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**Functional analysis of the group 3 sigma factors,
SigF1 and SigF2, in the cyanobacterium
Synechococcus elongatus PCC 7942**

By

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Abstract

Introduction

Cyanobacteria, including *Synechococcus elongatus* PCC 7942 (hereafter *Synechococcus*), produce oxygen via photosynthesis, a process sensitive to environmental changes. To respond to the stress, cells regulate the transcription of genes. This regulation is based on the modulation of various alternative sigma factors, which, together with RNA polymerase (RNAP), initiate the transcription of genes essential for environmental acclimation. This study focuses on the role of group 3 sigma factors SigF1 and SigF2 in *Synechococcus*, analyzing their impact on gene expression under diverse genetic backgrounds and environmental conditions.

Results

Phenotypic analysis of the SigF1-deficient strain showed that, in the absence of agitation, the cells lose their planktonic ability and settle on the bottom of the culture medium. This similarity to the homologous SigF-deficient phenotype in *Synechocystis* sp. PCC 6803, particularly in its association with the Type IV pilus formation gene, *pilA1*. Northern blotting confirmed that SigF1 activates *pilA1* transcription in response to illumination. Interestingly, it was found that light-induced changes in the PQ redox status may affect the *pilA1* promoter activation because the presence of photosynthetic inhibitor DBMIB inhibited the *pilA1* transcript accumulation. Although *pilA1* (*synpcc7942_0049*) and *pilA2* (*synpcc7942_0048*) constitute an operon and each open reading frame spans 435 bp in length, northern blotting results indicated that only the *pilA1* ORF, with a transcript size of approximately 700–800 nt, is included. Therefore, *pilA1* appears to be transcribed as a monocistronic operon rather than a bicistronic one. Additionally, a GFP-reporter strain was engineered by fusing the upstream promoter region of *pilA1* with the *mNG* gene, which encodes the GFP variant, and primer

extension analysis of this strain revealed a SigF1-dependent transcription start site. Site-directed scanning mutagenesis was employed to alter -3, -4, or -6 base pairs within the 50 bp region of the *pilA1* promoter in the reporter plasmid, which helped identify essential promoter regions. Consequently, I proposed the -35 and -10 elements of the SigF1-dependent promoter (G/C)TGA and GGGTAA. ChIP-qPCR analysis confirmed that SigF1, along with RNAP, is recruited to and accumulated on this promoter in the dark, suggesting the presence of a pre-activation state. Transcription is activated when PQ pool oxidation occurs under illumination.

RNA-seq analysis of strains overexpressing SigF1 indicated its role in regulating heat shock-related and mobility-associated genes. Examination of the *hspA* gene, which was significantly upregulated in these strains, showed that its transcription is induced by SigF1, although ChIP-qPCR demonstrated that SigF1 indirectly activates the *hspA* promoter.

Regarding SigF2, known for its role in rhythmic control and inducing gene expression in the dark, RNA-seq analysis of SigF2-overexpressing strains revealed a small number of genes upregulation, including *ccpB* and *digB*. Primer extension analysis confirmed the SigF2-dependent transcription start sites for *ccpB* and *digB*, leading to the proposal of the -35 and -10 promoter sequences. Furthermore, the -10 region of the promoter sequence recognized by SigF2 closely resembles that of SigF1, indicating evolutionary conservation between these two sigma factors. Additionally, the transcription of *ccpB* and *digB* was found to be activated under the PQ pool oxidized condition induced by the PSII inhibitor, DCMU, further validating the dependency on SigF2. Consistent with previous studies, full-length SigF1 can enhance the transcription of SigF2 downstream genes, suggesting the cross-talk of SigF1 and SigF2 promoter recognition specifics.

SigF1 and SigF2 do not influence each other's expression under the conditions of SigF1

and SigF2 overexpression. This suggested that additional mechanisms are involved in regulating the expression of downstream genes.

Discussion

The findings in this study have revealed that in *Synechococcus*, SigF1 and SigF2 play critical roles in the regulation of gene expression in response to environmental cues. SigF1 is shown to regulate the transcription of *pilA1* under illuminated conditions, underscoring its importance in the survival strategies of cyanobacteria. The phenomenon of SigF1 binding to the promoter prior to transcription initiation enhances our understanding of the cellular transcription initiation process. However, the specific mechanisms through which SigF1 activates transcription require further investigation. One explanation is that specific activation mechanisms are necessary to trigger the transcription after illumination. Alternatively, transcriptional activation may be inhibited in the dark, and this inhibition is canceled upon light exposure. Similarly, SigF2 activates the transcription of the downstream genes *ccpB* and *digB* under PQ pool oxidized condition, suggesting its involvement not only in circadian rhythm regulation but also in photosynthetic response. However, the precise transcriptional mechanisms employed by SigF2 remain to be elucidated. Moreover, the similarity in the promoter sequences dependent on SigF1 and SigF2 suggests potential functional similarities between these sigma factors. A joint study of their downstream genes could provide deeper insights into the regulatory functions of both SigF1 and SigF2.

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Abbreviations

RNAP	RNA polymerase
TSS	Transcription start stie
TUA	<i>Synechococcus elongatus</i> PCC 7942 (Wild type), derived from a stock from Tokyo University of Agriculture
NU	<i>Synechococcus elongatus</i> PCC 7942 (Wild type), derived from a stock from Nagoya University
TUAF1SP	<i>sigF1</i> deficient strain of TUA with <i>spc^R</i>
NUF1SP	<i>sigF1</i> deficient strain of NU with <i>spc^R</i>
NUF1KM	<i>sigF1</i> deficient strain of NU with <i>kan^R</i>
TUAF2KM	<i>sigF2</i> deficient strain of TUA with <i>kan^R</i>
TUAF1*SP/F2KM	<i>sigF1</i> * and <i>sigF2</i> double mutant strain of TUA
NUF1SP/F2KM	<i>sigF1</i> and <i>sigF2</i> double mutant strain of NU
F1F	SigF1-FLAG strain with <i>cm^R</i>
R2F	RpoC2-FLAG strain with <i>cm^R</i>
F1OX	Strain overexpressing SigF1
F2OX	Strain overexpressing SigF2
ATP	Adenosine triphosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
PQ	Plastoquinone
ORF	Open reading frame
TCSs	Two-component systems
IPTG	Isopropyl β -D-1-thiogalactopyranoside
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DBMIB	2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone
mNG	mNeonGreen, a green/yellow fluorescent protein derived from <i>Branchiostoma lanceolatum</i>

Chapter 1 Introduction

1.1. Cyanobacteria

Cyanobacteria are bacteria characterized by their ability to perform oxygenic photosynthesis (Sánchez-Baracaldo and Cardona, 2020). In the photosynthetic process, light energy from the sun is captured and used to energize electrons in the oxygen of a water molecule, producing adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH), which are then used for inorganic CO₂ fixation and other biosynthetic processes (Nikkanen et al., 2021). The advent of oxygenic photosynthesis radically transformed Earth's environment by increasing the levels of gaseous oxygen (O₂), significantly impacting the evolution of life forms (Sánchez-Baracaldo et al., 2022). The photosynthetic process is affected by diverse environmental changes, and cyanobacteria have evolved intricate mechanisms to acclimate to and survive under divergent stress conditions (Pandey et al., 2004; Sandrini et al., 2016; Tandeau de Marsac and Houmard, 1993). Regulation at the transcriptional level is the key mechanism underlying this adaptability, where differential activation of RNA polymerase (RNAP) sigma factors finely tunes gene expression in response to environmental changes (Gross et al., 1998; Gruber and Gross, 2003).

1.2. Transcription in Bacteria

Sigma factors play pivotal roles in the recruitment of RNAP to specific DNA promoter regions and transcriptional initiation (Browning and Busby, 2016). Bacterial transcription initiation is composed of multiple steps (Buc and McClure, 1985; Duchi et al., 2018; Mejía-Almont et al., 2020; Saecker et al., 2011). Before transcription initiation, a sigma factor binds to the RNAP core enzyme to make an RNAP holoenzyme, which can specifically recognize a promoter sequence and initiate transcription (Saecker et al., 2011). The interaction of the RNAP holoenzyme with promoter DNA leads to the formation of a closed complex (Friedman and Gelles, 2012; Plaschka et al., 2016). Subsequently, the two DNA strands around the transcriptional

start site (TSS) are separated to form an open complex (Buc and McClure, 1985; Duchi et al., 2018). The sigma factor function is thought to be critical for both steps (Helmann and Chamberlin, 1988). Transcription initiation begins with the commencement of RNA chain synthesis guided by the DNA template strand (Basu et al., 2014; Kapanidis et al., 2006). RNAP begins to move along with the DNA, leaving the promoter region behind and changing to the elongation complex, where the sigma factor is dissociated from RNAP (Basu et al., 2014). In the elongation step, the RNAP advances along with the DNA template, adding complementary ribonucleotides to the growing RNA chain (Vassilyev et al., 2007). Elongation proceeds until the RNAP reaches a specific termination signal on the DNA template, marking the end of transcription (Mejía-Almonte et al., 2020; Ray-Soni et al., 2016).

Promoters typically contain specific nucleotide sequences such as UP element, transcription start site (TSS), -10 , and -35 regions (Ruff et al., 2015). These regions are recognized and interacted with by different components of the RNAP holoenzyme. The RNAP holoenzyme comprises five subunits: α_2 , β , β' and ω , along with a sigma factor that binds to the RNAP (Burgess, 1969; Ruff et al., 2015). Typically, the up element is engaged by the alpha subunit of RNAP. In the sigma factor, region 4 recognizes the -35 element, and region 2 identifies and interacts with the -10 element (Campbell et al., 2002). Additionally, the -10 extension element and core recognition elements (CRE) also interact with various functional domains of the sigma factor (Dausse et al., 1972) (Figure 1.1).

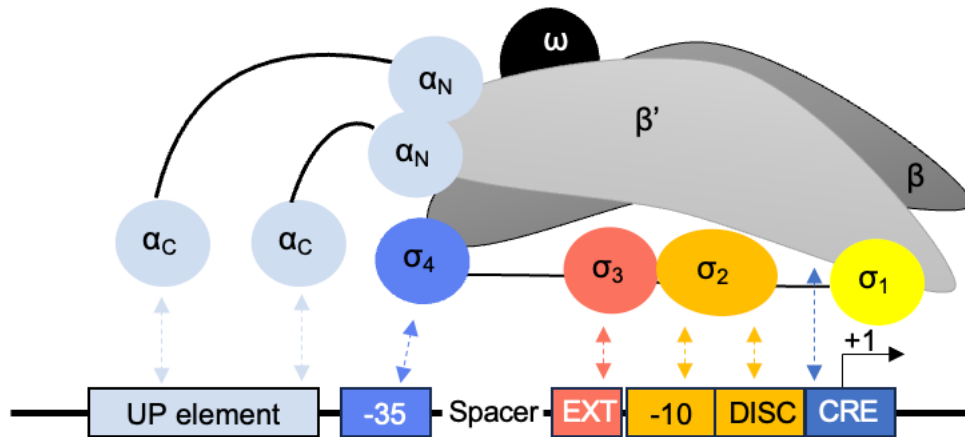


Figure 1.1 A scheme of interaction between RNAP core enzyme with $\sigma 70$ and promoter regions. Up element: cyan; -35 element: blue; extended -10 : red; -10 element and discriminator (DISC): orange; core recognition elements (CRE): dark blue; and TSS compose promoter. α_2 , β , β' and ω , along with a sigma factor, compose RNAP holoenzyme as shown. This figure was adapted from Mejía-Almont et al., 2020.

1.3. Regulatory of transcription initiation and Anti-sigma factor

The regulation of bacterial transcription initiation is a complex process involving multiple steps and factors aimed at precisely initiating gene expression at the right times and places. RNAP and sigma factor, promoter recognition, the small ligand, transcription factors such as activators and repressors, and the degree of chromosome folding are also part of the regulation of the initiation of transcription (Browning et al., 2004)

The regulation of transcription initiation by sigma factors typically involves an interaction with anti-sigma factors (Hughes and Mathee, 1998). Anti-sigma factors bind to sigma factors, preventing them from associating with RNAP and thus regulating the initiation of transcription (Alba and Gross, 2004). In turn, the activity of anti-sigma

factors is modulated by anti-anti-sigma factors. For example, in *Bacillus subtilis*, the anti-sigma factor RsbW regulates SigB as part of a partner-switching mechanism. Typically, RsbW binds to SigB, inhibiting its association with RNAP and thus preventing transcription initiation. RsbV, serving as an anti-anti-sigma factor, can be either phosphorylated or dephosphorylated by RsbU. In its dephosphorylated state, RsbV can bind to RsbW, releasing SigB to freely associate with RNAP and initiate transcription (Figure 1.2) (Pané-Farré et al., 2009).

Transcription factors such as activators and repressors modulate transcription initiation. Activators can enhance transcription by aiding RNA polymerase binding to promoters, often through direct interactions or by altering DNA conformation to facilitate access to essential promoter elements (Ebright, 1993; Stock et al., 2000; Sheridan et al., 1998). For instance, activators like CRP (cAMP receptor protein) bind to sites near promoters and help recruit RNA polymerase (Ebright, 1993). In contrast, repressors block transcription either by sterically hindering RNA polymerase binding or by altering the structure of the promoter so that it becomes less accessible to RNA polymerase (Müller-Hill, 1998).

Small ligands like ppGpp significantly influence transcription initiation by modulating RNAP activity, particularly under nutrient-limited conditions. They destabilize open complexes at specific promoters, primarily affecting genes crucial for translation machinery synthesis during amino acid scarcity. This mechanism allows bacteria to regulate gene expression in response to their metabolic state. Additionally, bacteria integrate multiple signals to control transcription initiation, involving interactions among sigma factors, transcription factors, and small ligands (Barker et al., 2001; Schneider et al., 2002). These interactions ensure a coordinated response to environmental and internal changes, optimizing gene expression and bacterial adaptability.

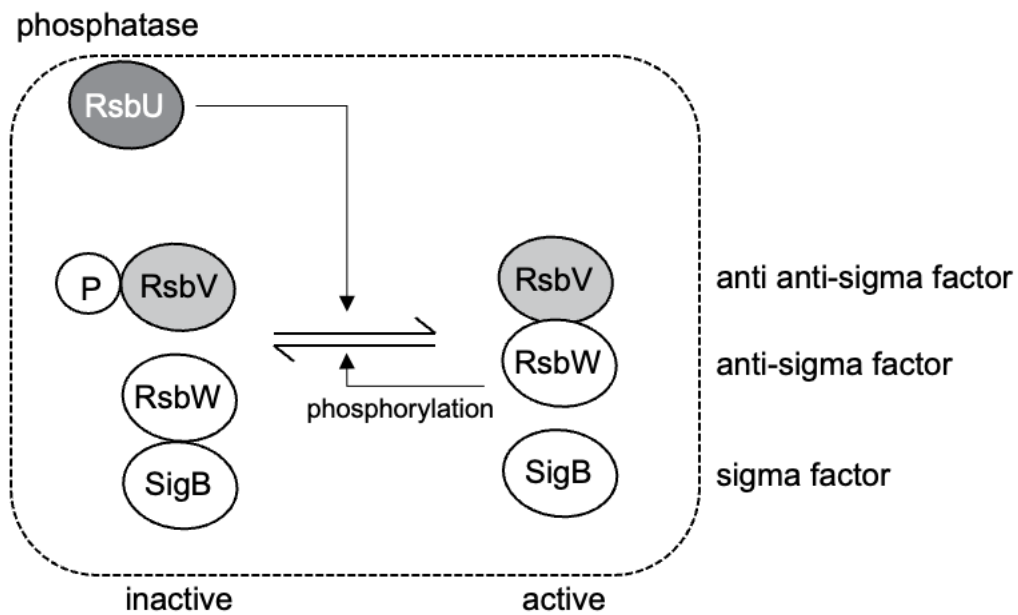


Figure 1.2 Model of SigB activation in *B. subtilis*. Environmental stress triggers the phosphatase RsbU to dephosphorylate RsbV, which is phosphorylated under normal conditions. RsbV, acting as an anti-anti-sigma factor, then binds to the anti-sigma factor RsbW. This interaction releases SigB from RsbW, allowing free SigB to associate with RNAP and bind to the promoter, thereby initiating transcription. This figure was adapted from Pané-Farré et al. 2009.

1.4. Sigma factors in cyanobacteria

Sigma factors are categorized into four groups based on their structure and function: groups 1, 2, 3, and extracytoplasmic function (ECF) (Feklistov et al., 2014; Paget and Helmann, 2003). *Synechococcus* has ten sigma factors, each belonging to one of these four groups (Imamura and Asayama, 2009). The group 1 sigma factor, RpoD1 in *Synechococcus*, functions as the principal sigma factor and is indispensable for growth (Sugimoto et al., 1997). Group 2 contains five sigma factors: RpoD2, RpoD3, RpoD4, RpoD5, and RpoD6. Of these, RpoD3 responds to high light and low temperatures under the control of the response regulator RpaB (Espinosa et al., 2015; Osanai et al.,

2008; Seki et al., 2007). RpoD2, RpoD5, and RpoD6, together with a group 3 sigma factor SigF2, orchestrate the genome-wide transcription by the Kai-dependent circadian rhythm (Fleming et al., 2018; Nair et al., 2002). The roles of the ECF sigma factors, SigG and SigI, remain mostly elusive.

Group 3 sigma factors in *Synechococcus* include SigF1 and SigF2 (Imamura and Asayama, 2009). Orthologous genes of both *sigF1* and *sigF2* are present in divergent cyanobacterial genomes. However, some strains, like *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) lacks the *sigF2* orthologue (Imamura and Asayama, 2009; Yoshimura et al., 2007) (Figure 1.3). In *Synechocystis*, the SigF1-ortholog SigF regulates Type IV pilus formation, which is involved in motility and phototaxis (Bhaya et al., 2000; Flores et al., 2019; Schuergers and Wilde, 2015).

As noted above, SigF2 is involved in the regulation of the circadian rhythm and is required for dark-inducible DNA competence (Taton et al., 2020). In the RpaA-controlled cascade reaction, the sigma factors are activated sequentially. The cascade begins with RpoD2, followed by RpoD6, and then RpoD5 and SigF2. Each sigma factor exhibits a peak expression at different circadian times, which corresponds to their role in the cascade. These sigma factors are not only sequentially activated but also show interdependency. The expression and activity of one sigma factor can influence the expression of others, creating a tightly regulated network. For example, the absence of SigF2 can significantly increase RpoD2 expression (Fleming et al., 2018). The expression of SigF2 changes with the circadian rhythm and reaches its peak in the dark. This was accompanied by increased expression of genes encoding components of Type IV pilus that are critical for DNA uptake by the cell, as well as other competence-related proteins (Taton et al., 2020).

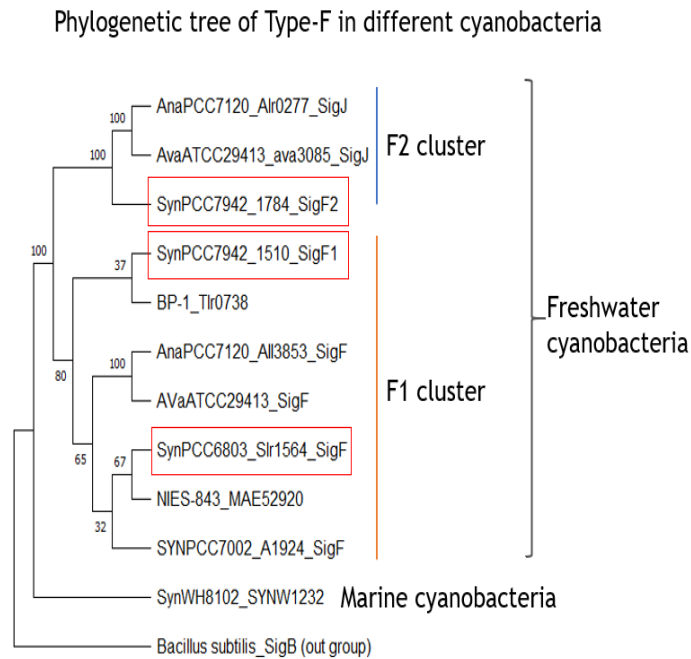


Figure 1.3 Phylogenetic tree of Type-F sigma factors in different cyanobacteria.

Type-F sigma factors are divided into two categories: F1 cluster and F2 cluster. The F2 cluster is found only in freshwater cyanobacteria.

1.5. Type IV pilus in *Synechococcus*

A critical feature of *Synechococcus* is its Type IV pilus, filamentous structures protruding from the cell surface. These pili are instrumental in various biological functions crucial to the survival and adaptability of cyanobacteria, such as motility, surface adhesion, and biofilm formation. Furthermore, Type IV pilus facilitate natural transformation by capturing external DNA molecules from the environment and transporting them to the cell interior, thereby contributing to genetic diversity and adaptation through horizontal gene transfer (Taton et al., 2020).

Type IV pili dynamics, including their morphology (length and number) and activities (extension and contraction), exhibit rhythmic oscillatory patterns that align with the light-dark cycle. This rhythmic regulation significantly influences the natural

transformation efficiency of the cells. According to (Zhang et al., 2024), these oscillatory behaviors of Type IV pilus and their expression are tightly regulated by the circadian clock, impacting Type IV pilus morphology and function directly. Notably, the gene *pilA1* is essential for pili formation; deletion of *pilA1* results in the loss of pili, although it does not affect biofilm formation. Cells devoid of pili tend to sediment at the bottom of liquid cultures, suggesting that pili might play a role in the survival strategies of *Synechococcus* (Aguilo-Ferretjans et al., 2021).

1.6. Photosynthesis in cyanobacteria

Photosynthesis in cyanobacteria is similar to photosynthesis in plants and involves two main stages: light-dependent reactions and the Calvin cycle.

In light-dependent reactions, cyanobacteria utilize the electron transport chain (ETC) to convert light energy into chemical energy, ultimately producing ATP and NADPH (Figure 1.4). The process also involves the photolysis of water molecules, resulting in the release of oxygen as a by-product (Angermayr S et al., 2008). This process is fundamental to life support on Earth and involves a complex series of protein complexes and electron carriers located within the thylakoid membranes of cyanobacterial cells (Blankenship et al., 2010). The following is an overview of the main components and steps involved in cyanobacterial ETC: Photosystem II (PSII) is the starting point of the electron transport chain. Here, light energy is absorbed by chlorophyll molecules, exciting electrons to higher energy levels (Komenda et al., 2012; Keren et al., 2005). This energy is used to split water molecules into oxygen, protons (H^+), and electrons. Oxygen is released as a by-product. Excited electrons from PSII are transferred to PQ, a small fat-soluble carrier molecule. PQ then delivers these electrons to the cytochrome b6f complex in the chain. The cytochrome b6f complex facilitates electron transfer from PQ to another electron carrier, plastocyanin (PC) (Vinyard et al., 2013). During this transfer, protons are pumped across the thylakoid membrane, helping

to create a proton gradient that is essential for ATP synthesis. The electrons arrive at Photosystem I (PSI) (Durán et al., 2004). They are re-excited by light absorbed by PSI and then transferred to ferredoxin (Fd), a small protein that carries the electrons to the final electron acceptor NADP^+ , which reduces them to NADPH (Jordan et al., 2005). Finally, the proton gradient established by the movement of electrons through the ETC drives the protons back across the membrane through ATP synthase, promoting the synthesis of ATP from ADP and inorganic phosphate (Shimakawa et al., 2015).

The energy and reducing power generated during the light-dependent reactions are utilized in the Calvin cycle to fix carbon dioxide into organic compounds. While this process occurs in the chloroplast stroma of higher plants, in cyanobacteria, it takes place in the cytoplasm. Here, the enzyme RuBisCO catalyzes the fixation of CO_2 into stable intermediates, ultimately facilitating the synthesis of glucose and other carbohydrates (Dai et al., 2018).

Cyanobacteria are distinctive among bacteria due to their ability to perform oxygenic photosynthesis. They harbor a variety of pigments, including chlorophyll a, phycocyanin, and allophycocyanin, which enable them to absorb diverse wavelengths of light, thus enhancing their photosynthetic efficiency (Campbell et al., 1998). As vital primary producers in aquatic ecosystems, cyanobacteria play a crucial role in the global carbon cycle and serve as a fundamental food source for a vast array of aquatic organisms.

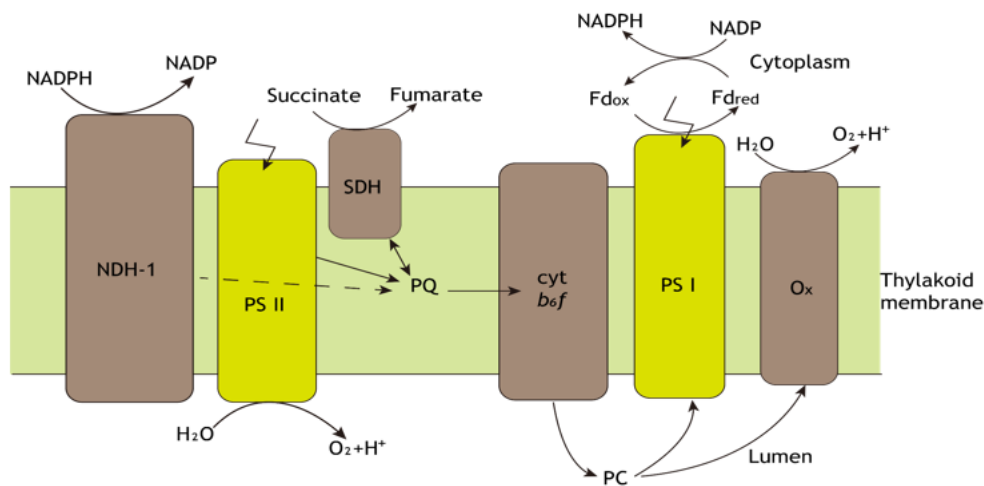


Figure 1.4 Schematic diagram of the electron transport chain of photosynthesis.

The diagram illustrates the main components and electron flow in the photosynthetic electron transport chain. The process begins with Photosystem II (PS II), which uses light energy to split H_2O into O_2 and H^+ , releasing electrons. These electrons are transferred through the plastoquinone (PQ) pool to the cyt *b₆f* complex. The electrons are then passed to PS I via PC. PS I uses light energy to reduce $NADP^+$ to NADPH. Additionally, the diagram shows the involvement of NADH dehydrogenase-like complex (NDH-1) and succinate dehydrogenase (SDH) in electron transfer, linking the cytoplasm and thylakoid lumen.

1.7. Two-component systems in *Synechococcus*

Two-component systems (TCSs) are prevalent modes of signal transduction in bacteria and archaea and are also found in some eukaryotes. These systems allow cells to detect and react to environmental variations, often via membrane-bound sensors, histidine kinases (HKs), and cytoplasmic response regulators (RRs). HKs can sense external stimuli, such as changes in light or temperature, leading to autophosphorylation; this involves the addition of a phosphate group to a histidine residue upon sensing a stimulus. The phosphate is subsequently transferred to an aspartic acid residue on RR,

altering RR conformation and activating it. The activated RR then binds to specific DNA sequences, regulating the transcription of genes in response to environmental cues (Ashby et al., 2006; Hoch et al., 2000).

