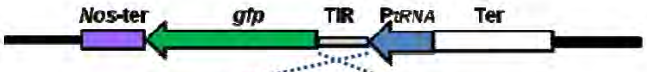
9.4 The translation efficiency of plasmids harboring SD/ non-SD sequences with translational enhancers

Results in Table 6 showed that the translation efficiencies of pSDC7-BoxA, pSDC7-46Y10 and pAC6-46Y10 were significantly lower than that of corresponding controls (pSDC7 and pAC6). Therefore, the present of the previously reported translational

enhancers, boxA and pyrimidine-rich sequences, did not enhance but decreased the translation efficiency in *Synechococcus.*



Plasmids	+1 Start codon	References
SD-like sequences		
pKT-GFP	CGATCCCCG**GG**T**GGT**CAGTCCCTTATG	Chungjatupornchai *et al.*, 2009
pSD-TBG2	CGGATCCCG**GG**T**GGT**CAGTCCCTTATG	Udpuay *et al.*, unpublished data
pSD-TBG3	CGGATCTCG**GG**T**GGT**CAGTCCCTTATG	Udpuay *et al.*, unpublished data
Consensus-SD sequences		
pSDC8	CGGATCCC**AAGGAGGT**TTTCCCTTATG	This work
pSDC7	CGGATCCCT**AGGAGGT**TTTCCCTTATG	This work
pSDC-S8	CGGATCC**AAAGGAGG**ATTCCCTTATG	This work
pSDC-S6	CGGATCCTT**AGGAGG**ATTCCCTTATG	This work
Non-SD sequences		
pSDR-F1	CGGATCTC**TTCAATATTT**-CCCTTATG	This work
pSDR-F2	CGGATCTC**ATTTACCTCCT**CCCTTATG	This work
pSDR-F3	CGGATCTC**CCAATCTAC**-TCCCTTATG	This work
pAC6	CGGATCCCT**ACCACCT**TTTCCCTTATG	This work
Translational enhancers/ consensus-SD and non-SD sequences		
pSDC7-BoxA	CGGATCC***GCTCTTTAACAATTTAT*** ***CAGA***TCCCT**AGGAGGT**TTTCCCTTATG	This work
pSDC7-46Y10	CGGATCCG***CTTTCTCCCCAGCCTTTCCCCT*** ***TTCTCTTTTT*****AGGAGGT**TTTCCCTTATG	This work
pAC7-46Y10	CGGATCCG***CTTTCTCCCCAGCCTTTCCCCT*** ***TTCTCTTTTT*****ACCACCT**TTTCCCTTATG	This work



SD-like sequences	+1	
pNG-PT	CGGATCTCG**GG**T**GGT**CAGTCCCTTATG	This work
Consensus-SD sequences		
pNG-SDC8	CGGATCCC**AAGGAGGT**TTTCCCTTATG	This work
Non-SD sequences		
pNSD	CGGATCTCHHHHHHHHHHHTCCCTTATG	This work

Fig. 13. Plasmids harboring various TIR upstream of *gfp* gene

Plasmids pKT-GFP, pSD-TBG2, pSD-TBG3 and pNG-PT were used as controls. TER, Transcription-translation terminator signal of phage T4; P_{tRNA}, $tRNA^{pro}$ promoter; TIR, translation initiation region; *gfp*, green fluorescent protein gene; *Nos-ter*, nopaline synthase

terminator. The first nucleotide is the transcription start site (+1). The restriction site *Bam*HI is underlined. The consensus-SD sequences (Vimberg *et al.,* 2007) in pSDC8, pSDC7, pSDC-S8, pSDC-S6, pSDC7-BoxA and pSDC7-46Y10 are in bold. The non-SD sequences in pSDR-F1, pSDR-F2, pSDR-F3, pAC6 and pNSD are also in bold. The randomized nucleotides (A, T and C) of plasmid libraries pNSD are marked by H. The boxA sequence (A/U-rich enhancer) of *rrnB* (Vimberg *et al.,* 2007) in pSDC7-BoxA and pyrimidine-rich sequence (C/U-rich enhancer) (Mutsuda and Sugiura 2006) in pSDC7-46Y10 and pAC6-46Y10 are in italic bold. The gene expression cassettes of plasmids pNG-PT, pNG-SDC8, and pNSD are inverse orientation.

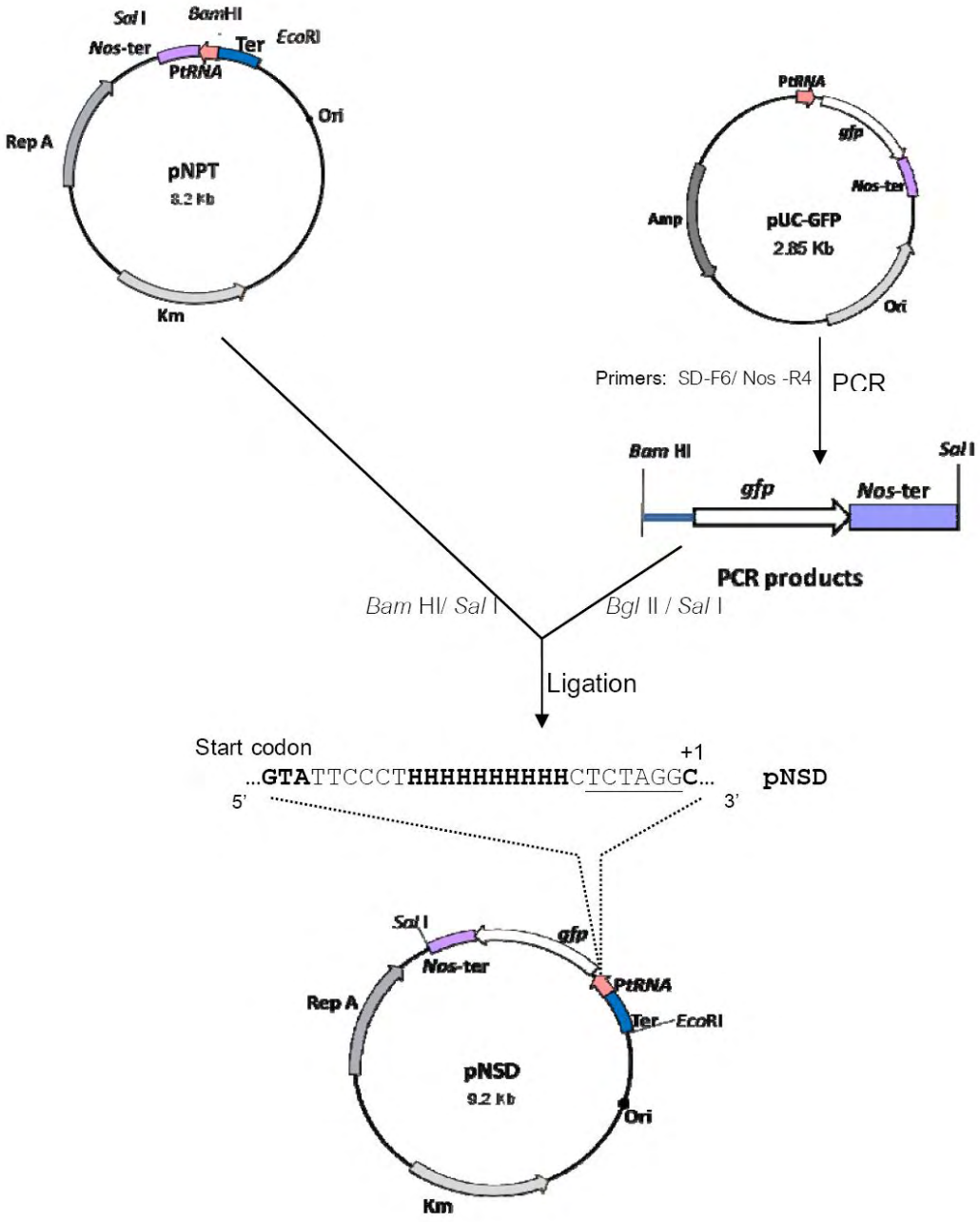


Fig. 14. Schematic diagram for construction of non-SD sequence library pNSD

The PCR product containing gene cassette, the (non-SD)–*gfp*-(*Nos*-ter), was cloned into a shuttle vector pNPT. The transcription start site (+1) and start codon (ATG) of *gfp* mRNA are indicated. The randomized nucleotides (A, T and C) of plasmid libraries pNSD are marked by H.

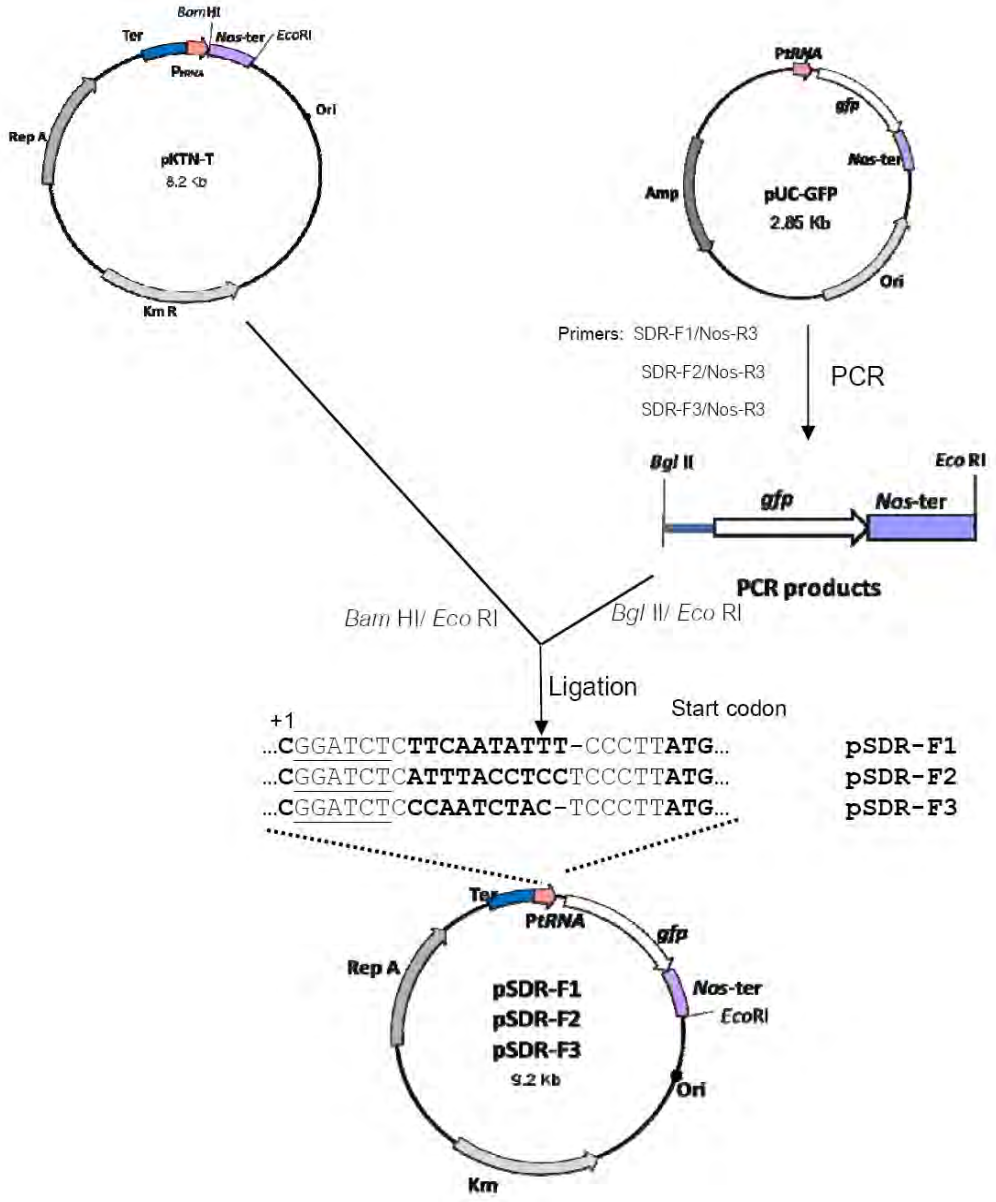


Fig. 15. Schematic diagram for construction of plasmids harboring functional non-SD sequence of *E.coli*

The PCR products containing gene cassette, the functional non-SD sequences of *E. coli*, green fluorescent protein (*gfp*) gene, and nopaline synthase polyadenylation site (*Nos*-ter), were cloned into a shuttle vector pKTN-T. The transcription start site (+1) and strat codon (ATG) of *gfp* mRNA are indicated.

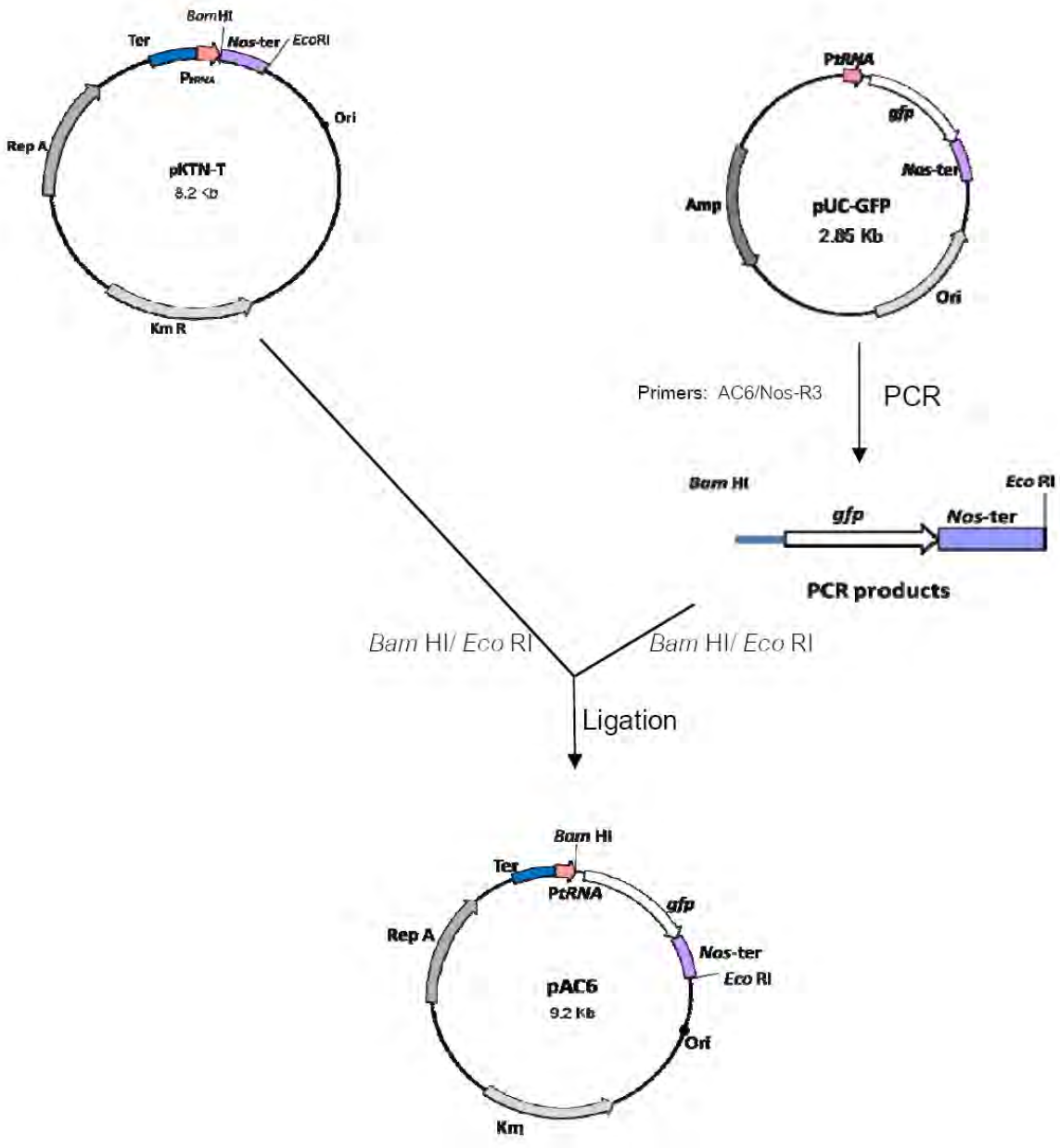


Fig. 16. Schematic diagram for construction of pAC6

The PCR product containing gene cassette, the non-SD sequence (C/U-rich: ACCACCU), green fluorescent protein (*gfp*) gene, and nopaline synthase polyadenylation site (*Nos*-ter), were cloned into a shuttle vector pKTN-T. The transcription start site (+1) and strat codon (ATG) of *gfp* mRNA are indicated.

