Studies on the transcriptional regulation in a toxic cyanobacterium *Microcystis aeruginosa*

TAKASHI HONDA

Laboratory of Marine Microbiology, Division of Applied Bioscience,

Graduate School of Agriculture, Kyoto University

Table of Contents

Chapter 1

General introduction

In the recent years toxic blooms of cyanobacteria have frequently occurred in eutrophic freshwater worldwide (Guo 2007; Paerl and Huisman 2008). Unicellular cyanobacterium *Microcystis aeruginosa* is the most notorious among bloom forming cyanobacteria (Oliver et al. 2012). Because many strains of *Microcystis* produce cyclic heptapeptide hepatotoxins, called microcystins, which specifically inhibit eukaryotic protein phosphatase type 1 and 2A and enhance liver tumor (MacKintosh et al. 1990; Namikoshi et al. 1992; Nishiwaki-Matsushima et al. 1992; Yoshizawa et al. 1990). *Microcystis* blooms have caused death of livestock (Huisman et al. 2005; Oberholster et al. 2009). Moreover, 60 patients in a haemodialysis unit in Brazil died probably due to use of microcystins-containing water for dialysate preparation in 1996 (Pouria et al. 1998). Therefore, the World health organization (WHO) determines a provisional guideline value of total microcystin-LR (free plus cell-bound) to 1 μg/L in 1998 (WHO 2011). Thus, it is world issues for maintaining safe water supplies to control and forecast the toxic cyanobacterial blooms (Backer and McGillicuddy 2006; Pearson and Neilan 2008).

Many studies have been performed on relationship between environmental factors (e.g., nutrient supply, light and temperature) and dynamics of cyanobacterial blooms (Oliver et al. 2012). For example, cyanobacteria tend to favor low N:P ratio (Smith 1983). However, cyanobacterial population was increased with increasing N:P ratio in Lake Okeechobee in USA (McCarthy et al. 2009). Therefore, the mechanisms for bloom

formation and termination have not been fully elucidated (Oliver et al. 2012). Although algal growth potential (AGP) test have been employed for evaluation of effect of physicochemical characters on the growth of *Microcystis* (Maestrinp et al. 1997), there is no strategy that can forecast the toxic cyanobacterial blooms based on environmental physicochemical characters (Oliver et al. 2012).

In bacteria, physiological changes which response to internal or external stimuli are mainly controlled at transcriptional levels (Seshasayee et al. 2006). This nature is applicable to evaluation of bacterial physiological status by monitoring expression of marker genes (Lindell and Post 2001). Therefore, I surveyed gene regulation during formation and termination of *Microcystis* bloom. This may provide important basic knowledge to understand physiological state of this species at genetic level. In addition, this also may contribute to development of the method for forecasting bloom dynamics of *Microcystis.*

Many factors are involved in termination of blooms, e.g., chytrid infection, grazing by zooplanckton and protozoan, cyanophage infection and nutrient depletion (Oliver et al. 2012). Recently, it has become known that large number of viruses (including phages) are exist in aquatic water, ca. ~ 10^7per mL (Bergh et al. 1989), and that viral lysis is major factor on microbial mortality which impact is approximately equal to grazing by zooplanckton and protozoan; ca. 20-40% of standing stock procaryotes are removed from surface water by viral lysis per day (Suttle 1994, 2005, 2007). *Microcystis* population is attacked by large number of unknown, diverse phages (Kuno et al. 2012) and also affected with its infectious phages (Yoshida et al. 2008a). Therefore, phage infection may be one of the major factor terminating *Microcystis* blooms (Suttle 1994, 2005, 2007). However, little is known about transcriptional

alterations of *Microcystis* genes during phage infection. In Chapter 2, I investigated the gene expression of *Microcystis* when cells were infected with phage to identify genes involved in bloom termination.

It is assumed that bloom formation is induced by increased growth of caynobacterial cells. Bacterial cell growth is followed by cell cycle progression. Bacterial cell cycle is traditionally divided into follow three stages as follows.1) The B period: the period between division and initiation of chromosome replication, 2) The C period: the period required for replication, and 3) The D period: the time between the end of replication and completion of division (Wang and Levin 2009). In order to impart correct genetic information to progeny cells, cell division requires fully replicated chromosome and begin with the end of chromosome replication (Clark 1968). Therefore, it is predicted that some kind of system for controlling cell cycle, so called “checkpoint”, exist between the C and D periods (Clark 1968). However, bacterial checkpoint is poorly understood (see Chapter 3-1). In *Microcystis*, chromosome replication and cell division are coordinated, at least partially, via transcriptional regulation of a cell division gene *ftsZ* (Yoshida et al. 2005). Therefore, I expected some transcriptional regulators of *ftsZ* in *Microcystis*. I hypothesized that this transcription factor could be a marker gene for normal growing cells, and that expression monitoring of this factor make it possible to forecast bloom formation. In Chapter 3, I purified and characterized this candidate for checkpoint factor to forecast bloom formation of *Microcystis*.