In cyanobacteria, TCSs play a critical role in regulating photosynthesis and adapting to changes in light conditions and nutrient availability. *Synechococcus* utilizes a variety of TCSs for environmental adaptation, including notable examples such as NblS-RpaB, Hik2/Hik34-Rre1, and SasA-RpaA.

NblS-RpaB regulates photosynthesis and stress responses. Upon detecting stress signals, the sensor kinase NblS activates RpaB, a response regulator that controls genes associated with light harvesting and stress defense, thus maintaining efficient photosynthesis under adverse conditions. This regulatory mechanism also involves the group 3 sigma factor RpoD3 (Kato et al., 2011).

SasA-RpaA is essential for the cyanobacterial circadian clock, where the sensor kinase SasA phosphorylates RpaA, the response regulator, affecting circadian rhythms. This TCS is crucial for synchronizing cellular activities such as photosynthesis and nitrogen fixation with the day-night cycle, enhancing metabolic efficiency (Takai et al., 2006).

Hik2/Hik34-Rre1 involves sensor kinases Hik2 and Hik34 interacting with the response regulator Rre1 to manage the cell's response to heat shock and redox stress in photosynthetic PQ. Hik34-Rre1 effectively manages heat shock to maintain cellular stability, while Hik2-Rre1 addresses both heat shock and redox stress, implementing negative feedback regulation through DnaK2. DnaK2, part of the highly conserved DnaK family of heat shock proteins, plays a crucial role in the cellular stress response across various organisms. As a molecular chaperone, DnaK aids in the proper folding of new polypeptides, stabilization of unfolded proteins, and prevention of protein

aggregation, particularly under conditions like high temperatures. In *Synechococcus*, DnaK2 has been demonstrated to modulate the heat shock response and PQ redox through overexpression, aiding in restoring stress-responsive cells to homeostasis (Kobayashi et al., 2017; Nachiketa et al., 2021; Hasegawa et al., 2023).

1.8. Aims

The primary aim of my study is to explore the regulatory roles of sigma factors SigF1 and SigF2 in the cyanobacterium *Synechococcus*, focusing on their influence on gene expression in response to environmental changes. My aim is to characterize the regulatory mechanisms of SigF1 and SigF2 through a series of targeted experiments.

In this study, the influence of SigF1 on the transcriptional activation of the *pilA1* gene during the dark-to-light shift was investigated. The functional interactions between SigF1-RNAP and the *pilA1* promoter were examined by ChIP-qPCR to detect the recruitment of sigma factors and RNAP on the *pilA1* promoter. The impact of SigF1 or SigF2 overexpression on the global transcription of *Synechococcus* was assessed using RNA sequencing. The modulation of gene expression by SigF2, including genes such as *ccpB* and *digB* under dark conditions and PQ pool oxidized conditions, was explored, along with the cross-talk between SigF1 and SigF2 to their downstream genes. Furthermore, the transcriptional start sites and promoter regions dependent on SigF1 and SigF2 were proposed.

Chapter 2 Material and Methods

2.1. Bacterial strains, media, and culture

Our laboratory wild-type strain (TUA), another wild-type strain derived from Nagoya University (NU), and their derivatives were used in this study (Table S1). The cultivation conditions for these strains were in BG-11 (Rippka, 1988) liquid medium with 2% CO₂ aeration and on BG-11 solid medium supplemented with 1.5% w/v agar at 30°C with 2% CO₂ aeration under continuous fluorescent light (30 $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$). Additionally, spectinomycin (40 $\mu\text{g ml}^{-1}$), kanamycin (10 $\mu\text{g ml}^{-1}$), or chloramphenicol (7.5 $\mu\text{g ml}^{-1}$) were added to the medium when needed. For detailed receipt of the reagents used, see Appendix at the end of the document.

2.2. Plasmids construction

All cloning procedures were performed in *E. coli* DH5 α following standard molecular biology protocols.

2.2.1. Construction of plasmids for mNG-reporter strains and 3-, 4-, 6- base pairs substitute plasmids

To generate PAG (NSI::PpilA1-mNG) reporter strains, a 200-bp region upstream of the *pilA1* ORF was amplified by PCR (primer set: pilA1-pro-smaI-F/pilA1-pro-smaI-R). Subsequently, it was inserted into the SmaI site of pNS Ω Sp-mNG, which had been modified from pNS-Pspec-gm-mNG by replacing the gentamicin-resistance gene cassette (*gm^R*) with a spectinomycin-resistance gene cassette (*spc^R*), resulting in pNS Ω Sp-PpilA1-mNG (Figure 3.7 and Figure 3.9A). Plasmids for the mNG-reporter strains (M1–M10) were constructed by site-directed mutagenesis (Liu and Naismith, 2008). To substitute three, four, and six base pairs in the *pilA1* promoter region (Figure 3.10A), the template plasmid pNS Ω Sp-PpilA1-mNG was amplified by PCR using the KOD One PCR Master Mix (Toyobo Co., Ltd, Osaka, Japan) with primer sets listed in Table S3. The obtained mutated plasmids are listed in Table S2.

2.2.2. Construction of F1F and R2F

To construct C-terminal FLAG-tagged SigF1, the *sigF1* open reading frames (ORFs) and downstream fragments were amplified by PCR from *Synechococcus* chromosomal DNA using specific primers (Table S3). The resulting amplicon *sigF1* fragment was digested by SacI and BglII and then inserted into the SacI/BamHI site of pFLAGΩCm, resulting in pFLAGΩCm-F1orf. The downstream fragment was digested by HincII and then inserted into the HincII site of pFLAGΩCm-F1orf, resulting in the plasmid pFLAGΩCm-F1F (Figure 2.1).

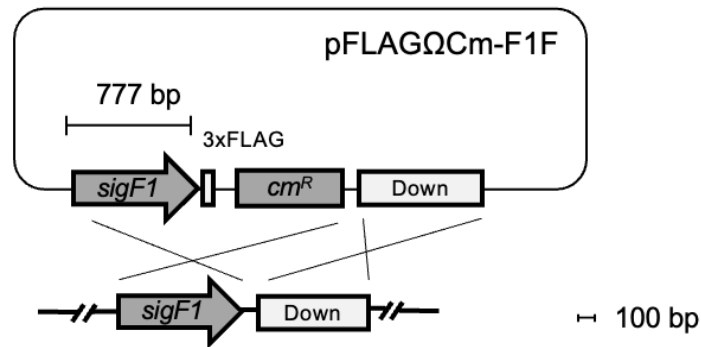


Figure 2.1 Construction of the F1F strain. The *sigF1* gene and its downstream region were inserted into pFLAGΩCm, constructing a fusion of *rpoC2* ORF with a 3×FLAG tag. The genomic *sigF1* gene was substituted with the *sigF1*-FLAG construct by selection for the attached *cm^R* marker. Primer sets used are listed in Table S1.

To construct C-terminal FLAG-tagged RpoC2, the *rpoC2* open reading frames (ORFs) and downstream fragments were respectively amplified by PCR from *Synechococcus* chromosomal DNA using specific primers (listed in Table S3). The pFLAGΩCm was linearized by PCR with primer sets (PFLAGcm-inverse-F and PFLAGcm-inverse-R). The resulting amplicon of the *rpoC2* fragment was ligated with the pFLAGΩCm by Gibson assembly, resulting in pFLAGΩCm-R2orf. The downstream fragment was

digested by SalI and ApaI and then inserted into the SalI/ApaI site of pFLAGΩCm-R2orf, resulting in the plasmid pFLAGΩCm-R2F (Figure 2.2).

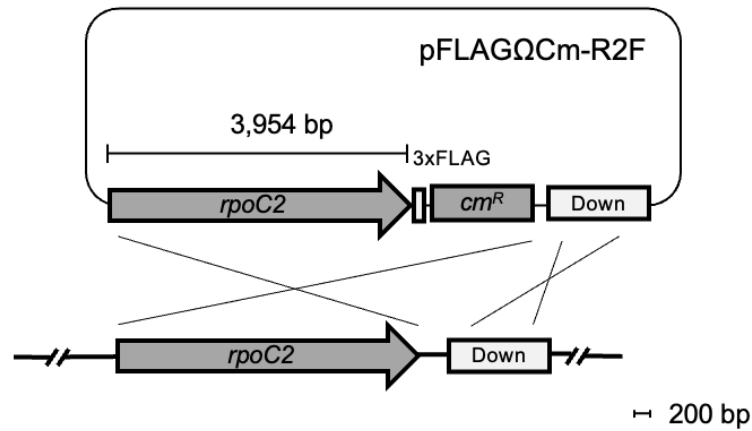


Figure 2.2 Construction of the R2F strain. The *rpoC2* gene and its downstream region were inserted into pFLAGΩCm, constructing a fusion of *rpoC2* ORF with a 3×FLAG tag. The genomic *rpoC2* gene was substituted with the *rpoC2*-FLAG construct by selection using the attached *cm^R* marker. The primer sets used are listed in Table.

2.2.3. Construction F1OX and F2OX

To construct *sigF1* overexpressed strain, the *sigF1* and *sigF2* open reading frames (ORFs) were respectively amplified by PCR from *Synechococcus* chromosomal DNA using specific primers (listed in Table S3). The resulting amplicon *sigF1* and *sigF2* fragments were digested by SacI and BglII and then inserted into the SacI/BamHI site of pNSEI, respectively, resulting in pNSEI-F1 and pNSEI-F2 (Figure 2.3, Figure 2.4).

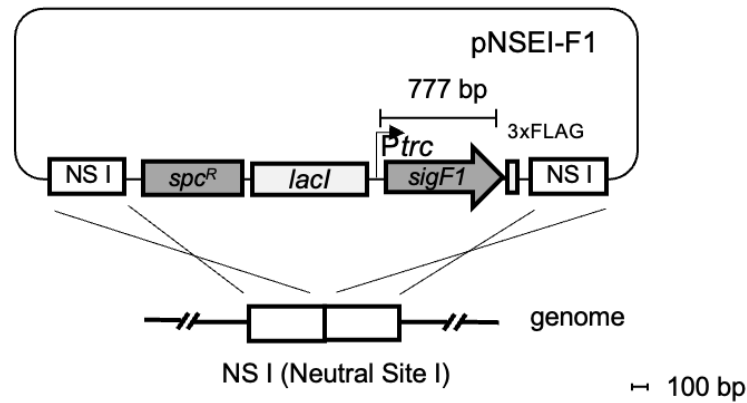


Figure 2.3 Construction of the F1OX strain. The *sigF1* gene were inserted into pNSEI, constructing a fusion of *sigF1* ORF with a 3×FLAG tag. The primer sets used are listed in Table S3.

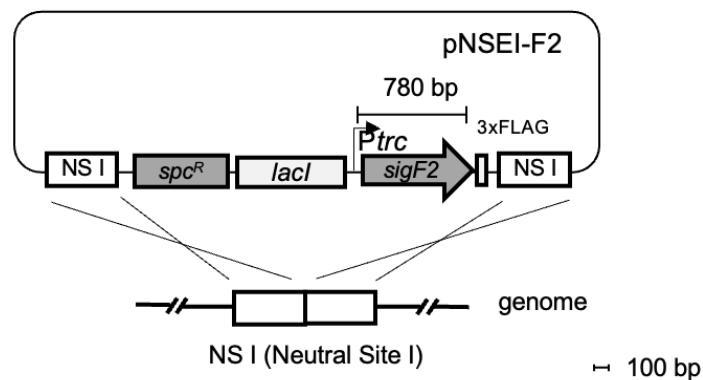


Figure 2.4 Construction of the F2OX strain. The *sigF2* gene were inserted into pNSEI, constructing a fusion of *sigF2* ORF with a 3×FLAG tag. The primer sets used are listed in Table S3.

2.3. Strains construction

2.3.1. *sigF1*-deficient strains

To generate a *sigF1/sigF1** (*synpcc7942_1510*) deletion mutant in NU and TUA (Figure 2.5), the upstream and downstream 0.5 kb of *sigF1/sigF1** were amplified from chromosomal DNA with specific primer sets (*sigF1-up500-f/sigF1-up-KXH-r* and *sigF1-dn-VXK-f/sigF1-dn500-r*, respectively). Subsequently, a gene cassette containing

resistance to spectinomycin was amplified by PCR from the pHP45Ω plasmid (Prentki and Krisch, 1984) (primer set: SpR-FW-KXH/SpR-RV-KXV). These PCR fragments were joined by recombinant PCR (primer set: sigF1-up500-f/sigF1-dn500-r). The resulting construct was used for the transformation of the wild-type strain to generate the corresponding resistant mutant. Spectinomycin-resistant mutants were selected and then verified by PCR analysis. To obtain PAGF1KM (PpliA1-mNG/F1KM), a gene cassette containing resistance to kanamycin was amplified by PCR from the plasmid pUC4K (Taylor and Rose, 1988) (primer set: KanR-RV-KXV/KanR-FW-KXH). The other steps were the same as described above.

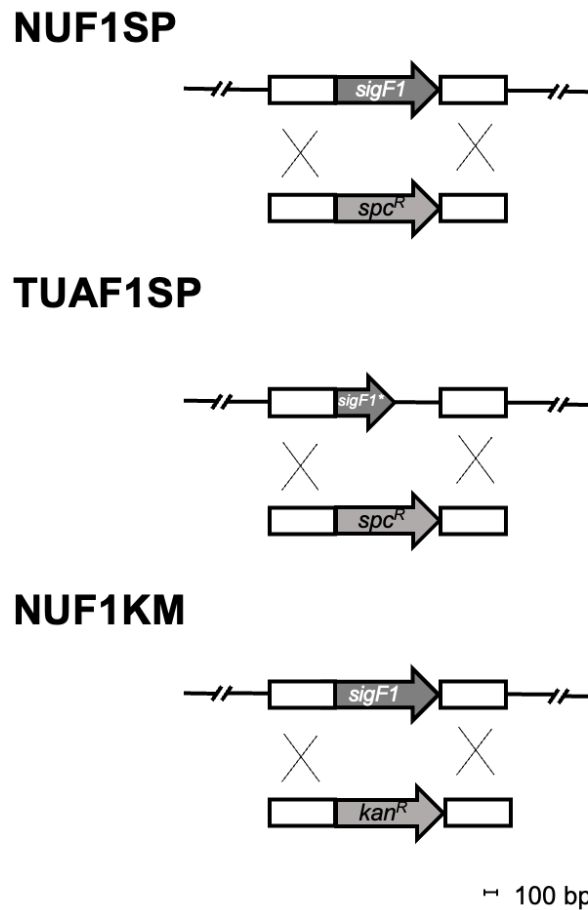


Figure 2.5 Construction of NUF1SP, TUAF1SP, and NUF1KM. DNA recombinant fragments were transformed into NU or TUA to make NUF1SP, TUAF1SP and NUF1KM.

2.3.2. *sigF2* deficient strain

To obtain *sigF2* (*synpcc7942_1784*) deletion mutant, the upstream and downstream 0.5 kb of *sigF2* were amplified from chromosomal DNA with specific primer sets (Figure 2.6). A gene cassette containing resistance to kanamycin was amplified by PCR from the plasmid pUC4K (Taylor and Rose, 1988) (primer set: KanR-RV-KXV/KanR-FW-KXH). The other steps were the same as described in Section 2.3.1. Primer sets for this experiment are listed in Table S3.

F2KM

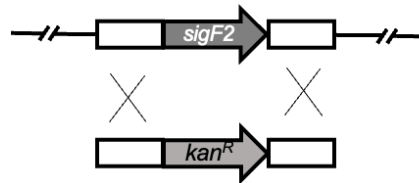


Figure 2.6 Construction of F2KM. DNA recombinant fragments were transformed into NU or TUA to make TUA F2KM and NU F2KM.

2.3.3. *sigF1* and *sigF2* double mutant strains

To obtain *sigF2* and *sigF1* double mutant strains, I used the recombinant fragment constructed in Section 2.3.1 and Section 2.3.2 and transformed them into TUA and NU (Figure 2.7).

F1SP/F2KM

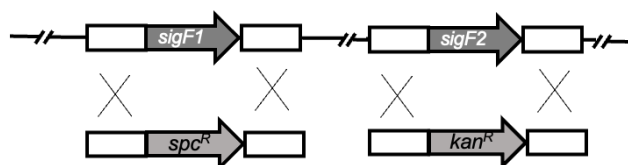


Figure 2.7 Construction of F1SP/F2KM. DNA recombinant fragments were transformed into TUA or NU to make TUA F1SP/F2KM and NU F1SP/F2KM.

2.4. Transformation of *Synechococcus*

To obtain recombinant strains, plasmids, and constructed DNA fragments were transformed into *Synechococcus* by natural transformation [41]. Liquid cultures were cultivated to OD₇₅₀ of 0.6-0.8. Subsequently, cells were harvested by centrifugation at 5,000 × rpm and washed once with BG-11 medium. Add 300 µl of BG-11 medium and approximately 600 ng of plasmid DNA or recombinant fragment. Cells were incubated overnight in the dark and then in the light for 3 h. Cells were plated on BG-11 solid medium containing antibiotics and cultured for 1 week at 30°C.

2.5. Northern blot analysis

Total RNA was extracted from cells using the hot phenol method, as described by Mironov and Los (2015). RNA concentration and quality were determined with NanoDrop (Thermo Fisher Scientific Inc). For the Northern blot analysis, 3 µg of RNA was utilized, adhering to the protocol by Josefsen and Nielsen (2011). The DIG-labeled DNA probe targeting *pilA1* was amplified by PCR. Primer sets used for the probes are listed in the supplementary table (Table S3).

2.6. RNA-seq Analysis

Total RNA was extracted from cells using the hot phenol method, as described by Mironov and Los (2015). RNA samples were analyzed by Dr. Yuu Hirose at Toyohashi University of Technology. Visualization of the RNA-seq results was performed using Morpheus (<https://software.broadinstitute.org/morpheus>).

2.7. Immunoblot analysis

2.7.1. Sampling

Cell culture started with an initial OD₇₅₀ of 0.15, and cells were harvested when they reached an OD₇₅₀ of approximately 0.5. Around 10 ml cells were centrifuged in 50 ml centrifuge tubes at 7,000 × g at 4°C for 2 min, resuspended in BG-11, and transferred to

a 1.5 ml microcentrifuge tube. The resuspension was centrifuged at $15,000 \times \text{rpm}$ at 4°C for 20 sec, removed the supernatant and stored at -80°C .

2.7.2. Whole protein extraction

Cell pellets were resuspended in PBS buffer (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl) and lysed by vortex with glass beads ($\leq 106 \mu\text{m}$, Sigma-Aldrich Co. LLC, St Louis, USA) at 4°C for 10 min and rested for 5 min 3 times. Lysate was centrifuged at $5,000 \times g$ at 4°C for 5 min and transferred supernatant to a 1.5 ml microcentrifuge tube. The supernatant was centrifuged by $15,000 \times g$ at 4°C for 20 min, and the supernatant was transferred to a 1.5 ml microcentrifuge tube. Protein concentration was measured by Biorad (Bio-Rad Laboratories, Inc.).

2.7.3. SDS-PAGE

Protein samples are mixed with $2\times$ SDS sample buffer. This mixture is then heated at 95°C for 10 min to further denature the proteins. Prepare the separating gel and stacking gel according to the table below. Put the separating gel on the lower layer and add the stacking gel after it solidifies. The prepared samples are loaded into wells in the stacking gel and electrophoresis at 30 mA for approximately 50 min.

2.7.4. Western blotting

The PVDF membrane was shaken in methanol for 15 min, and then transferred to the transfer buffer and shaken for 15 min. Place three layers of filter paper, glue, membrane and three layers of filter paper on the blotter in sequence. Apply current ($1 \text{ mA}/\text{cm}^2$) for approximately 60 min to transfer proteins from the gel to the membrane. The transferred membrane was blocked in a blocking buffer for 4 days overnight. The next day, add primary antibody and shake at room temperature for 60 min. Wash twice with TBST at room temperature, 15 min each time. Transfer the membrane to blocking buffer containing secondary antibody and incubate with shaking for 30 min. Wash twice again

with TBST at room temperature for 15 min each time. Use immunostain zeta drops on the membrane and detect chemiluminescence under luminous graph I. Anti-FLAG antibody (Sigma, Munich, Germany) was used at 1: 10,000 dilution as the primary antibody, and the secondary antibody was anti-mouse-HRP (Promega Corp. Madison, WI, USA). For detailed receipt of the reagents used, see Appendix at the end of the document.

2.8. Phos-tag analysis

Cells were harvested from the culture tubes at each experimental time point and collected by centrifugation at $7,000 \times g$ for 2 min at 4°C. The collected cells were then stored at -80°C for further analysis. Total proteins were extracted from cells, and the protein concentration was determined using the Bradford protein assay. For electrophoresis, 20 µg of the protein extract was loaded onto a 12% polyacrylamide gel containing 25 µM Phos-tag AAL-107 reagent (Wako, Tokyo, Japan) and 50 µM MnCl₂. The gel was run at a constant current of 100 V and 12 mA at 4°C for 3 hours. Following electrophoresis, the gel was treated with a transfer buffer containing 10 mM EDTA to remove Mn²⁺ ions. Protein transfer to the membrane was performed at 2 mA/cm² for 60 minutes. The membrane was then blocked overnight with a blocking buffer. For detection, an anti-Rre1 polyclonal antibody was used as the primary antibody, and an anti-rabbit-HRP secondary antibody (Promega Corporation, USA) was applied at a 1:4,000 dilution.

2.9. ChIP-qPCR analysis

Cell culture was started with an initial OD₇₅₀ of 0.15, and cells were harvested when they reached an OD₇₅₀ of approximately 0.5. The whole cell extraction procedure was performed as described by Hanaoka and Tanaka (2008). For chromatin immunoprecipitation, 800 ng of protein was processed using an anti-FLAG antibody (Sigma, Munich, Germany), according to Hanaoka and Tanaka (2008). Quantitative

real-time PCR analysis used the primer sets pilA1-qPCR-F1/pilA1-qPCR-R1 and pilA1-qPCR-F2/pilA1-qPCR-R2.

2.10. Primer extension

Primer extension analysis was performed by the ReverTra Ace qPCR RT kit (Toyobo Co., Ltd, Osaka, Japan) with a FAM-labeled mNG-PEx-R primer on 8 µg RNA, following protocols described by Sambrook (1989) and Shimizu et al. (2017). For marker sequencing ladders, the DNA fragment with the *pilA1* promoter region was amplified from pNSΩSp-PpilA1-mNG using the primer set Omega-seq2/mNG-probe-R. Subsequently, four dideoxy chain termination reactions, each with one of ddA, ddG, ddC, and ddT, were performed using this DNA fragment and the FAM-labeled mNG-PEx-R primer with a USB Thermo Sequenase Cycle Sequencing Kit (Thermo Fisher Scientific Inc., Japan), producing a sequence ladder. DNA sample analysis was performed by the Biomaterial Analysis Center at Tokyo Institute of Technology. GeneScan 600 LIZ size standard (Thermo Fisher Scientific Inc., Japan) was added before the analysis to compare the sizes of the primer extension products with the sequence ladder. Sequencing results were analyzed with Peak Scanner software version 1.0 (Applied Biosystems).

2.11. Fluorescent activity measurement

Cell culture was started with an initial OD₇₅₀ of 0.15, and cells were measured at an OD₇₅₀ of approximately 0.5. To detect fluorescence activity, 2 ml of the culture was sampled. Fluorescence activity, with excitation at 506 nm and emission at 517 nm, was determined using a spectrofluorometer (JASCO Corporation), while OD₇₅₀ measurements were conducted with a UV/Vis spectrophotometer (Beckman Coulter, Inc.). Relative fluorescence activity was calculated as the ratio of fluorescence activity to OD₇₅₀.

**Chapter 3 Structure of the SigF1-dependent
pilA1 gene promoter and the light activation
mechanism**

3.1. Introduction

In *Synechocystis*, SigF directs the transcription of the *pilA1* gene encoding a pilin protein that constitutes Type IV pili (Flores et al., 2019). Type IV pilin is encoded by two homologous genes, *pilA1* (*synpcc7942_0049*) and *pilA2* (*synpcc7942_0048*), which are tandemly aligned in the genome. Each gene measures 435 bp, as shown in Figure 3.1. Consequently, I examined whether the Type IV pilus expression in *Synechococcus* is under the control of SigF1, a homolog of *Synechocystis* SigF.

It has been noted that cyanobacterial wild-type strains maintained in each laboratory accumulate unexpected SNPs, for example, as revealed by a detailed analysis in *Synechocystis*. A whole genome sequence analysis of *Synechococcus* also revealed that our laboratory wild-type strain (hereafter TUA), originally derived from stock in a laboratory at Tokyo University of Agriculture, harbors a mutation (C to A) in the *sigF1* gene (*synpcc7942_1510*) to generate a nonsense codon at the 146th amino acid (Q149*; Figure 3.2) (Bairagi et al., 2022; Watanabe et al., 2012). This mutation is expected to result in a truncated SigF1 protein (SigF1*) devoid of conserved region 4, which can recognize –35 regions (Figure 3.3). The *sigF1* gene in another wild-type strain (NU, derived from a laboratory stock at Nagoya University) does not include this mutation, and thus, the NU strain was used as the wild-type for *sigF1* gene analysis.

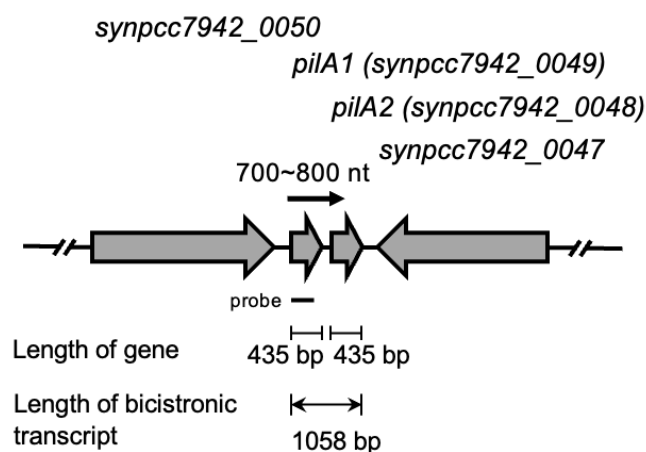


Figure 3.1 The predicted structure of the *pilA1* operon. The predicted structure of the *pilA1* operon. The labels “435 bp” under *pilA1* and *pilA2* denote the length of each gene. The length of the bicistronic transcript is 1,058 bp is shown. Because the *pilA1* transcript size was observed as 700–800 nt, the *pilA1* gene was considered a monocistronic operon. The location of the probe used in the northern blot analysis is shown.