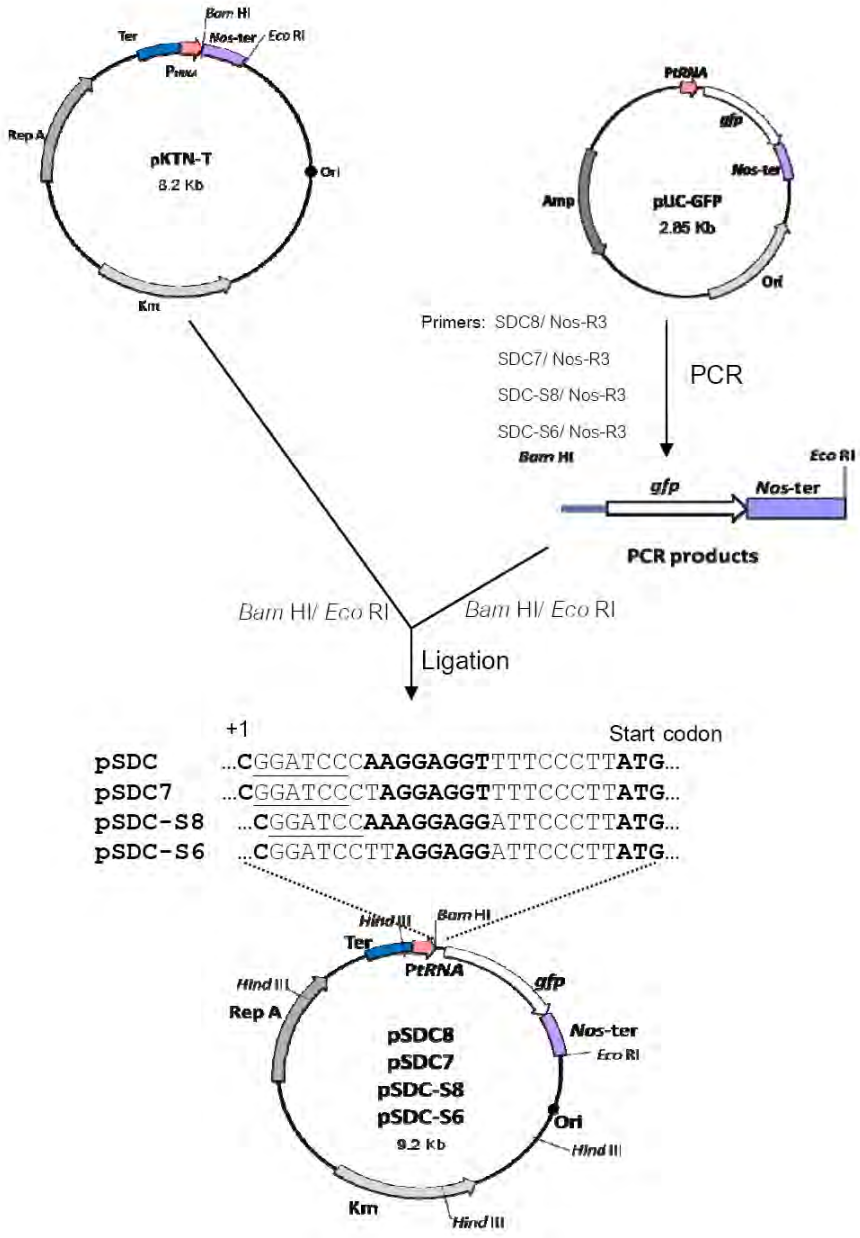


Fig. 17. Schematic diagram for construction of pSDC8, pSDC7, pSDC-S8 and pSDC-S6

The PCR products containing gene cassettes, the (consensus-SD)– *gfp*-(*Nos-ter*), were cloned into the *Bam*HI/ *Eco*RI sites of the shuttle vector pKTN-T. The transcription start site (+1) and start codon (ATG) of *gfp* are indicated.

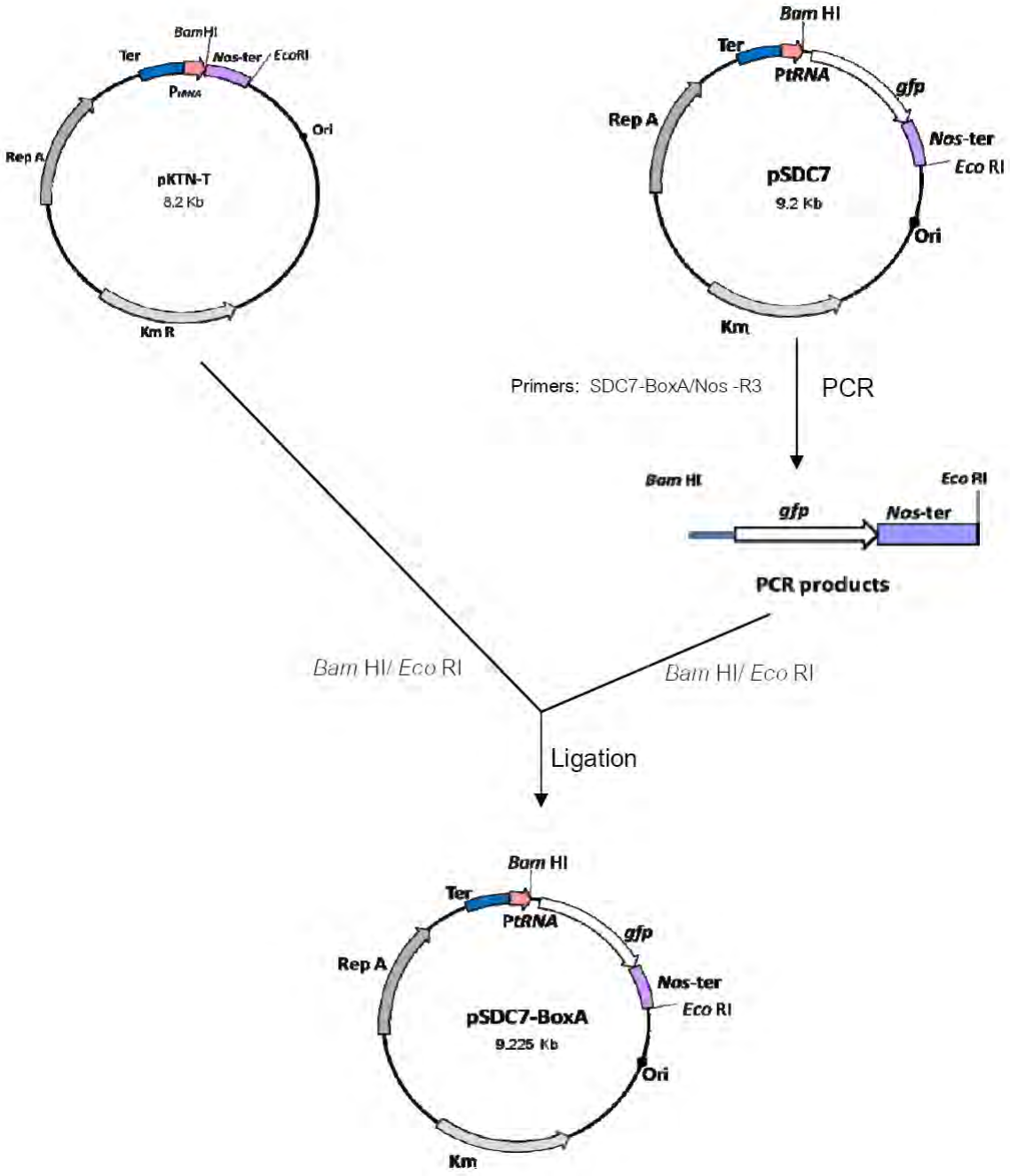


Fig. 18. Schematic diagram for construction of pSDC7-BoxA

The PCR product, containing the A/U-rich enhancer (boxA sequence of *rrnB* (25) upstream of consensus-SD sequence was amplified form pSDC7 with primers SDC7-BoxA and NOS-R3. The gene cassette (A/U-rich)-SD –*gfp*-(*Nos*-ter), was cloned into the *Bam*HI/ *Eco*RI site of the shuttle vector pKTN-T. The resulting plasmid is pSDC7-BoxA.

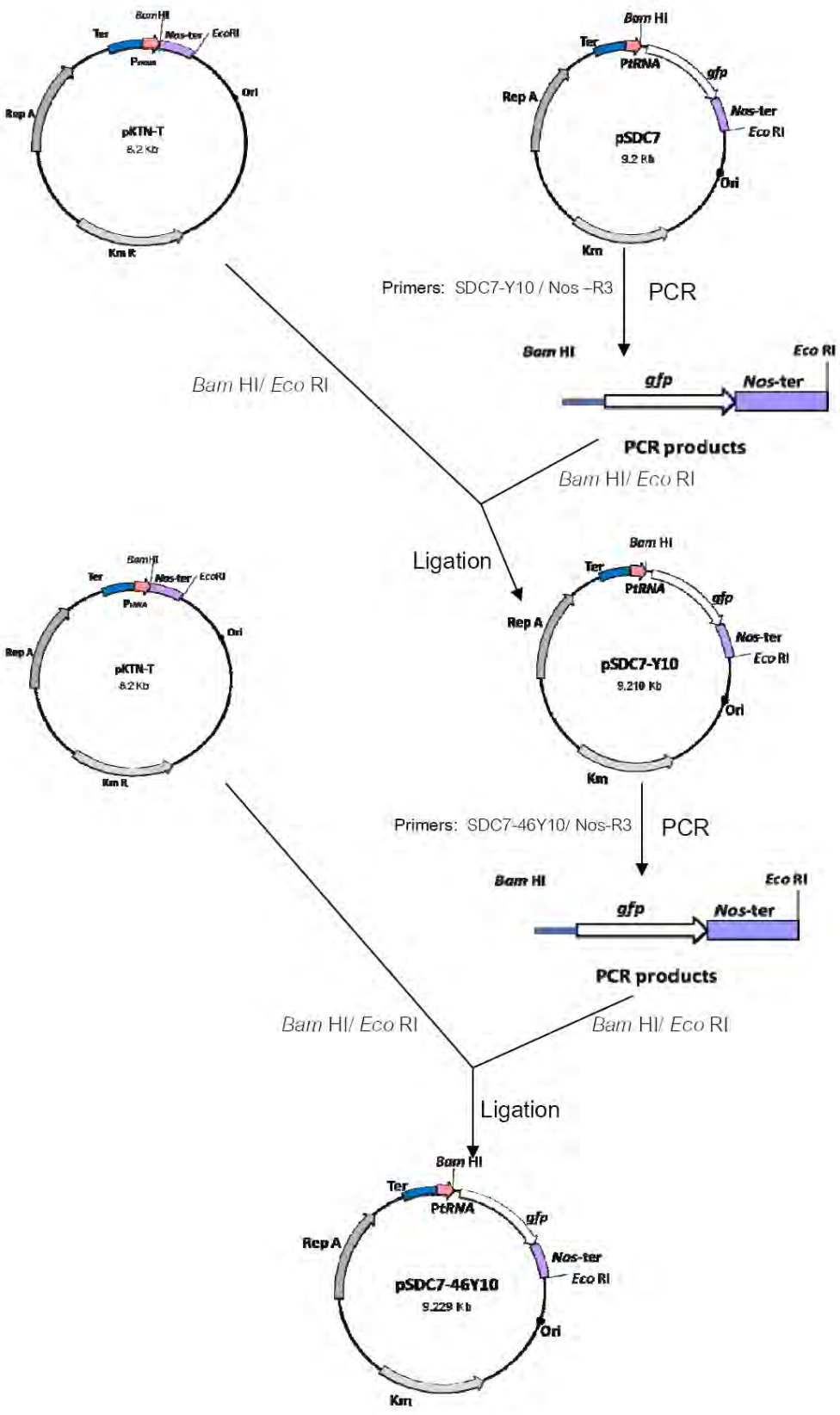

Bam HI
Ter
Nos-ter
Eco RI
Ori
Rep A
pKTN-T
8.2 Kb
Km R
PtRNA
gfp
pSDC7
9.2 Kb
Km
Primers: SDC7-Y10 / Nos –R3
PCR
Bam HI/ Eco RI
PCR products
Ligation
pSDC7-Y10
9.210 Kb
Primers: SDC7-46Y10/ Nos-R3
pSDC7-46Y10
9.229 Kb

Fig. 19. Schematic diagram for construction of pSDC7-46Y10

The PCR product containing Y10 sequence (38) upstream of gene cassette SD –*gfp*-(*Nos*-ter) was amplified from pSDC7 using primers SDC7-Y10 and Nos-R3 and was cloned into the *Bam*HI/ EcoRI site of the shuttle vector pKTN-T. The resulting plasmid is pSDC7-Y10. The gene cassette containing the pyrimidine rich sequence (C/U-rich enhancer) was amplified from pSDC7-Y10 using primers SDC7-46Y10 and Nos-R3. The gene cassette (C/U-rich enhancer)-SD –*gfp*-(*Nos*-ter) was cloned into the *Bam*HI/ EcoRI site of the shuttle vector pKTN-T. The plasmid pSDC7-46Y10 was obtained.

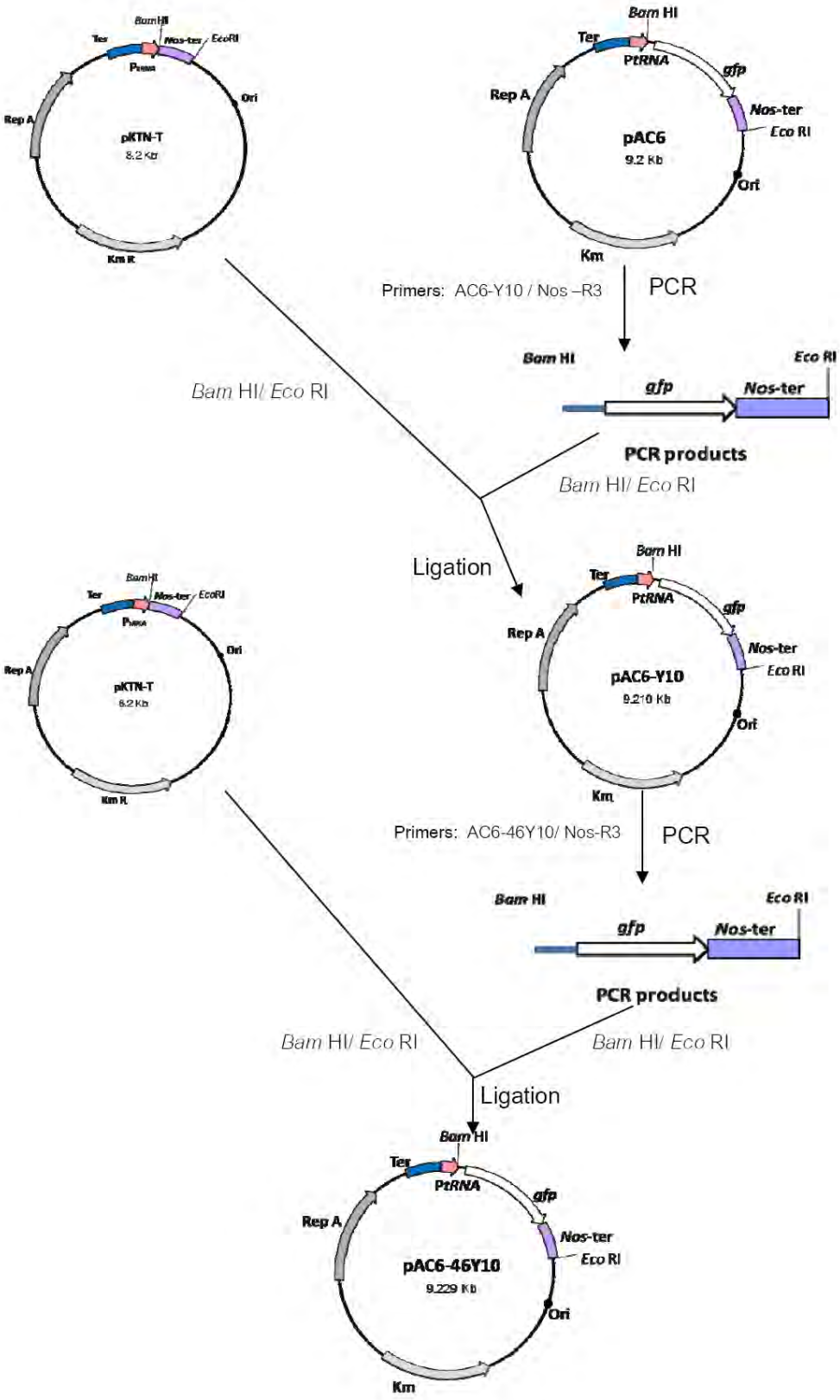

Bam HI
Ter
PtRNA
gfp
Nos-ter
Eco RI
Rep A
pAC6
9.2 Kb
Ori
Km
Primers: AC6-Y10 / Nos –R3
PCR
Bam HI
Eco RI
gfp
Nos-ter
PCR products
Bam HI/ Eco RI
Bam HI/ Eco RI
Ligation
pKTN-T
8.2 Kb
Km R
pAC6-Y10
9.210 Kb
Primers: AC6-46Y10/ Nos-R3
PCR
pAC6-46Y10
9.229 Kb
Bam HI/ Eco RI
Bam HI/ Eco RI
Ligation

Fig. 20. Schematic diagram for construction of pAC-46Y10

The PCR product of Y10 sequence (38) upstream of gene cassette (non-SD) –*gfp*-(*Nos*-ter) was amplified from pAC6 using primers AC6-Y10 and Nos-R3 and was cloned into the *Bam*HI/ EcoRI site of the shuttle vector pKTN-T. The resulting plasmid is pAC6-Y10. Then, the gene cassette containing the pyrimidine rich sequence (C/U-rich enhancer) was amplified from pAC-Y10 using primers AC6-46Y10 and Nos-R3. The gene cassette (C/U-rich enhancer)-(non-SD) –*gfp*-(*Nos*-ter) was cloned into the *Bam*HI/ EcoRI site of the shuttle vector pKTN-T. The plasmid pAC6-46Y10 was obtained.