Chapter 2

Gene expression of *Microcystis aeruginosa* during infection of cyanophage

Introduction

Viral reproduction relies on host cell components, including metabolic substrates and the machinery for replication, transcription, and translation. In addition, many cyanophages carry multiple host-like genes termed auxiliary metabolic genes (AMGs) that play key roles in redirecting host cell metabolism to phage production (Thompson et al. 2011). For example, marine cyanophages infecting *Prochlorococcus* or *Synechococcus* often harbor the *psbA* [photosystem II (PSII) D1], *cp12* (Calvin cycle inhibitor), *talC* (transaldolase), *gnd* (6-phosphogluconate dehydrogenase), and *zwf* (glucose-6-phosphate dehydrogenase) genes (Thompson et al. 2011). One hypothesis is that these gene products maintain host photosynthetic activity but redirect the host's metabolism to decrease activity of the Calvin cycle and increase activity of the pentose phosphate pathway. This redirection of cell resources may increase fitness of the phages infecting cyanobacteria that possess a small genome and inhabit the oligotrophic ocean (Thompson et al. 2011).

Ma-LMM01 has been assigned to a new lineage of the *Myoviridae* family (Carstens and Ball 2009; Lavigne et al. 2009; Yoshida et al. 2008b). It is a lytic cyanomyovirus infecting the toxic strain of freshwater cyanobacterium *Microcystis aeruginosa* (Yoshida et al. 2006; Yoshida et al. 2008b). The Ma-LMM01 genome has no homologs of AMGs, which have been found in many marine cyanophages. Alternatively, the

phage carries an *nblA* homolog that is involved in the degradation of the major light-harvesting complexes, phycobilisomes (Gao et al. 2012; Yoshida-Takashima et al. 2012; Yoshida et al. 2008b). In natural environments, *M. aeruginosa* floats near the surface using a gas vacuole (Mlouka et al. 2004) and is intensively and frequently exposed to light that may rapidly lead to photoinhibition upon phage infection (Yoshida-Takashima et al. 2012; Yoshida et al. 2008b). This behavior has led us to propose that phycobilisome degradation is beneficial to the phages infecting the cyanobacteria inhabiting near-surface waters (Yoshida-Takashima et al. 2012; Yoshida et al. 2008b). In fact, we have observed that the level of phycobilisomes decreased in *Microcystis* during Ma-LMM01 infection (our unpublished data). In *Planktothrix* phage PaV-LD, phage NblA is indeed active and degrades the host's phycobilisomes (Gao et al. 2009). However, little is known about transcriptional alterations of host genes involved in other cellular processes during Ma-LMM01–*M. aeruginosa* infection, in particular.

To search changes of gene expression specific to phage infection, we investigated the expression pattern of four categories of *M. aeruginosa* genes during Ma-LMM01 infection: (1) the housekeeping genes as indicators of transcription activity in general; (2) the stress response genes as indicators of stress status; (3) carbohydrate metabolic genes; (4) photosynthetic genes (Table 2-1). Our results show that infection with Ma-LMM01 did not shut down host transcription activity-rather it directly or indirectly induced the general stress response genes.

Table 2-1. Description of the genes monitored in this study.

Organisms	Functional category, pathway	Gene ID[a]	Gene	Description of gene products or function
Microcystis				
	Housekeeping			
	DNA repair and recombination protein	MAE_48230	*gyrB*	DNA gyrase subunit B
	Homologous recombination/ DNA repair	MAE_16070	*recA*	Double-stranded DNA binding protein RecA
	Transcription	MAE_54470	*sigA*	Principal sigma factor SigA
	Stress response genes			
	Chaperone	MAE_46070	*groES*	Small subunit of co-chaperonin GroES
	Chaperone	MAE_45710	*hspA*	16.6 kDa small heat shock protein
	Chaperone	MAE_11440	*hspA2*	16.6 kDa small heat shock protein
	Chaperone	MAE_39990	*htpG*	Molecular chaperone HtpG
	Degradation of protein, peptides, and glycopeptides	MAE_30510	*htrA*	Serine protease HtrA
	Transcription	MAE_14230	*sigB*	Group 2 sigma factor SigB
	Anti oxidation			
	Scavenging enzyme for reactive oxygen species	MAE_53990	*sodB*	Superoxide dismutase
	Photosynthetic apparatus			
	Photosystem II	MAE_10220	*psbAI*	PSII D1 protein
		MAE_10380	*psbAII*	PSII D1 protein
		MAE_10510	*psbAIII*	PSII D1 protein
		MAE_10800	*psbAIV*	PSII D1 protein
		MAE_58140	*psbAV*	PSII D1 protein
	Carbohydrate metabolism			
	Pentose phosphate pathway	MAE_15220	*gnd*	6-phosphogluconate dehydrogenase
	Glycolysis / Gluconeogenesis	MAE_34890	*gap1*	Glyceraldehyde 3-phosphate dehydrogenase
	Calvin cycle	MAE_47890	*rbcL*	Ribulose 1, 5-bisphosphate carboxylase/Oxygenase large subunit (RuBisCO)
Ma-LMM01				
	Degradation of phycobilisome	MaLMM01_gp005	*nblA*	Phycobilisome degradation protein NblA
	Phage structurel protein	MaLMM01_gp091	*g91*	Putative phage tail sheath protein