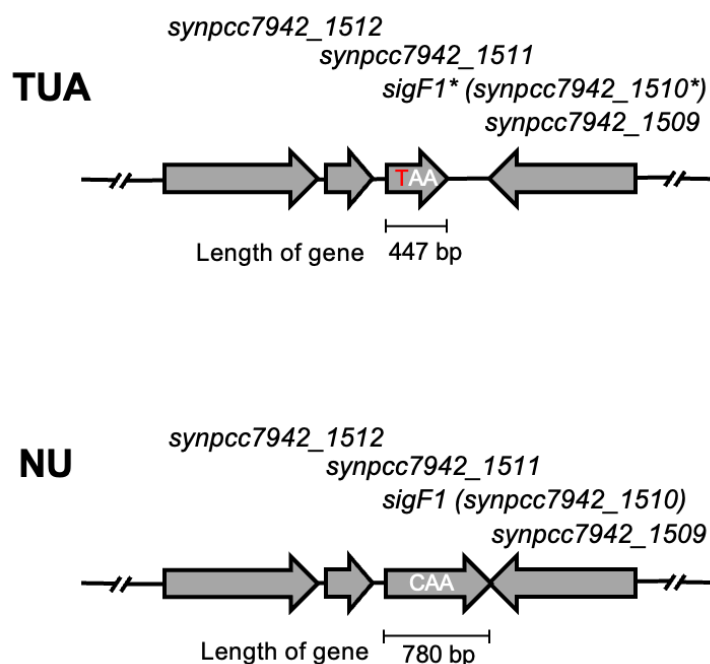
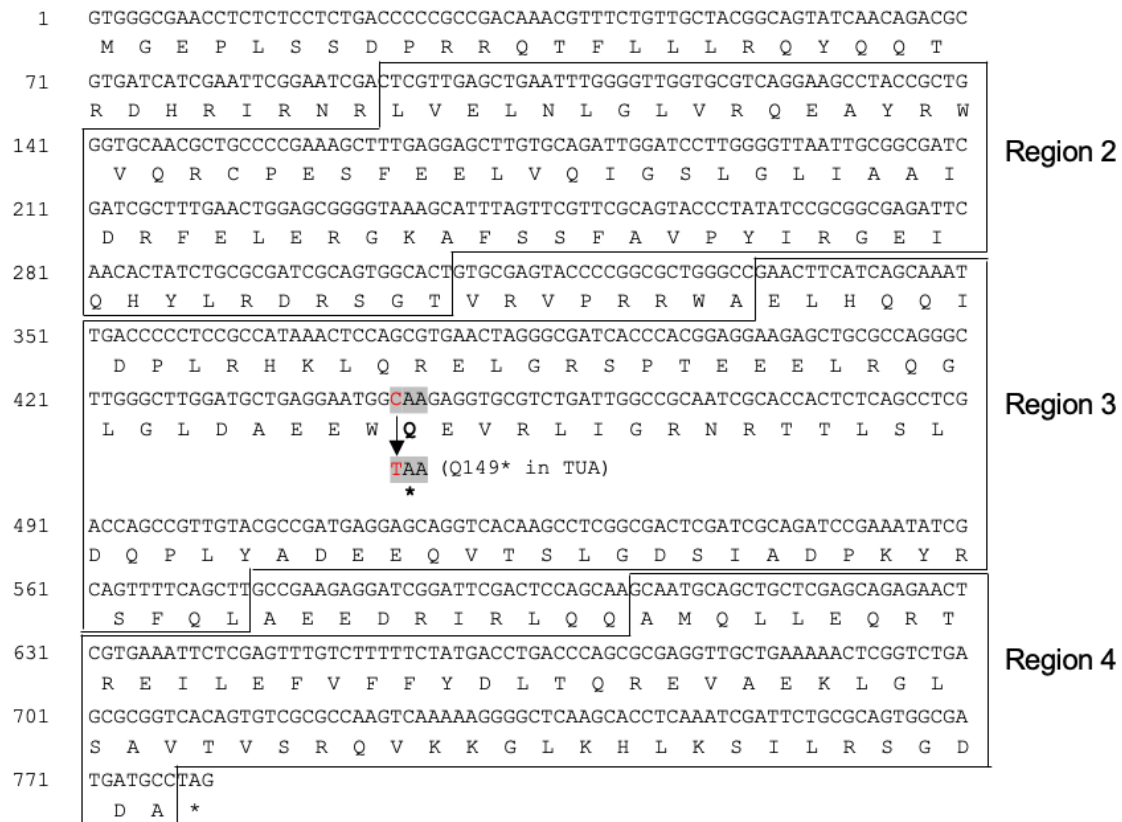


Figure 3.2 The *sigF1* gene regions of the TUA and NU strains.



The structure of the *sigF1* open reading frame

Figure 3.3 Nucleotide sequence of the *sigF1* gene. The amino acid sequence is shown below the DNA sequence. The gray area contains three bases, with the site of the single nucleotide polymorphism (SNP) marked in red. This SNP leads to a base change that results in the substitution of an amino acid with a stop codon, which is highlighted in bold. The boxed area represents the segment unique to the full-length SigF1, which includes region 4.

3.2. Results

3.2.1. Phenotypes of SigF1 deficient mutants

To determine whether both *sigF1** and *sigF1* can initiate the transcription of *pilA1*, I used TUA and NU and their *sigF1* deficient mutant. Firstly, I constructed two *sigF1* deletion mutants, TUA Δ F1SP (TUA but *sigF1::spcR*) and NUF1SP (NU but

sigF1::spcR), respectively. Because of the phenotypic correlation between SigF1 and pilin, I cultured TUA, NU, and these *sigF1*-deficient strains in liquid media for 25 days without agitation. Among these bacteria, I observed SigF1 deficient strains easily precipitated at the bottom in the liquid culture without agitation (Figure 3.4), as is consistent with a recent independent observation (Zedler et al., 2023).

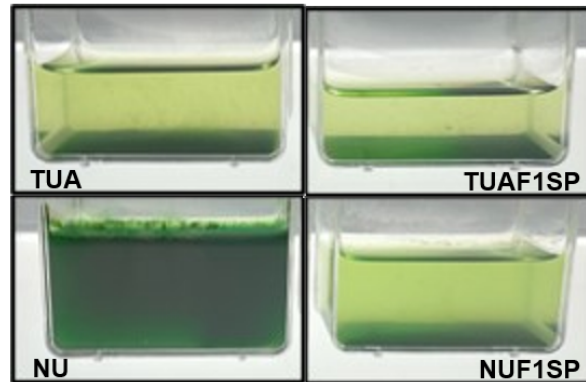


Figure 3.4 The phenotypes of TUA and NU and the relevant *sigF1* mutants. Cells were cultivated without agitation under constant light at 30°C for 25 days. *sigF1* mutants showed sedimentation at the bottom.

3.2.2. The *pilA1* operon transcripts accumulate under illumination dependent on SigF1

Previous research showed that pilin is usually produced under light, and I first studied the relationship between *pilA1* transcription and illumination. To examine the effect of *sigF1* on *pilA1* expression, I conducted the northern blotting as described in Section 2.5. TUA, NU, and their *sigF1* deficient strains were cultured in a liquid culture medium in light condition for 24 h, dark-adapted for 18 h, and then transferred to light conditions. Cells were collected according to the time course shown in Figure 3.5A. Northern blotting analysis using *pilA1* ORF sequence as a probe revealed that this gene is transcribed as a transcript of around 700–800 nt. Considering that *pilA1* and *pilA2* are

each 435 bp in length (Figure 3.1), the transcript I detected in northern blotting likely includes only *pilA1* ORF. Therefore, *pilA1* appears to be transcribed as a monocistronic operon rather than a bicistronic one since co-transcription of *pilA1* and *pilA2* would result in a transcript length of 1058 bp. Northern blotting analysis showed that the expression of the *pilA1* gene was positively regulated by *sigF1*, and the expression was activated by light (Figure 3.5).

To further figure out if the *pilA1* transcription relates to photosynthesis or not, I used photosynthesis inhibitors, DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) and DBMIB (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone) to confirm. DCMU exerts its function by inhibiting the electron transport chain in photosynthesis. Specifically, it binds to the plastoquinone binding site of PSII, blocking the transfer of electrons from the reaction center of PSII to PQ and making the PQ pool oxidized. DBMIB functions by interfering with the electron transport chain in photosynthesis. It binds to the PQ binding site, blocking the transfer of electrons from plastoquinone to cytochrome *b₆f* complex. This inhibits the electron transport chain and leads to PQ pool reduction. It was found that this activation was strongly inhibited by the presence of DBMIB but not by DCMU. These results suggest that light-induced changes in the PQ redox status may be involved in the promoter activation (Figure 3.6).

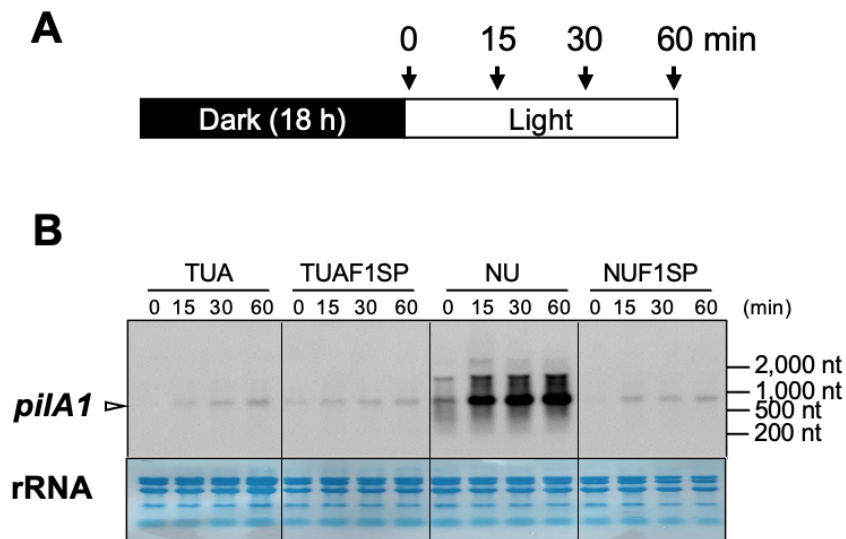


Figure 3.5 Transcription of the *pilA1* gene is activated by illumination in a manner dependent on SigF1. (A) Experimental scheme for the dark-to-light shift experiments. Cells were cultivated under illumination with an initial OD₇₅₀ of 0.15 for 24 h, dark-adapted for 18 h, and then transferred to light conditions. Cells were collected at 0, 15, 30, and 60 min. (B) Analysis of *pilA1* transcripts in TUA and NU and their *sigF1* deficient mutants in the dark-to-light shift. Total RNAs were extracted, and 3 μ g RNA per lane was analyzed by northern analysis. The *pilA1* transcript was detected by a probe made by primers listed in Table S3. rRNA (ribosomal RNA) is shown as the loading control. The bars on the right indicate RNA molecule sizes. All experiments were replicated at least three times to ensure reproducibility.

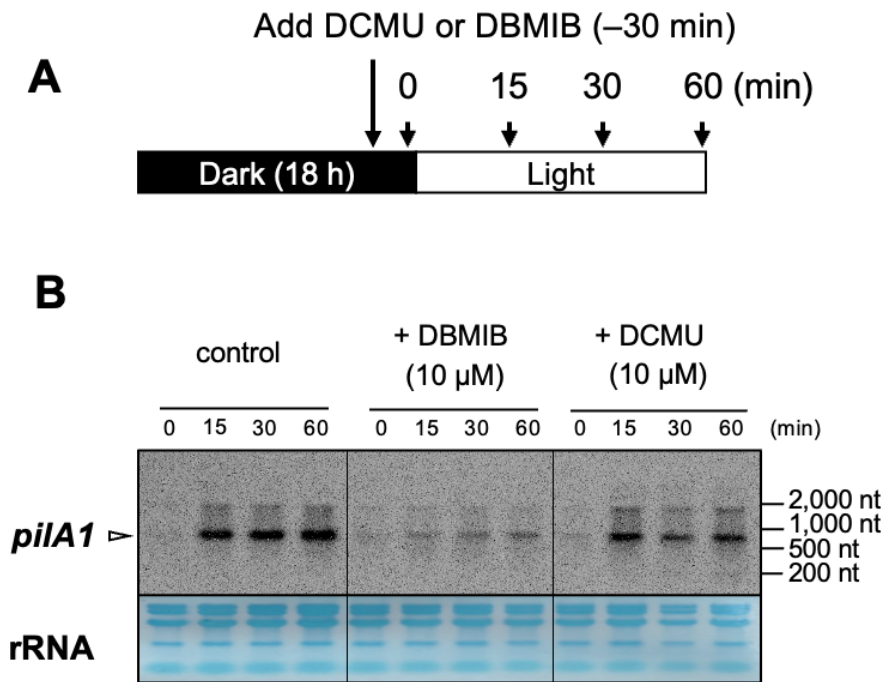


Figure 3.6 Transcription of *pilA1* in the presence of DCMU and DBMIB. (A)

Experimental scheme for the DCMU and DBMIB experiments. Cells were cultivated under illumination with an initial OD₇₅₀ of 0.15 for 24 h. Following this, the cells were adapted to dark conditions for 18 hours to stabilize their response to light deprivation. Prior to re-exposure to light, the cells were treated with DCMU. Samples were collected at four time points after treatment with DCMU. (B) Analysis of *pilA1* transcripts in TUA and NU and their *sigF1* mutants in DL+DCMU. Total RNAs were extracted, and 3 μ g RNA per lane was analyzed by northern analysis. The *pilA1* transcript was detected by a probe made by primers listed in Table S3. rRNA (ribosomal RNA) is shown as the loading control. The bars on the right indicate RNA molecule sizes. All experiments were replicated at least three times to ensure reproducibility.

3.2.3. Identification of TSS of *pilA1*

To analyze the *pilA1* promoter structure in detail, I constructed a fusion of the *pilA1* promoter with the mNG reporter gene, which encodes the green fluorescent protein (GFP), as described by Shaner et al., 2013. Here, the *pilA1* promoter region from the –200 to –1 positions (with respect to the 1st A of the *pilA1* initiation codon) was fused to the 5' region upstream of the mNG ribosome binding site and ORF (Figure 3.7). Following the procedure outlined in Section 2.2.1, I constructed plasmids for the PAG and PAGF1KM reporter strains and introduced them into a neutral site in the genomes of NU and NUF1KM (NU but *sigF1::kanR*), respectively.

To evaluate this monitoring system, I implemented the fluorescence activity assay described in Section 2.11 and observed the changes in GFP fluorescence during the transition from dark to light. I first determined that the transcription of mNG in the PAG strain is dependent on light activation and is dependent on SigF1 (Figure 3.8). However, its fluorescence activity remained almost unchanged from dark to light conditions (Figure 3.8B). Thus, while GFP fluorescence is not suitable for time-course analysis, it effectively monitors promoter SigF1 dependence.

After confirming the functionality of the PAG fluorescent reporter strain, I determined the transcription start site (TSS) of the *pilA1* promoter using primer extension analysis. Fluorescently labeled oligonucleotide primers targeting the mNG ORF were utilized (Table S3), following the primer extension procedure detailed in Section 2.10. This analysis identified a unique SigF1-dependent site at position –31, relative to the first 'A' of the *pilA1* start codon as +1 (Figure 3.9).

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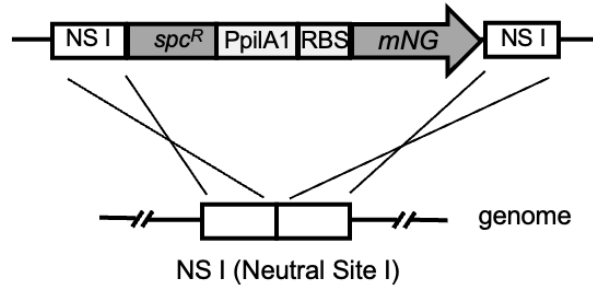
1  TCCTCAGTACCTGCCGTATCAATCAACAGACTGGCTGTTTCCTGAGTGACTTGTTCAGTGCTGCACTGA
71  TTTGCAGGACTGCTTTTTTGATCAGGAGAAGCCCAATGCGGACGGAGCCAAAGAAGAGCCATGCGGAAATT
                                +1
141  TTCAGGAACCTGAAATAAAAGCCTGCTGTAGGGTAAGTTACTTCAGAACAGCTCTCTTGCTGAGGATC
211  TCCTGACCCGGGTCTCAAGTAGGAGATTAATTCAATGGTGTCTAAAGGTGAGGAGGATAACATGGCTAG
                                M V S K G E E D N M A S
281  TTTACCGGCCACTCACGAATTGCACATCTTTGGGAGTATTAAACGGCGTCGACTTTGATATGGTCGGGCAA
                                L P A T H E L H I F G S I N G V D F D M V G Q
351  GGCACCGGAATCCTAACGACGGCTATGAGGAAGTGAACCTCAAGAGTACGAAGGGCGACCTCCAATTTT
                                G T G N P N D G Y E E L N L K S T K G D L Q F
421  CTCCTTGGATTCTCGTACCACATATTGGATACGGTTTTTACCAGTACCTGCCGTACCCCGACGGCATGTC
                                S P W I L V P H I G Y G F H Q Y L P Y P D G M S
491  CCCCTTTCAGGCGCAATGGTGGATGGCAGTGGCTATCAAGTGCACCGTACCATGCAGTTTGAAGATGGG
                                P F Q A A M V D G S G Y Q V H R T M Q F E D G
561  GCGTCTTTGACCGTCAATTACCGCTATACCTATGAAGGGTCCCATATTAAAGGTGAAGCGCAAGTGAAGG
                                A S L T V N Y R Y T Y E G S H I K G E A Q V K
631  GTACGGGGTTCCCGCGGATGGACCTGTATGACCAATTCGCTCACGGCCGCTGATTGGTGTGGTCGAA
                                G T G F P A D G P V M T N S L T A A D W C R S K
701  GAAGACCTATCCCAACGATAAGACCATCATCAGCACGTTTAAATGGAGCTACACAACCGGTAATGGTAAA
                                K T Y P N D K T I I S T F K W S Y T T G N G K
771  CGTTATCGCAGCACCGCACGCACTTACACGTTTCGCCAAACCCATGGCCGCAATTACCTGAAAAATC
                                R Y R S T A R T T Y T F A K P M A A N Y L K N
841  AACCCATGTATGTTTTTCGGAACAGAATTGAAACATTCAAAAGTGAAGTAAATTTCAAAGAATGGCA
                                Q P M Y V F R K T E L K H S K T E L N F K E W Q
911  GAAAGCCTTACAGATGTTATGGGTATGGATGAATTATATAAATAA
                                K A F T D V M G M D E L Y K *

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The sequence of the *PpilA1-mNG* fusion

Figure 3.7 Nucleotide sequence of *PpilA1-mNG*. The underlining shows the upstream sequence of the *pilA1* gene fused with the *mNG* gene. The amino acid sequence of mNG is shown below the DNA sequence. The grey area indicates the primer location for primer extension analysis. The TSS is marked in red with +1.

A



B

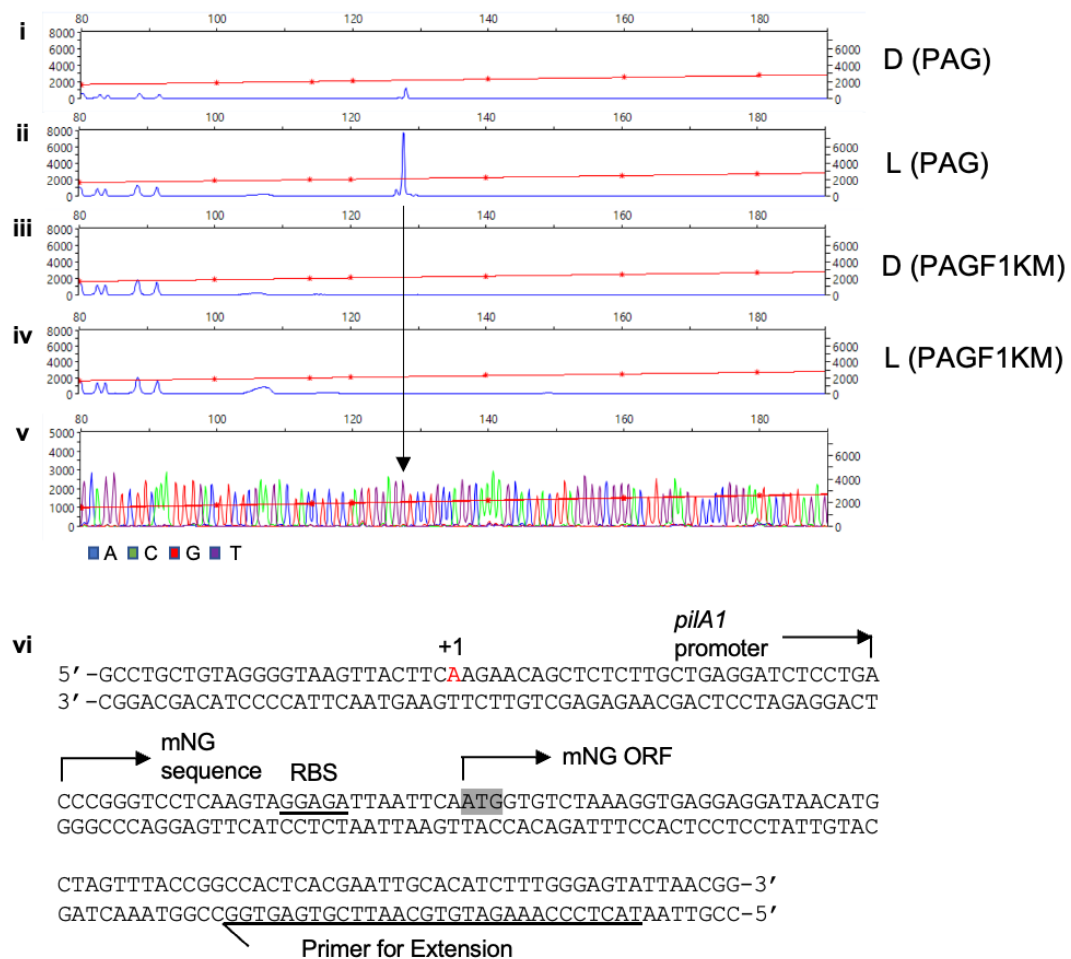


Figure 3.9 Analysis of the *pilA1* promoter structure. (A) Construction of *pilA1* promoter-activity reporter strains. The 200 bp 5'-upstream region (−200 to −1 relative to the ORF start A) of the *pilA1* ORF, including the ribosome binding site (RBS) for the

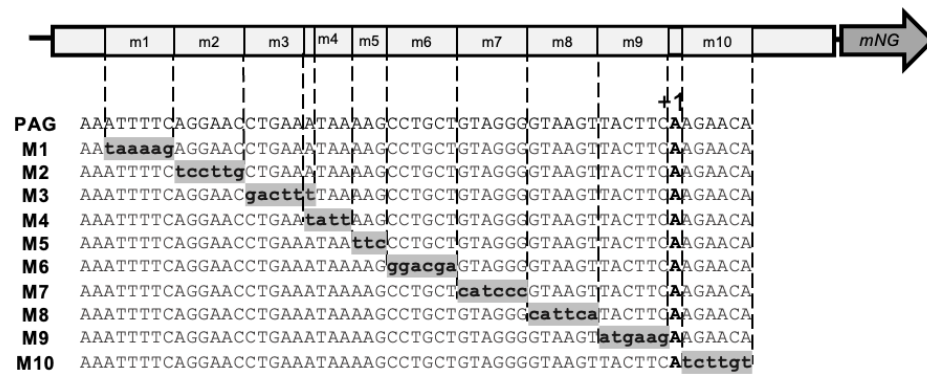
translation, was fused to the *mNG* ORF. This plasmid was subsequently transformed into NU and F1KM, constructing the PAG and PAGF1KM strains. (B) Primer extension analysis. Cells grown under standard conditions were used for RNA preparation at an OD₇₅₀ of approximately 0.5. Primer extension products and the sequence ladder were analyzed after the addition of the size marker, positions indicated by red asterisks, to compare and estimate the product sizes. From top to bottom: **i.** PAG, dark-adapted for 18 h; **ii.** PAG, grown under illumination; **iii.** PAGF1KM, dark-adapted for 18 h; **iv.** PAGF1KM, grown under illumination; **v.** Sequence ladder to compare and estimate the sizes of the primer extension products; and **vi.** The DNA sequence of the *pilA1* promoter-*mNG* fused region. The arrow indicates the location of the primer for primer extension.

3.2.4. The critical sequence of the *pilA1* promoter recognized by SigF1 in

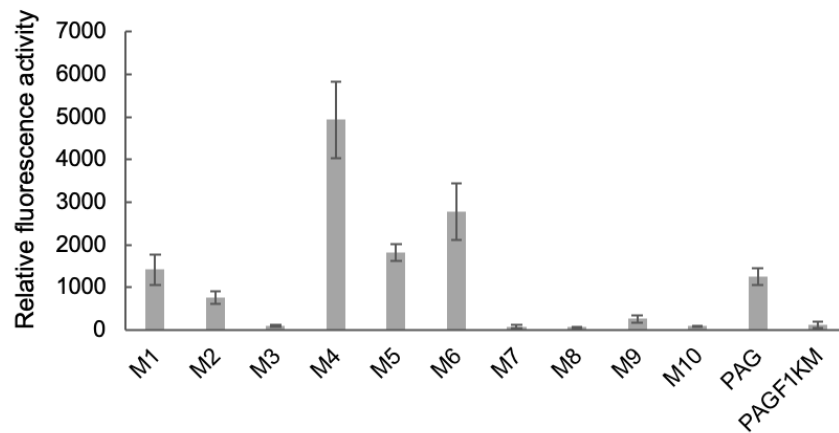
Synechococcus

To identify sequence elements required for promoter activity, scanning mutagenesis analysis was performed on the 5' upstream sequence of the TSS. As shown in Figure 3.10A, I introduced a series of 3-, 4-, or 6- base pair substitutions in the target region and examined the fluorescence of the reporter strains. The results showed that although the fluorescence activity levels of the M1, M2, and M4–M6 mutants remained similar or considerably increased compared with the PAG strain, the fluorescence activities of the M3 and M7–M9 mutants almost disappeared (Figure 3.10B). Thus, it is suggested that critical sequences for transcription exist within m3 and m7–m10. I expected the –35 and –10 elements of the SigF1-dependent promoter (G/C)TGA and GGGTAA (Figure 3.10C).

A



B



C

-35 -10 +1
 GGAAC**CTGAAA**TAAAGCCTGCTGTAG**GGGTAA**GTTACTTCA *pilA1*

Figure 3.10 Effects of 3-, 4- or 6-base-pair substitutions on *pilA1* promoter region activity. (A) Sequences of the mutated promoter region. Modifications in nucleotide sequences are highlighted in lowercase. (B) Fluorescent activities of reporter strains. Cells grown under standard conditions were sampled at an OD₇₅₀ of approximately 0.5. Relative fluorescence activities are the means of three independent measurements and are normalized by OD₇₅₀. Error bars indicate standard deviations. (C) The TSS (+1) is indicated in bold. The proposed consensus promoter sequences, the -10 and -35 boxes, are shown in grey.