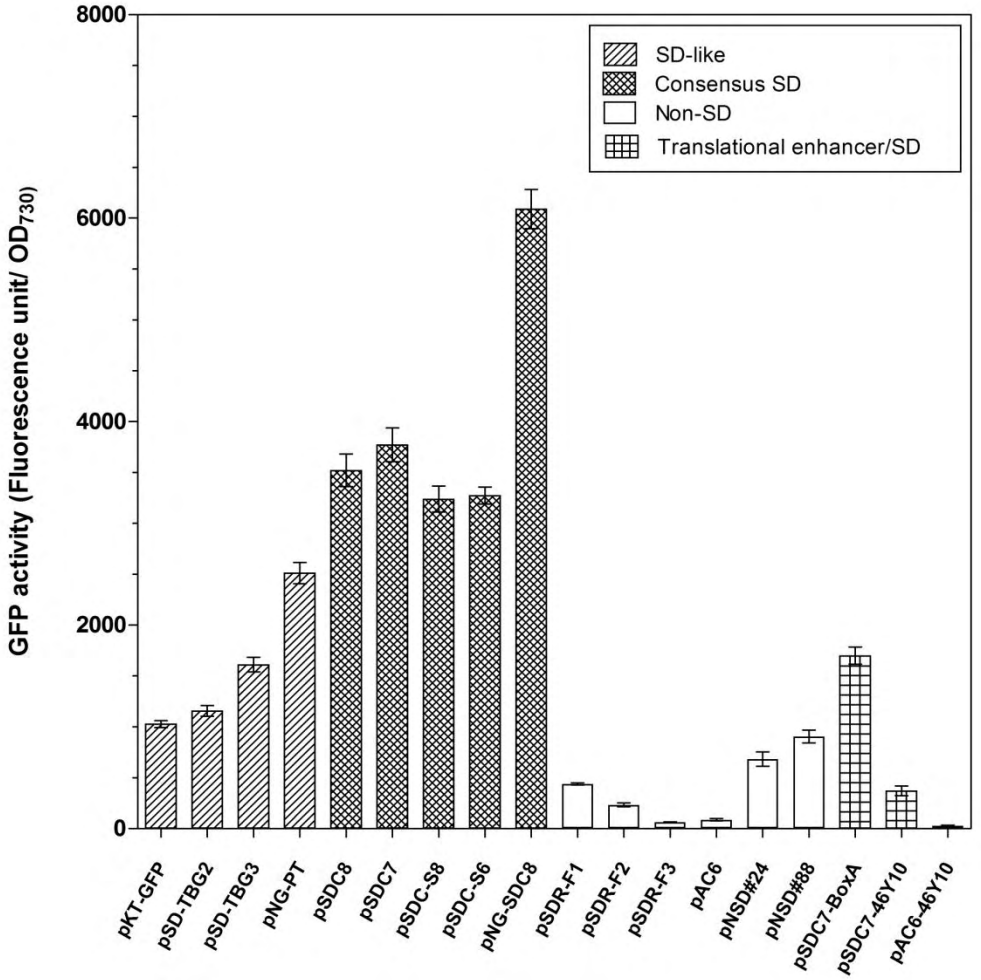


Fig. 21. The GFP activities of *Synechococcus* harboring plasmids with various TIR upstream of *gfp* gene

Cell cultures of *Synechococcus* harboring harboring plasmids with various TIR upstream of *gfp* gene grown under continuous light of 4000-4500 lux for 1 day were harvested. The GFP activities of *Synechococcus* cultures were determined. Each value and error bar represents the average of three independent experiments and its standard deviation.

Table 5. The relative *gfp* mRNA level of *Synechococcus* harboring plasmids with various TIR upstream of *gfp* gene

Plasmids	*gfp* mRNA levels (Fold difference)	*gfp* mRNA levels (%)				
SD-like sequences						
pKT-GFP	1.00	100				
pSD-TBG2	0.98	98	100			
pSD-TBG3	0.82	82	83	100		
pNG-PT	0.70	70	72	86	100	
Consensus-SD sequences						
pSDC8	0.25	25	25	30	35	100
pSDC7	0.30	30	31	37	43	122
pSDC-S8	0.43	43	44	52	61	174
pSDC-S6	0.73	73	75	89	104	298
pNG-SDC8	0.21	21	22	26	30	86
Non-SD sequences						
pSDR-F1	0.84	84	85	102	119	341
pSDR-F2	1.08	108	110	131	153	439
pSDR-F3	1.91	191	195	233	272	778
pAC6	1.54	154	158	188	219	629
pNSD#24	0.86	86	97	104	122	349
pNSD#88	0.95	95	158	116	135	387
Translational enhancer/ consensus-SD sequences						
pSDC7-BoxA	0.94	94	95	113	132	378
pSDC7-46Y10	2.28	228	233	278	324	929
Translational enhancer/ Non-SD sequences						
pAC6-46Y10	1.39	139	142	169	197	565

Plasmid pKT-GFP was used as control for plasmids harboring SD-like sequences, pSD-TBG2 for consensus-SD sequences and pAC6, pSD-TBG3 for non-SD sequences (pSDR-F1, pSDR-F2 and pSDR-F3) and pNG-PT for the synthetic non-SD library (pNSD).

Table 6. Translation efficiency of various TIR

Plasmids	TIR Sequence (5'-3')	Translational Effiency (%)				
SD-like sequences						
pKT-GFP	CGAUCCCCG**GGUGGU**CAGUCCCUUAUG	100				
pSD-TBG2	CGGAUCCCG**GGUGGU**CAGUCCCUUAUG	115	100			
pSD-TBG3	CGGAUCUCG**GGUGGU**CAGUCCCUUAUG	191	167	100		
pNG-PT	CGGAUCUCG**GGUGGU**CAGUCCCUUAUG	350	301	181	100	
Consensus SD sequences						
pSDC8	CGGAUCCC**AAGGAGGU**UUUCCCUUAUG	1380	1224	733	403	100
pSDC7	CGGAUCCCU**AGGAGGU**UUUCCCUUAUG	1223	1052	632	349	88
pSDC-S8	CGGAUCC**AAAGGAGG**AUUCCCUUAUG	735	636	387	211	53
pSDC-S6	CGGAUCCUU**AGGAGG**AUUCCCUUAUG	437	377	228	125	31
pNG-SDC8	CGGAUCCC**AAGGAGGU**UUUCCCUUAUG	2824	2395	1454	807	200
Non-SD sequences						
pSDR-F1	CGGAUCUC**UUCAAUAUUU**-CCCUUAUG	51	45	26	14	4
pSDR-F2	CGGAUCUC**AUUUACCUCCU**CCCUUAUG	21	18	11	6	2
pSDR-F3	CGGAUCUC**CCAAUCUAC**-UCCCUUAUG	3	3	2	1	0
pAC6	CGGAUCCC**AACCACCU**UUUCCCUUAUG	6	5	3	1	0
pNSD#24	CGGAUCUC**UAAUUUUUUU**UCCCUUAUG	78	61	40	22	5
pNSD#88	CGGAUCUC**UACCUAUUUU**UCCCUUAUG	93	49	48	27	7
Translational enhancer/ consensus SD sequences						
pSDC7-BoxA	CGGAUCC***GCUCUUUAACAAUUUAU CAGA***UCCCU**AGGAGGU**UUUCCCUUAUG	177	155	93	52	13
pSDC7-46Y10	CGGAUCCG***CUUUCUCCCCAGCCUUUCCCCUU UCUCUUUUUAGGAGGU***UUUCCCUUAUG	16	14	8	5	1
Translational enhancer/ Non-SD sequences						
pAC6-46Y10	CGGAUCCG***CUUUCUCCCCAGCCUUUCCCCUU UCUCUUUUUACCACCU***UUUCCCUUAUG	2	2	1	1	0

The translation efficiency (%) of the TIR is defined as the GFP activity divided by the level of *gfp* mRNA. Plasmids pKT-GFP, pSD-TBG2, pSD-TBG3 and pNG-PT were used as controls. The first nucleotide is the transcription start site (+1). The restriction site *Bam*HI is underlined. The consensus-SD sequences (Vimberg *et al.,* 2007) in pSDC8, pSDC7, pSDC-S8, pSDC-S6, pSDC7-BoxA and pSDC7-46Y10 are in bold. The non-SD sequences in pSDR-F1, pSDR-F2, pSDR-F3, pAC6 and pNSD are also in bold. The randomized nucleotides (A, T and C) of plasmid libraries pNSD are marked by H. The boxA sequence (A/U-rich enhancer) of *rrnB* (Vimberg *et al.,* 2007) in pSDC7-BoxA and pyrimidine-rich sequence (C/U-rich enhancer) (Mutsuda and Sugiura 2006) in pSDC7-46Y10 and pAC6-46Y10 are in italic bold. Plasmid pKT-GFP was used as control for plasmids harboring SD-like sequences, pSD-TBG2 for consensus-SD sequences and pAC6, pSD-TBG3 for non-SD sequences (pSDR-F1, pSDR-F2 and pSDR-F3) and pNG-PT for the synthetic non-SD library (pNSD).

10. Highly active synthetic regulatory sequences containing tandem enhancer/ promoter and translation initiator.

10.1 Deletion analysis of the *rrnA* regulatory sequence of *Synechococcus.*

The rRNA A operon (*rrnA*) is highly active in *Synechococcus*. So far, there has no report concerning the regulatory sequence of *rrnA*. In order to analyze regulatory sequence of *rrnA*, plasmids harboring various lengths of *rrnA* regulatory sequence upstream of the SD-like sequence (link) and promoterless *gfp* reporter gene were constructed. Results in Fig. 22 showed that deletions of the putative box A/B (pRNG2) and LysR binding sequence (pRG2D1) increased the GFP activities in *Synechococcus* but decreased in *E. coli*. Thus, the putative box A/B and LysR binding sequences function as inhibitors *Synechococcus* but as enhancers in *E. coli*. Deletion of the 26 bp upstream of the putative UP element (pRG2D3) decreased the GFP activity in *Synechococcus* but has no effect in *E. coli*. Therefore, the 26 bp upstream of the putative UP element is necessary for high GFP activity in *Synechococcus.* Deletion of the putative UP element (pRG2D2) decreased the GFP activity in both *Synechococcus* and *E. coli*. The results indicated that the UP element can function as enhancer in both *Synechococcus* and *E. coli*. The GFP activity of active *rrnA* regulatory sequence upstream of the consensus-SD sequence (pRSS) was 23.97-fold higher than that of control (Fig. 24).

10.2 Investigation of activity of *E. coli* σ^{70} promoter in *Synechococcus.*

The σ^{70} promoter containing the -35 (TTGACA) and -10 (TATAAT) region is highly active in *E. coli*. In order to investigate the activity of the *E. coli* σ^{70} promoter (P_{sig70}) in *Synechococcus*, plasmid pKT-Sig70 harboring the -35 (TTGACA) and -10 (TATAAT) region of P_{sig70} upstream of the consensus-SD sequence was constructed (Fig. 23). Results in Fig. 25 showed that the GFP activities of P_{sig70} was 6.17-fold higher than that of control. The P_{sig70} is highly active in both *Synechococcus* and *E. coli*.

10.3 Combination of tandem enhancer/ promoter and consensus-SD sequence.

In order to construct regulatory sequences which are highly active exceeding the natural cyanobacterial regulatory sequence, plasmids harboring combination of tandem enhancer/ promoter and consensus-SD sequence upstream of the promoterless *gfp* reporter

gene were constructed. Results in Fig. 25 showed that the tandem P_{tRNA}-P_{sig70} promoter (pTS70) was apparently more active than each promoter alone (pKT-GFP and pSDC-Sig70), however, was still less active than P_{rnnA} with consensus-SD sequence (pRSC). The tandem promoters P_{rnnA} -P_{rnnA} (pRRSC), P_{sig70}- P_{rnnA} (pS70-RSC) and P_{sig70}- P_{rnnA}- P_{rnnA} (pS70-2RSC) were apparently more active than P_{rnnA} (pRSC) in both *Synechococcus* and *E. coli*. The GFP activities of *Synechococcus* harboring plasmids with tandem promoters P_{rnnA} -P_{rnnA} (pRRSC), P_{sig70}- P_{rnnA} (pS70-RSC) and P_{sig70}- P_{rnnA}- P_{rnnA} (pS70-2RSC) were 32.33-, 36.66- and 35.88-fold, respectively, higher than that of control (P_{tRNA}). To investigate the level of GFP in the cells, the total proteins extracted from *Synechococcus* and *E. coli* were subjected to SDS-PAGE. The results in Fig. 26 showed that the GFP (approximately 27 kDa) was detected in total proteins extracted from *Synechococcus* and *E. coli* harboring plasmids pS70-2RSC and pS70-RSC (lanes 3 and 4, respectively). To our knowledge, this is the first report that the expression of heterologous protein in *Synechococcus* is high enough to be detected by SDS-PAGE stained with Comassie brilliant blue. Thus, we have successfully constructed the synthetic regulatory sequences which are highly active exceeding the natural cyanobacterial regulatory sequences.

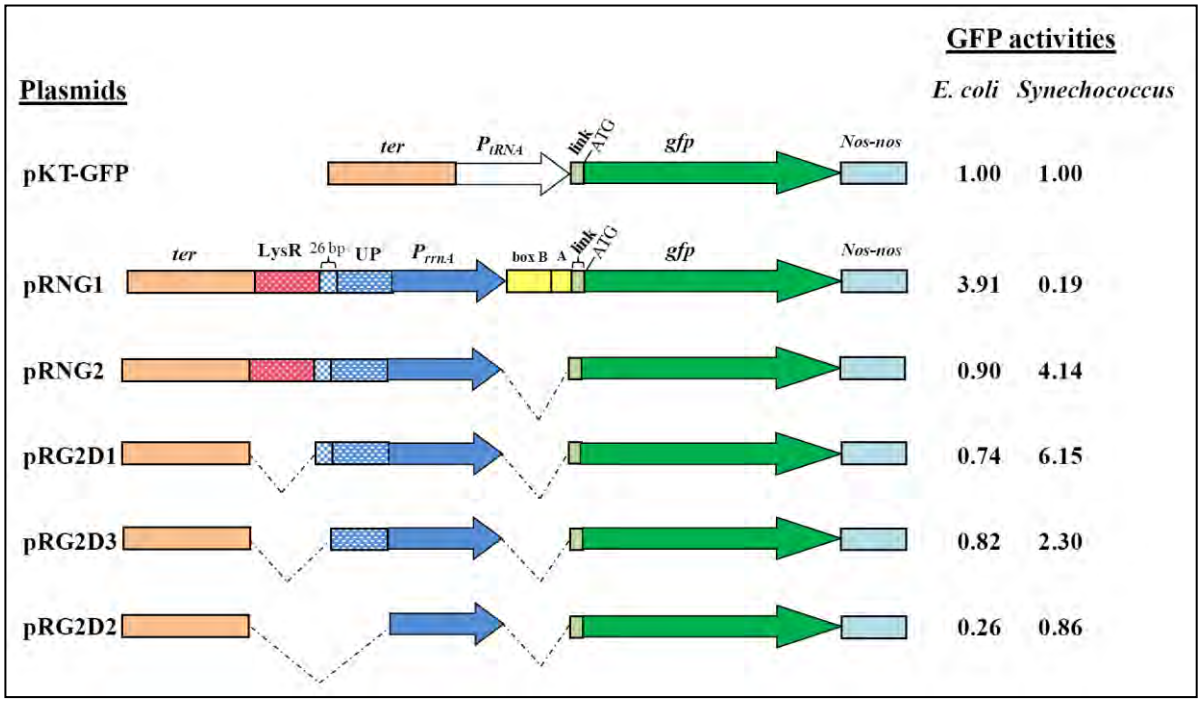


Fig. 22. Deletion analysis of the *rrnA* regulatory sequence.

The regulatory sequence of *Synechococcus rrnA* gene located upstream of the promoterless *gfp* reporter gene is shown. TER, Transcription-translation terminator signal of phage T4; P_{tRNA}, $tRNA^{pro}$ promoter; link, SD-like sequence; *gfp*, green fluorescent protein gene; *Nos-ter*, nopaline synthase terminator; LysR, putative target-binding sequence of LysR; UP, UP element; box A and B, putative box A and B sequence. GFP activities were derived from GFP fluorescence unit per OD_{730} and normalized to that of pKT-GFP. Each value derived from average of four independent experiments.