[a] For *M. aeruginosa*, IDs in CyanoBase is indicated. For Ma-LMM01, GenBank locus tags were indicated.

Materials and methods

Host strain, growth conditions, phage infection, and sample preparation

Microcystis aeruginosa strain NIES-298 was obtained from the National Institute for Environmental Studies (NIES). The *Microcystis* cells were inoculated into CB medium (Kasai et al. 2004) (Table 2-2) and cultured under a 12/12-h light/dark photocycle (light intensity: 40 μmol photons/m^2/s) at 30 °C with 0.5 % CO_2 (v/v) aeration. Exponentially growing *Microcystis* cells (2,000 ml; 7.8×10^5 cells/mL) were synchronized by the block-release method as described previously (Yoshida et al. 2005).

Table 2-2. Composition of CB medium

$Ca(NO_3)_2 \cdot 4H_2O$	150.0 mg
KNO_3	100.0 mg
$MgSO_4 \cdot 7H_2O$	40.0 mg
K_2HPO_4	30.0 mg
VitaminB12(1mg/mL)	0.1 mL
Biotin(1mg/mL)	1.0 mL
Thiamine HCl(2.5mg/mL)	0.4 mL
PIV metals*	3.0 mL
Bicine	500.0 mg
Distilled water	1000.0 mL

pH 9.0

*PIV metals

$FeCl_3 \cdot 6H_2O$	19.60 mg
$MnCl_2 \cdot 4H_2O$	3.60 mg
$ZnSO_4 \cdot 7H_2O$	2.20 mg
$CoCl_2 \cdot 6H_2O$	0.40 mg
$Na_2MoO_4 \cdot 2H_2O$	0.25 mg
$Na_2EDTA \cdot 2H_2O$	100.00 mg
Distilled water	100.00 mL

To prepare the phage lysate, we infected 200 ml of *Microcystis* cells (2×10^6–7×10^6 cells/mL) with Ma-LMM01 and incubated the infected cells for 3 days. The resultant lysate was filtered through a 3.0-μm polycarbonate membrane filter (Millipore, Billerica, MA) and stored at 4 °C. The titer of the phage lysate was determined using the most probable number method (approx. 8.6×10^6–3.2×10^6 infectious units/mL).

When the light was turned on [0 h = 0 h post-infection (pi)], the 2,000-mL culture of synchronized *Microcystis* cells was divided over nine different 200-mL flasks and into three different treatments as follows. In the phage-infected culture treatment, 50 ml of the phage lysate was added to 200 mL of *Microcystis* cells at 0 h pi, resulting in a multiplicity of infection of 1.5–5.6. In the control culture, 50 ml of CB medium was added to the 200 ml of *Microcystis* cells. The third treatment was a high light (HL) intensity culture treatment which was prepared to evaluate the transcription level of stress response genes. For this treatment we added 50 ml of CB medium to 200 mL of *Microcystis* cells and incubated the cells under a light intensity of 200 μmol photons/m^2/s. The level of HL irradiance was determined based on data showing that the growth rate of *Microcystis* peaked at about 80 μmol photons/m^2/s and fell at about 135 μmol photons/m^2/s (Yagi et al. 1984). After the *Microcystis* cells had been inoculated with the phage, an aliquot of the culture was collected every 2 h for 16 h and at 24 h; the samples were immediately fixed in 25 % glutaraldehyde at a final concentration of 1 % and stored at 4 °C until analysis. The number of host cells and phage particles in the fixed samples was determined using epifluorescence microscopy (Nicon ECLIPSE E800; Nicon, Tokyo, Japan). The host cells were stained with 4′,6-diamidino-2-phenylindole. To count phage particles, I stained particles that had been passed through a 0.2-μm polycarbonate filter (ADVANTEC, Tokyo, Japan) with

SYBR Gold (Molecular Probes, Eugene, OR) and then filtered the particles through a 0.02-μm Al_2O_3 Anodisc membrane filter (Whatman, Piscataway, NJ). The expression profiles from 0 to 6 h pi were determined because the host cells began lysis at 6 h pi (Yoshida et al. 2006). For RNA extraction, cells at 0, 1, 2, 3, 4, and 6 h pi were collected on 3.0-μm PTFE membrane filters (Millipore, Billerica, MA), re-suspended in 5 ml of stop solution (phenol:ethanol, 5:95 v/v), and stored at −80 °C (Sato 1995).