3.2.5. Interaction between the SigF1-containing RNAP and the *pilA1* promoter

To check whether SigF1-containing RNAP directly recognizes the *pilA1* promoter, I constructed a modified TUA strain with a carboxyl-terminal FLAG-tagged SigF1 protein and replaced the chromosomal *sigF1* gene to make strain F1F. Similarly, I also selected the *rpoC2* gene, which encodes the subunit β' of the RNAP core enzyme and constructed a modified NU strain with a carboxy-terminal FLAG-tagged RpoC2 protein and replaced the chromosomal *rpoC2* gene to make strain R2F. I constructed the plasmids as described in the experimental procedures of Section 2.2.2 and subsequently transferred the target fragments into *Synechococcus* following the transformation protocol outlined in Section 2.4.

In the F1F strain, the northern blotting analysis (Section 2.5) demonstrated that *pilA1* transcripts accumulated in a light-dependent manner, indicating that FLAG-tagged SigF1 protein complements the original SigF1 function (Figure 3.11A). Immunoblot analysis (Section 2.7) using α -FLAG antibody showed that SigF1 protein and RpoC2 protein continued to accumulate in cells regardless of light conditions (Figure 3.11B and Figure 3.11C). Therefore, light-driven *pilA1* promoter activation is unlikely to be the result of SigF1 protein accumulation.

The interaction between SigF1-containing RNAP and the *pilA1* promoter was detected by ChIP-qPCR (chromatin immunoprecipitation quantitative PCR) analysis (Section 2.9) in dark-to-light conditions. By using the primer sets located in the *pilA1* promoter region (A1), the end of the *pilA1* ORF (A2), and the *rpoD1* (*synpcc7942_0649*) promoter region (D1), I checked the interaction of SigF1 with these sites (Figure 3.12A). The results clearly show that specific SigF1-promoter interactions are more pronounced under dark conditions than after light (Figure 3.12B). This suggests that SigF1-containing RNAP is recruited to the *pilA1* promoter under dark conditions and remains there until the light signal occurs. The interaction of RpoC2

with the promoter also showed that not only SigF1 but also the RNAP holoenzyme is recruited to the promoter under dark conditions (Figure 3.12D). Following transcription initiation, SigF1 rapidly dissociates from the *pilA1* gene while the RNAP core enzyme proceeds along the gene, a process consistent with transcription elongation (Figure 3.12C and D).

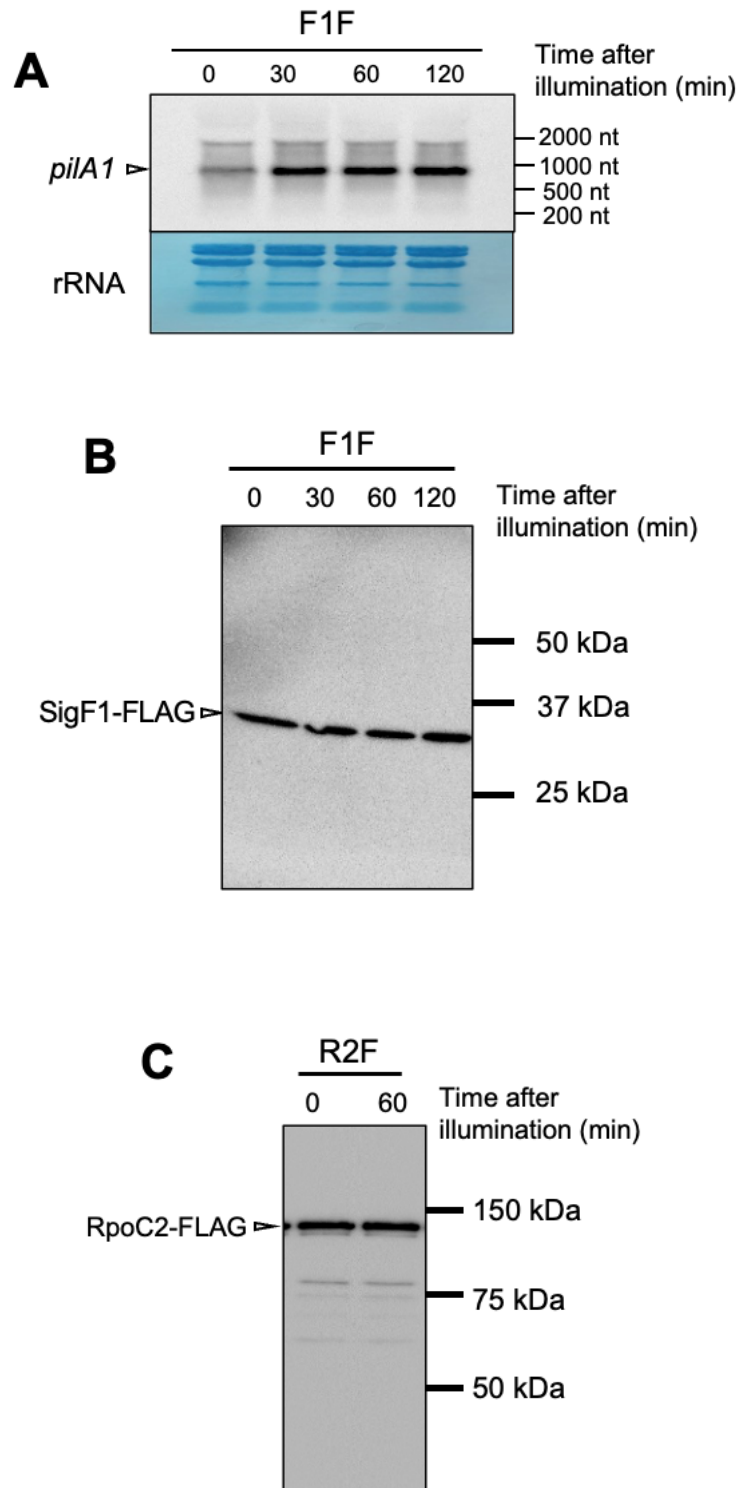
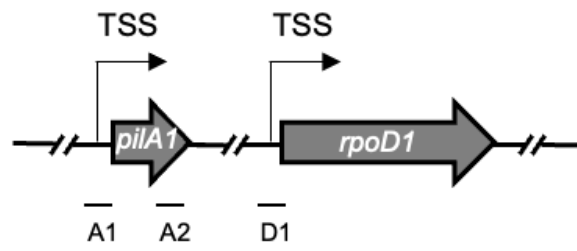
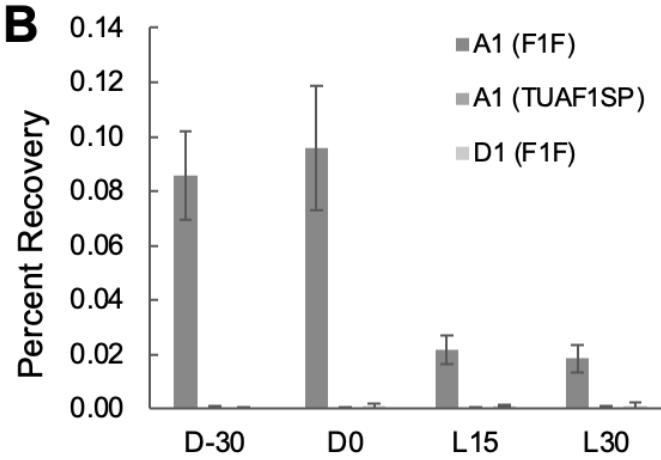


Figure 3.11 Analysis of F1F and R2F strains. (A) Accumulation of *pilA1* transcripts during the dark-to-light shift. After cultivating the cells to OD₇₅₀ of approximately 0.5, total RNAs were extracted during the indicated light regime, and 3 µg of RNA was used for each analysis. The *pilA1* transcript was detected by the *pilA1* probe made by primers listed in Table S3. rRNA (ribosomal RNA) is shown as the loading control. The bars on the right indicate RNA molecule sizes. (B) SigF1 protein accumulation during the dark-to-light shift. The graph depicts the consistent SigF1 expression levels before and after the onset of illumination. The SigF1-FLAG protein (33.28 kDa) was detected using α-FLAG antibody. The bars on the right indicate marker sizes. (C) Accumulation of the RpoC2 protein in NU during the dark-to-light shift. The RpoC2-FLAG protein (146.08 kDa) was detected using α-FLAG antibody, with the right bars indicating the marker ladder sizes.

A

Locations of qPCR primer sets

B

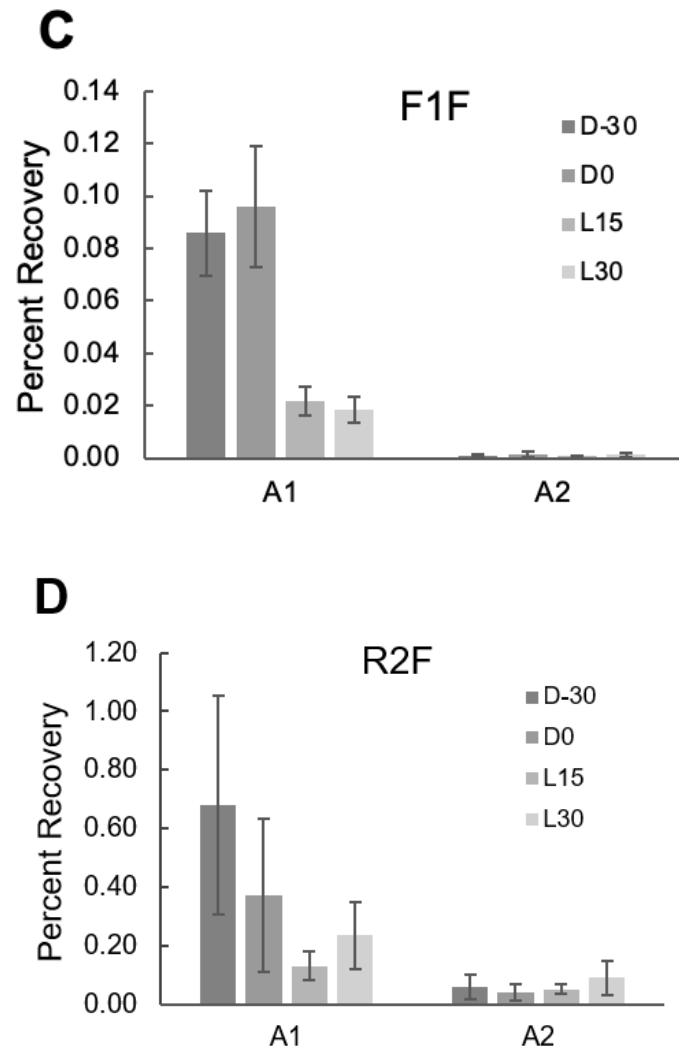


Figure 3.12 ChIP-qPCR analysis of the *pilA1* gene with FLAG-tagged SigF1 and RNAP. (A) Locations targeted in the ChIP-qPCR analysis are detailed. A1 and A2 mark positions within the promoter region and the 3'-end of the *pilA1* ORF, respectively, while D1 identifies positions within the *rpoD1* (*synpcc7942_0649*) promoter region. Primers used for ChIP-qPCR are detailed in Table S3.

(B) Interaction between FLAG-tagged SigF1 and the *pilA1* promoter was analyzed by ChIP-qPCR during the dark-to-light shift. Whole-cell extracts were prepared from the F1F and TUA1SP strains grown under the same conditions as in Figure 3.5A. Cells were collected at the time point 30 min before transferring to light (D-30), and 0 min (D0), 15 min (L15), and 30 min (L30) after transferring to light. The levels of DNA immunoprecipitated by α -FLAG antibody for the *pilA1* (A1) and *rpoD1* (D1) promoter

regions were quantified by qPCR as the percent recovery relative to the total input DNA and expressed as the means of three independently repeated samplings. Error bars indicate standard deviations.

(C) The interactions between the FLAG-tagged SigF1 and the *pilA1* promoter (A1) or downstream region (A2) were analyzed by ChIP-qPCR analysis with α -FLAG antibody. The F1F strain was cultivated and analyzed under the same conditions as in (B). The percent recovery relative to the total input DNA was calculated and expressed as the means of three independent repeated samplings. Error bars indicate standard deviations.

(D) The interactions between the FLAG-tagged RNAP and the *pilA1* promoter (A1) or downstream region (A2) were analyzed by ChIP-qPCR analysis with α -FLAG antibody. The R2F strain was analyzed under the same conditions as in (B). The percent recovery relative to the total input DNA was calculated and expressed as the means of three independent repeated samplings. Error bars indicate standard deviations.

3.3. Discussion

This chapter establishes that SigF1 plays a crucial role in the transcription of *pilA1*. During the dark-to-light transition, SigF1 containing RNA polymerase is recruited to the *pilA1* promoter, suggesting a role in transcriptional activation. Consequently, regulatory activities likely occur post-recruitment. One explanation is that certain activation mechanisms necessary for transcription are triggered post-illumination (Figure 3.13A). Alternatively, it is possible that some aspects of transcriptional activation are inhibited in the dark, and this inhibition is alleviated upon exposure to light (Figure 3.13B). Similarly, in *E. coli*, transcriptional activation involves the activator protein interacting with RNAP post-promoter recruitment to induce conformational changes necessary for transcription initiation. Furthermore, SigF in *Synechocystis* and SigF1 in *Synechococcus* exhibit functional conservation. Although SigF1 does not recognize the target promoter sequence of SigB, the structural similarities between SigB and SigF suggest that the activity of SigF1 may also be modulated by the combined action of anti-sigma and anti-anti-sigma factors, such as homologs of RsbW and RsbV. The role of these factors in regulating SigF1 remains unclear, necessitating further research to clarify these mechanisms.

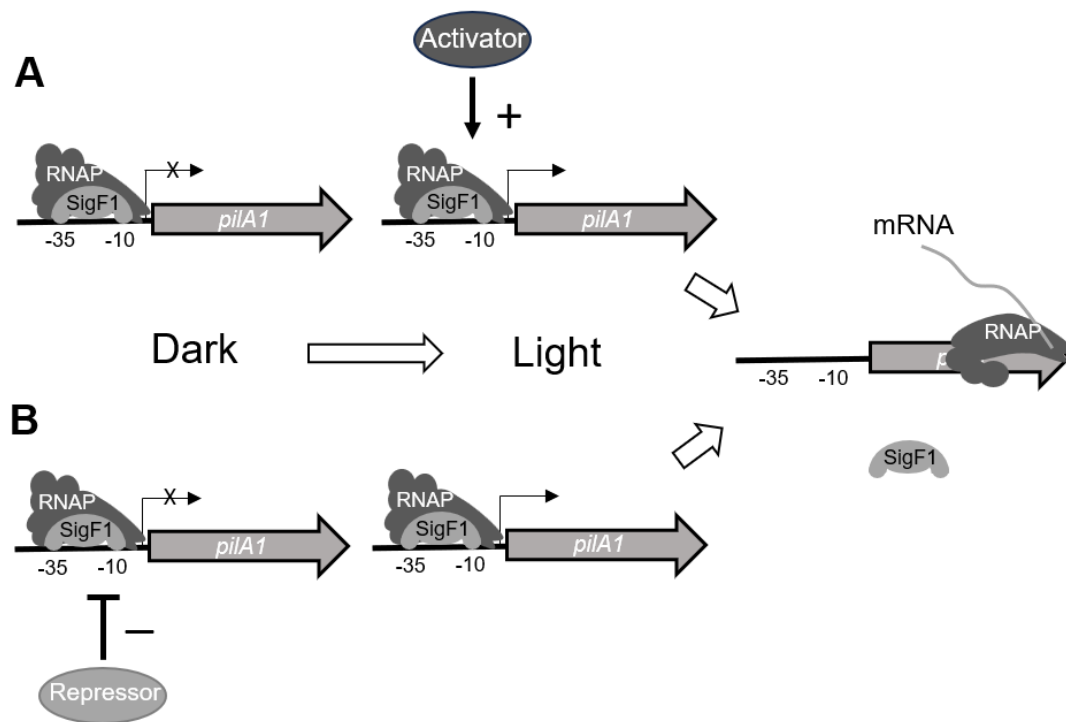


Figure 3.13 Hypothetical models for the activation of *pilA1* transcription by SigF1 during a dark-to-light shift. Two possibilities are considered to explain the obtained result. (A) Under dark conditions, RNAP is recruited to the promoter. However, the initiation of transcription requires further activation by an activator, which is unknown and functions dependent on light. (B) RNAP is recruited to the promoter under the dark. However, an unknown repressor inhibits a transcriptional initiation step after promoter recruitment, which is released by illumination.

Chapter 4 Exploring the Regulatory Roles of SigF1 in Gene Expression

4.1. Introduction

In Chapter 3, I demonstrated that the transcription initiation of the Type IV pilus gene, *pilA1*, is directly dependent on SigF1. To date, little research has been conducted on other genes that may also be regulated by SigF1. To explore this, I constructed the F1OX strain, which overexpresses SigF1, and utilized RNA-seq to examine changes in the transcriptome before and after IPTG (Isopropyl β -D-1-thiogalactopyranoside) induction. After categorizing these downstream genes, I selected representative ones for further investigation. In this chapter, I examined the regulatory relationship between SigF1 and heat-shock-related gene *hspA*.

4.2. Results

4.2.1. Confirmation of SigF1 overexpressed strain, F1OX

To investigate other downstream genes regulated by SigF1, I constructed the F1OX strain that overexpresses SigF1, as described in Section 2.2.3. Initially, I inserted the sigF1 ORF fragment into the overexpression vector pNSEI to construct the plasmid pNSEI-F1 (Figure 2.3). Subsequently, as detailed in section 2.4, I employed natural transformation to introduce the plasmid into TUA. The successful construction of the strain was verified by comparing the expression of SigF1-FLAG before and after the addition of IPTG. To confirm the successful overexpression of the SigF1-FLAG fusion protein, I performed a western blotting analysis using an anti-FLAG antibody. The results confirmed that IPTG successfully induced SigF1 overexpression in TUA (Figure 4.1). The overexpression strain F1OX is now prepared for further studies to explore the role of SigF1 in gene expression regulation.

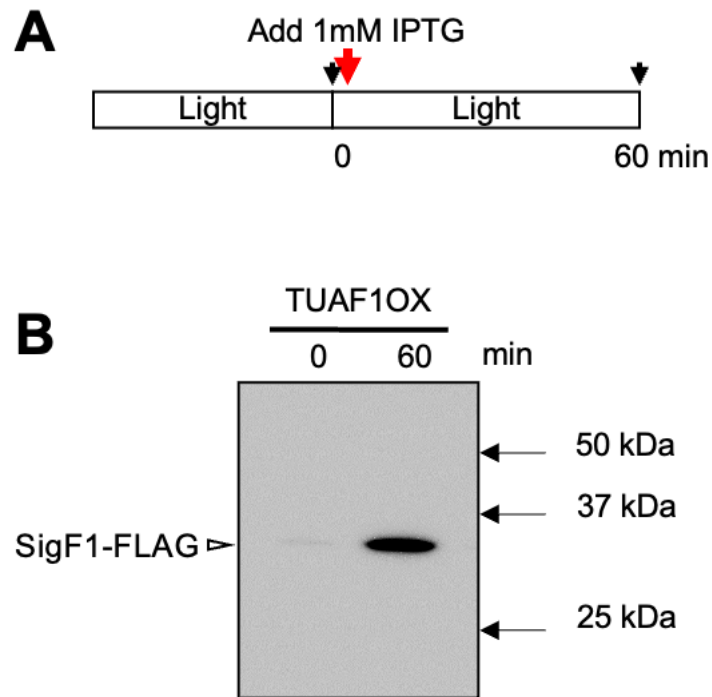


Figure 4.1 The expression of SigF1-FLAG is induced by IPTG. (A) Experimental scheme for the IPTG induction experiments. Cells were cultivated under illumination with an initial OD₇₅₀ of 0.15 for 24 h to an OD₇₅₀ of approximately 0.5. Cells were collected for analysis before and 60 minutes after adding IPTG. (B) SigF1 protein accumulation after adding IPTG. The SigF1-FLAG protein (33.28 kDa) was detected using α -FLAG antibody. The bars on the right indicate marker sizes.

4.2.2. Analysis of downstream genes of SigF1 by RNA-seq

To identify the genes regulated by SigF1, RNA-seq was utilized to examine the transcriptomic changes in the F1OX strain before and after IPTG induction. Total RNA samples were extracted from F1OX under both conditions, following the protocols outlined in Section 2.6. The results delineated two principal categories of genes that were upregulated by SigF1: those involved in pilus assembly and those associated with the heat shock response (Figure 4.2). Specifically, pilus assembly genes such as *pilT*, *pilB1*, and *pilC* were identified. In the category of heat shock response, genes, including *hspA*, *hspG*, and *groEL2*, were upregulated. Notably, overexpression of SigF1 led to a significant increase in *hspA* expression, establishing a previously unreported link between SigF1 and heat shock response genes.

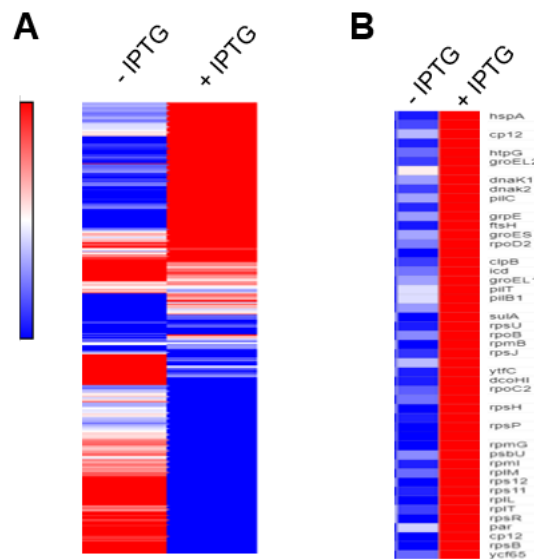


Figure 4.2 Heatmap of gene expression in F1OX by IPTG induction. (A) This heatmap displays the transcriptional responses of the F1OX strain to IPTG induction. Each row represents an individual gene, and the columns compare conditions before and after IPTG induction. The expression levels are depicted in a color gradient from blue (low expression) to red (high expression), with the scale provided in the legend. (B) Top 50 genes are annotated for easy reference.

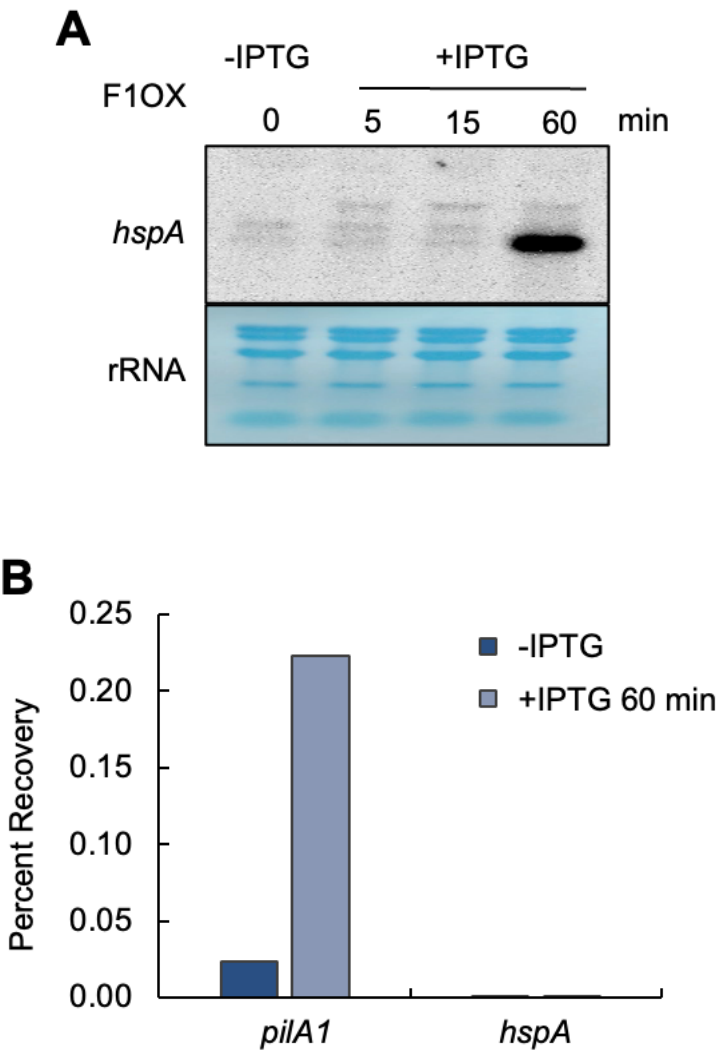
4.2.3. SigF1 did not affect *hspA* transcription directly

hspA is a gene related to heat shock response regulated by the Hik2/Hik34-Rre1 components of the two-component system. When cells undergo heat shock and experience PQ peroxidation or PQ overreduction, Rre1 becomes phosphorylated. This phosphorylated Rre1 then binds to the upstream region of the *hspA* promoter to activate transcription.

To determine whether SigF1 directly regulates *hspA* transcription, I initially conducted a Northern blotting analysis on strains overexpressing *sigF1*, as described in Section 2.5. The results indicated that *hspA* transcription commenced 60 minutes after IPTG induction, aligning with the RNA-seq data (Figure 4.2). This observation suggests that overexpressed SigF1 influences *hspA* transcription (Figure 4.3A); however, the effect might not be direct, as *hspA* expression was not detected until one hour after IPTG addition.

To investigate whether SigF1 interacts directly with the *hspA* promoter, I employed ChIP-qPCR, following the methodology outlined in Section 2.9. This involved extracting total cells from fixed samples before and after IPTG addition, followed by cell lysis, chromatin shearing, immunoprecipitation, washing, and decross-linking. I used specific primer sets for the *pilA1* and *hspA* promoter regions for the qPCR analysis. As previously found in Chapter 3, the results confirmed that SigF1 binds to the *pilA1* promoter. However, no interaction between SigF1 and the *hspA* promoter was detected 60 minutes after IPTG induction, indicating that SigF1 does not directly bind to the *hspA* promoter to initiate transcription (Figure 4.3B). Additionally, considering *hspA* transcription was regulated by phosphorylated Rre1, I followed the protocol described in Section 2.8 and performed the Phos-tag to check the Rre1 phosphorylation status in SigF1 overexpressed condition. The result showed overexpression of SigF1 did

not result in phosphorylation of Rre1 (Figure 4.3C). This suggests that SigF1 contributes to *hspA* transcription through an alternative mechanism.