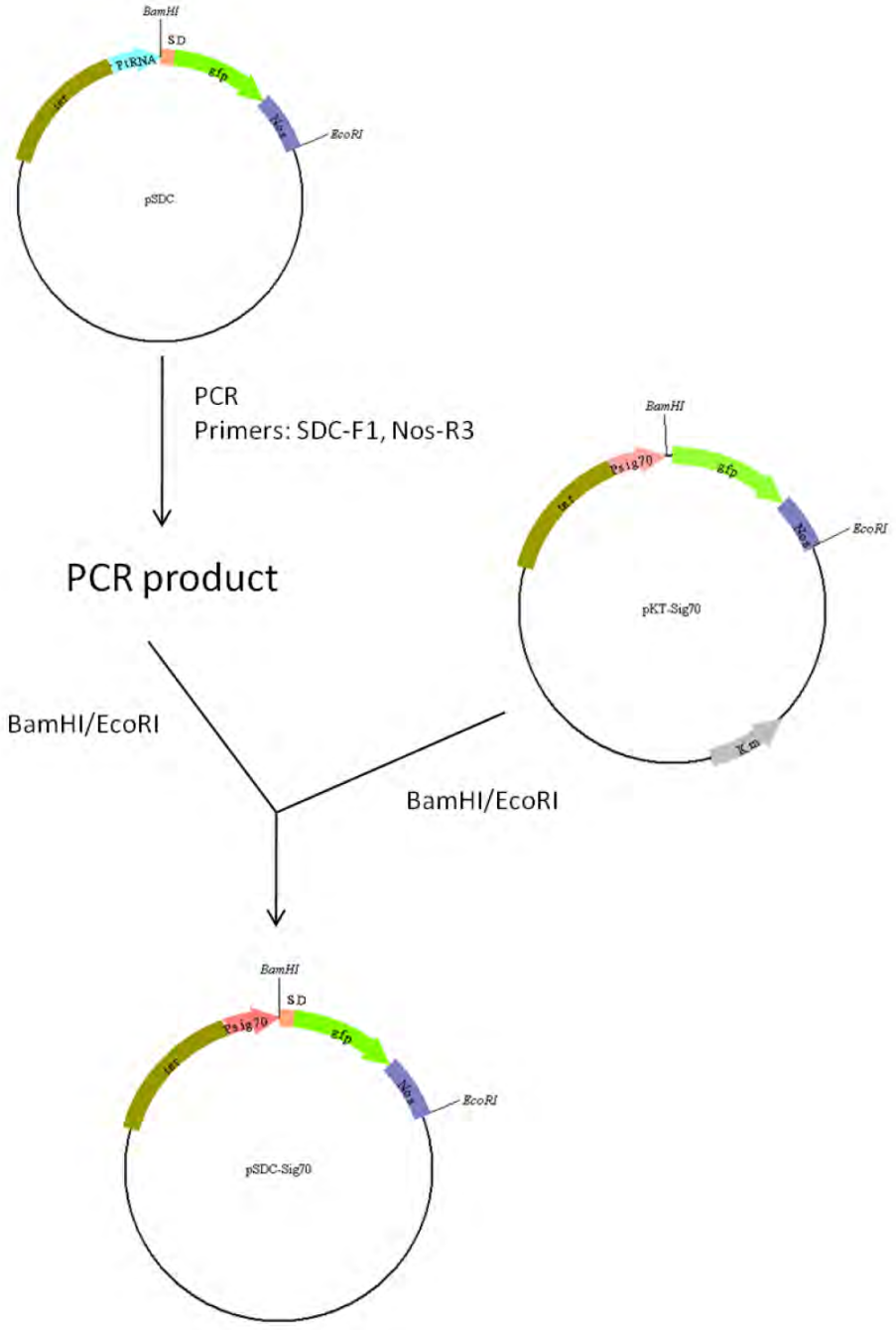


Fig. 23. Schematic diagram for construction of pSDC-Sig70

The PCR product, containing the cassette (consensus-SD) –*gfp*-(*Nos*-ter) amplified from pSDC8 using primer set: SDC-F1 and Nos-R3, was cloned into the *Bam*HI/ EcoRI site of pKT-Sig70.

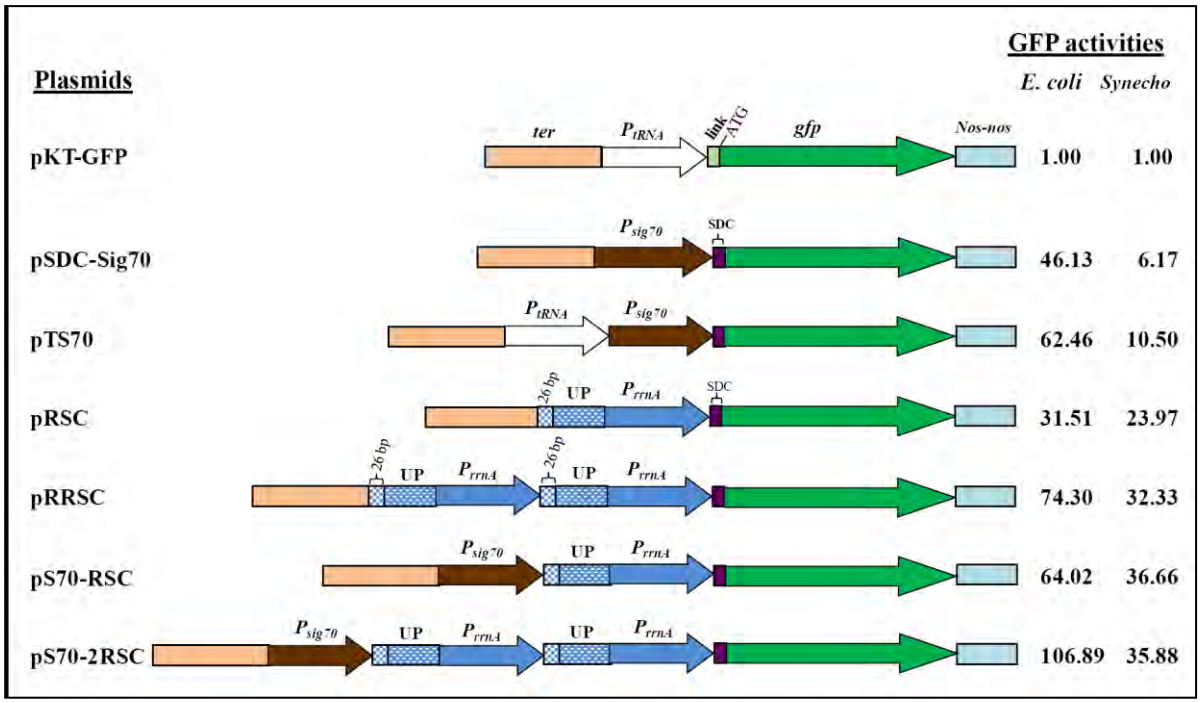


Fig. 24. Combination of tandam enhancer/promoter and consensus-SD sequence.

Tandam enhancer/ promoter located upstream of the promoterless *gfp* reporter gene is shown. TER, Transcription-translation terminator signal of phage T4; P_{tRNA}, $tRNA^{pro}$ promoter; link, SD-like sequence; SDC, consensus-SD sequences; *gfp*, green fluorescent protein gene; *Nos-ter*, nopaline synthase terminator; UP, UP element. GFP activities were GFP fluorescence units per OD_{730} and normalized to that of pKT-GFP. Each value derived from average of four independent experiments.

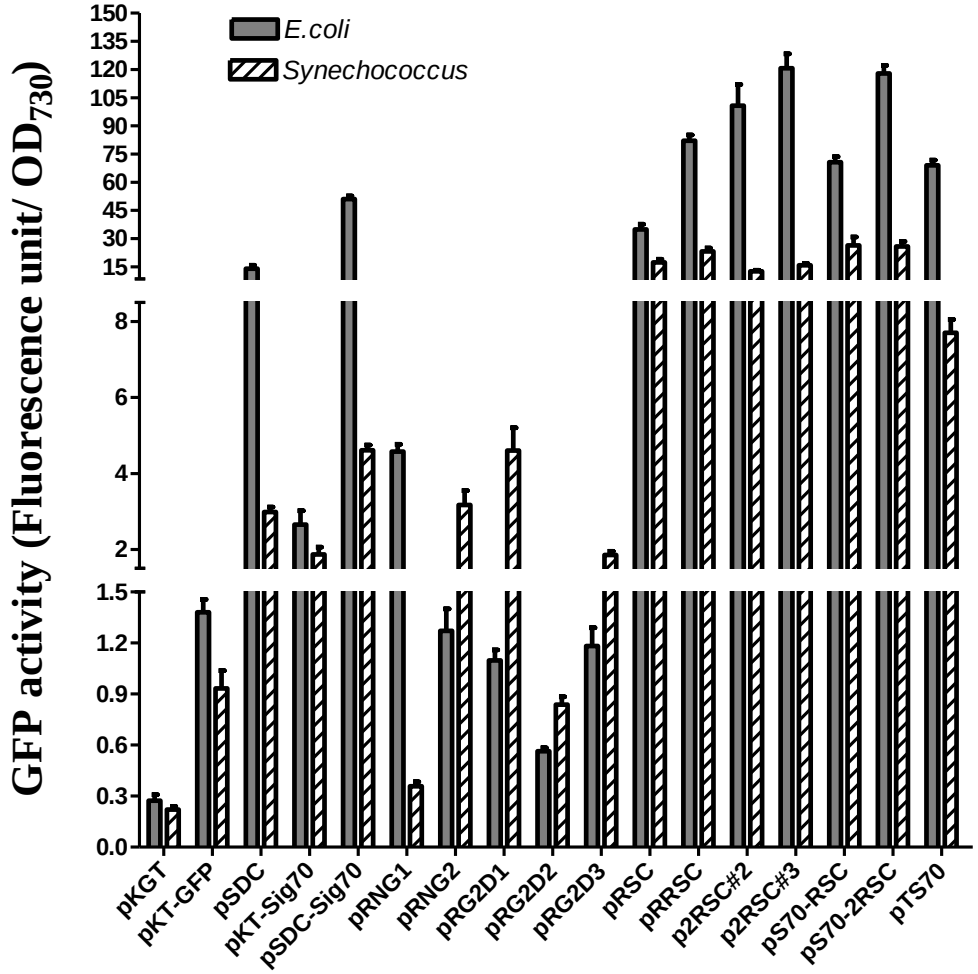


Fig. 25. The GFP activities of *gfp* under the control of synthetic regulatory sequences.

The GFP activities of *E. coli* and *Synechococcus* harboring the recombinant plasmids were determined. Plasmid pKGT was used as negative control and pKT-GFP was used as reference plasmid. Each value and error bar represents the average of four independent experiments and its standard deviation.

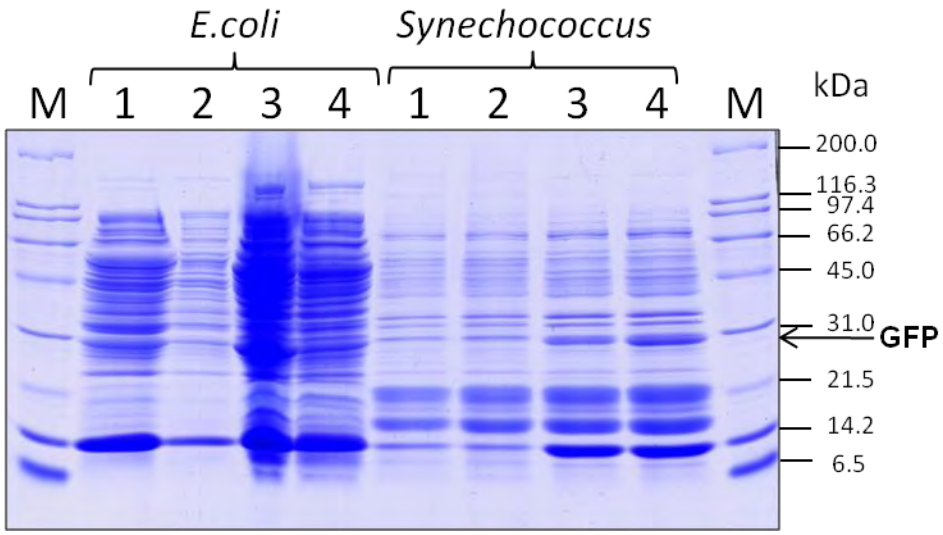


Fig. 26. Expression of *gfp* gene under the control of synthetic regulatory sequences.

12.5 % SDS-PAGE gel staining with Comassie brilliant blue R-250. Proteins from whole cell extracts of *E. coli* and *Synechococcus* were analyzed. Lanes 1, 2, 3 and 4 are from cells harboring plasmids pKGT (negative control), pKT-GFP (reference plasmid), pS70-2RSC and pS70-RSC, respectively. The GFP band is indicated.

References

1. Abelson J (1990) Directed evolution of nucleic acids by independent replication and selection. Science 249:488-489.

2. Asato Y (2005) Control of ribosome synthesis during the cell division cycles of *E. coli* and *Synechococcus.* Curr Issues Mol Biol 7:109-17.

3. Chang B, Halgamuge S and Tang SL (2006) Analysis of SD sequences in completed microbial genomes: Non-SD-led genes are as common as SD-led genes. Gene 373: 90–99

4. Cormack BP, Valdivia RH and Falkow S (1996) FACS-optimized mutants of the green fluorescent protein (GFP). Gene 173: 33-8.

5. Chungjatupornchai W (1990) Expression of mosquitocidal protein genes of *Bacillus thuringiensis* subsp. *israelensis* and the herbicide resistance gene *bar* in *Synechocystis* PCC6803. Curr Microbiol 21:283-288.

6. Chungjatupornchai W, Senawong T and Panyim S (1999) Isolation and characterization of *Synechococcus* PCC7942 promoters: $tRNA^{PRO}$ gene functions as a promoter. Curr Microbiol 38(4): 210-216.

7. Chungjatupornchai W, Fa-aroonsawat S and Panyim S (2002) Characterization of regions of the cyanobacterial $tRNA^{pro}$ gene that affect the expression of a β-glucuronidase reporter gene. FEMS Microbiol Lett 211: 57-64.

8. Chungjatupornchai W and Fa-aroonsawat S (2008) Biodegradation of organophosphate pesticide using recombinant cyanobacteria with surface- and intracellular-expressed organophosphorus hydrolase. J Microbiol Biotechnol 18:946-951.

9. Chungjatupornchai W and Fa-aroonsawat S (2009) Translocation of green fluorescent protein to cyanobacterial periplasm using ice nucleation protein. J. Microbiol 47:187-192.

10. Chungjatupornchai W, Kamlangdee A and Fa-aroonsawat S (2011) Display of organophosphorus hydrolase on the cyanobacterial cell surface using Synechococcus outer membrane protein A. Appl Biochem Biotech 164: 1048-1057.

11. Crameri A, Whitehorn EA, Tate E and Stemmer WPC (1996) Improved green fluorescent protein by molecular evolution using DNA shuffling. Nat Biotechnol 14:315-319.

12. Fukuda H, Sakai M, Nagahama K, Fujii K, Matsuoka M, Inoue Y and Ogawa T (1994) Heterologous expression of the gene for the ethylene-forming enzyme from *Pseudomonas syringae* in the cyanobacterium *Synechococcus*. Biotechnol Lett 16:1–6.

13. Giver L, Gershenson A, Freskgard PO and Arnold FH. (1998) Directed evolution of a thermostable esterase. Proc Natl Acad Sci USA 95:12809-12813.

14. Hammer K, Mijakovic I and Jensen PR (2006) Synthetic promoter libraries- tuning of gene expression. Trend Biotechnol 24:53-55.

15. Hansel A, Pattus F, Jurgens UJ and Tadros MH (1998) Cloning and characterization of the genes coding for two porins in the unicellular cyanobacterium *Synechococcus* PCC 6301. Biochim Biophys Acta 1399:31-39.

16. Jefferson RA, Burgess SM and Hirsh D (1986) β–gGlucuronidase from *Escherichia coli* as a gene-fusion marker. Proc Natl Acad Sci USA 83:8447-8451.

17. Kagiya G, Ogawa, R, Hatashita M, Takagi K, Kodaki T, Hiroshi S and Yamamoto K. (2005) Generation of a strong promoter for *E. coli* from eukaryotic genome DNA. J Biotechnol 115:239-248.

18. Kawata Y, Yamano N, Kojima H and Itoh S (1991) Expression of salmon growth hormone in the cyanobacterium *Agmenellum quadruplicatum*. Biotechnol Lett 13:851–856.

19. Kolev V, Ivanov I, Berzal-Herranz A and Ivanov GI (2003) Non-Shine-Dalgarno initiators of translation selected from combinatorial DNA libraries. J Mol Microbiol Biotechnol 5:154-160.

20. Komarova AV, Tchufistova LS, Supina EV and Boni IV (2002) Protein S1 counteracts the inhibitory effect of the extended Shine-Dalgarno sequence on translation. RNA. 8(9):1137-1147.

21. Li X, Eastman EM, Schwartz RJ and Draghia-Alki R (1999) Synthetic muscle promoters: activities exceeding naturally occurring regulatory sequences. Nat Biotechnol 17:241-245.

22. Ma J, Campbell A and Karlin S (2002) Correlation between Shine–Dalgarno sequence and gene features such as predicted expression levels and operon structure. J Bacteriol 184:5733–5745.

23. Maeda S, Kawaguchi Y, Ohe T and Omata T (1998) cis-Acting sequences required for NtcB-dependent, nitrite-responsive positive regulation for nitrate assimilation operon in the cyanobacterium *Synechococcus* sp. strain PCC7942. J Bacteriol 180:4080-4088.

24. Martinelli R and De Simone V (2005) Short and highly efficient synthetic promoters for melanoma-specific gene expression. FEBS letters 579:153-156

25. Mutsuda, M., & Sugiura, M. (2006) Translation initiation of cyanobacterial *rbcS* mRNAs requires the 38-kDa ribosomal protein S1 but not the Shine-Dalgarno sequence. J. Biol. Chem. 281(50):38314–38321.

26. Okawa R, Kagiya G, Kodai T, Fukuda S and Yamamoto K (2007) Construction of strong mammalian promoters by random cis-acting element elongation. Biotechnique 42:628-633

27. Plansangkate P, Fa-aroonsawat S, Panyim S, and Chungjatupornchai W (2004) The light-responsive promoter of cyanobacterial *ORF76* gene overlaps with the *htpG* terminator. FEMS Microbiol Lett 235: 341–347.

28. Sauer J, Schreiber U, Schmid R, Volker U and Forchhammer K (2001) Nitrogen starvation-Induced chlorosis in *Synechococcus* PCC 7942: Low-level photosynthesis as a mechanism of long-term survival. Plant Physiology 126:233-243.

29. Sazuka T and Ohara O (1996) Sequence features surrounding the translation initiation sites assigned on the genome sequence of *Synechocystis* sp. strain PCC6803 by amino-terminal protein sequencing. DNA Res 3:225-232.

30. Schneider GJ, Haselkorn R (1988) RNA polymerase subunit homology among cyanobacteria, other eubacteria, and archaebacteria. J Bacteriol 170:4136–4140.

31. Shao Z and Arnold FH (1996) Engineering new functions and altering existing functions. Curr Opin Struct Biol 6:513-518.

32. Solem C and Jensen PR (2002) Modulation of gene expression made easy. Appl Environ Microbiol 68:2397-2403.

33. Umeda H, Aiba H and Mizuno H (1996) SomA, a novel gene that encodes a major outer-membrane protein of *Synechococcus* sp. PCC 7942. Microbiology 142: 2121-2128.

34. Van den Burg B, Vriend G, Veltman OR, Venema G and Eijsink VG (1998) Engineering an enzyme to resist boiling. Proc Natl Acad Sci USA 95:2056-2060.

35. Vimberg V, Tats A, Remm M, and Tenson T (2007) Translation initiation region sequence preferences in *Escherichia coli.* BMC Mol Biol 8**:**100-113.

Output ที่ได้จากโครงการ

Presentation at conferences:

1. Chungjatupornchai W, Kitraksa P, Udpuay N and Fa-aroonsawat S. Improvement of heterologous gene expression in blue green alga using synthetic regulatory-sequence libraries. Abstract in: Proceeding of The 9th International Phycological Congress, August 2-8, 2009, Tokyo, Japan.

2. Chungjatupornchai W, Kamlangdee A and Fa-aroonsawat S. Surface display of organophosphorus hydrolase on *Synechococcus* PCC7942. Abstract In: Proceeding of The 8th European Workshop on Molecular Biology of Cyanobacteria, August 28- September 1, 2011, Naatali, Finland.

Publication

@= corresponding author

1. Chungjatupornchai W@ and Fa-aroonsawat S (2009) Translocation of green fluorescent protein to cyanobacterial periplasm using ice nucleation protein. Journal of Microbiology, 47: 187-192.

2. Chungjatupornchai W@, Kamlangdee A and Fa-aroonsawat S (2011) Display of organophosphorus hydrolase on the cyanobacterial cell surface using *Synechococcus* outer membrane protein A. Applied Biochemistry and Biotechnology, 164: 1048-1057.

3. Chungjatupornchai W@ and Fa-aroonsawat S. (2011) Characterization of the regulatory sequence of *rrnA* operon of Synechococcus PCC7942. (In preparation)

4. Chungjatupornchai W@ and Fa-aroonsawat S. (2011) Enhance constitutive expression of heterologous gene in cyanobacteria using tandem promoters. (In preparation)

ภาคผนวก

IMPROVEMENT OF HETEROLOGOUS GENE EXPRESSION IN BLUE GREEN ALGA USING SYNTHETIC REGULATORY-SEQUENCE LIBRARIES

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Since blue green algae (cyanobacteria) are photoautotrophic prokaryotes and have simple growth requirements and inexpensive to maintain, they have been used as hosts to express several heterologous genes. However, the level of heterologous gene expression in cyanobacteria is low when compared with that in *E. coli*. Current knowledge of the relationship between nucleotide sequence structure and function of promoters and ribosome binding site for translation initiation recognized within cyanobacteria is still limited. Recently, large-scale analysis of distribution of the Shine-Dalgarno (SD) sequences in completed microbial genomes (10 species) reveals that genes led by SD are as common as genes not led by SD. This project is aimed to develop highly active regulatory sequences in cyanobacterium *Synechococcus* PCC7942 by generating strong promoters and optimal translation initiation sequences from synthetic DNA libraries. The synthetic regulatory-sequence libraries containing randomized DNA sequences of LysR binding sequence and SD/ non-SD sequence were constructed in the shuttle vector using green fluorescence protein (GFP) as reporter. The recombinant plasmids were transformed into *Synechococcus* and the transformants were screened for GFP activity. Preliminary results showed that the GFP activities were 1.5 to 3- fold higher than that of control. DNA sequencing of the recombinant plasmids was performed at the randomized DNA regions. Analysis of the relationship between resulting DNA sequences and the GFP activities is undertaken and the results will be presented. Our results indicate that highly efficient regulatory sequences can be generated from the synthetic libraries.

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SURFACE DISPLAY OF ORGANOPHOSPHORUS HYDROLASE ON *SYNECHOCOCCUS* PCC 7942

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The display of proteins to cyanobacterial cell surface is made complex by combination of Gram-positive and Gram-negative features of cyanobacterial cell wall. We previously attempted to use the truncated ice nucleation protein (InpNC) from *Pseudomonas syringae* as an anchoring motif to display organophosphorus hydrolase (OPH), capable of degrading a wide range of organophosphate pesticides, and green fluorescent protein (GFP) on cell surface of *Synechococcus* PCC 7942. We found that a minor fraction of OPH is displayed onto the outermost cell surface with a substantial fraction buried in the cell wall, whereas GFP was found almost entirely in periplasm and not able to display on the outermost cell surface. Here, we showed that *Synechococcus* outer membrane protein A (SomA) can be used as an anchoring motif for the display of OPH on cyanobacterial cell surface. The OPH was fused in frame to the carboxyl-terminus of different cell-surface exposed loops of SomA. Proteinase K accessibility assay and immunostaining visualized under confocal laser scanning microscopy demonstrated that a minor fraction of OPH with 12 histidines fused in frame with the 3rd cell-surface exposed loop of SomA (SomAL3-OPH12H) was displayed onto the outermost cell surface with a substantial fraction buried in the cell wall, whereas, OPH fused in frame with the 5th cell-surface exposed loop of SomA (SomAL5-OPH) was successfully translocated across the membrane and completely displayed onto the outermost surface of *Synechococcus*. The successful display of the functional heterologous protein on cell surface provides a useful model for variety of applications in cyanobacteria including screening of polypeptide libraries and whole-cell biocatalysts by immobilizing enzymes.

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Translocation of Green Fluorescent Protein to Cyanobacterial Periplasm Using Ice Nucleation Protein

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The translocation of proteins to cyanobacterial cell envelope is made complex by the presence of a highly differentiated membrane system. To investigate the protein translocation in cyanobacterium *Synechococcus* PCC 7942 using the truncated ice nucleation protein (InpNC) from *Pseudomonas syringae* KCTC 1832, the green fluorescent protein (GFP) was fused in frame to the carboxyl-terminus of InpNC. The fluorescence of GFP was found almost entirely as a halo in the outer regions of cells which appeared to correspond to the periplasm as demonstrated by confocal laser scanning microscopy, however, GFP was not displayed on the outermost cell surface. Western blotting analysis revealed that InpNC-GFP fusion protein was partially degraded. The N-terminal domain of InpNC may be susceptible to protease attack; the remaining C-terminal domain conjugated with GFP lost the ability to direct translocation across outer membrane and to act as a surface display motif. The fluorescence intensity of cells with periplasmic GFP was approximately 6-fold lower than that of cells with cytoplasmic GFP. The successful translocation of the active GFP to the periplasm may provide a potential means to study the property of cyanobacterial periplasmic substances in response to environmental changes in a non-invasive manner.

***Keywords*:** cyanobacteria, *Synechococcus*, translocation, periplasm, ice nucleation protein, green fluorescent protein (GFP)

Cyanobacteria are oxygenic photosynthetic microorganisms that are ultrastructurally similar to both bacteria and higher plant chloroplasts. Cyanobacterial cell envelope is a combination of Gram-negative and Gram-positive features i.e. outer membrane and plasma membrane separated by a periplasmic space, and the thick peptidoglycan layer, respectively (Hoiczyk and Hansel, 2000). Cyanobacteria have an internal system of thylakoid membranes where the photosynthetic light capture and electron transport take place (Gantt, 1994). The presence of highly differentiated membrane systems lends cyanobacterial cells a unique complexity among eubacteria. The targeting of proteins into and across the correct cyanobacterial membrane systems remains a great challenge; the knowledge on this subject is limited, because (i) no *in vitro* assays are available, the thylakoid and plasma membranes are found to be highly fragmented after isolation (Spence *et al.*, 2003) and (ii) no cyanobacterial protein translocation mutants have been isolated.

Attempts have been made to target the chloramphenical acetyltransferase to periplasmic space and thylakoid lumen of *Synechococcus* PCC 7942 using various signal peptides (Mackle and Zilinskas, 1994), and the green fluorescent protein to periplasmic space of *Synechocystis* PCC 6803 using the Tat pathway (Spence *et al*., 2003). We previously reported that using truncated ice nucleation protein (Inp) containing only the N- and C-terminal portion (InpNC) from *Pseudomonas syringae* KCTC 1832 as an anchoring motif, a minor fraction of organophosphorus hydrolase (OPH) is displayed onto the outermost cell surface of *Synechococcus* PCC 7942, however, a substantial fraction of OPH is buried in the cell wall (Chungjatupornchai and Fa-aroonsawat, 2008). The InpNC has been successfully used for targeting OPH (Shimazu *et al.*, 2001) and green fluorescent protein (GFP) (Li *et al.*, 2004) to cell surface of *E. coli*, and viral antigens to cell surface of *Salmonella* (Lee *et al*., 2000). The N-terminal domain of Inp contains three or four potential transmembrane spans but no signal sequence. The Inp is not linked to an oligosaccharide and therefore not anchored to the outer membrane via phosphatidylinositol as proposed previously (Schmid *et al*., 1997). The secretion mechanism of Inp is still unknown.

GFP is a popular reporter for gene expression and protein localization studies. GFP does not require the addition of any substrate or co-factor, it promises real time visualization of gene expression. The GFPmut2, a GFP variant with a triple amino acid substitution (S65A/V68L/S72A), has been shown to express in *E. coli* as a soluble protein with improved fluorescence (Cormack *et al.*, 1996).

In this study, we have used the GFPmut2 as a reporter to investigate the protein translocation in cyanobacterium *Synechococcus* PCC 7942. The GFP was fused in frame to the carboxyl-terminus of InpNC. We demonstrated that the InpNC was able to direct translocation across the intracellular membranes and that the transported GFP accumulated almost entirely in the periplasm.

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Materials and Methods

Strains, growth conditions, and plasmids

Synechococcus PCC 7942 strain R2-SPc (hereafter, referred to as *Synechococcus*) (Kuhlemeier *et al.*, 1983) was grown on BG-11 medium containing 1.5% agar at 30°C under constant illumination of 3,000 lux (i.e. 38 μE/m^2/sec). *E. coli* strain MC1061 (Casadaban and Cohen, 1980) was grown in LB broth or on agar. Plasmid pGF101, containing the truncated *inaKnc* gene encoding N- and C-terminal domains (InpNC) of ice nucleation protein from *P. syringae* KCTC 1832 (Jung, 1998), was kindly provided by E. J. Kim (Genefocus, Korea). Plasmid pBCgfp harboring *gfp* gene encoding a GFP variant, GFPmut2 (Cormack *et al*., 1996), has been described (Matthysse *et al.*, 1996).

Plasmid construction

The *gfp* gene encoding the GFP was amplified from pBCgfp using primers gfp-F1 and gfp-R2 (Table 1). The *Bam*HI/*Sac*I-digested PCR product was cloned into the corresponding sites in pUC18-GUS (Chungjatupornchai and Fa-aroonsawat, 2008) to obtain pUC-GFP. For translocation expression of GFP, the *inaKnc* gene amplified from pGF101 using primers ina-F1 and ina-R1 was digested with *Sal*I and cloned into the corresponding site in pUC-GFP to obtain pUC-InpNC-GFP. The PCR product containing cassette P_{tRNA}-*inaKnc-gfp* was amplified using primers 24-mer and gfp-R2 with template derived from overlap extension PCR of two PCR products: i) P_{tRNA} promoter amplified using primers 24-mer and E3-R4 with pUC-T1R1 derived from *Bam*HI/*Eco*RI fragment of pKGT-T1R1 (Chungjatupornchai *et al*., 2002) cloned into the corresponding sites in pUC18-GUS as template; ii) *inaKnc-gfp* amplified from pUC-InpNC-GFP using primers Lk-ina-F2 and gfp-R2. For cytoplasmic expression of GFP, the PCR product containing gene cassette P_{tRNA}-*gfp* was amplified using primers T1 and gfp-R2 with template derived from overlap extension PCR of two PCR products: i) P_{tRNA} promoter amplified from pKGT-T1R1 (Chungjatupornchai *et al*., 2002) using primers T1 and E3-R4; ii) *gfp* gene amplified from pBCgfp using primers Lk-gfp-F2 and gfp-R2. The gene cassettes P_{tRNA}-*gfp* digested with *Bam*HI/*Sac*I and P_{tRNA}-*inaKnc-gfp* digested with *Hin*dIII/*Sac*I were cloned into the corresponding sites of pUC18-GUS. The resulting P_{tRNA}-*gfp*-(Nos-ter) digested with *Bam*HI/*Eco*RI and P_{tRNA}-*inaKnc-gfp*-(Nos-ter) digested with *Hin*dIII filled-in/*Eco*RI were cloned into the similar sites of shuttle vector pKGT (Chungjatupornchai *et al*., 2002) resulted in pKT-GFP and pKT-InpNC-GFP, respectively. The resulting plasmids were transformed into *Synechococcus* as described (Kuhlemeier *et al*., 1983).

GFP fluorescence analysis

Synechococcus cells were harvested and resuspended in phosphate-buffered saline (PBS). The intensity of fluorescence was measured using spectrofluorometer (Model FP-6300, Jasco, Japan) with excitation at 488 nm and emission measured at 510 nm. Specific fluorescence intensity was defined as whole cell fluorescence intensity divided by the optical density measured at 730 nm. For pronase accessibility assays, cells were suspended in 1 ml (OD_{730}=1) of PBS containing 16.24 units of pronase (Sigma, USA), and then incubated at 37°C for 2 h. The treated cells were washed with PBS and the fluorescence intensities were measured as described above. For measurement of cell fractions, *Synechococcus* cells were suspended in 50 mM Tris-HCl, 50 mM NaCl, 5% glycerol, pH 8.0, and subjected to three rounds of French press treatment. The whole cell lysate was centrifuged to obtain supernatant (soluble fraction) and pellet (total membrane fraction). The fluorescence intensity of cell fractions was measured as described above. The images of GFP fluorescence in *Synechococcus* cells were obtained using a confocal laser scanning microscope (CLSM) (Fluoview FV1000, Olympus) with excitation at 488 nm and emission measured at 520 nm.

Western blot analysis

Proteins from whole cell extracts were separated by 15% SDS-PAGE and immobilized onto a polyvinylidene-difluoride membrane. For immunodetection, primary GFP monoclonal antibody (Clonetech, USA) was used at 1:2,000 dilution. The secondary antibody, goat anti-mouse IgG conjugated with horseradish peroxidase (Sigma), was used at 1:5,000 dilution. Reaction of horseradish peroxidase was detected using ECL plus Western Blotting Detection System (GE Healthcare, UK) as described by manufacturer.

Results and Discussion

Construction of plasmids harboring the *gfp* gene

We reported previously that using the InpNC as an anchoring motif, a minor fraction of OPH is displayed onto the outermost surface of cyanobacterial cells, but a substantial frac-

Table 1. Primers used in this study. Locations of the primers are indicated in Fig. 1. The restriction sites *Bam*HI, *Sac*I, *Sal*I, and *Sma*I are underlined

Primer	Sequence (5'-3')	Target sequence
T1	CGGGATCCTTGCCCTCGCCTCCTAGTCC	P_{tRNA}
E3-R4	CATAAGGGACTGACCACCCGGGGATCGTGACAAGTTACCAATGTAGC	P_{tRNA}
Lk-gfp-F2	GATCCCCGGGTGGTCAGTCCCTT/ATGAGTAAAGGAGAAGAAC	P_{tRNA} / *gfp*
gfp-F1	CGGGATCCATGAGTAAAGGAGAAGAAC	*gfp*
gfp-R2	CGCGAGCTCTCATTTGTATAGTTCATCCATGC	*gfp*
Lk-ina-F2	GATCCCCGGGTGGTCAGTCCCTT/ATGACTCTCGACAAGGCG	P_{tRNA} / *inaK*
ina-F1	ACGCGTCGACATGACTCTCGACAAGGCG	*inaK*
ina-R1	ACGCGTCGAC CCCGGGCTTTACCTCTATC	*inaK*
24-mer	CGCCAGGGTTTTCCCAGTCACGAC	pUC18

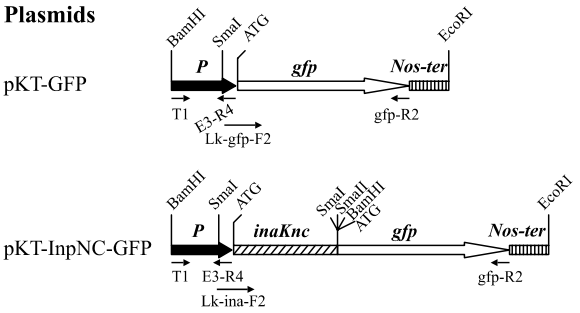

Fig. 1. Constructs encoding GFP. Plasmid pKT-GFP and pKT-InpNC-GFP were used for cytoplasmic and translocation expression of GFP in *Synechococcus*, respectively. P, tRNA promoter; *inaKnc,* truncated ice nucleation protein gene; *gfp*, green fluorescent protein gene; Nos-ter, nopaline synthase terminator. Locations of primers used in this study are indicated. The figure is not drawn to scale.