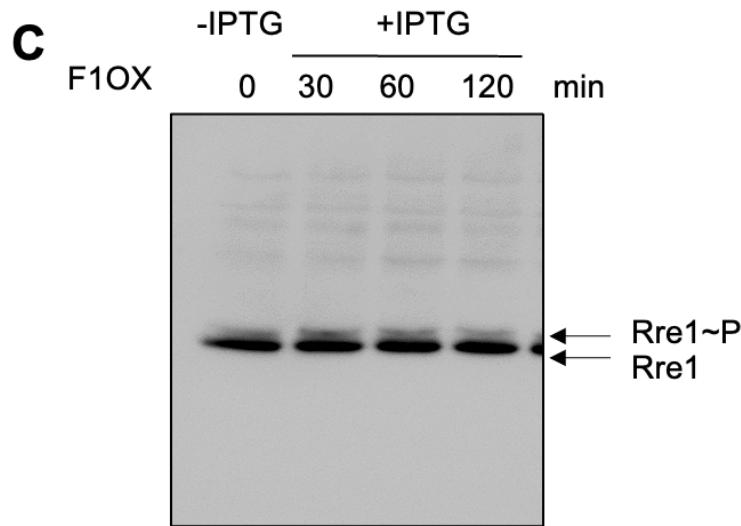


Figure 4.3 Analyze the interaction of SigF1 with *hspA* promoter. (A) Analysis of *hspA* transcripts in F1OX before and after adding IPTG. Total RNAs were extracted, and 3 μ g RNA per lane was analyzed by northern analysis. The *hspA* transcript was detected by a probe made by primers listed in Table S3. rRNA (ribosomal RNA) is shown as the loading control. (B) Interaction between FLAG-tagged SigF1 and the *hspA* promoter was analyzed by ChIP-qPCR before and after adding IPTG. Whole-cell extracts were prepared from F1OX strains grown under illumination with an initial OD₇₅₀ of 0.15 for 24 h to an OD₇₅₀ of approximately 0.5. Cells were collected at the time point 0 min before and 60 min after adding IPTG. The levels of DNA immunoprecipitated by α -FLAG antibody for the *pilA1* and *hspA* promoter regions were quantified by qPCR as the percent recovery relative to the total input DNA and expressed as the means of three independently repeated samplings. Error bars indicate standard deviations. (C) Phos-tag Gel Analysis of Protein Phosphorylation. This figure presents a Phos-tag gel electrophoresis used to detect the phosphorylation states of Rre1 before and after IPTG induction. Upper-bands indicate phosphorylated Rre1, and lower-bands correspond to unphosphorylated Rre1. The result shows that Rre1 was unphosphorylated after IPTG induction.

4.3. Discussion

In this chapter, I employed RNA-seq to investigate the genes potentially regulated by SigF1. This analysis not only confirmed the role of SigF1 in upregulating pili-related genes but also revealed its influence on the expression of heat shock-related genes. Importantly, while SigF1 does not directly interact with the *hspA* promoter, the data suggest that SigF1 may be involved in a more intricate regulatory mechanism affecting *hspA* expression. The absence of Rre1 phosphorylation following SigF1 overexpression further supports the hypothesis that SigF1 influences gene expression through secondary regulatory pathways, potentially involving other transcription factors or signaling mechanisms.

Previous studies have documented that *hspA* expression is also modulated by the circadian regulator, Kai, with *hspA* levels initially increasing and then decreasing during the dark phase. Conversely, this pattern is reversed in Kai mutant strains, illustrating the complex regulation of *hspA* (Iwasaki et al., 2011). The involvement of SigF1 in this regulatory mechanism indicates that its regulatory scope may extend to interactions with other genes or sigma factors, contributing to the dynamic regulation of *hspA*.

The complex nature of *hspA* regulation, impacted by both environmental cues and intrinsic cellular rhythms, underscores the sophistication of cyanobacterial gene regulation. The findings here highlight the need for further investigation to fully elucidate the mechanisms through which SigF1 modulates *hspA* transcription and determine whether similar regulatory frameworks influence other stress-responsive genes. Such studies are essential to deepen our understanding of sigma factor functionality and the regulatory networks within cyanobacteria.

Chapter 5 Investigating the Role of SigF2 in Gene Regulation and Stress Response in *Synechococcus*

5.1. Introduction

As photosynthetic organisms, Cyanobacteria rely on photosynthesis, making the circadian rhythm crucial for coordinating gene expression within the organism. At the core of this rhythm is the Kai clock, regulated by a series of responses and numerous transcription factors, with RpaA serving as a principal transcription factor. As detailed in the introduction, SigF2 is regulated by RpaA and is part of a cascade of sigma factors (Fleming and O'Shea, 2018). SigF2 is induced in the dark, binding to the promoters of downstream genes to initiate transcription (Ishi, 2017).

Previous DNA microarray analysis of the genome revealed that 7% of the genes exhibit dark-induced transcription dependent on the circadian rhythm (Hosokawa et al. 2011). Among these, the dark-inducible gene *digB* was found to be regulated by SigF2, with its expression increasing as light transitions to darkness (Figure 5.1) (Kashima, 2014). Another gene associated with SigF2 is *ccpB*, whose function remains unidentified, though it shares sequence similarity with CP12 (*synpcc7942_0361*), a key player in carbon assimilation. Notably, CP12 is expressed in light conditions, whereas *ccpB* is induced in darkness, suggesting that *ccpB* function might complement that of *cp12* (Ishi, 2017). SigF2 directly controls the transcription of *ccpB*. The role of SigF2, like other F-type sigma factors, SigF1 analyzed in the first two chapters, remains largely unexplored.

In this chapter, I continue the analysis of the SigF2 function. Firstly, RNA sequencing was employed to investigate the transcriptome changes before and after overexpression of *sigF2*. Subsequently, primer extension was used to identify the TSS recognized by SigF2, and a putative promoter sequence dependent on SigF2 was proposed.

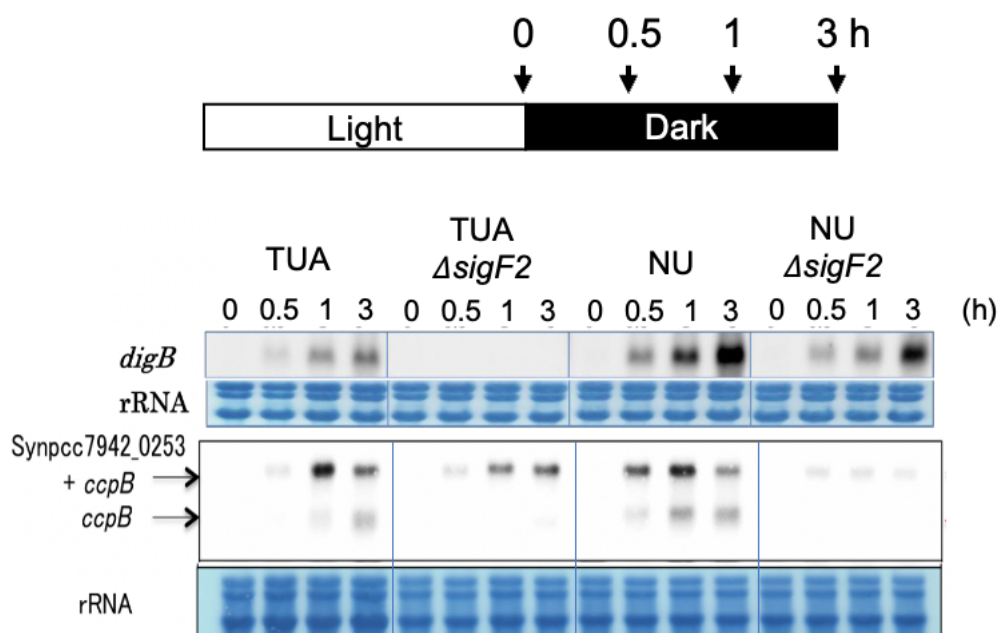


Figure 5.1 Expression of SigF2 downstream genes in TUA and NU (This figure was adapted from Kashima, Thesis, 2014 and Ishi, Master Thesis, 2017) Previous study showed SigF2 could induce the *digB* and *ccpB* transcription in the dark.

5.2. Results

5.2.1. Confirmation of SigF2 overexpressed strain, F2OX

To further investigate the downstream genes under the control of SigF2, I constructed the F2OX strain that overexpresses SigF2, as described in Section 2.2.3. Similar to constructing the F1OX strain, I inserted the *sigF2* ORF fragment into the overexpression vector pNSEI to construct the plasmid pNSEI-F2 (Figure 2.4). As detailed in Section 2.4, I employed natural transformation to introduce the plasmid into TUA and got the F2OX strain. The construction of the strain was verified by analyzing the expression levels of SigF2-FLAG before and after IPTG induction. To confirm the successful overexpression of the SigF2-FLAG fusion protein, I conducted Western blotting analysis using anti-FLAG antibody. The results demonstrated that IPTG effectively induced the overexpression of SigF2 in TUA (Figure 5.2). With the overexpression strain F2OX now established, it is ready for subsequent investigations into the role of SigF2 in regulating gene expression.

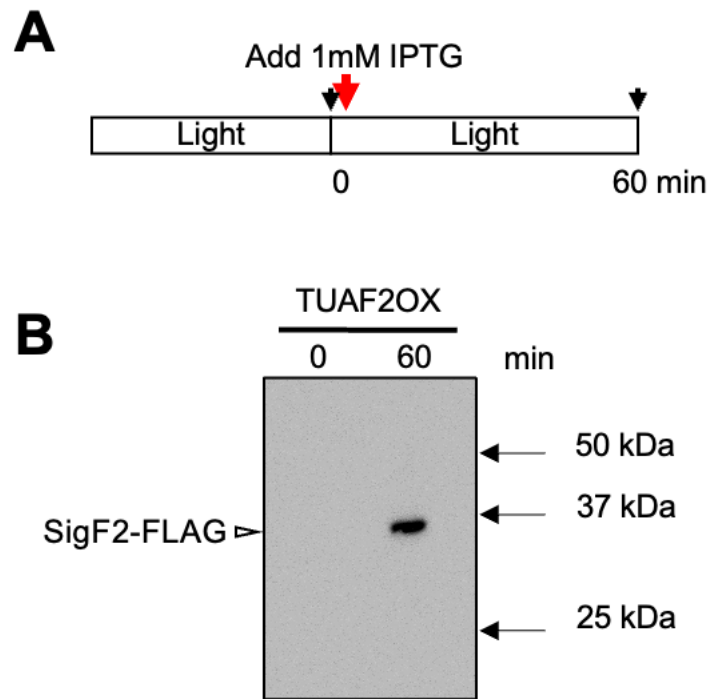


Figure 5.2 The expression of SigF2-FLAG is induced by IPTG. (A) Experimental scheme for the IPTG induction experiments. Cells were cultivated under illumination with an initial OD₇₅₀ of 0.15 for 24 h to an OD₇₅₀ of approximately 0.5. Cells were collected for analysis before and 60 minutes after adding IPTG. (B) SigF1 protein accumulation after adding IPTG. The SigF2-FLAG protein (33 kDa) was detected using α -FLAG antibody. The bars on the right indicate marker sizes.

5.2.2. Analysis of downstream genes of SigF2 by RNA-seq

To identify the genes regulated by SigF2, RNA-seq was utilized to examine the transcriptomic changes in the F2OX strain before and after IPTG induction. Total RNA samples were extracted from F2OX under both conditions, following the protocols outlined in Section 2.6. The results showed that SigF2 upregulated only a few genes (Figure 5.3). Basically, they are genes related to induction in the dark.

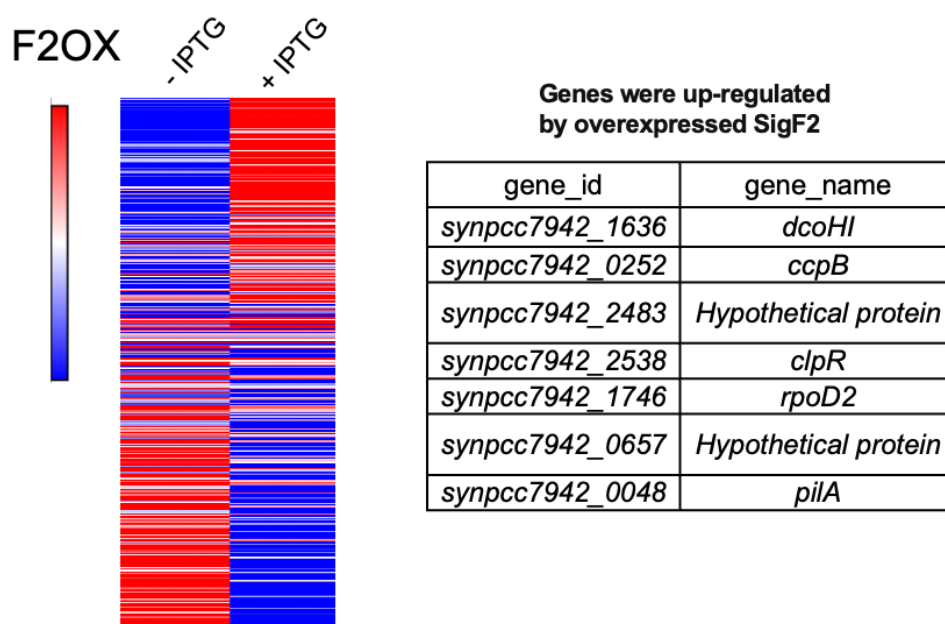


Figure 5.3 Heatmap of gene expression in F2OX by IPTG induction. (A) This heatmap displays the transcriptional responses of the F2OX strain to IPTG induction. Each row represents an individual gene, and the columns compare conditions before and after IPTG induction. The expression levels are depicted in a color gradient from blue (low expression) to red (high expression), with the scale provided in the legend. Top 7 genes are annotated in the table for easy reference.

5.2.3. Identification of TSS of *synpcc7942_0253-ccpB* operon

To analyze the promoter sequence recognized by SigF2, I employed the primer extension method described in Section 2.10 to identify the TSS of the downstream gene of SigF2. RNA-seq results suggest that *ccpB* is under the control of SigF2. Supporting this, previous functional analyses of SigF2 indicated that it initiates transcription of *ccpB* in the dark (Kashima, 2013). Although not directly regulated by SigF2, gene *synpcc7942_0253*, which forms an operon with *ccpB* (Figure 5.4), also appeared responsive to the light-to-dark shift and accumulated in the dark. Transcription of both *ccpB* and the *synpcc7942_0253-ccpB* transcript was observed, and this transcription was reduced in the *sigF2* mutant strain (Figure 5.1).

To further investigate the role of SigF2 in regulating these two genes, I designed specific primers, which were listed in Table S3, for separate primer extension experiments. The results revealed a SigF2-dependent TSS at -110 upstream of the *ccpB* ORF, with the start codon 'A' of *ccpB* designated as $+1$ (Figure 5.5). A TSS was also detected in the upstream region of the *synpcc7942_0253* ORF. When the 'A' of the start codon of *synpcc7942_0253* is defined as $+1$, the TSS is located at -21 (Figure 5.6); however, this promoter is not dependent on SigF2.

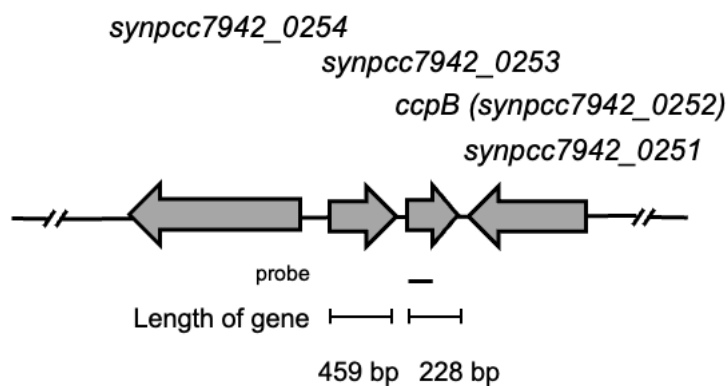


Figure 5.4 The predicted structure of the *synpcc7942_0253-ccpB* operon. The labels under *synpcc7942_0253* and *ccpB* denote the length of each gene.

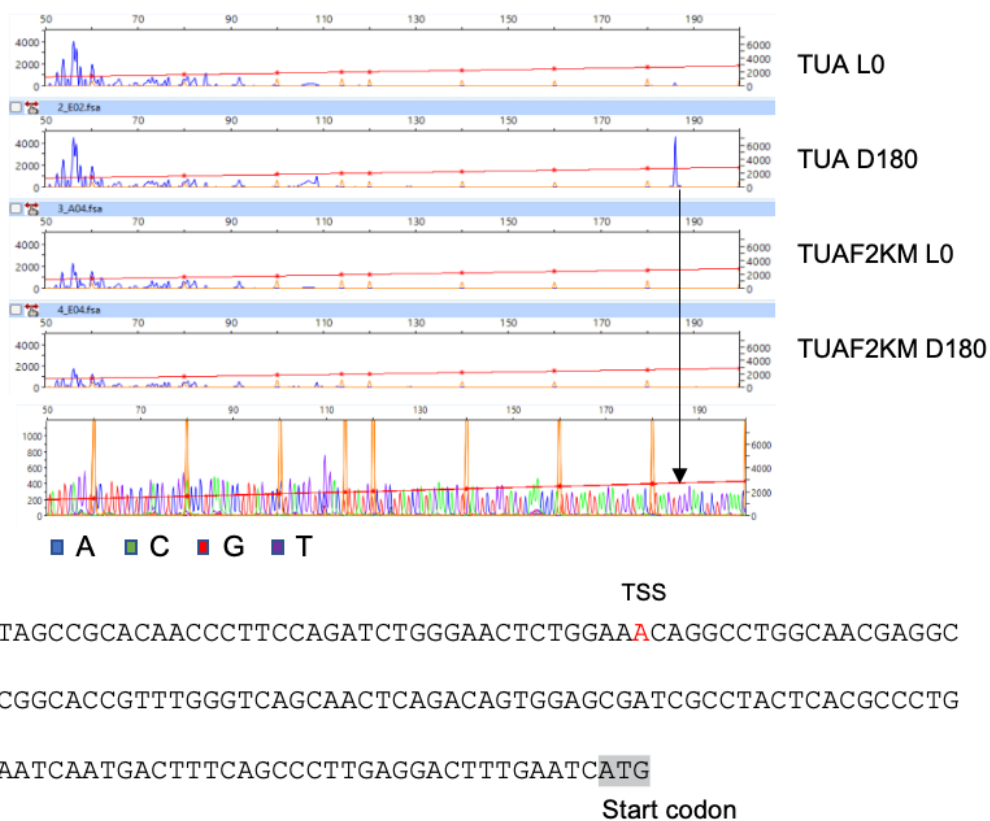


Figure 5.5 Analysis of the *ccpB* promoter structure. Primer extension analysis. Cells grown under standard conditions were used for RNA preparation at an OD₇₅₀ of approximately 0.5. Primer extension products and the sequence ladder were analyzed after the addition of the size marker, positions indicated by red asterisks, to compare and estimate the product sizes. Sequence ladder to compare and estimate the sizes of the primer extension products. The sequence shows the position of the TSS relative to the start codon of *ccpB*.

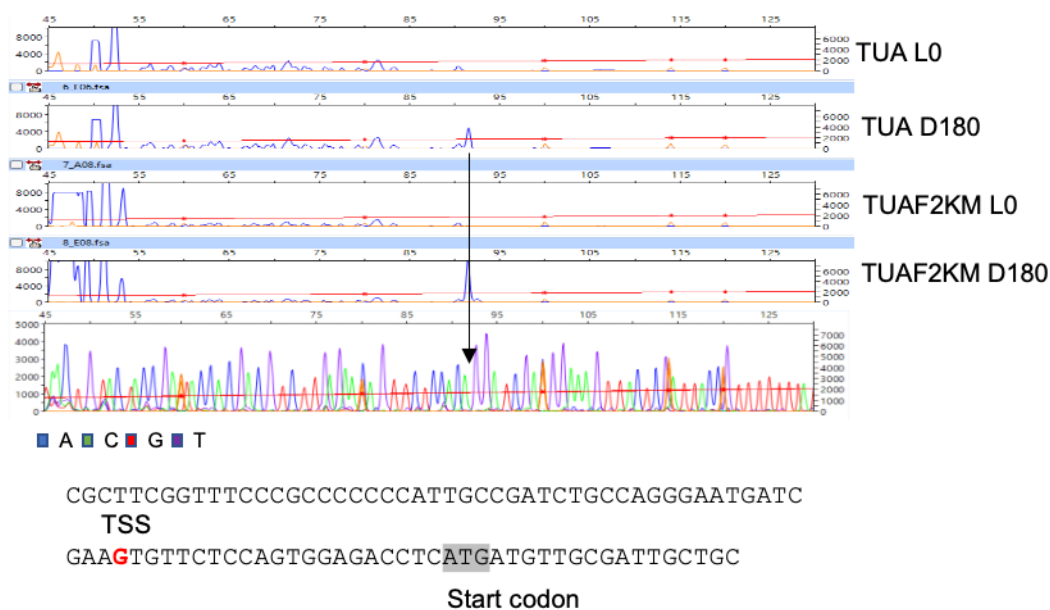


Figure 5.6 Analysis of the *synpcc7942_0253* promoter structure. Primer extension analysis. Cells grown under standard conditions were used for RNA preparation at an OD₇₅₀ of approximately 0.5. Primer extension products and the sequence ladder were analyzed after the addition of the size marker, positions indicated by red asterisks, to compare and estimate the product sizes. Sequence ladder to compare and estimate the sizes of the primer extension products. The sequence shows the position of the TSS relative to the start codon of *synpcc7942_0253*.

5.2.4. Identification of TSS of *digB*

digB is one of several genes induced by darkness, the functions of which remain unknown. These dark-induced genes are regulated by circadian rhythms. Notably, *digB* is regulated by SigF2 in the dark (Figure 5.1). To identify the promoter of *digB*, I utilized the primer extension method outlined in Section 2.10. For this purpose, I designed specific primers listed in Table X targeting the promoter region of *digB*. The results revealed a SigF2-dependent TSS at –31 upstream of the *digB* ORF, with the start codon 'A' of *digB* designated as +1 (Figure 5.7).

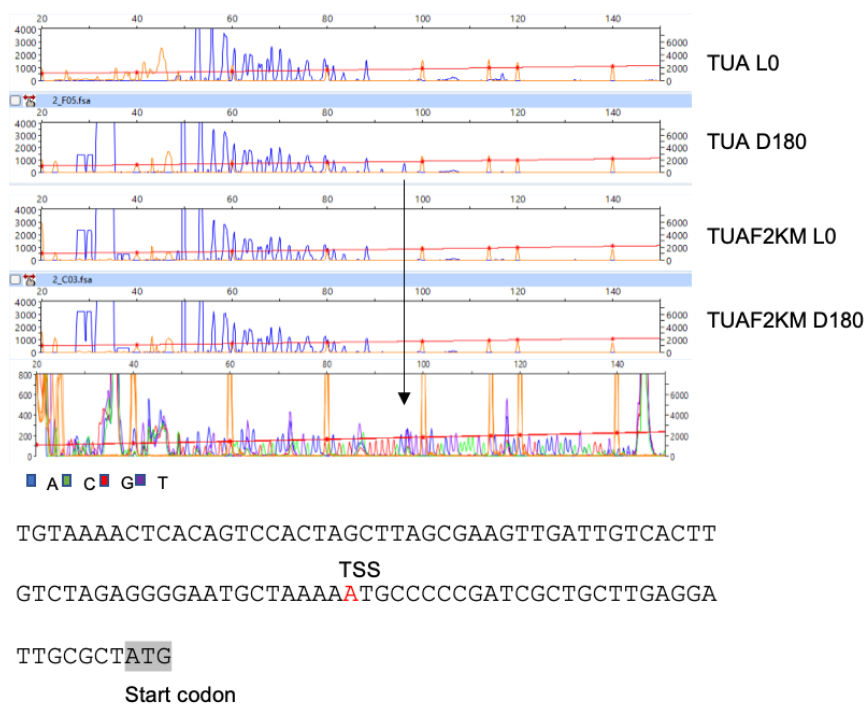


Figure 5.7 Analysis of the *digB* promoter structure. Primer extension analysis. Cells grown under standard conditions were used for RNA preparation at an OD₇₅₀ of approximately 0.5. Primer extension products and the sequence ladder were analyzed after the addition of the size marker, positions indicated by red asterisks, to compare and estimate the product sizes. Sequence ladder to compare and estimate the sizes of the primer extension products. The sequence shows the position of the TSS relative to the start codon of *digB*.

5.2.5. Putative consensus promoters recognized by SigF2

Based on the transcription start site (TSS) identified through primer extension, I proposed putative SigF2-dependent conserved promoter elements at positions –35 and –10, designated as AG(T/C)G and GGGAA, respectively (Figure 5.8). Although the promoter of the *synpcc7942_0253* gene does not directly depend on SigF2, its –10 region exhibits a high degree of sequence conservation with the –10 regions of *ccpB* and *digB*. This suggests that this sequence motif may be a common feature among promoters of genes that are induced in darkness. Additionally, the promoter sequence –10 region recognized by SigF2 also closely resembles the –10 region of the promoter sequence recognized by SigF1, indicating evolutionary conservation between these two sigma factors. This conservation may explain the ability of SigF1 to enhance the transcriptional activity of genes downstream of SigF2.

–35	–10	+1	
AGCGAA AGTTGA	TTGTCACTTGTCTAGAG GGGAAT	GC-TAAAAA	<i>digB</i>
TCCCT AGCCGC	ACAACCCTTCCAGATCT GGGAAC	TC-TGGAAA	<i>ccpB</i>
TTCCC GCCCCC	CCATTGCCGATCTGCCA GGGAAT	GA-TCGAAG	<i>synpcc7942_0253</i>
GGAAC CTGAAA	TAA-AAGCCTGCTGTAG GGGTAA	GTTACTTCA	<i>pilA1_SigF1</i>

Figure 5.8 Comparison of promoter sequences recognized by SigF1 and SigF2, respectively. The TSS (+1) is indicated in bold. The proposed consensus promoter sequences, the –10 and –35 boxes, are shown in grey.

5.3. Discussion

The findings presented in this chapter enhance our understanding of the regulatory role of SigF2 in the dark. Previous studies have established SigF2 as a sigma factor associated with dark induction and essential for natural competence. In this chapter, I utilized RNA sequencing to investigate downstream genes potentially regulated by SigF2. The RNA-seq analysis and subsequent primer extension experiments not only confirmed the involvement of SigF2 in the regulation of dark-induction-related genes such as *ccpB* but also identified specific transcription start sites (TSSs) recognized by SigF2. SigF2-dependent TSSs were pinpointed at distinct positions upstream of the *ccpB* and *digB* ORFs. These findings align with earlier results that depicted SigF2's nocturnal expression in the circadian rhythm and its role in inducing gene transcription, thus reinforcing the notion that SigF2 directly initiates transcription in response to darkness.

Interestingly, the similarity between the -10 promoter region recognized by SigF2 and that recognized by SigF1 indicates evolutionary conservation and suggests potential shared ancestral functions between these two sigma factors. This evolutionary link implies that although the sigma factors may have evolved to respond to different environmental cues (light and dark transitions), they have preserved the fundamental mechanism that governs their binding affinity to promoter DNA. The observed ability of SigF1 to enhance the transcriptional activity of genes downstream of SigF2 further suggests synergistic interactions between these sigma factors, which may amplify cellular responses to environmental changes.

Despite significant advancements in delineating the genes regulated by SigF2 and their regulatory conditions, the functions of many of these genes, such as *digB*, remain elusive. These genes are activated by darkness and regulated by circadian rhythms, indicating their involvement in critical nocturnal processes or responses to the absence

of light. Elucidating these functions could uncover new dimensions of cyanobacterial adaptation to diurnal cycles.

**Chapter 6 Regulatory Interaction of Sigma
Factors SigF1 and SigF2 and their impact on
downstream gene transcription Under PQ Pool
Oxidized Condition in *Synechococcus***

6.1. Introduction

In previous chapters, I examined the roles of group 3 sigma factors SigF1 and SigF2. I found that SigF1 initiates the transcription of *pilA1* during the transition from dark to light. Conversely, my study in Chapter 5 and the previous study demonstrated that SigF2 activates the transcription of *digB*, *ccpB*, and several genes associated with pili composition during the transition from light to dark. Notably, in transitions from light to dark, the presence of full-length SigF1 can either enhance the transcription of *ccpB* and *digB* or enable their transcription in the *sigF2* mutant strain (Figure 5.1). These observations indicate that SigF1 and SigF2 share interconnected regulatory mechanisms in response to light and dark transitions. Given this interconnection, the possibility that the expression of SigF1 and SigF2 mutually affect each other could explain their influence on the transcription of downstream genes. Additionally, beyond the changes induced by light and dark conditions, SigF1 and SigF2 may exert complementary effects on the transcriptional regulation of downstream genes in response to other signals

Therefore, in this chapter, I investigated whether the expression of SigF1 and SigF2 affects each other. Since photosynthesis is a critical physiological process in cyanobacteria. I further explored the impact of SigF1 and SigF2 on downstream gene transcription under PQ pool oxidized conditions using the photosynthetic electron transport inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea).

6.2. Results

6.2.1. *ccpB* and *digB* transcription under DCMU condition depend on SigF2 and SigF1

In this chapter, the roles of the group 3 sigma factors SigF1 and SigF2 were further explored to understand their regulatory effects under PQ pool oxidized conditions induced by the photosynthetic electron transport inhibitor DCMU. TUA strains, NU strains, and their respective SigF1 and SigF2 double mutants were cultured, starting from an OD₇₅₀ of 0.15 until approximately 0.5. Samples were collected according to the time course detailed in Figure 6.1A, with DCMU added after the first sampling and subsequent samples taken at designated times.

As described in Section 2.5, Northern blotting analysis was used to analyze the expression of *ccpB* and *digB* across different samples. The results revealed that in both TUA and NU strains, the transcription of *ccpB* and *digB* was activated under the oxidized state of plastoquinone (PQ). Consistent with previous observations, the expression levels of these genes were higher in the NU strain containing full-length SigF1 compared to the TUA strain, which only has a truncated SigF1. In the double mutants, neither of these genes was expressed, indicating the crucial role of SigF1 and SigF2 in their regulation (Figure 6.1B).

Interestingly, the transcription of the upstream gene *synpcc7942_0253* was also activated under PQ pool oxidized conditions. However, unlike the consistent activation pattern with *ccpB* observed during the light-to-dark transition, the transcription levels of *synpcc7942_0253* showed an inverse relationship with *ccpB* in the TUA and NU strains (Figure 6.1B). Specifically, *synpcc7942_0253* transcription levels were higher in TUA compared to NU, and unexpectedly, the NU double mutant showed higher levels of *synpcc7942_0253* transcription than NU itself. This suggests a potential repressive role of SigF1 in the regulation of *synpcc7942_0253* transcription.

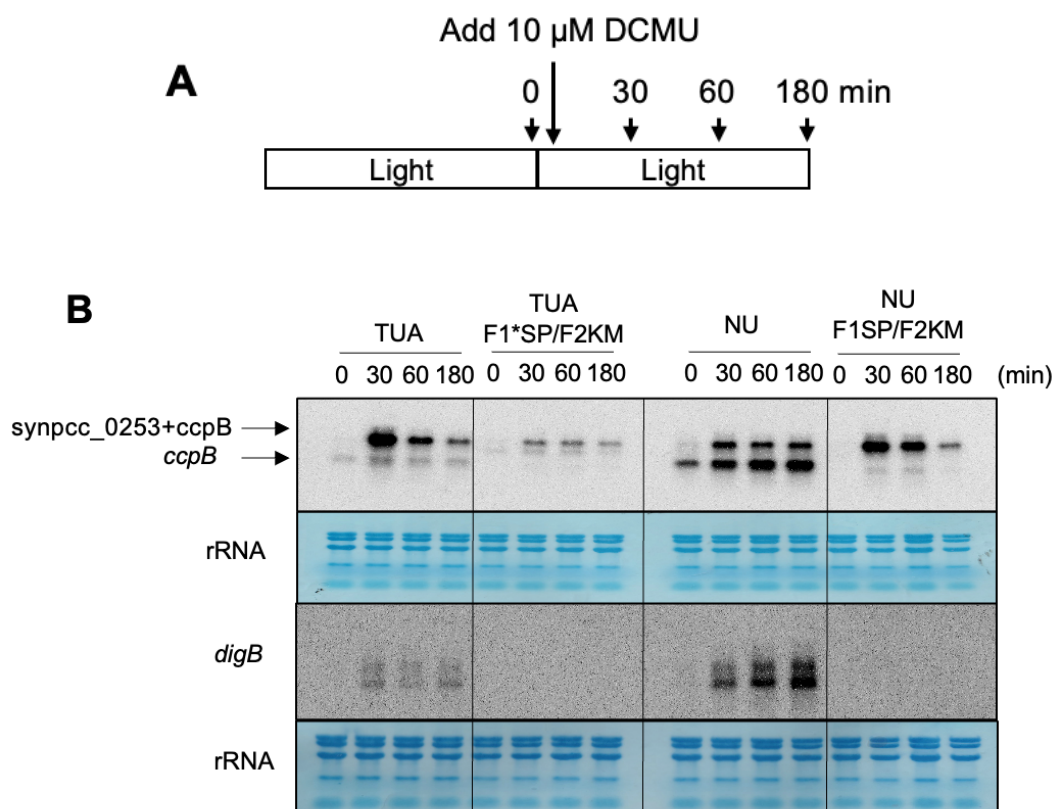


Figure 6.1 Northern blotting analysis of *ccpB*, *digB*, and *synpcc7942_0253* expression under PQ pool oxidized conditions induced by DCMU. (A) Experimental scheme for PQ pool oxidized condition induced by DCMU experiments. Cells were cultivated under illumination with an initial OD₇₅₀ of 0.15 for 24 h, and then added DCMU. Cells were collected at 0, 15, 30, and 60 min. (B) This figure illustrates the transcriptional response of *ccpB*, *digB*, and *synpcc7942_0253* in TUA strains, NU strains, and their respective SigF1 and SigF2 double mutants to PQ pool oxidized conditions. Samples were collected before and at various times after DCMU treatment, as shown in the time course. The results demonstrate that *ccpB* and *digB* are actively transcribed under the oxidized plastoquinone (PQ) state, with enhanced expression levels observed in the NU strain, which contains full-length SigF1, compared to the TUA strain harboring truncated SigF1. Notably, transcription of these genes was absent

in the double mutants, underscoring the essential roles of SigF1 and SigF2 in their regulatory pathways. Conversely, transcription of *synpcc7942_0253* was also activated under PQ pool oxidized conditions, but with a distinct pattern; it was higher in the TUA strain than in the NU strain, suggesting an inhibitory influence of SigF1 on this gene. Surprisingly, *synpcc7942_0253* transcription levels were elevated in the NU double mutant relative to the NU strain, indicating complex regulatory interactions that may involve repression by SigF1.

6.2.2. Overexpression of SigF1 and SigF2 did not affect the expression of each other

To determine whether the mutual influence of SigF1 and SigF2 expression is the cause of cross-talk, I used F1OX and F2OX strains. Following the procedures in Section 2.5, I performed northern blotting analysis on *sigF1* and *sigF2* transcripts during the overexpression of SigF1 or SigF2, respectively. The results showed that in the F1OX strain, the overexpression of SigF1 did not affect the transcription of *sigF2*. Similarly, in the F2OX strain, the overexpression of SigF2 did not induce the transcription of *sigF1* (Figure 6.2). Therefore, the influence of these two sigma factors on downstream gene expression is likely mediated by other regulatory mechanisms.

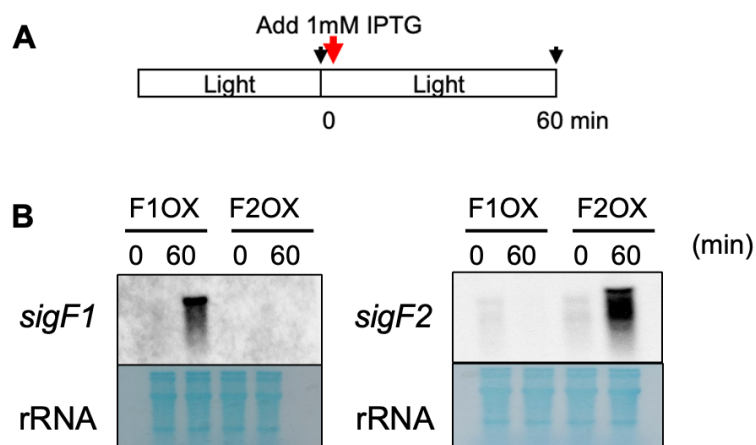


Figure 6.2 Northern blotting analysis of *sigF1* and *sigF2* transcripts in F1OX and F2OX strains (A) Experimental scheme for the IPTG induction experiments. Cells were cultivated under illumination with an initial OD₇₅₀ of 0.15 for 24 h to an OD₇₅₀ of approximately 0.5. Cells were collected for analysis before and 60 minutes after adding IPTG. (B) Analysis of *sigF1* and *sigF2* transcripts in F1OX and F2OX before and after adding IPTG. Total RNAs were extracted, and 3 µg RNA per lane was analyzed by northern analysis. The *sigF1* and *sigF2* transcripts were detected by probes made by primers listed in Table S3. rRNA (ribosomal RNA) is shown as the loading control.

6.3. Discussion

The results discussed in this chapter further clarify the roles of the sigma factors SigF1 and SigF2, emphasizing their regulatory functions under DCMU-induced PQ pool oxidized conditions. These findings demonstrate that SigF1 and SigF2 are integral to a regulatory mechanism that manages stress during photosynthesis. Notably, gene expression was significantly enhanced in the NU strain harboring full-length SigF1 compared to the TUA strain with truncated SigF1, suggesting that full-length SigF1 facilitates increased transcriptional activation under stress. This enhancement illustrates the synergistic interaction between SigF1 and SigF2 in the photosynthetic stress response. The fact that SigF1 and SigF2 do not affect each other's expression also indicates that other mechanisms are involved in regulating the expression of downstream genes.

The analysis of *synpcc7942_0253* under PQ pool oxidized conditions reveals an intriguing dimension of sigma factor activity. The transcriptional patterns of *synpcc7942_0253*, as compared to *ccpB* between the TUA and NU strains—especially the heightened transcription in the NU double mutant—imply that SigF1 may have a repressive influence on this gene. Similar transcriptional responses were observed in *hspA* during experiments on PQ pool oxidized conditions, indicating that the regulatory mechanism involving SigF1 is pervasive. Further research is required to elucidate this complex regulatory mechanism.

Chapter 7 General Discussion

In this study, I studied the functions of two Sigma factors, SigF1 and SigF2, in cyanobacteria and their role in regulation.

In the functional analysis of SigF1, it was observed that the *pilA1* gene, which encodes type IV pilin in *Synechococcus*, is transcribed by RNA polymerase associated with the group 3 sigma factor SigF1. This transcriptional activity is linked to the sedimentation phenotype observed in these cyanobacteria. Phenotypic analysis of the SigF1-deficient strain revealed significant parallels with its homolog, SigF, in *Synechocystis*, particularly in relation to the type IV pilus formation gene *pilA1* (Flores et al., 2019). Unlike *Synechocystis*, *Synechococcus* does not exhibit swimming capabilities but possesses pili that facilitate twitching movements and phototropism, which are vital for navigating light gradients and avoiding predation (Conradi et al., 2020).

Interestingly, *pilA1* has been shown to be essential for pilus formation in cyanobacteria. Previous studies have indicated that knockout of *pilA1* results in rapid cell sedimentation, a phenotype not directly linked to biofilm formation (Zedler et al., 2023). However, this rapid sedimentation phenotype has recently been associated with SigF1, a finding that was corroborated in this study (Figure 3.4). This phenotype, observed both in *Synechocystis* and *Synechococcus*, underscores the critical role of motility mechanisms that cells rely on for survival. The analysis presented here reveals complex interactions between gene expression patterns and sigma factors under specific environmental stress conditions.

SigF1 has been demonstrated to activate *pilA1* transcription in response to light. This transcriptional activation correlates with previous reports indicating that *pilA1* transcription is rhythmic, regulated by the Kai circadian clock, accumulating during the day and diminishing at night. These findings align with my experimental observations, where SigF1 activation of *pilA1* occurs upon light exposure.

Furthermore, my investigations into SigF1 protein expression revealed that its levels remain constant regardless of light changes (Figure 3.11B). This suggests that while *pilA1* transcription initiation by SigF1 is likely regulated by Kai or a light-activated regulatory mechanism, the protein expression of SigF1 itself is not influenced by light or rhythmic changes.

In studying the interaction between SigF1 and the *pilA1* promoter during the transcription process using ChIP-qPCR, I found that SigF1, together with RNAP, is already recruited to the promoter in the dark. However, transcription does not commence until the cell perceives a light signal. This observation deviates from the typical cellular transcription processes where recruitment of specific sigma factors usually coincides with environmental stimuli prompting transcription initiation.

This regulatory mechanism might be influenced by anti-sigma factors. Given the homology between SigF and SigF1 with SigB in *B. subtilis*, it is plausible that they share functional similarities and are subject to analogous regulatory mechanisms. In *B. subtilis*, RsbW binds to SigB, preventing its association with RNAP and thus inactivating it. RsbU can dephosphorylate RsbV, permitting RsbV to bind RsbW and facilitate the release of SigB (Huckauf et al., 2000; Alper et al., 1996 ; Voelker et al., 1995). Although the involvement of these factors in regulating SigF1 remains speculative, a similar mechanism could potentially operate in the photoactivation process of *Synechococcus*. If anti-sigma factors inhibit sigma factor function, their interaction with SigF1 in the dark might suppress transcriptional activation following promoter recruitment. However, further research is required to elucidate these underlying mechanisms thoroughly.

Additionally, I engineered a GFP reporter strain by fusing the upstream promoter region of *pilA1* with mNG, a GFP protein, to investigate the transcriptional regulation by SigF1. Primer extension analysis of this reporter strain identified a SigF1-dependent

transcription start site (TSS). Subsequently, site-directed scanning mutagenesis was employed to modify the -3, -4, or -6 base pairs within the 50 bp region of the *pilA1* promoter in the reporter plasmid. The fluorescence activity of these reporter strains was then measured to pinpoint critical areas of the promoter, specifically the -35 and -10 regions, confirming their SigF1 dependence.

While the GFP reporter system facilitated the successful determination of the *pilA1* promoter structure, it proved less effective in detecting dynamic changes in promoter activity in response to light variations. Despite these limitations, Northern blotting confirmed that the *pilA1* promoter could be normally activated by light to transcribe the fused *mNG* gene (Figure 3.8A). The observed stability of the mNG protein fluorescence, regardless of light conditions (Figure 3.8B), suggests that high stability of mNG protein within the cellular environment may mask short-term changes in promoter activity. This stability impedes the effectiveness of system in real-time detection of transcriptional responses to environmental changes. However, this GFP reporter system holds significant potential for broader applications in studying gene promoter structures. Its utility lies in the simplicity with which only the promoter region upstream of the *mNG* gene needs to be altered to assess different promoter dynamics. Thus, despite its limitations in dynamic light condition studies, this approach can be effectively applied to dissect promoter architectures and understand transcriptional regulation mechanisms across various genes.

RNA-seq analysis of strains overexpressing SigF1 highlighted its role in regulating genes associated with heat shock responses and cellular migration. Among the upregulated genes, *hspA*, a gene encoding a heat shock protein, was notably induced by SigF1, as confirmed by Northern blotting. Despite this induction, ChIP-qPCR analysis revealed that SigF1 does not bind directly to the *hspA* promoter, suggesting an indirect regulatory mechanism. Previous studies have shown that *hspA* is regulated by two-

component systems (TCS), where the phosphorylated transcription factor Rre1 targets the upstream promoter region. However, in the context of SigF1 overexpression, no phosphorylation of Rre1 was detected during *hspA* transcription. This observation implies that other TCS components might be involved in regulating *hspA* under these conditions. Furthermore, the involvement of the RpaA-SasA pathway is considered a potential regulatory mechanism, given that RpaA, a rhythm-regulated protein, has been linked to rhythmic control of gene expression in previous studies (Takai et al., 2006; Iwasaki et al., 2011). Although the connection between RpaA-SasA and *hspA* regulation in response to SigF1 activity requires further investigation, it presents a plausible link due to the rhythmic nature of *hspA* expression.

SigF2 is recognized for its pivotal role in rhythmic control and the induction of gene expression in darkness. Prior studies have established that SigF2 regulates two genes, *ccpB* and *digB*, which are linked to the natural competencies of *Synechococcus*. RNA-seq analysis of strains overexpressing SigF2 revealed only a modest upregulation of genes, prompting further investigations into *ccpB* and *digB*. Primer extension was employed to identify SigF2-dependent transcription start sites on the promoters of these genes, leading to the proposal of the –35 and –10 promoter regions. A comparative analysis with SigF1-dependent promoter regions showed notable conservation in their –10 regions, suggesting a functional overlap. Both SigF1 and SigF2 belong to the type F sigma factor class and have been shown to synergistically enhance the transcription of *ccpB* and *digB*, hinting at a shared regulatory mechanism that may be rooted in the conservation of these promoter regions. Furthermore, the lack of mutual influence on the expression of SigF1 and SigF2 indicates that the regulation of downstream gene expression involves other mechanisms, possibly including anti-sigma factors or two-component systems.

Further experimentation revealed that transcription of *ccpB* and *digB* could be activated under PQ pool oxidized induced by DCMU, affirming their reliance on SigF2. Interestingly, full-length SigF1 also augmented the transcription of these genes, consistent with previous findings. However, a novel observation was made regarding the expression pattern of *synpcc7942_0253*, the upstream gene of *ccpB*. Under the PQ pool oxidized, *synpcc7942_0253* exhibited an expression pattern inverse to that of *ccpB*, suggesting differential functions and regulatory mechanisms despite their operonic configuration. Moreover, comparative expression analysis in TUA and NU strains revealed that in the presence of full-length SigF1, the expression level of *synpcc7942_0253* was reduced compared to the SigF1-deficient strain, indicating a potential inhibitory role of SigF1 in this context.

This investigation led to the proposition of two sigma factors-dependent promoter regions, enriching our understanding of how SigF1 and SigF2 functionally interplay to regulate gene expression under varying environmental stresses.

References

- Aguilo-Ferretjans, M. D. M., Bosch, R., Puxty, R. J., Latva, M., Zadjelovic, V., Chhun, A., Sousoni, D., Polin, M., Scanlan, D. J., & Christie-Oleza, J. A. (2021). Pili allow dominant marine cyanobacteria to avoid sinking and evade predation. *Nature communications*, **12**, 1857.
- Alba, B. M., & Gross, C. A. (2004) Regulation of the *Escherichia coli* σ^E -dependent envelope stress response. *Molecular microbiology*, **52**, 613–619.
- Alper, S., Dufour, A., Garsin, D. A., Duncan, L., & Losick, R. (1996) Role of adenosine nucleotides in the regulation of a stress-response transcription factor in *Bacillus subtilis*. *J. Mol. Biol.*, **260**, 165–177.
- Angermayr, S. A., Hellingwerf, K. J., Lindblad, P., & de Mattos, M. J. (2009) Energy biotechnology with cyanobacteria. *Current opinion in biotechnology*, **20**, 257–263.
- Asayama, M., & Imamura, S. (2008) Stringent promoter recognition and autoregulation by the group 3 σ -factor SigF in the cyanobacterium *Synechocystis* sp. strain PCC 6803. *Nucleic Acids Res.*, **36**, 5297–5305.
- Ashby, M. K., & Houmard, J. (2006) Cyanobacterial two-component proteins: structure, diversity, distribution, and evolution. *Microbiol. Mol. Biol. R.*, **70**, 472–509.
- Bairagi, N., Watanabe, S., Nimura-Matsune, K., Tanaka, K., Tsurumaki, T., Nakanishi, S., & Tanaka, K. (2022) Conserved Two-component Hik2-Rre1 Signaling Is Activated Under Temperature Upshift and Plastoquinone-reducing Conditions in the Cyanobacterium *Synechococcus elongatus* PCC 7942. *Plant Cell Physiol.*, **63**, 176–188.
- Barker, M. M., Gaal, T., Josaitis, C. A. & Gourse, R. L. (2001) Mechanism of regulation of transcription initiation by ppGpp. I. Effects of ppGpp on transcription initiation in vivo and in vitro. *J. Mol. Biol.* **305**, 673–688.

- Basu, R. S., Warner, B. A., Molodtsov, V., Pupov, D., Esyunina, D., Fernández-Tornero, C., Kulbachinskiy, A., & Murakami, K. S. (2014) Structural basis of transcription initiation by bacterial RNA polymerase holoenzyme. *J. Biol. Chem.*, **289**, 24549–24559.
- Bhaya, D., Bianco, N. R., Bryant, D., & Grossman, A. (2000) Type IV pilus biogenesis and motility in the cyanobacterium *Synechocystis* sp. PCC6803. *Mol. Microbiol.*, **37**, 941–951.
- Bhaya, D., Watanabe, N., Ogawa, T., & Grossman, A. R. (1999) The role of an alternative sigma factor in motility and pilus formation in the cyanobacterium *Synechocystis* sp. strain PCC6803. *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 3188–3193.
- Blankenship, R.E. (2010) Early evolution of photosynthesis. *Plant Physiol.* **154**, 434–438.
- Browning, D. F., & Busby, S. J. (2016) Local and global regulation of transcription initiation in bacteria. *Nat. Rev. Microbiol.*, **14**, 638–650.
- Browning, D. F., & Busby, S. J. (2004). The regulation of bacterial transcription initiation. *Nat. Rev. Microbiol.*, **2**, 57–65.
- Buc, H., & McClure, W. R. (1985) Kinetics of open complex formation between *Escherichia coli* RNA polymerase and the lac UV5 promoter. Evidence for a sequential mechanism involving three steps. *Biochemistry*, **24**, 2712–2723.
- Burgess R. R. (1969) Separation and characterization of the subunits of ribonucleic acid polymerase. *J. Biol. Chem.*, **244**, 6168–6176.
- Campbell, D., Hurry, V., Clarke, A. K., Gustafsson, P., & Oquist, G. (1998) Chlorophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation. *Microbiol. Mol. Biol. R.*, **62**, 667–683.

- Campbell, E. A., Muzzin, O., Chlenov, M., Sun, J. L., Olson, C. A., Weinman, O., Trester-Zedlitz, M. L., & Darst, S. A. (2002) Structure of the bacterial RNA polymerase promoter specificity sigma subunit. *Mol. Cell*, **9**, 527–539.
- Clerico, E. M., Ditty, J. L., & Golden, S. S. (2007) Specialized techniques for site-directed mutagenesis in cyanobacteria. *Methods Mol. Biol.*, **362**, 155–171.
- Dai, W., Chen, M., Myers, C., Ludtke, S. J., Pettitt, B. M., King, J. A., Schmid, M. F., & Chiu, W. (2018). Visualizing Individual RuBisCO and Its Assembly into Carboxysomes in Marine Cyanobacteria by Cryo-Electron Tomography. *J. Mol. Biol.*, **430**, 4156–4167.
- Dausse, J. P., Sentenac, A. & Fromageot, P. (1972) Interaction of RNA polymerase from *Escherichia coli* with DNA: influence of DNA scissions on RNA–polymerase binding and chain initiation. *Eur. J. Biochem.* **31**, 394–404
- Duchi, D., Gryte, K., Robb, N. C., Morichaud, Z., Sheppard, C., Brodolin, K., Wigneshweraraj, S., & Kapanidis, A. N. (2018) Conformational heterogeneity and bubble dynamics in single bacterial transcription initiation complexes. *Nucleic Acids Res.*, **46**, 677–688.
- Durán, R. V., Hervás, M., De La Rosa, M. A., & Navarro, J. A. (2004). The efficient functioning of photosynthesis and respiration in *Synechocystis* sp. PCC 6803 strictly requires the presence of either cytochrome c6 or plastocyanin. *J. Biol. Chem.*, **279**, 7229–7233.
- Ebright, R. H. (1993) Transcription activation at Class I CAP-dependent promoters. *Mol. Microbiol.* **8**, 797–802
- Espinosa, J., Boyd, J. S., Cantos, R., Salinas, P., Golden, S. S., & Contreras, A. (2015) Cross-talk and regulatory interactions between the essential response regulator RpaB

and cyanobacterial circadian clock output. *Proc. Natl. Acad. Sci. U.S.A.*, **112**, 2198–2203.

Feklístov, A., Sharon, B. D., Darst, S. A., & Gross, C. A. (2014) Bacterial sigma factors: a historical, structural, and genomic perspective. *Annu. Rev. Microbiol.*, **68**, 357–376.

Fleming, K. E., & O'Shea, E. K. (2018) An RpaA-dependent sigma factor cascade sets the timing of circadian transcriptional rhythms in *Synechococcus elongatus*. *Cell Rep.*, **25**, 2937–2945.e3.

Flores, C., Santos, M., Pereira, S. B., Mota, R., Rossi, F., De Philippis, R., Couto, N., Karunakaran, E., Wright, P. C., Oliveira, P., & Tamagnini, P. (2019) The alternative sigma factor SigF is a key player in the control of secretion mechanisms in *Synechocystis* sp. PCC 6803. *Environ. Microbiol.*, **21**, 343–359.

Friedman, L. J., & Gelles, J. (2012) Mechanism of transcription initiation at an activator-dependent promoter defined by single-molecule observation. *Cell*, **148**, 679–689.