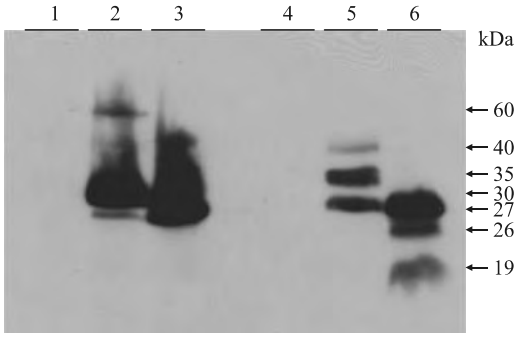

Fig. 3. Western blot analysis of GFP expressed in *Synechococcus*. Proteins from whole cell extracts of *E. coli* at OD_{600}=1.2 (lanes 1, 2, and 3) and *Synechococcus* at OD_{730}=2.0 (lanes 4, 5, and 6) were analyzed by immunoblotting using GFP monoclonal antibody. Lanes 1 and 4 are from cells harboring pKGT; lanes 2 and 5 are pKT-InpNC-GFP; lanes 3 and 6 are pKT-GFP.

tion of OPH is buried in the cell wall (Chungjatupornchai and Fa-aroonsawat, 2008). In this study, in order to investigate the protein translocation in *Synechococcus* PCC 7942, the same construct of InpNC (Chungjatupornchai and Fa-aroonsawat, 2008) was fused in frame with the coding region of a GFP variant, GFPmut2. The GFPmut2 was chosen because it is more fluorescent than wild-type GFP and has been shown to express in *E. coli* as a soluble protein (Cormack *et al*., 1996). Plasmids pKT-InpNC-GFP harboring gene cassette P_{tRNA}-*inaKnc-gfp* for translocation of GFP was constructed (Fig. 1) and transformed into *Synechococcus*. Plasmid pKT-GFP harboring gene cassette P_{tRNA}-*gfp* for cytoplasmic expression of GFP was used as control. Both plasmids contained cyanobacterial $tRNA^{pro}$ promoter (P_{tRNA}) (Chungjatupornchai *et al.*, 1999; Chungjatupornchai *et al*., 2002) enabling constitutive expression of GFP.

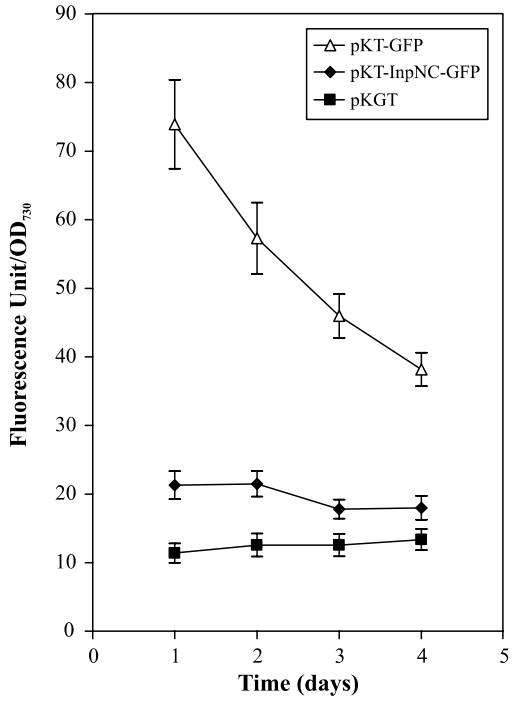

Fig. 2. Fluorescence intensity of GFP in *Synechococcus* cultures at various time courses. Cell cultures of *Synechococcus* harboring pKGT, pKT-GFP, and pKT-InpNC-GFP were harvested each day. The fluorescence intensity of whole cells was measured using spectrofluorometer. Each value and error bar represents the means of three independent experiments and its standard deviation.

Expression of the InpNC-GFP fusion protein in *Synechococcus*

To determine the optimal expression of GFP in *Synechococcus*, the fluorescence intensities of cell cultures harvested at various time courses were measured. The results in Fig. 2 showed that for cells harboring pKT-GFP, the levels of fluorescence intensity reached a maximum in 1-day culture and decreased thereafter in 2-, 3-, and 4-day cultures. For cells harboring pKT-InpNC-GFP, the levels of fluorescence intensity were not significantly different in 1- and 2-day cultures, and then slightly decreased in 3- and 4-day cultures. Therefore, all subsequent GFP fluorescence determination was performed using 1-day culture. Very little background fluorescence was detected in control cells harboring pKGT vector. After background fluorescence was subtracted, the levels of fluorescence intensity of cells harboring pKT-GFP are approximately 6-fold (for 1-day culture) and 5-fold (for 4-day culture) higher than that of corresponding cells harboring pKT-InpNC-GFP, indicating that the GFP of cells harboring pKT-GFP degraded faster than that of pKT- InpNC-GFP. The results in Fig. 2 indicated that GFP is functionally expressed in *Synechococcus*. The growth of cells expressing GFP is not significantly different from that of wild type (data not shown).

Western blotting analysis was performed to monitor the expression of GFP. The *E. coli* harboring the same plasmids as *Synechococcus* were used as control. As shown in Fig. 3, no band was detected with the GFP antibody in control extracts derived from cells harboring pKGT vector (lanes 1 and 4). In *E. coli* extracts, a band of expected size of the InpNC-GFP (60 kDa) was detected (lane 2). However, the

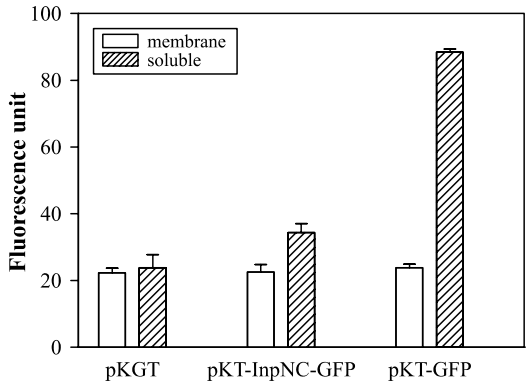

Fig. 4. Fluorescence intensities of *Synechococcus* cell fractions. The fluorescence intensities of soluble and total membrane fractions were measured using spectrofluorometer. The data are means of three experiments with standard deviation. Fluorescence intensities of total membrane fractions of pKT-InpNC-GFP and pKT-GFP were not significantly different from that of pKGT (control). However, fluorescence intensities of soluble fractions of pKT-InpNC-GFP and pKT-GFP were significantly higher than that of pKGT ($P<0.05$, t test).

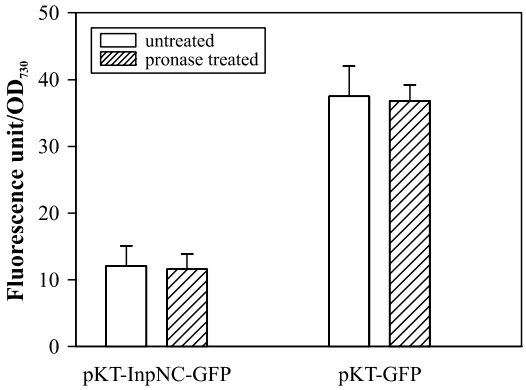

Fig. 5. Pronase accessibility assays of *Synechococcus* expressing GFP. Whole cells harboring pKT-InpNC-GFP and pKT-GFP were treated with pronase prior to determine fluorescence intensities. Values of the control without GFP (pKGT) were subtracted. The data are means of three experiments with standard deviation.

presence of relatively large portion of the degraded InpNC-GFP fusion protein indicated that most of the fusion protein had been subjected to proteolytic degradation. Almost all of the cytoplasmic GFP (with expected size of 27 kDa) had not been subjected to proteolytic degradation (lane 3). In *Synechococcus* extracts, all the bands with molecular weight less than the expected size of the InpNC-GFP fusion protein were detected (lane 5), indicating that almost all of the fusion protein had been subjected to proteolytic degradation. The smallest degradation product was the same size as free GFP (~27 kDa). The degradation products with approximate size of 40 and 35 kDa were also detected. The GFP fluorescence was observed in cells harboring pKT-InpNC-GFP (Fig. 2). Taken together, the results suggest that the degradation products may be GFP conjugated with the C-terminal domain of InpNC. The N-terminal of InpNC-GFP may be susceptible to protease attack and/or structurally unstable. However, most of the cytoplasmic GFP with expected size (~27 kDa) and very small amount of degradation product were detected (lane 6), indicating that most of the cytoplasmic GFP had not been subjected to proteolytic degradation. The immunobloting also showed cytoplasmic GFP (pKT-GFP) bands as thicker than translocated GFP (pKT-InpNC-GFP) bands. The results agreed well with the results in Fig. 2 that total expression of the cytoplasmic GFP is much higher.

In order to determine the fluorescence intensities of cell fractions, *Synechococcus* cells were disrupted by French press treatment and centrifuged to obtain the soluble and total membrane fractions. Figure 4 showed that very little background fluorescence was detected in both fractions of cells harboring pKGT vector without *gfp* gene. For cells harboring translocated GFP (pKT-InpNC-GFP), virtually all of the fluorescence was found in the soluble fraction corresponding to cytoplasmic and/or periplasmic contents. Thus, the translocated GFP is present as a soluble protein. The level of fluorescence detected in total membrane fraction was not significantly different from that of pKGT, indicating that the GFP is not located in cell membrane but in cytoplasm and/or periplasmic space. In cells harboring cytoplasmic GFP (pKT-GFP), high level of fluorescence was found in the soluble fraction. It has been reported that when exported by the Sec pathway, GFP was shown to be completely incapable of folding correctly and no fluorescence was observed (Feilmeier *et al*., 2000), however, the Tat system successfully exported correctly folded GFP to the periplasms of *E. coli* (Santini *et al*., 2001), and cyanobacterium *Synechocystis* PCC 6803 (Spence *et al*., 2003). Our observation of translocated GFP fluorescence in this study strongly suggested that GFP was capable of folding correctly and forming its chromophore in *Synechococcus*.

Probing the translocated GFP

Immunostaining microscopy of whole cells has been used successfully to detect the presence of OPH displayed on the outermost cell surface of *Synechococcus* (Chungjatupornchai and Fa-aroonsawat, 2008). In this study, we used the immunostaining microscopy method described previously (Chungjatupornchai and Fa-aroonsawat, 2008) with GFP monoclonal antibody as first antibody to investigate whether the GFP was present on the outermost cell surface. Under confocal laser scanning microscopy, no immunoreactivity of anti-GFP was detected on the surface of cells harbouring pKT-InpNC-GFP (data not shown). Thus, the GFP was not displayed on the outermost of cell surface.

Pronase accessibility assay of intact cells has been used to provide the evidence for the surface location of GFP protein, since pronase can not readily penetrate through the outer membrane (Shi and Wen Su, 2001; Li *et al.*, 2004). Therefore, *Synechococcus* whole cells were treated with pronase. Figure 5 showed that GFP fluorescence intensities of pronase treated cells harboring pKT-InpNC-GFP and pKT-GFP were not significantly different from those of corresponding untreated cells. The results confirmed that the

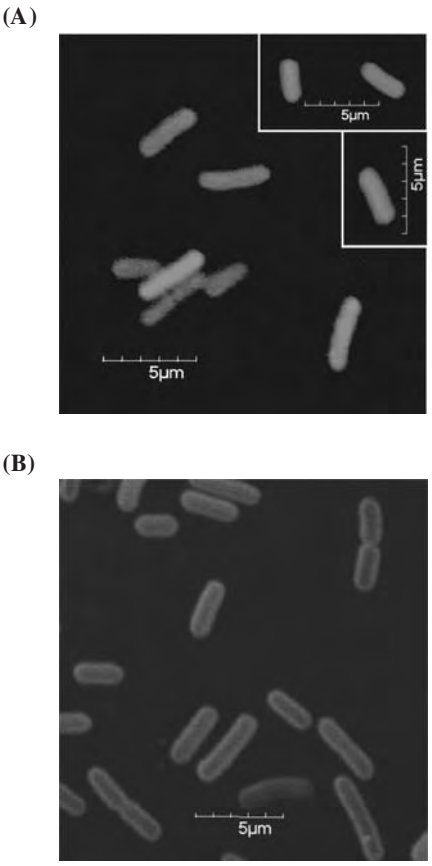

Fig. 6. CLSM images of *Synechococcus* cells expressing GFP. The location of GFP in *Synechococcus* cells harboring pKT-GFP (A) and pKT-InpNC-GFP (B) were visualized under CLSM with excitation at 488 nm and emission measured at 520 nm.

GFP of cells harboring pKT-InpNC-GFP was not located on the outermost cell surface.

The targeting of proteins into and across the correct membrane systems of cyanobacteria is poorly understood because of formidable technical problems that encountered in studies on these organisms. The thylakoid and plasma membranes are highly fragmented after isolation (Spence *et al*., 2003). So far, there are no techniques available to efficiently separate the cytoplasmic and periplasmic content. Therefore, *Synechococcus* harboring pKT-InpNC-GFP was further analyzed to identify the subcellular location of the GFP by CLSM with excitation at 488 nm and emission measured at 520 nm. The autofluorescence of *Synechococcus* photosynthetic pigments in the thylakoid membranes emits primarily in the red region of spectrum, therefore does not interfere with GFP signals. No fluorescence signal was detected in control cells harboring pKGT vector (data not shown). In cells harboring pKT-GFP, very bright fluorescence was distributed uniformly throughout the cytoplasm with no hint of enrichment of the signal at the periphery of the cells (Fig. 6A). In cells harboring pKT-InpNC-GFP, the fluorescence was found almost entirely as a halo in the outer regions of cells which appeared to correspond to the periplasm (Fig. 6B). Very little fluorescence was found in the cell interior, possibly representing untranslocated GFP derived from degradation of InpNC-GFP fusion protein. All of these results collectively suggest that InpNC can direct translocation of GFP across plasma membrane to periplasm but not across outer membrane to display on the outermost cell surface.

Several possibilities might lead to the observed translocation of GFP in cells harbouring pKT-InpNC-GFP. First, the level of periplasmic GFP was much lower than that of cytoplasmic GFP (Fig. 2, 3, 4, 5, and 6), although both plasmids pKT-InpNC-GFP and pKT-GFP contained the *gfp* gene under the control of P_{tRNA} promoter and were derived from pKGT vector with approximately 30 copies in the cells (Monshupanee *et al.*, 2006). Similar results have been observed in the previous study that the surface-expressed InpNC-OPH has a much lower activity than cytoplasmic OPH (Chungjatupornchai and Fa-aroonsawat, 2008). Taken together, these results suggest that some of the instability of InpNC-GFP can be attributed to InpNC moiety. Second, no full-length InpNC-GFP fusion protein was detected (Fig. 3). The N-terminal domain of InpNC may be susceptible to protease attack; the remaining C-terminal domain conjugated with GFP lost the ability to direct translocation across outer membrane and to act as a surface display motif. It has been suggested that the secretion signal may be located in the N-terminal domain of Inp, since truncated Inp containing only the N-terminal domain can be successfully employed as a display motif on *E. coli* cell surface (Li *et al*., 2004). Therefore, better GFP translocation efficiencies might be achieved, if InpNC-GFP fusion protein could be protected from proteolysis. Whether proteolysis of InpNC-GFP fusion protein occurred in cytoplasm and/or periplasm remains to be investigated. Finally, the cell wall of cyanobacteria can be an obstacle; it is a combination of Gram-positive and Gram-negative features. In addition, cyanobacterial outer membranes are normally surrounded by a fibrous sheath and in many cases a surface layer (Hoiczyk and Hansel, 2000).

It has been shown that in *E. coli*, GFP translocated to periplasm can be used as a reporter to study the property of periplasm in response to osmotic up-shock (Santini *et al*., 2001). The successful translocation of the active GFP to the periplasm of *Synechococcus* in this study may provide a potential means to investigate the property of cyanobacterial periplasmic substances in response to environmental changes in a non-invasive manner.

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References

Casadaban, M.J. and S.N. Cohen. 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli. J. Mol. Biol.* 138, 179-207.

Chungjatupornchai, W. and S. Fa-aroonsawat. 2008. Biodegradation of organophosphate pesticide organophosphorus hydrolase. *J. Microbiol. Biotechnol.* 18, 946-951.

Chungjatupornchai, W., S. Fa-aroonsawat, and S. Panyim. 2002. Characterization of regions of the cyanobacterial $tRNA^{pro}$ gene that affect the expression of a beta-glucuronidase reporter gene. *FEMS Microbiol. Lett.* 211, 57-64.

Chungjatupornchai, W., T. Senawong, and S. Panyim. 1999. Isolation and characterization of *Synechococcus* PCC 7942 promoters: $tRNA^{pro}$ gene functions as a promoter. *Curr. Microbiol.* 38, 210-216.

Cormack, B.P., R.H. Valdivia, and S. Falkow. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* 173, 33-38.

Feilmeier, B.J., G. Iseminger, D. Schroeder, H. Webber, and G.J. Phillips. 2000. Green fluorescent protein functions as a reporter for protein localization in *Escherichia coli. J. Bacteriol.* 182, 4068-4076.

Gantt, E. 1994. Supramolecular Membrane Organization, p. 119-138. *In* D.A. Bryant (ed.), Molecular Biology of Cyanobacteria, Kluwer Academic Publishers, Netherlands.

Hoiczyk, E. and A. Hansel. 2000. Cyanobacterial cell walls: news from an unusual prokaryotic envelope. *J. Bacteriol.* 182, 1191-1199.

Jung, H.C., J.M. Lebeault, and J.G. Pan. 1998. Surface display of *Zymomonas mobilis* levansucrase by using the ice-nucleation protein of *Pseudomonas syringae. Nat. Biotechnol.* 16, 576-580.

Kuhlemeier, C.J., A.A. Thomas, A. Van Der Ende, R.W. Van Leen, W.E. Borrias, C.A. Van Den Hondel, and G.A. Van Arkel. 1983. A host-vector system for gene cloning in the cyanobacterium *Anacystis nidulans* R2. *Plasmid* 10, 156-163.

Lee, J.S., K.S. Shin, J.G. Pan, and C.J. Kim. 2000. Surface-displayed viral antigens on *Salmonella* carrier vaccine. *Nat. Biotechnol.* 18, 645-648.

Li, L., D.G. Kang, and H.J. Cha. 2004. Functional display of foreign protein on surface of *Escherichia coli* using N-terminal domain of ice nucleation protein. *Biotechnol. Bioeng.* 85, 214-221.

Mackle, M.M. and B.A. Zilinskas. 1994. Role of signal peptides in targeting of proteins in cyanobacteria. *J. Bacteriol*. 176, 1857-1864.