Gross, C. A., Chan, C., Dombroski, A., Gruber, T., Sharp, M., Tupy, J., & Young, B. (1998) The functional and regulatory roles of sigma factors in transcription. *Cold Spring Harb. Symp. Quant. Biol.*, **63**, 141–155.

Gruber, T. M., & Gross, C. A. (2003) Multiple sigma subunits and the partitioning of bacterial transcription space. *Annu. Rev. Microbiol.*, **57**, 441–466.

Hanaoka, M., & Tanaka, K. (2008) Dynamics of RpaB–promoter interaction during high light stress, revealed by chromatin immunoprecipitation (ChIP) analysis in *Synechococcus elongatus* PCC 7942. *Plant J.*, **56**, 327–335.

- Hasegawa, H., Tsurumaki, T., Kobayashi, I., Imamura, S., & Tanaka, K. (2020) Identification and analysis of a principal sigma factor interacting protein SinA, essential for growth at high temperatures in a cyanobacterium *Synechococcus elongatus* PCC 7942. *J. Gen. Appl. Microbiol.*, **66**, 66–72.
- Helmann, J. D., & Chamberlin, M. J. (1988) Structure and function of bacterial sigma factors. *Annu. Rev. Biochem.*, **57**, 839–872.
- Hoch, J. A. (2000) Two-component and phosphorelay signal transduction. *Curr. Opin. Microbiol.*, **3**, 165-170.
- Hosokawa, N., Hatakeyama, T. S., Kojima, T., Kikuchi, Y., Ito, H., & Iwasaki, H. (2011). Circadian transcriptional regulation by the posttranslational oscillator without de novo clock gene expression in *Synechococcus*. *Proc. Natl. Acad. Sci. U. S. A.*, **108**, 15396–15401.
- Huckauf, J., Nomura, C., Forchhammer, K., & Hagemann, M. (2000) Stress responses of *Synechocystis* sp. strain PCC 6803 mutants impaired in genes encoding putative alternative sigma factors. *Microbiology (Reading, Engl.)*, **146**, 2877–2889.
- Hughes, K. & Mathee, K. (1998) The anti-sigma factors. *Annu. Rev. Microbiol.*, **52**, 231–286
- Imamura, S., & Asayama, M. (2009) Sigma factors for cyanobacterial transcription. *Gene Regul. Syst. Biol.*, **3**, 65–87.
- Imamura, S., Yoshihara, S., Nakano, S., Shiozaki, N., Yamada, A., Tanaka, K., Takahashi, H., Asayama, M., & Shirai, M. (2003) Purification, characterization, and gene expression of all sigma factors of RNA polymerase in a cyanobacterium. *J. Mol. Biol.*, **325**, 857–872.
- Ishi master thesis (2017) Tokyo Institute of Technology, Life Science and Technology

- Jordan, P., Fromme, P., Witt, H. T., Klukas, O., Saenger, W., & Krauss, N. (2001). Three-dimensional structure of cyanobacterial photosystem I at 2.5 Å resolution. *Nature*, **411**, 909–917.
- Josefsen, K., & Nielsen, H. (2011) Northern blotting analysis. *Methods Mol. Biol.*, **703**, 87–105.
- Kapanidis, A. N., Margeat, E., Ho, S. O., Kortkhonjia, E., Weiss, S., & Ebright, R. H. (2006) Initial transcription by RNA polymerase proceeds through a DNA-scrunching mechanism. *Science*, **314**, 1144–1147.
- Kashima master thesis (2014) Chiba University, Graduate School of Horticulture
- Kato, H., Kubo, T., Hayashi, M., Kobayashi, I., Yagasaki, T., Chibazakura, T., Watanabe, S., & Yoshikawa, H. (2011). Interactions between histidine kinase NblS and the response regulators RpaB and SrrA are involved in the bleaching process of the cyanobacterium *Synechococcus elongatus* PCC 7942. *Plant & cell physiology*, **52**, 2115–2122.
- Keren, N., Ohkawa, H., Welsh, E. A., Liberton, M., & Pakrasi, H. B. (2005). Psb29, a conserved 22-kD protein, functions in the biogenesis of Photosystem II complexes in *Synechocystis* and *Arabidopsis*. *Plant Cell*, **17**, 2768–2781.
- Komenda, J., Sobotka, R. & Nixon, P.J. (2012) Assembling and maintaining the photosystem II complex in chloroplasts and cyanobacteria. *Curr. Opin. Plant Biol.*, **15**, 245–251.
- Liu, H., & Naismith, J. H. (2008) An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol. *BMC Biotechnol.*, **8**, 91.
- Losick, R., & Pero, J. (1981) Cascades of sigma factors. *Cell*, **25**, 582–584.

- Mahmood, T., & Yang, P. C. (2012) Western blot: technique, theory, and trouble shooting. *North Am. J. Med. Sci.*, **4**, 429–434.
- Mejía-Almonte, C., Busby, S. J. W., Wade, J. T., van Helden, J., Arkin, A. P., Stormo, G. D., Eilbeck, K., Palsson, B. O., Galagan, J. E., & Collado-Vides, J. (2020) Redefining fundamental concepts of transcription initiation in bacteria. *Nat. Rev. Genet.*, **21**, 699–714.
- Miller, A., Wood, D., Ebricht, R. H., & Rothman-Denes, L. B. (1997) RNA polymerase beta' subunit: a target of DNA binding-independent activation. *Science*, **275**, 1655–1657.
- Mironov, K. S., & Los, D. A. (2015) RNA isolation from *Synechocystis*. *BIO-Protoc.*, **5**, e1428–e1428.
- Müller-Hill, B. (1998) Some repressors of bacterial transcription. *Curr. Opin. Microbiol.* **1**, 145–151
- Nair, U., Ditty, J. L., Min, H., & Golden, S. S. (2002) Roles for sigma factors in global circadian regulation of the cyanobacterial genome. *J. Bacteriol.*, **184**, 3530–3538.
- Nikkanen, L., Solymosi, D., Jokel, M., & Allahverdiyeva, Y. (2021) Regulatory electron transport pathways of photosynthesis in cyanobacteria and microalgae: Recent advances and biotechnological prospects. *Physiol. Plant.*, **173**, 514–525.
- Osanai, T., Ikeuchi, M., & Tanaka, K. (2008) Group 2 sigma factors in cyanobacteria. *Physiol. Plant.*, **133**, 490–506.
- Paget, M. S., & Helmann, J. D. (2003) The σ^{70} family of sigma factors. *Genome Biol.*, **4**, 203.

- Pandey, K. D., Shukla, S. P., Shukla, P. N., Giri, D. D., Singh, J. S., Singh, P., & Kashyap, A. K. (2004) Cyanobacteria in Antarctica: ecology, physiology and cold adaptation. *Cell. Mol. Biol. (Noisy-le-grand)*, **50**, 575–584.
- Pané-Farré, J., Jonas, B., Hardwick, S. W., Gronau, K., Lewis, R. J., Hecker, M., & Engelmann, S. (2009) Role of RsbU in controlling SigB activity in *Staphylococcus aureus* following alkaline stress. *J. Bacteriol.*, **191**, 2561–2573.
- Petersohn, A., Bernhardt, J., Gerth, U., Höper, D., Koburger, T., Völker, U., & Hecker, M. (1999) Identification of sigma(B)-dependent genes in *Bacillus subtilis* using a promoter consensus-directed search and oligonucleotide hybridization. *J. Bacteriol.*, **181**, 5718–5724.
- Plaschka, C., Hantsche, M., Dienemann, C., Burzinski, C., Plitzko, J., & Cramer, P. (2016) Transcription initiation complex structures elucidate DNA opening. *Nature*, **533**, 353–358.
- Prentki, P., & Krisch, H. M. (1984) In vitro insertional mutagenesis with a selectable DNA fragment. *Gene*, **29**, 303–313.
- Ray-Soni, A., Bellecourt, M. J., & Landick, R. (2016) Mechanisms of bacterial transcription termination: all good things must end. *Annu. Rev. Biochem.*, **85**, 319–347.
- Rippka R. (1988). Isolation and purification of cyanobacteria. *Methods Enzymol.*, **167**, 3–27.
- Ruff, E. F., Record, M. T., Jr, & Artsimovitch, I. (2015) Initial events in bacterial transcription initiation. *Biomolecules*, **5**, 1035–1062.
- Saecker, R. M., Record Jr, M. T., & Dehaseth, P. L. (2011) Mechanism of bacterial transcription initiation: RNA polymerase-promoter binding, isomerization to initiation-

competent open complexes, and initiation of RNA synthesis. *J. Mol. Biol.*, **412**, 754–771.

Sambrook, J. (1989) Extraction, purification, and analysis of messenger RNA from eukaryotic cells. *Molecular cloning. A laboratory manual*, **7**, 37–53.

Sánchez-Baracaldo, P., & Cardona, T. (2020) On the origin of oxygenic photosynthesis and Cyanobacteria. *New Phytol.*, **225**, 1440–1446.

Sánchez-Baracaldo, P., Bianchini, G., Wilson, J. D., & Knoll, A. H. (2022) Cyanobacteria and biogeochemical cycles through Earth history. *Trends Microbiol.*, **30**, 143–157.

Sandrini, G., Ji, X., Verspagen, J. M., Tann, R. P., Slot, P. C., Luimstra, V. M., Schuurmans, J. M., Matthijs, H. C., & Huisman, J. (2016) Rapid adaptation of harmful cyanobacteria to rising CO₂. *Proc. Natl. Acad. Sci. U.S.A.*, **113**, 9315–9320.

Schneider, D. A., Gaal, T. & Gourse, R. L. (2002) NTP-sensing by rRNA promoters in *Escherichia coli* is direct. *Proc. Natl Acad. Sci. USA* **99**, 8602–8607

Schuerger, N., & Wilde, A. (2015). Appendages of the cyanobacterial cell. *Life (Basel)*, **5**, 700–715.

Seki, A., Hanaoka, M., Akimoto, Y., Masuda, S., Iwasaki, H., & Tanaka, K. (2007) Induction of a group 2 σ factor, RPOD3, by high light and the underlying mechanism in *Synechococcus elongatus* PCC 7942. *J. Biol. Chem.*, **282**, 36887–36894.

Shaner, N. C., Lambert, G. G., Chammas, A., Ni, Y., Cranfill, P. J., Baird, M. A., Sell, B. R., Allen, J. R., Day, R. N., Israelsson, M., Davidson, M. W., & Wang, J. (2013) A bright monomeric green fluorescent protein derived from *Branchiostoma lanceolatum*. *Nat. Methods*, **10**, 407–409.

- Sheridan, S. D., Benham, C. J. & Hatfield, G. W. (1998) Activation of gene expression by a novel DNA structural transmission mechanism that requires supercoiling-induced DNA duplex destabilization in an upstream activating sequence. *J. Biol. Chem.* **273**, 21298–21308
- Shimakawa, G., Shaku, K., Nishi, A., Hayashi, R., Yamamoto, H., Sakamoto, K. et al. (2015) FLAVODIIRON2 and FLAVODIIRON4 proteins mediate an oxygen-dependent alternative electron flow in *Synechocystis* sp PCC 6803 under CO₂-limited conditions. *Plant Physiol.*, **167**, 472–U732.
- Shimizu, T., Shen, J., Fang, M., Zhang, Y., Hori, K., Trinidad, J. C., Bauer, C. E., Giedroc, D. P., & Masuda, S. (2017) Sulfide-responsive transcriptional repressor SqrR functions as a master regulator of sulfide-dependent photosynthesis. *Proc. Natl. Acad. Sci. U.S.A.*, **114**, 2355–2360.
- Stock, A. M., Robinson, V. L. & Goudreau, P. N. (2000) Two-component signal transduction. *Annu. Rev. Biochem.* **69**, 183–215
- Sugimoto, Y., Tanaka, K., Masuda, S., & Takahashi, H. (1997) The *rpoD1* gene of *Synechococcus* sp. strain PCC 7942 encodes the principal sigma factor of RNA polymerase. *J. Gen. Appl. Microbiol.*, **43**, 17–21.
- Takai, N., Nakajima, M., Oyama, T., Kito, R., Sugita, C., Sugita, M., Kondo, T., & Iwasaki, H. (2006). A KaiC-associating SasA-RpaA two-component regulatory system as a major circadian timing mediator in cyanobacteria. *Proc. Natl. Acad. Sci. U.S.A.*, **103**, 12109–12114.
- Tandeau de Marsac, N., & Houmard, J. (1993) Adaptation of cyanobacteria to environmental stimuli: new steps towards molecular mechanisms. *FEMS Microbiol. Rev.*, **10**, 119–189.

- Taton, A., Erikson, C., Yang, Y., Rubin, B. E., Rifkin, S. A., Golden, J. W., & Golden, S. S. (2020) The circadian clock and darkness control natural competence in cyanobacteria. *Nat. Commun.*, **11**, 1688.
- Taylor, L. A., & Rose, R. E. (1988) A correction in the nucleotide sequence of the Tn903 kanamycin resistance determinant in pUC4K. *Nucleic Acids Res.*, **16**, 358.
- Trautmann, D., Voss, B., Wilde, A., Al-Babili, S., & Hess, W. R. (2012) Microevolution in cyanobacteria: re-sequencing a motile substrain of *Synechocystis* sp. PCC 6803. *DNA Res.*, **19**, 435–448.
- Vassilyev, D. G., Vassilyeva, M. N., Perederina, A., Tahirov, T. H., & Artsimovitch, I. (2007) Structural basis for transcription elongation by bacterial RNA polymerase. *Nature*, **448**, 157–162.
- Vinyard, D. J., Ananyev, G. M., & Dismukes, G. C. (2013). Photosystem II: the reaction center of oxygenic photosynthesis. *Annu. Rev. Biochem.*, **82**, 577–606.
- Voelker, U., Dufour, A., & Haldenwang, W. G. (1995) The *Bacillus subtilis* *rsbU* gene product is necessary for RsbX-dependent regulation of sigma B. *J. Bacteriol.*, **177**, 114–122.
- Watanabe, S., Ohbayashi, R., Shiwa, Y., Noda, A., Kanesaki, Y., Chibazakura, T., & Yoshikawa, H. (2012) Light-dependent and asynchronous replication of cyanobacterial multi-copy chromosomes. *Mol. Microbiol.*, **83**, 856–865.
- Westpheling, J., Raney, M. & Losick, R. (1985) RNA polymerase heterogeneity in *Streptomyces coelicolor*. *Nature*, **313**, 22–27.
- Yoshihara, S., Geng, X., Okamoto, S., Yura, K., Murata, T., Go, M., Ohmori, M., & Ikeuchi, M. (2001) Mutational analysis of genes involved in pilus structure, motility and

transformation competency in the unicellular motile cyanobacterium *Synechocystis* sp. PCC6803. *Plant Cell Physiol.*, **42**, 63–73.

Yoshimura, H., Okamoto, S., Tsumuraya, Y., & Ohmori, M. (2007) Group 3 sigma factor gene, *sigJ*, a key regulator of desiccation tolerance, regulates the synthesis of extracellular polysaccharide in cyanobacterium *Anabaena* sp. strain PCC 7120. *DNA Res.*, **14**, 13–24.

Zedler, J. A. Z., Michel, M., Pohnert, G., & Russo, D. A. (2023) Cell surface composition, released polysaccharides, and ionic strength mediate fast sedimentation in the cyanobacterium *Synechococcus elongatus* PCC 7942. *Environ. Microbiol.*, **25**, 1955–1966.

Supplemental Tables

Table 1. Strains were used in this study.

Strain	Genotype or relevant characteristics	Source
TUA	<i>Synechococcus elongatus</i> PCC 7942 (Wild type), derived from a stock from Tokyo University of Agriculture	Laboratory stock
NU	<i>Synechococcus elongatus</i> PCC 7942 (Wild type), derived from a stock from Nagoya University	Laboratory stock
TUAF1SP	<i>sigF1::spc^R</i>	This study
NUF1SP	<i>sigF1::spc^R</i>	This study
NUF1KM	<i>sigF1::kan^R</i>	This study
F1F	<i>sigF1-3×FLAG-tag::cm^R</i>	This study
R2F	<i>rpoC2-3×FLAG-tag::cm^R</i>	This study
F1OX	<i>sigF1-3×FLAG-tag::spc^R</i> (IPTG-OE)	This study
F2OX	<i>sigF2-3×FLAG-tag::spc^R</i> (IPTG-OE)	This study
PAG	NSI::PpilA1-mNG	This study
PAGF1KM	NSI::PpilA1-mNG <i>sigF1::kan^R</i>	This study
M1	NSI::PpilA1-mNG (m1)	This study
M2	NSI::PpilA1-mNG (m2)	This study
M3	NSI::PpilA1-mNG (m3)	This study
M4	NSI::PpilA1-mNG (m4)	This study
M5	NSI::PpilA1-mNG (m5)	This study
M6	NSI::PpilA1-mNG (m6)	This study
M7	NSI::PpilA1-mNG (m7)	This study
M8	NSI::PpilA1-mNG (m8)	This study
M9	NSI::PpilA1-mNG (m9)	This study
M10	NSI::PpilA1-mNG (m10)	This study

Table 2. Plasmids were used in this study.

Plasmid	Description	Source
pBluescriptSK+	Vector for construction of pFLAGΩSp	Agilent Technologies Japan
pBluescriptKS	Vector for construction of pKScatKE	Laboratory stock
pHSG399	Template for <i>cm^R</i>	Laboratory stock
pKScatKE	pBluescriptKS with <i>cm^R</i>	Laboratory stock
psinAFΩup	Template for 3×FLAG with omega fragment, <i>spc^R</i>	Hasegawa et al., 2020
pFLAGΩSp	pFLAG with omega fragment, <i>spc^R</i>	This study
pFLAGΩCm	pFLAG with omega fragment, <i>cm^R</i>	This study
pFLAGΩCm-F1orf	pFLAGΩCm with <i>sigF1</i>	This study
pFLAGΩCm-F1F	pFLAGΩCm with <i>sigF1</i> and its downstream	This study
pFLAGΩCm-R2orf	pFLAGΩCm with <i>rpoC2</i>	This study
pFLAGΩCm-R2F	pFLAGΩCm with <i>rpoC2</i> and its downstream	This study
pNSEI-F1	pNSEI with <i>sigF1</i> -FLAG	This study
pNSEI-F2	pNSEI with <i>sigF2</i> -FLAG	This study
pNS-Pspec-gm-mNG	pNSmNG with <i>gm^R</i>	A gift from Dr. Ryudo Ohbayashi
pNSΩSp-mNG	pNSmNG with omega fragment, <i>spc^R</i>	This study
pNSΩSp-PpilA1-mNG	pNSΩSp-mNG with PpilA1	This study

pHP45Ω	Template for omega fragment, <i>spc^R</i>	Prentki and Krisch, 1984
pUC4k	Template for omega fragment, <i>kan^R</i>	Taylor and Rose, 1988

Table 3. Primer sets were used in this study.

Name and purpose	Sequence (5'~3')
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For qPCR and Northern blotting	
pilA1-probe-f	atgtctgactccctccgtct
pilA1-probe-r	ctagcattgccgcaagttatgt
hspA-probe-f	atggcactcgttcgattcagtc
hspA-probe-r	ctatcgctcgcaagcttcagc
mNG-probe-f	ATGGTGTCTAAAGGTGAGGAGG
mNG-probe-r	TCACTTGCCTTCACCTTTAATATG
ccpB-probe-f	atgactctcaccctcaagaccg
ccpB-probe-r	ctaaacgtcgatcatccggcatt
digB-probe-f	ATGGCTCATCTCTCATCTCTATGG
digB-probe-r	TTACTCGACGATTAAGTGTCTG
sigF1-probe-f	gtggcactgtgcgagtac
sigF1-probe-r	ctaggcatcatcgccactg
sigF2-probe-f	tacgcgacagttgtca
sigF2-probe-r	ctaggctaagggtcggttg
pilA1-qPCR-F1	ccaaagaagagccatgcggaaat
pilA1-qPCR-R1	tcaggagatcctcagcaagagag

pilA1-qPCR-F2	ctggaagtggtaaagcagcgaat
pilA1-qPCR-R2	ttaaagagttgcgctgccttgg

For FLAG report	
strains and mutant	
construction	
Omega-seq1	cgagcaggggaattgatccg
Omega-seq2	gggctttactaagctgatccgg
CATpro-KXH-FW	ggaGGTACCTCTAGAGTTAACGGGCCAACTTTTGGC GAAAATG
CAT-RV-KXV	ggaGGTACCTCTAGAGATATCaaaattacgccccgccctg
CAT-F	gggggtaccTGTTGATACCGGGAAGCCCT
CAT-R	ggggaattcAGGCGTAGCACCAGGC
PFLAGcm-inverse-R	TGGAGCTCCAGCTTTTGTTC
PFLAGcm-inverse-F	AGAACTAGTGGATCCgactacaaag
rpoC2-gb-F	AAAGCTGGAGCTCCAatggcagaagcgaaaagc
rpoC2-gb-R	GGATCCACTAGTTCTgtcttcgtcaacatccaccag
rpoC2-dn-SHX-F	ggaGTCGACCTCGAGtaggtgctttctgccggt
rpoC2-dn1000-ApaI-r	aatGGGCCCgaataatcgagggtcgtcacc
sigF1-SacI-F	ggaGAGCTCgtgggcgaacctctctct
BglII-sigF1-3'	ggccagatctggcatcatcgccactgcg
sigF1-sacB-LRV	ggaaAGATCTactggctccgttggtcgtc
sigF1-sacB-LFW	ggaaGTCGACtgcgcgagaatcctgaaacg
sigF1-up500-f	tccagtgggagtcagtgga
sigF1-up-KXH-R	catGTTAACTCTAGAGGTACctccattgctaaccaagcagaggca
sigF1-dn-VXK-F	GATATCTCTAGAGGTACctccactggctccgttggtcgc

sigF1-dn500-r	gatctttgcttggcagaagtcca
sigF2-sacB-LRV	ggaaAGATCTtatcaggcctccgaggactg
sigF2-sacB-RFW	ggaaAGATCTctagcgccgttgcaaaaactg
sigF2-f	ggaGAGCTCgtggtgaaccaagcagcggttac
sigF2-r	aatGGATCCggctaagggctcgtttgcc
SpR-FW-KXH	ggaGGTACCTCTAGAGTTAACatgaggggaagcggatgcgc
SpR-RV-KXV	ggaGGTACCTCTAGAGATATCttatttgccgactaccttggtgatc
KanR-FW-KXH	ggaGGTACCTCTAGAGTTAACatgagccatattcaacgggaaacg
KanR-RV-KXV	ggaGGTACCTCTAGAGATATCctgattagaaaaactcatcgagcatc aaatg

For primer extension and fluorescence analysis	
mNG-hindIII-inv-R	agcataaagcttctcTCGAGCTTCTGGAGCAGGAA
pilA1-pro-smaI-F	aggCCCGGGtatcaatcaacagactggctgt
pilA1-pro-smaI-R	aggCCCGGGtcaggagatcctcagcaaga
mNG-NdeISmaI-inv-F	CATATGCCCCGGGtccTCAAGTAGGAGATTAATTC AATGGTG
Omega-NedaSmaI-mNG	ggaCCCGGGCATATGaagctttatgcttgtaaaccgtttg
Omega-HindIII-mNG	gagaagctttatgcttgtaaaccgtttt
mNG-probe-R	TCACTTGCGCTTCACCTTTAATATG
mNG-PEx-R	TACTCCCAAAGATGTGCAATTCGTGAGTGG
ccpB-PEx-R	acgcggcattgcggatcatctttgccgaag
0253-PEx-R	tgtgcccatacacaatcggagtccaagcaag
digB-PEx-R	AGAGCACGATCGCCAACCATAGCAGCCAGT
mNG-pilA1-Gib-sub-1-F	aagagccatgcggaataaaaagaggaacctgaaataaaaagcct

mNG-pilA1-Gib-sub-1-R	ttatttcaggttcctcttttattccgcatggctcttct
mNG-pilA1-Gib-sub-2-F	catgcggaaattttctccttgctgaaataaaagcctgctgtag
mNG-pilA1-Gib-sub-2-R	aggcttttatttcagcaaggagaaaattccgcatggctc
mNG-pilA1-Gib-sub-3-F	gaaatttcaggaacgacttttaaagcctgctgtagggg
mNG-pilA1-Gib-sub-3-R	tacagcaggcttttaaagtcgttcctgaaaattccgcatg
mNG-pilA1-Gib-sub-4-F	ttcaggaacctgaatattaagcctgctgtaggg
mNG-pilA1-Gib-sub-4-R	ccctacagcaggcttaatttcaggttcctgaaaattcc
mNG-pilA1-Gib-sub-5-F	aggaacctgaaataattccctgctgtaggggtaagt
mNG-pilA1-Gib-sub-5-R	taccctacagcagggaattatttcaggttcctgaaaattc
mNG-pilA1-Gib-sub-6-F	aacctgaaataaaagggacgagtaggggtaagtacttcaagaa
mNG-pilA1-Gib-sub-6-R	gtaacttaccctactcgtcccttttatttcaggttcctgaaaattt
mNG-pilA1-Gib-sub-7-F	aaataaaagcctgctcatcccgtaagttacttcaagaacagctct
mNG-pilA1-Gib-sub-7-R	cttgaagtaacttacgggatgagcaggcttttatttcaggt
mNG-pilA1-Gib-sub-8-F	aagcctgctgtagggcattcatacttcaagaacagctctcttg
mNG-pilA1-Gib-sub-8-R	gctgttcttgaagtatgaatgccctacagcaggcttttatt
mNG-pilA1-Gib-sub-9-F	gctgtaggggtaagtatgaagaagaacagctctcttgctg
mNG-pilA1-Gib-sub-9-R	aagagagctgttcttcttcatacttaccctacagcagg
mNG-pilA1-Gib-sub-10-F	gggtaagtacttcatcttgtgctctcttgctgaggatct
mNG-pilA1-Gib-sub-10-R	cctcagcaagagagcacaagatgaagtaacttaccctacagc

Appendix

BG-11 Media Preparation

Liquid BG-11

Solution (Vol.)	Vol.
Stock solution 1 (ml)	50
Stock solution 2 (μl)	1 *
Stock solution 3 (μl)	1
Stock solution 4 (μl)	1
Stock solution 6 (μl)	1
Micro element A6 (μl)	1 *
1M HEPES KOH pH 8.0 (ml)	20
H ₂ O (ml)	925
Total (ml)	1,000
Autoclave (121°C,15 min)	*add after autoclave

BG-11 plate

Solution (Vol.)	Vol.
Stock solution 1 (ml)	15
Stock solution 2 (μl)	300 *
Stock solution 3 (μl)	300
Stock solution 4 (μl)	300
Stock solution 5 (μl)	300
Stock solution 6 (μl)	300
Micro element A6 (μl)	300 *
1M HEPES KOH pH 8.0 (ml)	6
H ₂ O (ml)	277.5
Agar (g)	4.5
Total (ml)	300
Autoclave (121°C,15 min)	*add after autoclave

BG-11 Stock Solutions

Stock solution 1 (20×)

Component	Molecular Mass	Amount (g)
Na ₂ NO ₃ (Sodium Nitrate)	84.99	30
MgSO ₄ ·7H ₂ O (Magnesium Sulfate Heptahydrate)	246.47	1.5
Total volume to 1,000 ml		Autoclave (121°C, 15 min)

Stock solution 2 (1000×)

Component	Molecular Mass	Amount (g)
Citric Acid·H ₂ O	210.14	0.6
Ammonium Iron (III) Citrate, Brown	261.98	0.6
Na ₂ EDTA·2H ₂ O	372.24	0.1
Total volume to 100 ml		Fertilize by filter (0.22 µm) and store at 4°C

Stock solution 3 (1000×)

Component	Molecular Mass	Amount (g)
CaCl ₂ ·2H ₂ O (Calcium Chloride Dihydrate)	147.01	3.7
Total volume to 1,000 ml		Autoclave (121°C, 15 min)

Stock solution 4 (1000×)

Component	Molecular Mass	Amount (g)
Na ₂ CO ₃ (Sodium Carbonate)	105.9885	2.0
Total volume to 1,000 ml	Autoclave (121°C, 15min)	

Stock solution 5 (1000×)

Component	Molecular Mass	Amount (g)
Na ₂ S ₂ O ₃ (Sodium Thiosulfate)	158.11	15.8
Total volume to 1,000 ml	Autoclave (121°C, 15 min)	

Stock solution 6 (1000×)

Component	Molecular Mass	Amount (g)
K ₂ HPO ₄ (Potassium Hydrogen Phosphate)	174.18	3.8
Total volume to 1,000 ml	Autoclave (121°C, 15 min)	

1M HEPES -KOH (pH 8.0) (50×)

Component	Molecular Mass	Amount (g)
HEPES	238.3012	238.3

* Adjust pH to 8.0 with KOH (Potassium Hydroxide)

Total volume to 1,000 ml Autoclave (121°C, 15 min)

Microelements A6 (1000×)

Component	Molecular Mass	Amount (g)
H ₃ BO ₃ (Boric Acid)	61.83	2.86
MnCl ₂ ·4H ₂ O (Manganese (II) Chloride Tetrahydrate)	197.9	1.81
ZnSO ₄ ·7H ₂ O (Zinc Sulfate Heptahydrate)	287.56	0.222
Na ₂ MoO ₄ ·2H ₂ O (Sodium Molybdate Dihydrate)	241.95	0.39
CuSO ₄ ·5H ₂ O (Copper Sulfate Pentahydrate)	249.68	0.079
Co (NO ₃) ₂ ·6H ₂ O (Cobaltous Nitrate Hexahydrate)	291.03	0.0494

Total volume to 1,000 ml and store at 4°C

PCR Mix	For colony PCR	
KOD One	25 µl	2.5 µl
Forward Primer (10 pmol/µl)	1.5 µl	0.75 µl
Reverse Primer (10 pmol/µl)	1.5 µl	0.75 µl
DNA template (10 ng)	1 µl	-
H ₂ O	21 µl	1 µl
Total	50 µl	5 µl

PCR Reaction Cycle

Temperature	Time
94°C	10 sec.
Tm-5°C	5 sec.
68°C	1~10 sec./kb
25 ~ 30 cycles	

Gibson assembly

Reagent	Amount (μl)
Linearized vector	0.5 pmol
DNA fragment	1 pmol
2×Gibson assembly	5
Master mix	
H ₂ O	Up to 10 μl

Reaction Mix incubates in 50°C for 20 min.

Northern blotting

RNA extraction from Cyanobacteria

Procedure

1. Add 450 μl RNA extraction buffer and an equal amount of acidic phenol to the cell pellet and incubate at 65°C for 8 min.
2. Centrifuge at 15,000 × rpm for 5 min at room temperature
3. Transfer the supernatant to a new 1.5 ml tube, add 400 μl of acidic PCI, and mix by inverting 4–6 times.
4. Centrifuge at 15,000 × rpm for 5 min at room temperature
5. Repeat steps 3–4
6. Transfer the supernatant to a new 1.5 ml tube, add 1/10 volume (about 40 μl) of 5 M LiCl and 2–2.5 times volume (about 1 ml) 99.5% ethanol, and store at –30 °C for 30 min or 1 h.
7. Centrifuge at 15,000 × rpm for 30 min, at 4 °C.
8. Discard the supernatant and rinse it with 70% ethanol.
9. Centrifuge at 15,000 × rpm for 5 min, at 4 °C.
10. Remove the supernatant and dry up on the bench.

11. Add 35 μ l H₂O to the tube, resolve the RNA pellet by tapping, then measure the concentration with a spectrophotometer (Thermo Fisher Scientific NDL Nano Drop Lite).

Solutions for RNA extraction

RNA extraction buffer

Stock	Final Conc.
1 M Tris-HCl pH 6.5	50 mM
0.5 M EDTA pH 8.0	5 mM
10% SDS	0.5%
H ₂ O	

Acidic phenol

Stock	Amount
Phenol crystals	Proper amount
H ₂ O	Equal amount to phenol

Acidic PCI

Stock	Volume
Acidic phenol	25 ml
Chloroform	24 ml
Isoamyl alcohol	1 ml

Procedure for Northern Blotting

1. Prepare 5 μ g RNA sample with 7.5 μ l H₂O and 7.5 μ l RNA sample mix, incubate 65 °C for 10 min, Rapid cooling for 2 min on ice. Then add 10 $\times$ RNA Loading buffer 1.5 μ l.
2. Load samples to 1.0% denature agarose gel and electrophoresis in 1 $\times$ MOPS buffer.
3. Preparing Blotter follow this order: Container for Northern blot, Stand for Northern blot, Whatmann 3MM (12 $\times$ 25) 3 sheets, Gel, Membrane, Whatmann 3MM (12 $\times$ 12)

- 3 sheets, Comfort towel (11 × 11). Then, pore 20× SSC to the container and transfer RNA from gel to membrane overnight.
4. Remove the membrane from Blotter and crosslink by UV (CL-1000 Ultraviolet Crosslinker, Funakoshi, 1,200×100 mJ / cm²).
 5. Stain by Methylene blue solution and wash by MiliQ.
 6. Pack the membrane in hybrid pack and add hybridization buffer 12 ml, incubate at 48 °C >30 min.
 7. Incubate DNA probe at 65°C for 5 min and rapid cool for 3 min on ice.
 8. Add DNA probe for hybridization buffer (100 ng/ml) and incubate at 48°C overnight.
 9. Wash membrane with low stringency buffer at room temperature for 5 min × 2.
 10. Wash the membrane with a high stringency buffer at 68°C for 15 min × 2.
 11. Wash the membrane by washing the buffer briefly.
 12. Incubate in buffer 2 at room temperature for 30 min.
 13. Add DIG-AP antibody (Roche) 1/10,000 vol. against buffer 2 vol. Incubate at room temperature for 1 h.
 14. Wash membrane by wash buffer at room temperature for 15 min × 2.
 15. Equilibrate membrane in Buffer 3 for 5 min.
 16. Prepare Saran wrap and put the membrane. Drop CDP-star ready-to-use (Roche) 7 or 8 drops equally, then incubate at room temperature for 5 min in dark.

Detect by luminograph

DIG-labelled DNA probe

PCR reaction

KOD plus Neo PCR buffer (10×)	2 µl
DIG-dNTPs (2 mM)	2 µl
MgSO ₄ (25 µM)	1.2 µl
DNA template (10 ng)	0.4 µl
Primer-F (10 pmol/µl)	0.6 µl
Primer-R (10 pmol/µl)	0.6 µl
KOD plus Neo polymerase	0.4 µl
H ₂ O	12.8 µl
Total	20 µl

Solutions for Northern Blotting

20× SSC

Stock	Final Conc.
NaCl	3.0 M
Trisodium Citrate Dihydrate	0.3 M
H ₂ O	

10×MOPS Buffer (Running Buffer)

Stock	Final Conc.
MOPS	200 mM
Sodium acetate	50 mM
EDTA	10 mM
H ₂ O	

10× Maleic Acid Buffer

Stock	Final Conc.
Maleic Acid	1.0 M
NaCl	1.0 M
Adjust pH7.5 by NaOH	
H ₂ O	

10× Blocking Solution

Stock	Final Conc.
Blocking reagent (Roche)	10%
10× Maleic Acid Buffer	1×

Wash Buffer

Stock	Final Conc.
10× Maleic Acid Buffer	1×
Tween 20	0.3%

Methylene blue solution

Stock	Final Conc.
Sodium acetate	0.5 M
Methylene blue	0.04%

Hybridization Buffer

Stock	Volume
20× SSC	25 ml
Formamide	50 ml
2M Sodium phosphate Buffer	2.5 ml
10× Blocking Solution	20 ml
10% N-Lauroylsarcosine	1 ml
SDS	7 g
Yeast Total RNA	5 mg
H ₂ O	up to 100 ml

Low Stringency Buffer

Stock	Final Conc.
20× SSC	2×
10% SDS	0.1%
H ₂ O	

High Stringency Buffer

Stock	Final Conc.
20× SSC	0.5×
10% SDS	0.1%
H ₂ O	

Buffer 2

Stock	Final Conc.
10× Blocking stock solution	1×
10× Maleic Acid Buffer	1×
H ₂ O	

Buffer 3

Stock	Final Conc.
Tris-HCl pH 9.5	100 mM
NaCl	100 mM
MgCl ₂	50 mM
H ₂ O	

RNA Sample Buffer

Stock	Volume
10× MOPS	2 ml
Formaldehyde:	3 ml
Formamide	10 ml

Store at 4°C

1.0% denature agarose gel

Stock	Volume
10× MOPS	10 ml
Agarose ME	1 g
H ₂ O	87 ml

Melt agarose by microwave and cool down to about 50°C; add formaldehyde 3 ml.

Western blotting analysis**Protein extraction from Cyanobacteria**

1. Add 80 µl of 1 × PBS (pH 7.4) and 0.1 mg of Glass Beads ≥ 106 µm (Sigma) to the cell pellet.
2. vortex at 4°C for 5 min.
3. on ice for 5 min.

4. 2–3 repeat twice.
5. centrifuge at $5000 \times g$ for 5 min, at 4°C .
6. Transfer the supernatant to a new 1.5 ml tube, then centrifuge at $15000 \times g$ for 20 min, at 4°C .
7. Transfer the supernatant to a new 1.5 ml tube, perform protein assay using Bradford, then measure the protein concentration of the samples.

Solutions for protein extraction

1×PBS Buffer

Stock	Volume
NaCl	137 mM
Phosphate	10 mM
KCl	2.7 mM

SDS-PAGE

1. Make a gel with the above composition.
2. When the gel becomes hardened, remove the comb and wash the wells with H_2O .
3. Take a sample that contains 20 μg protein, then mix it with the sample buffer to denature the protein. Denatured the protein sample mix at 95°C for 5 min, then add samples to the well.
4. Run at 30 mA for 55 min with 1× Running buffer.

Solutions for SDS-PAGE

4× Separating buffer (pH 8.8)

Stock	Final Conc.
Trizma base	1.6M
Sodium Dodecyl Sulfate	0.4%
Adjust pH to 8.8 with HCl	
H_2O	

2× Stacking buffer (pH 6.8)

Stock	Final Conc.
Trizma base	120 mM
Sodium Dodecyl Sulfate	0.2%
Adjust pH to 6.8 with HCl	
H ₂ O	

10× Running buffer

Stock	Final Conc.
Trizma base	250 mM
Glycine	1.92 M
Sodium Dodecyl Sulfate	10%
H ₂ O	

SDS Polyacrylamide gel lower

Stock	10%	12%	15%
4× Separating buffer (pH 8.8)	1.5 ml	1.5 ml	1.5 ml
30% Acrylamide	2.0 ml	2.4 ml	3.0 ml
H ₂ O	2.5 ml	2.1 ml	1.5 ml
TEMED	6 µl	6 µl	6 µl
10% APS	60 µl	60 µl	60 µl

SDS Polyacrylamide gel upper

Stock	
2 × Stacking buffer (pH 6.8)	1.5 ml
30% Acrylamide	0.39 ml
H ₂ O	1.11 ml
TEMED	3 µl
10% APS	30 µl

2× Sample buffer

Stock	Final Conc.
Tris-HCl	0.125 M
Sodium Dodecyl Sulfate	4%
Bromophenol Blue	0.01%
Sucrose	10%
2-Mercaptoethanol	10%
H ₂ O	

Western blot analysis

1. Immerse the membrane (6 cm × 9 cm) in methanol to remove hydrophobic groups.
2. Immerse the membrane and 6 sheets of filter paper (6 cm × 9 cm) in Blotting Buffer.
3. Set 3 sheets of filter paper, gel, membrane, and 3 sheets of filter paper in this order in the Blotter, then perform 1 h blotting at 2 mA /cm².
4. Transfer the membrane to 3.5% blocking solution and shake for 1 h at room temperature.
5. Add the primary antibody to a concentration 10000 times and shake for 1 h at room temperature.
6. 1× TBS-T wash 10 min × 3 at room temperature.
7. Transfer the membrane to a new 3.5% blocking solution, add the secondary antibody to a concentration 10000 times, and shake for 30 min at room temperature.
8. 1× TBS-T wash 10 min × 3 at room temperature.
9. Mix equal amounts of Solution A and B of ImmunoStar Zeta (Wako), drop them on the membrane, and incubate in the dark for 5 min.
10. Detect by Luminograph.

Solutions for Western blotting

10× Native PAGE buffer

Stock	Final Conc.
Trizma base	250 mM
Glycine	1.92 M
H ₂ O	

Blotting buffer

Stock	Final Conc.
10× Native PAGE Buffer	1×
Methanol	20%
H ₂ O	

10× TBS-T

Stock	Final Conc.
Trizma base	250 mM
NaCl	1.54 M
Autoclave at 121°C for 15 min	

3.5% blocking buffer

Stock	Volume
Skim milk	3 g
1× TBS-T	100 ml

Phos-tag analysis

Procedure for protein extraction

1. Add 100 µl lysis buffer and 1 µl phosphatase inhibitor cocktail.
2. Add 0.1 g glass beads (212–300 µm).
3. Vortex at 4°C for 30 seconds, then place on ice for 30 seconds. Repeat this process 10 times.
4. Centrifuge at 15,000 × rpm for 5 min at 4°C.
5. Remove the supernatant, determine the protein concentration, and store the samples at –80°C.

Procedure for Phos-tag SDS-PAGE

1. Make a gel with the following composition.
2. When the gel becomes hardened, remove the comb and wash the wells with H₂O.
3. Take a sample that contains 10 µg protein, then mix it with the sample buffer to denature the protein.
4. Incubate samples at 25°C for 30 min.
5. Run at 100V for 3.5h with 1× Running buffer at 4°C.

Procedure for Phos-tag western blotting

1. After electrophoresis, remove the PAGE gel from the plate and transfer it to a container with 10 mM EDTA Transfer Buffer. Shake for 10 min. (Make sure to adjust so that the buffer can move up and down.)
2. Discard the buffer and add new 10 mM EDTA Transfer Buffer. Shake for 10 min.
3. Discard the buffer and add new Transfer Buffer. Shake for 10 min.
4. Discard the buffer and add new Transfer Buffer. Shake for 10 min.
5. Immerse the PVDF membrane in methanol. Shake for 15 min to remove the methanol base.
6. Immerse the membrane in Transfer Buffer. Shake for 15 min.
7. Perform the transfer as usual.
8. Block the membrane with Blocking buffer for 30–60 min.
9. Add the primary antibody and shake for more than 60 min at 4°C
10. Wash the membrane with TBS-T at room temperature (RT) for 15 min and repeat twice.
11. Add HRP-conjugated secondary antibody to the Blocking buffer and shake the membrane for 30 min.
12. Wash the membrane with TBS-T at room temperature for 15 min, repeat twice.
13. Mount the membrane on film, mix ImmunoStar Zeta (Wako) Solution A and Solution B at a 1:1 ratio, drop it onto the membrane, and detect chemiluminescence using Lumino Graph I (ATTO).

Solutions for Phos-tag

Lysis buffer

Stock	Final Conc.
1 M Tris-HCl (pH 8.0)	25 mM
0.5 M EDTA	0.5 mM
H ₂ O	

2× SDS Sample buffer

Stock	Final Conc.
1 M Tris-HCl (pH 8.0)	0.125 M
10% Sodium Dodecyl Sulfate	4%
Sucrose	10%
H ₂ O	

5 mM Phos-tag solution

Stock	Final Conc.
Phos-tag	5 mM
Methanol	3%
H ₂ O	

10 mM MnCl₂ solution

Stock	Final Conc.
Manganese (II) Chloride Tetrahydrate	0.125 M
10% Sodium Dodecyl Sulfate	4%
Sucrose	10%
Bromophenol blue	0.01%

10 mM EDTA transfer buffer

Stock	Final Conc.
10× Native PAGE buffer	1 ×
Methanol	20%
0.5 M EDTA	10 mM
H ₂ O	

SDS Polyacrylamide gel lower

Stock	10%	12%	15%
4× Separating buffer (pH 8.8)	1.57 ml	1.75 ml	1.75 ml
30% Acrylamide	2.33 ml	2.8 ml	3.5 ml
H ₂ O	2.92 ml	2.45 ml	1.75 ml
TEMED	7 µl	7 µl	7 µl
10% APS	70 µl	70 µl	70 µl

SDS Polyacrylamide gel upper

Stock	
2× Stacking buffer (pH 6.8)	1.5 ml
30% Acrylamide	0.39 ml
H ₂ O	1.11 ml
TEMED	3 µl
10% APS	30 µl

Chromatin immunoprecipitation (ChIP)

Cross link

1. Collect 40 ml cells (OD₇₅₀ of 0.5).
2. Cross-link with 1 ml formaldehyde (final concentration of 1%) .
3. Rotate at room temperature for 10 min.
4. Add 2.5 M glycine 2 ml (final concentration 125 mM).
5. Rotate at room temperature for 5 min.
6. Centrifuge at 15,000 × rpm for 2 min, at 4°C.
7. Discard the supernatant, keep it on ice, and add 1 ml TBS to suspend the pellet.
Then move to a new 1.5 ml tube.
8. Centrifuge at 15,000 × rpm for 1 min, at 4°C.
9. Repeat 7–8
10. Discard the supernatant and store it at –80°C.

Modulation

11. Dissolved the sample on ice.
12. Add Glass beads, acid-washed ≤ 106 µm (SIGMA), 0.5g. Add 500 µl cold lysis buffer (contain Complete cocktail).

13. Vortex for 10 min at 4°C.
14. On ice for 5 min
15. Repeat 13–14 for 4 times
16. Spin down and move supernatant to a new 1.5 ml tube.
17. Sonicate the cells to an average DNA size of 0.6–0.7 kb by Branson Sonifier 250 (set at 20–30% output, 90% duty cycle, ten times for 5 sec with 1 min intervals)
18. Centrifuge at 15,000 × rpm for 5 min, at 4°C.
19. Move the supernatant to a new 1.5 ml tube.
20. Centrifuge at 15,000 × rpm for 15 min, at 4°C and move the supernatant to a new 1.5 ml tube.
21. Take 1 µl for protein assay, and store the rest at –80°C.

Chromatin immunoprecipitation

22. Balance the protein G Sepharose Fast Flow (GE Healthcare Bio-Sciences) with lysis buffer (contain Complete cocktail) to a slurry final concentration of 40%.
23. 0.5 mg of protein brought to a volume of 500 µl with cold lysis buffer (contain Complete cocktail).
24. Add balanced protein G Sepharose resin (40% slurry) 40 µl. Rotate for 60 min at 4°C.
25. Centrifuge at 15,000 × rpm for 15 seconds, at 4°C.
26. Move the supernatant to a new 1.5 ml tube.
27. Add 5 µg Anti-FLAG antibody
28. Rotate for overnight (16 h) at 4°C.
29. Centrifuge at 15,000 × rpm for 1 min, at 4°C.
30. Move the supernatant to a new 1.5 ml tube.
31. Add balanced protein G Sepharose resin (40% slurry) 60 µl. Rotate for 60 min at 4°C.
32. Centrifuge at 15,000 × rpm for 15 sec, at 4°C.
33. Discard the supernatant.
34. Add 1 ml lysis buffer, rotate at room temperature for 5 min.
35. Repeat 33–35 with wash buffer 1, wash buffer 2 and TE buffer.
36. Discard the supernatant.
37. Add 100 µl TE buffer (contain RNaseA 20 µg).
38. Incubate at 37°C for 30 min.
39. Add 900 µl TE buffer, rotate at room temperature for 5 min.
40. Centrifuge at 15,000 × rpm for 15 s, at 4°C.

41. Discard the supernatant.
42. Add 250 μ l IP elution solution.
43. Stand still for 15 min.
44. Centrifuge at 15,000 $\times$ rpm for 1 min, at 4°C.
45. Move the supernatant to a new 1.5 ml tube.
46. Repeat 43–46, final liquid should be 500 μ l in total.
47. Input DNA sample: 20 μ g of protein brought to a volume of 500 μ l with cold lysis buffer (contain Complete cocktail). Add 450 μ l IP elution solution. Add 20 μ l 5 M NaCl, mix well.
48. Incubate at 65°C for 5 h.
49. Add 1 ml 99% ethanol
50. Keep in at –30°C overnight.
51. Centrifuge at 15,000 $\times$ rpm for 15 min, at 4°C.
52. Discard the supernatant and rinse with 70% ethanol.
53. Dry up
54. Dissolve in 100 μ l TE buffer.
55. Add 100 μ l 2 $\times$ proteinase K buffer (contain 20 mg/ml proteinase K 1 μ l)
56. Incubate at 50°C for 30 min.
57. Add 200 μ l PCI, vortex.
58. Centrifuge at 15,000 $\times$ rpm for 5 min, at 4°C.
59. Move the supernatant to a new 1.5 ml tube.
60. Add 200 μ l TE buffer to PCI, vortex.
61. Centrifuge at 15,000 $\times$ rpm for 5 min, at 4°C.
62. Move the supernatant to the 1.5 ml tube (total 400 μ l)
63. Add 40 μ l 3 M NaOAC, 1 ml 99% ethanol. Store in –80°C for 1 h.
64. Centrifuge at 15,000 $\times$ rpm for 10 min, at 4°C.
65. Discard the supernatant and rinse with 70% ethanol.
66. Dissolve in 100 μ l TE buffer. Store at –80°C.

Solutions for ChIP

MQ (+ complete Mini EDTA free cocktail)

Stock	Volume
H ₂ O	10 ml
complete Mini EDTA free cocktail	1 tablet

TBS

Stock	Final Conc.
1 M Tris-HCl (pH 7.4)	20 mM
5 M NaCl	150 mM
H ₂ O	

Lysis buffer

Stock	Final Conc.
1 M HEPES-KOH (pH 7.5)	50 mM
5 M NaCl	140 mM
0.5 M EDTA	1 mM
10% Triton	1.0%
10% sodium deoxycholate	0.1%
H ₂ O	

Wash Buffer 1

Stock	Final Conc.
1 M HEPES-KOH (pH 7.5)	50 mM
5 M NaCl	500 mM
0.5 M EDTA	1 mM
10% Triton	1.0%
10% sodium deoxycholate	0.1%
H ₂ O	

Wash Buffer 2

Stock	Final Conc.
1 M Tris-HCl (pH 8.0)	10 mM
4 M LiCl	250 mM
10% NP-40	0.5%
0.5 M EDTA	1 mM
10% sodium deoxycholate	0.1%
H ₂ O	

TE

Stock	Final Conc.
1 M Tris-HCl (pH 8.0)	10 mM
0.5 M EDTA	1 mM
H ₂ O	

IP elution solution

Stock	Final Conc.
10% Sodium Dodecyl Sulfate	1.0%
1 M NaHCO ₃	100 mM
H ₂ O	

2× proteinase K buffer

Stock	Final Conc.
1 M Tris-HCl (pH 8.0)	20 mM
0.5 M EDTA	10 mM
10% Sodium Dodecyl Sulfate	1.0%
H ₂ O	

Primer extension

Procedure for sequence ladder (USB Thermo Sequenase Cycle Sequencing Kit)

1. Prepare 4 tubes labeled G, A, T, and C. Mixture solution as follows

DNA	2 μ l
Reaction buffer	2 μ l
Labeled primer	1 μ l
Thermo sequenase DNA polymerase	2 μ l
H ₂ O	10.5 μ l
Total	17.5 μ l

2. Transfer 4 μ l to the tube containing ddG, ddA, ddT, and ddC separately. Mix gently.
3. Cycling program as follows

95°C	30 sec
60°C	60 sec
40–60 cycles	

4. Add 4 μ l of stop solution to each of the termination reactions.
5. Use 1 μ l DNA sample for sequence and mix with size standard ratios as follows

DNA	1 μ l
GeneScan 600 liZ size standard v2.0	0.2 μ l
Hi-Di Formamide	10.8
Total	12

Procedure for primer extension

1. Mix 10 μ g RNA sample with 30 pmol labeled primer, 0.1 volume of 3 M sodium acetate (pH 5.2), and 2.5 volumes of ethanol. Store at –20°C for 30 min.
2. Centrifuge at 12,000 g for 10 min at 4°C.
3. Wash the pellet with 70% ethanol and recentrifuge.
4. Store at room temperature until the ethanol has evaporated.
5. Dissolve the pellets in 30 μ l hybridization buffer.
6. Incubate the mixture at 85°C for 10 min

7. Transfer to 30°C for overnight
8. Add 170 µl of water and 400 µl of ethanol. Store at 4°C for 1 hour.
9. Centrifuge at 12,000g for 15 min at 4°C.
10. Discard the supernatant and wash the pellet with 500 µl of 70 µl ethanol.
Recentrifuge and discard the supernatant.
11. Dry up at room temperature.
12. Dissolve in 15 µl H₂O.
13. Add 5× RT buffer and RT enzyme mix from ReverTra Ace qPCR RT kit
14. Reverse PCR reaction as follows:

37°C	15 min
98°C	5 min

15. Add 150 µl of TE (pH 7.6), 0.1 M NaCl and 200 µl of Phenol: Chloroform.
16. Vortex for 30 seconds
17. Centrifuge at 12,000 × g for 5 min at room temperature.
18. Transfer the upper to a new 1.5 ml tube.
19. Add 500 µl of ethanol and store at 4°C for 1 hour.
20. Centrifuge at 12,000 × g for 15 min at 4°C.
21. Wash the pellet with 500 µl of 70% ethanol.
22. Dry up at room temperature.
23. Dissolve the pellet in 6 µl of TE (pH 7.4) and add 8 µl of formamide.
24. Add 0.2 µl GeneScan 600 LIZ size standard v2.0.

Solution for primer extension

Hybridization buffer

Stock	Final Conc.
PIPES (pH 4.0)	40 mM
EDTA (pH 8.0)	1 mM
NaCl	0.4 M
80% formamide	

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