Matthysse, A.G., S. Stretton, C. Dandie, N.C. McClure, and A.E. Goodman. 1996. Construction of GFP vectors for use in gram-negative bacteria other than *Escherichia coli. FEMS Microbiol. Lett*. 145, 87-94.

Monshupanee, T., S. Fa-aroonsawat, and W. Chungjatupornchai. 2006. A cyanobacterial strain with all chromosomal rRNA operons inactivated: a single nucleotide mutation of 23S rRNA confers temperature-sensitive phenotypes. *Microbiology* 152, 1417-1425.

Santini, C.L., A. Bernadac, M. Zhang, A. Chanal, B. Ize, C. Blanco, and L.F. Wu. 2001. Translocation of jellyfish green fluorescent protein via the Tat system of *Escherichia coli* and change of its periplasmic localization in response to osmotic up-shock. *J. Biol. Chem.* 276, 8159-8164.

Schmid, D., D. Pridmore, G. Capitani, R. Battistutta, J.R. Neeser, and A. Jann. 1997. Molecular organisation of the ice nucleation protein InaV from *Pseudomonas syringae. FEBS Lett.* 414, 590-594.

Shi, H. and W. Wen Su. 2001. Display of green fluorescent protein on *Escherichia coli* cell surface. *Enzyme Microb. Technol.* 28, 25-34.

Shimazu, M., A. Mulchandani, and W. Chen. 2001. Cell surface display of organophosphorus hydrolase using ice nucleation protein. *Biotechnol. Prog*. 17, 76-80.

Spence, E., M. Sarcina, N. Ray, S.G. Moller, C.W. Mullineaux, and C. Robinson. 2003. Membrane-specific targeting of green fluorescent protein by the Tat pathway in the cyanobacterium *Synechocystis* PCC6803. *Mol. Microbiol.* 48, 1481-1489.

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Display of Organophosphorus Hydrolase on the Cyanobacterial Cell Surface Using *Synechococcus* Outer Membrane Protein A as an Anchoring Motif

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Abstract The display of proteins to cyanobacterial cell surface is made complex by combination of Gram-positive and Gram-negative features of cyanobacterial cell wall. Here, we showed that *Synechococcus* outer membrane protein A (SomA) can be used as an anchoring motif for the display of organophosphorus hydrolase (OPH) on cyanobacterial cell surface. The OPH, capable of degrading a wide range of organophosphate pesticides, was fused in frame to the carboxyl-terminus of different cell-surface exposed loops of SomA. Proteinase K accessibility assay and immunostaining visualized under confocal laser scanning microscopy demonstrated that a minor fraction of OPH with 12 histidines fused in frame with the third cell-surface exposed loop of SomA (SomAL3-OPH12H) was displayed onto the outermost cell surface with a substantial fraction buried in the cell wall, whereas OPH fused in frame with the fifth cell-surface exposed loop of SomA (SomAL5-OPH) was successfully translocated across the membrane and completely displayed onto the outermost surface of *Synechococcus*. The successful display of the functional heterologous protein on cell surface provides a useful model for variety of applications in cyanobacteria including screening of polypeptide libraries and whole-cell biocatalysts by immobilizing enzymes.

Keywords Cyanobacteria · *Synechococcus* PCC 7942 · Organophosphorus hydrolase · SomA · Porin · Surface display

Introduction

Cyanobacteria are oxygenic photosynthetic microorganisms. Their cell envelope possesses structural elements typical for both Gram-negative and Gram-positive bacterial envelopes, i.e., outer membrane and plasma membrane separated by a periplasmic space and the thick

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peptidoglycan layer, respectively [1]. In addition, cyanobacteria have an internal photosynthetic membrane system (thylakoids). Targeting of proteins into and across the correct cyanobacterial membrane system remains a great challenge, since the knowledge on this subject is limited. For example, various signal peptides have been used to target the chloramphenical acetyltransferase to periplasmic space and thylakoid lumen of *Synechococcus* PCC 7942 [2]. The Tat pathway has been used to target the green fluorescent protein (GFP) to periplasmic space of *Synechocystis* PCC 6803 [3]. We previously attempted to use the truncated ice nucleation protein (InpNC) from *Pseudomonas syringae* as an anchoring motif to display organophosphorus hydrolase (OPH) and GFP on cell surface of *Synechococcus* PCC 7942. We found that a minor fraction of OPH is displayed onto the outermost cell surface with a substantial fraction buried in the cell wall [4], whereas GFP was found almost entirely in periplasm and not able to display on the outermost cell surface [5]. Various surface expression systems have been developed for bacteria, since display of heterologous proteins has a wide range of biotechnological applications including screening of polypeptide libraries, bioadsorbents for removal of heavy metals, and whole-cell biocatalysts by immobilizing enzymes [6]; however, there is no report of cyanobacterial surface display system.

Synechococcus outer membrane protein A (SomA), a porin, is one of the most abundant proteins of the total envelope proteins of *Synechococcus* PCC 7942 [7]. SomA of *Synechococcus* PCC 7942 showed an overall homology of 97% to that of the closely related strain *Synechococcus* PCC 6301 [8]. The N-terminus of SomA contains a typical signal peptide and a highly conserved surface layer homology (SLH) domain; therefore, it may have a dual function: the formation of diffusion pores as well as act as linkers connecting the outer membrane with the peptidoglycan layer [8].

OPH encoded by the *opd* gene of *Flavobacterium* sp. [9] is a homodimeric organophosphotriesterase that can hydrolyze a wide range of organophosphorus pesticides [10]. OPH has been successfully displayed onto the cell surface of *Escherichia coli* [11], *Moraxella* sp. [12], and *Saccharomyces cerevisiae* [13] to enhance OPH biodegradation efficiency.

In this study, we have used the OPH as a reporter to investigate the ability of SomA to act as an anchoring motif for cyanobacterial surface display system. The OPH was fused in frame to the carboxyl-terminus of different cell-surface exposed loops of SomA. We demonstrated that OPH was successfully translocated across the membrane and completely displayed onto the outermost cyanobacterial cell surface.

Materials and Methods

Strain and Growth Condition

Synechococcus PCC 7942 strain R2-SPc (hereafter, referred to as *Synechococcus*) [14] was grown in liquid or on solid (1.5% agar) BG-11 medium [15] at 30 °C under constant illumination of 3,000 lx (i.e., 38 $\mu E/m^2/s$).

Plasmid Construction

The *somA* gene was amplified from genomic DNA of *Synechococcus* using primers somA-F1 and somA-R2 (Table 1) based on the GenBank sequence (D64077). The resulting BamHI/SalI-digested polymerase chain reaction (PCR) product was cloned into the

Table 1 Primers

Primer	Sequence (5′–3′)	Target sequence
somA-F1	CGGGATCCGAGGGTGGGGTCCGCAAG	Upstream of *somA*
somA-R1	CCGATCGCCTGTGCCGATCGACAT/TGATAAC CTCACACCATGTAGGG	*opd*/*somA*
somA-R2	ACGCGTCGACTCGCCCATCCCTAACTAA	Downstream of *somA*
somA-R3	CCGATCGCCTGTGCCGATCGACAT/GGTAGC ACCAAAAGGCTGGAAGGC	*opd*/*somA*
somA-R6	CCGATCGCCTGTGCCGATCGACAT/GCCACT GAAGAAGGTAGCGTCG	*opd*/*somA*
somA-R7	CCGATCGCCTGTGCCGATCGACAT/GCCCGA GGGATTAAAGCCAGG	*opd*/*somA*
opd-F1	CGGGATCCCTGGATCGATCGGCACAGGCG	*opd*
opd-F2	ATGTCGATCGGCACAGGCGATCGG	*opd*
opd-R5	CGGGATCCTCAATGGTGATGATGGTGATG/TG ACGCCCGCAAGGTCGGTGAC	6His/*opd*
his-R1	CGGGATCCTCAGTGATGGTGATGGTGGTGA TGGTGATGATGGTGATGTGACGC	12His
Nos-R2	CGGGATCCATCTAGTAACATAGATGACACCG	Nos-ter

Locations of the primers are indicated in Fig. 1. The restriction sites BamHI and SalI are *underlined*

corresponding sites of pUC18 to obtain plasmid pUC18-SomA. The *opd6H* gene encoding OPH including six histidines at C-terminus was amplified from pUC18-OPH [4] using primers opd-F1 and opd-R5. The resulting BamHI-digested PCR product was cloned into the BamHI sites of pKTN to obtain pKT-opd6H. Plasmid pKTN and pKTB were derived from shuttle vector pKGT [16] with the deletion of *GUS* gene. For intracellular expression of OPH, the PCR product containing gene cassette *Ps-opd12H* was amplified using primers somA-F1 and his-R1 with template derived from overlap extension PCR of two PCR products: (1) *Ps* promoter amplified from pUC18-SomA using primers somA-F1 and somA-R1 and (2) *opd12H* gene amplified from pKT-opd6H using primers opd-F2 and his-R1. The resulting BamHI-digested PCR product was inserted into BamHI in pKTN to obtain plasmid pPs-opd12H. For surface expression of OPH, the PCR products containing gene cassettes *Ps-L5opd12H*, *Ps-L3opd12H*, and *Ps-L2opd12H* were amplified using primers somA-F1 and his-R1 with template derived from overlap extension PCR of two PCR products: (1) truncated *somA* gene amplified from plasmid pUC18-SomA using primer sets somA-F1 and somA-R3 for Loop 5 (L5), somA-F1 and somA-R6 for L3, and somA-F1 and somA-R7 for L2, and (2) *opd12H* gene amplified from pPs-opd12H using primers opd-F2 and his-R1. The resulting BamHI-digested PCR products were inserted into the corresponding site in pKTN to obtain plasmids pPs-L5opd12H, pPs-L3opd12H, and pPs-L2opd12H, respectively. To construct plasmid pPs-L5opd, the PCR product containing gene cassette *Ps-L5opd* was amplified using primers somA-F1 and Nos-R2 with template derived from overlap extension PCR of two PCR products: (1) *Ps* promoter and truncated *somA* gene amplified from pUC18-SomA using primers somA-F1 and somA-R3, and (2) *opd* gene amplified from pUC18-OPH [4] using primers opd-F2 and Nos-R2. The resulting BamHI-digested PCR product was inserted into the BamHI in pKTB to obtain plasmids pPs-L5opd. The resulting plasmids extracted from *E. coli* were transformed into *Synechococcus* as described [14].

OPH Assay

For each assay, *Synechococcus* fresh whole cells (OD_{730}=1) were resuspended in 1 ml of CHES/$CoCl_2$ buffer (50 mM 2-[*N*-cyclohexylamino] ethane-sulfonic acid, pH 9.0, 50 μM $CoCl_2$) containing 2 mM paraoxon (Sigma). Reaction mixtures were incubated at 37 °C. OPH activity was measured by following the increase in absorbance of *p*-nitrophenol from the hydrolysis of substrate (paraoxon) at 400 nm ($\varepsilon_{400} = 17,000/\mathrm{M/cm}$). Specific activities were expressed as units (nanomoles of paraoxon hydrolyzed per minute) per OD_{730} of cells.

$CoCl_2$ Treatment

The 3-day cultures were resuspended (OD_{730}=1) in BG-11 liquid medium containing various concentrations of $CoCl_2$ and further grown for 18 h. The cells were assayed for OPH activity as described above.

Nitrogen Deprivation Treatment

Cells were suspended (OD_{730}=1) in BG-11^N liquid medium in which the ferric ammonia was replaced by ferric citrate or in BG-11^0 liquid medium in which the ferric ammonia and $NaNO_3$ were replaced by ferric citrate and $NaHCO_3$, respectively. The cultures were further grown for 24 h and assayed for OPH activity as described above.

Proteinase K Accessibility Assay

Cells were suspended in 1 ml (OD_{730}=1) of 15% sucrose, 15 mM Tris–HCl, 0.1 mM EDTA, pH 7.8 containing 200 μg of proteinase K (US Biological), then incubated at room temperature for 1 h. The treated cells were assayed for OPH activity as described above.

Immunostaining CLSM Images

Cells grown for 3 days were harvested and resuspended in phosphate-buffer saline (PBS). The procedure for immunostaining of cells was carried out essentially as described [4]. In brief, anti-His monoclonal antibody (GE Healthcare, USA) or anti-OPH antiserum [4] was used as first antibody. Goat anti-mouse IgG conjugated with horseradish peroxidase (Zymed, USA) was used as second antibody. Reactivity of the immune complexes was visualized by 0.05% diaminobenzidine and 0.1 M imidazole under confocal laser scanning microscopy (CLSM; Olympus FV1000). Cells of which first antibody was omitted served as negative controls.

Results and Discussion

Construction of Plasmids Harboring *somA-opd* Fusion Gene

The predicted topology of SomA based on typical features of porins has been reported that the polypeptide might transverse the outer membrane 14 times with the predicted β-strands, which are connected by six short internal loops on the periplasmic side and seven irregular cell-surface exposed loops [8].

In order to investigate the ability of SomA to act as an anchoring motif for the display of OPH onto the cyanobacterial cell surface, plasmids harboring *somA* promoter (*Ps*), N-terminal signal peptide, and truncated *somA* gene fused in frame with the *opd* gene were constructed (Fig. 1). The truncated *somA* gene encoded SomA with C-terminus at amino acid positions 382, 266, and 207 located at the fifth, third, and second cell-surface exposed loop (L5, L3, and L2), respectively. The 12-histidine peptide (12H), a good chelator for the divalent metal ions such as Ni^{2+} [17], was fused in frame with the C-terminus of SomA-OPH fusion protein. The resulting plasmids are pPs-L5opd12H, pPs-L3opd12H, and pPs-L2opd12H encoding fusion proteins SomAL5-OPH12H, SomAL3-OPH12H, and SomAL2-OPH12H, respectively (Fig. 1). In addition, plasmid pPs-L5opd encoding SomAL5-OPH (without 12 H) was constructed. For intracellular expression of OPH, plasmid pPs-opd12H encoding OPH12H was constructed (Fig. 1).

Optimal Expression of SomA-OPH Fusion Protein in *Synechococcus*

To determine the optimal SomA-OPH expression in *Synechococcus*, the OPH activities of cell cultures harvested at various time courses were determined. The results in Fig. 2 showed that for cells harboring pPs-L3opd12H and pPs-opd12H, the levels of OPH activity reached the maximum in 3-day culture. Therefore, further OPH activity determination was performed using 3-day culture.

Catalytically active OPH requires metal ions, such as Co^{2+}, as a cofactor [18]. Addition of a low concentration of cobalt chloride in the growth medium increased OPH activity of recombinant *E. coli* [19] and *S. cerevisiae* [13]. To investigate the effect of $CoCl_2$ on OPH activity of *Synechococcus*, cells were grown in BG-11 liquid medium containing various concentrations of $CoCl_2$. The results in Fig. 3 showed that for cells harboring pPs-L3opd12H

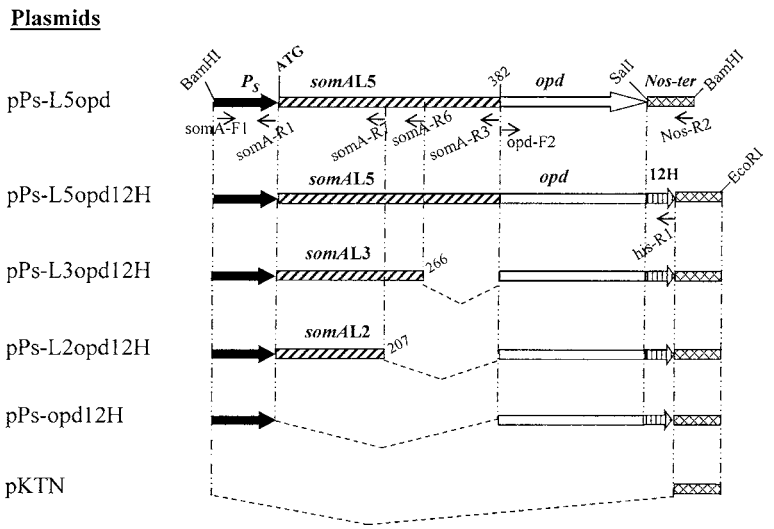

Fig. 1 Constructs encoding OPH. The 3′ end truncated *somA* genes: *somA*L5, L3, and L2 genes (located at the fifth, third, and second cell-surface exposed loop, respectively) were fused in frame with *opd* gene. The numbers of amino acids at the gene fusion positions are with respect to the start codon of SomA. *Ps somA* promoter, *somA* gene encoding *Synechococcus* outer membrane protein A, *opd* gene encoding organophosphorus hydrolase, *12H* 12 histidine peptide, *Nos-ter* nopaline synthase terminator. Locations of primers used in this study are indicated. The figure is not drawn to scale

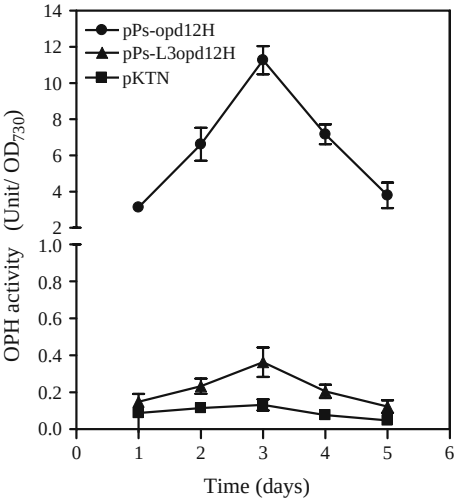

Fig. 2 OPH activities of *Synechococcus* cultures at various time courses. Cell cultures of *Synechococcus* harboring pKTN, pPs-L3opd12H, and pPs-opd12H were harvested each day. Fresh whole-cell suspensions (OD_{730}=1) were assayed for OPH activities. Each value and *error bar* represents the means of three independent experiments and its standard deviation

and pPs-opd12H, the levels of OPH activities reached the maximum at 2 mM $CoCl_2$. Therefore, further OPH activity determination was performed using cells grown in medium containing 2 mM $CoCl_2$.

It has been reported that under the condition of nitrogen deprivation, *somA* promoter is induced, and SomA increases slightly [20]. To investigate the effect of nitrogen deprivation on the expression of *somA–opd* fusion gene under the control of *somA* promoter (*Ps*), the OPH activities of cells grown in medium with or without nitrogen were performed. Figure 4 shows that for the cells harboring pPs-L3opd12H and pPs-opd12H, the level of OPH activity of cells grown in medium supplemented with nitrogen ($BG\text{-}11^N$) was not significantly different from that of corresponding cells grown in nitrogen deprivation medium ($BG\text{-}11^0$). The results indicated that nitrogen deprivation did not affect the expression of *opd* gene. Since the size of *somA* promoter fragment in the gene cassettes is 204 bp (upstream of *somA* start codon), this fragment might not contain the regulatory sequence responding to nitrogen deprivation. The regulatory sequence of the *somA* remains

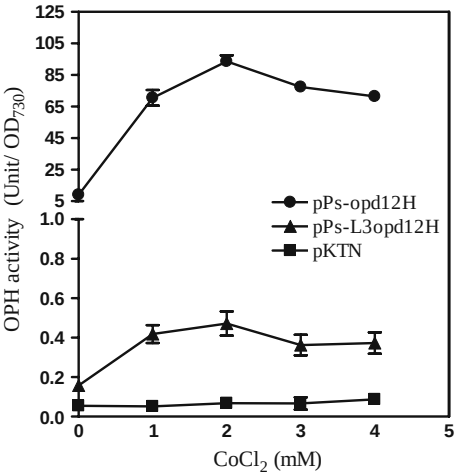

Fig. 3 Effect of $CoCl_2$ on OPH activities of *Synechococcus*. *Synechococcus* harboring pKTN, pPs-L3opd12H, and pPs-opd12H grown in BG-11 liquid medium including various concentration of $CoCl_2$ were harvested. Fresh whole-cell suspensions (OD_{730}=1) were assayed for OPH activities. Each value and *error bar* represents the mean of three independent experiments and its standard deviation

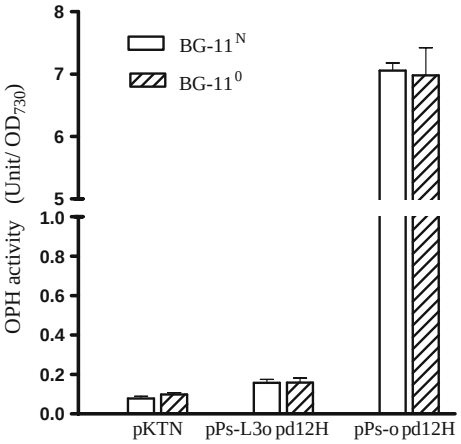

Fig. 4 Effect of nitrogen-depleted medium on OPH activities. *Synechococcus* harboring pKTN, pPs-L3opd12H, and pPs-opd12H were grown in liquid medium BG-11^N (including nitrogen) and BG-11^0 (excluding nitrogen). Whole-cell suspensions (OD_{730}=1) were assayed for OPH activities. Each value and *error bar* represents the mean of three independent experiments and its standard deviation

to be investigated. Therefore, further OPH activity determination was performed using cells grown in medium including nitrogen (BG-11).

Very little background OPH activity was detected in control cells harboring pKTN vector (Figs. 2, 3, 4, and 5). The results agreed well with previous report that *Synechococcus* has natural ability to degrade paraoxon, although at a very low level [4].

Region of SomA Required for OPH Activity of SomA-OPH Fusion Protein

The OPH activities of whole cells harboring the recombinant plasmids were compared. Results in Fig. 5 showed that the OPH activity of cells harboring pPs-L3opd12H was significantly higher than those of pPs-L2opd12H and pPs-L5opd12H. Therefore, the 266 amino acids of N-terminus (SomAL3, Fig. 1) is the optimal length required for OPH activity of the fusion protein. However, we observed that the OPH activity of cells harboring pPs-L5opd12H was 11-fold lower than that of pPs-L5opd (Fig. 5). Therefore, the addition of 12-histidine peptide at the C-terminus of SomAL5-OPH protein decreased the OPH activity.

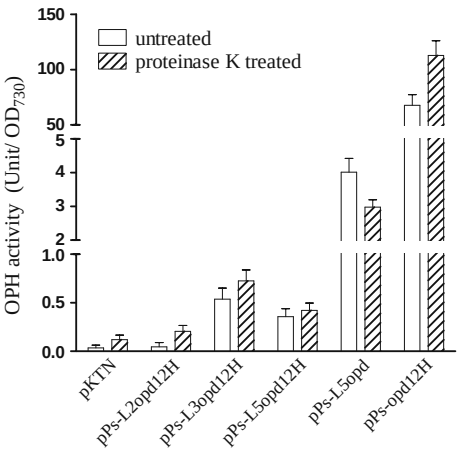

Fig. 5 OPH activities of whole cells treated with proteinase K. *Synechococcus* harboring pKTN, pPs-L2opd12H, pPs-L3opd12H, pPs-L5opd12H, pPs-L5opd, and pPs-opd12H were treated with proteinase K. The proteinase K-treated whole-cell suspensions (OD_{730}=1) were assayed for OPH activities. Each value and *error bar* represents the mean of three independent experiments and its standard deviation

Probing the Surface Location of OPH

Proteinase K accessibility assay of intact cells has been used to provide the evidence for the surface location of OPH [4, 11, 21], since proteinase K can not readily penetrate through the outer membrane. To investigate the surface location of the SomA-OPH fusion protein, proteinase K accessibility assay was performed. The results in Fig. 5 showed that for cells with intracellular-expressed OPH (pPs-opd12H), the OPH activity of proteinase K-treated cells was 1.7-fold higher than that of untreated cells. Similar results were observed in cells harboring pPs-L2opd12H, pPs-L3opd12H, and pPs-L5opd12H that the OPH activity of proteinase K-treated cells was slightly higher than that of corresponding untreated cells (Fig. 5). The results suggested that fusion proteins SomAL2-OPH12H, SomAL3-OPH12H, and SomAL5-OPH12H might not completely display on outermost cell surface. The increases of OPH activities might be simply due to the increases of outer membrane permeability caused by proteinase K. However, the OPH activity of proteinase K-treated cells harboring pPs-L5opd decreased by 25% when compared with that of untreated cells. The results indicated that SomAL5-OPH protein was successfully displayed on the outermost cell surface. Addition of 12H at the C-terminus of SomAL5-OPH protein decreased the OPH activity (pPs-L5opd and pPs-L5opd12H, Fig. 5). It is possible that only properly translocated OPH onto cell surface could retain functionality.

It has been showed that *E. coli* displaying polyhistidine peptides on cell surface can bind to Ni-NTA-agarose beads [17, 22]. In this study, adhersion of 12H to Ni-NTA-agarose beads (Qiagen GmbH, Germany) was performed as described [17] and visualized under phase-contrast microscopy. We found that adhersion of *Synechococcus* harboring pPs-L3opd12H to Ni-NTA-agarose beads was not significantly different from that of control cells with intracellular-expressed OPH (pPs-opd12H; data not shown). Thus, the results also suggested that 12H of SomAL3-OPH12H protein might not completely display on outermost cell surface to access restricted nickel ions. The low expression of SomAL3-OPH12H protein (Figs. 2, 3, and 4) might be another reason.

To determine the OPH activity in cytoplasm of *Synechococcus*, cells were disrupted by French press treatment and centrifuged to obtain the soluble fraction. The OPH activities of the soluble fraction from cells harboring pPs-L3opd12H and pPs-L5opd were barely detected and not significantly different from that of vector pKTN, whereas the OPH activity of soluble fraction from cells with intracellular-expressed OPH (pPs-opd12H) was 44± 10 unit/mg total proteins. Therefore, the OPH of cells harboring pPs-L3opd12H and pPs-L5opd was not located in cytoplasm, implying that the OPH was located in membrane fraction.

Immunostaining CLMS was performed to verify the surface location of the SomAL3-OPH12H and SomAL5-OPH protein. No immunoreactivities of anti-His and anti-OPH were detected on the surface of cells with intracellular-expressed OPH (pPs-opd12H) used as control (Fig. 6b, d, respectively) because these antibodies cannot access the intracellular-expressed OPH under the condition employed here. Immunoreactivities of anti-His and anti-OPH were detected as dark inclusions on the surface of cells harboring pPs-L3opd12H and pPs-L5opd (Fig. 6a, c, respectively). The results indicated that SomAL3-OPH12H and SomAL5-OPH were translocated across the membrane and anchored onto the outermost surface of *Synechococcus*.

Taken together, results from proteinase K accessibility assay (Fig. 5) and immunostaining CLSM (Fig. 6) revealed that (1) a minor fraction of SomAL3-OPH12H was displayed onto the outermost cell surface with a substantial fraction buried in the cell wall. Similar result has been reported for displaying OPH onto the cell surface of *Synechococcus* using InpNC as

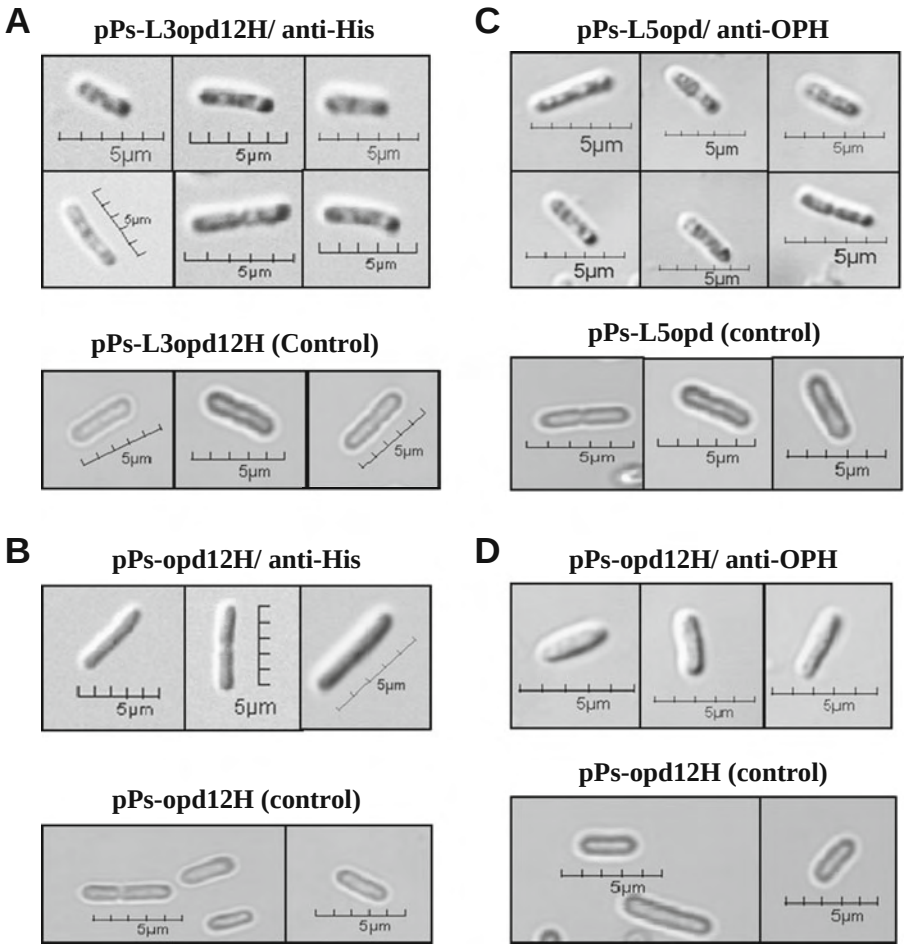

Fig. 6 CLSM images of *Synechococcus* cells expressing OPH. *Synechococcus* harboring plasmids pPs-L3opd12H, pPs-L5opd, and pPs-opd12H were cultured in BG-11 containing 2 mM $CoCl_2$. Whole cells exposed to first antibody, anti-His monoclonal antibody (**a**, **b**), or anti-OPH antiserum (**c**, **d**) are indicated, whereas cells of which first antibody was omitted are indicated as control. The cells were subsequently treated with goat anti-mouse IgG conjugated with horseradish peroxidase. Reactivity of immune complexes was visualized using diaminobenzidine and amidazole under CSLM (Olympus FV1000). The immunoreactivities of anti-His and anti-OPH were detected as dark inclusions on the surface of cells

anchoring motif [4]. (2) Substantial fraction of SomAL5-OPH was translocated across the membrane and completely displayed onto the outermost surface of *Synechococcus*.

Cells with surface-expressed OPH (pPs-L3opd12H and pPs-L5opd) exhibited lower activity than that of cells with intracellular-expressed OPH (pPs-opd12H; Figs. 2, 3, 4, and 5), although all the plasmids containing the *opd* gene under the control of *somA* promoter were derived from pKGT vector. It is possible that only properly translocated OPH onto cell surface could retain functionality. Similar results have been shown in the previous study that the surface-expressed InpNC-OPH has much lower activity than intracellular-expressed OPH [4]. It has been reported that cyanobacteria play an important role on bioremediation processes, for example, removal of the heavy metals from polluted water

[23]; biotransformation of mercury (Hg[II]) [24], and degradation of methyl parathion [25]. Since cyanobacteria, free-living photoautotrophic microorganisms, have simple growth requirements [15] and inexpensive to maintain when compared with *E. coli*, *Pseudomonas*, and *Saccharomyces*, expression of OPH in cyanobacteria may lead to the development of a low-cost and low-maintenance biocatalyst useful for detoxification of OP.

In conclusion, we developed a novel cell surface displaying system in which SomA was used as an anchoring motif for the display of OPH on the cyanobacterial outermost surface. The successful display of the functional heterologous protein on cell surface provides a useful model for variety of applications in cyanobacteria including screening of polypeptide libraries and whole-cell biocatalysts by immobilizing enzymes.

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References

1. Hoiczyk, E., & Hansel, A. (2000). *Journal of Bacteriology, 182*, 1191–1199.
2. Mackle, M. M., & Zilinskas, B. A. (1994). *Journal of Bacteriology, 176*, 1857–1864.
3. Spence, E., Sarcina, M., Ray, N., Moller, S. G., Mullineaux, C. W., & Robinson, C. (2003). *Molecular Microbiology, 48*, 1481–1489.
4. Chungjatupornchai, W., & Fa-aroonsawat, S. (2008). *Journal of Microbiology and Biotechnology, 18*, 946–951.
5. Chungjatupornchai, W., & Fa-aroonsawat, S. (2009). *Journal of Microbiology, 47*, 187–192.
6. Samuelson, P., Gunneriusson, E., Nygren, P. A., & Stahl, S. (2002). *Journal of Biotechnology, 96*, 129–154.
7. Umeda, H., Aiba, H., & Mizuno, H. (1996). *Microbiology, 142*, 2121–2128.
8. Hansel, A., Pattus, F., Jurgens, U. J., & Tadros, M. H. (1998). *Biochimistry Biophyics Acta, 1399*, 31–39.
9. Harper, L. L., McDaniel, C. S., Miller, C. E., & Wild, J. R. (1988). *Applied and Environmental Microbiology, 54*, 2586–2589.
10. Grimsley, J. K., Scholtz, J. M., Pace, C. N., & Wild, J. R. (1997). *Biochemistry, 36*, 14366–14374.
11. Richins, R. D., Kaneva, I., Mulchandani, A., & Chen, W. (1997). *Nature Biotechnology, 15*, 984–987.
12. Shimazu, M., Mulchandani, A., & Chen, W. (2001). *Biotechnology and Bioengineering, 76*, 318–324.
13. Takayama, K., Suye, S., Kuroda, K., Ueda, M., Kitaguchi, T., Tsuchiyama, K., et al. (2006). *Biotechnology Progress, 22*, 939–943.
14. Kuhlemeier, C. J., Thomas, A. A., van der Ende, A., van Leen, R. W., Borrias, W. E., van den Hondel, C. A., et al. (1983). *Plasmid, 10*, 156–163.
15. Williams, J. G. K. (1988). *Methods in Enzymology, 167*, 766–778.
16. Chungjatupornchai, W., Fa-aroonsawat, S., & Panyim, S. (2002). *FEMS Microbiology Letters, 211*, 57–64.
17. Sousa, C., Cebolla, A., & de Lorenzo, V. (1996). *Nature Biotechnology, 14*, 1017–1020.
18. Omburo, G. A., Kuo, J. M., Mullins, L. S., & Raushel, F. M. (1992). *Journal of Biology and Chemistry, 267*, 13278–13283.
19. Kaneva, I., Mulchandani, A., & Chen, W. (1998). *Biotechnology Progress, 14*, 275–278.
20. Sauer, J., Schreiber, U., Schmid, R., Volker, U., & Forchhammer, K. (2001). *Plant Physiology, 126*, 233–243.
21. Shimazu, M., Mulchandani, A., & Chen, W. (2001). *Biotechnology Progress, 17*, 76–80.
22. Xu, Z., & Lee, S. Y. (1999). *Applied and Environmental Microbiology, 65*, 5142–5147.
23. El-Enany, A. E., & Issa, A. A. (2000). *Environmental Toxicology and Pharmacology, 8*, 95–101.
24. Lefebvre, D. D., Kelly, D., & Budd, K. (2007). *Applied and Environmental Microbiology, 73*, 243–249.
25. Barton, J. W., Kuritz, T., O'Connor, L. E., Ma, C. Y., Maskarinec, M. P., & Davison, B. H. (2004). *Applied Microbiology and Biotechnology, 65*, 330–335.