Total RNA extraction, purification, and cDNA synthesis

Total RNA was extracted from 1 mL of the stored cell suspension as previously described by Yoshida et al. (2005). Briefly, stored cell pellets were treated with 5 % sodium dodecyl sulfate, and the RNA was extracted using Sepazol RNA I Super G (Nakarai Tesque, Kyoto, Japan) followed by two additional extractions with phenol and chloroform/isoamyl alcohol (49:1). The RNA was precipitated using isopropanol and washed with 70 % ethanol. The pellet was dissolved in dimethyl dicarbonate-treated water, and contaminating DNA was removed using TURBO DNase (Ambion, Austin, TX). A two hundreds ng of total RNA was reverse transcribed (RT) with random hexamers using a SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Real-time RT-PCR

Real-time RT-PCR primers were designed from the genome sequence of *M. aeruginosa* strain NIES-843 (GenBank accession no. NC_010296) and from Ma-LMM01 (GenBank accession no. NC_008562) using Primer-BLAST (Ye et al. 2012) (Table 2-3). Primers were tested using *M. aeruginosa* strain NIES-298 DNA as a template and showed specific amplification of the target genes.

SYBR Premix Ex Taq (Tli RNaseH Plus; TaKaRa Bio, Otsu, Japan) was used to determine the DNA copy number. A ten-fold dilution series of PCR-amplified DNA fragments using each RT-PCR primer set was amplified with the real-time PCR assay (Yoshida et al. 2010). Each reaction mixture contained 6.25 μL of SYBR Premix Ex Taq (Tli RNaseH Plus) and 5 pmol of forward and reverse primers. PCR amplification was performed using the Thermal Cycler Dice Real Time System mode TP815 (TaKaRa Bio, Otsu, Japan), and the cycling program consisted of an initial denaturing for 30 s at 95 °C, followed by 35 cycles of 5 s at 95 °C, 30 s at 58 °C, and 30 s at 78 °C. A melting curve analysis was performed at 60–95 °C. Linear-regression equations to obtain cycle threshold (Ct) values were calculated as a function of the known DNA copy numbers using Cycler Dice Real Time System Single Software (TaKaRa Bio, Otsu, Japan). Relative transcription amounts were calculated using the *rnpB* (RNase P RNA gene) transcripts as an internal control gene (Yoshida et al. 2010).

Statistical analysis

Data from the real-time RT-PCR analyses obtained from three independent biological experiments were averaged and presented as the mean ± standard deviation. Statistical analysis was performed (control cultures vs. phage-infected culture; control culture vs. HL culture) at each time point by using Student's t test or Welch's t test following an F test. P values of <0.05 were considered to be significant.

Results

In the Ma-LMM01-infected culture, the cell density of *Microcystis* decreased from 6.3×10^5 cells/mL at 0 h pi to 3.8×10^4 cells/mL at 24 h pi. Coincidentally, the number

Table 2-3. Primers used in this study

Organisms	Target gene	Primer direction	Primer sequence (5' to 3')	Reference
Microcystis				
	gyrB	F	TTACACGGAGTCGGGATTTC	This study
		R	AAACGTCCGGAGAGGGTACT	
	recA	F	CCGTGCCTAGTGGTGCTTTA	This study
		R	CTAAAGCGTGTTCCGCATCG	
	sigA	F	CGCCGATCAATCCCGCACCA	This study
		R	CTCGGTGGGTTTGCGACGCA	
	sigB	F	AGATCGCTTGGAGTGGGCGGA	This study
		R	CGCAACCACCAAGCGGAGGT	
	groES	F	TGGTATTTTCCTTCCCGACGC	This study
		R	CTTTGTCACCCACACCCACT	
	hspA	F	CGCTTTCGGGAGAGTGAGAT	This study
		R	CCCGCACTGAGTTCCGTTAT	
	hspA2	F	CGATGTGGAGGTTAGCAAGCA	This study
		R	GGCAGGGTAATCAAGCGACT	
	sodB	F	ATGCCGCCCAAGCATGGAAT	This study
		R	CCAACCAAGCCCAACCGCTA	
	htpG	F	CACATCTCCCCAACAACGAA	This study
		R	CGCCGTATTGCCGATTTTAT	
	htrA	F	GATAGCGATCGCCCATTCTC	This study
		R	GTGGCAGTGGTACAGGTGGA	
	psbAI-V	F	TCTACAACAGCGCGAGAGCG	This study
		R	TGATGAAGCAGGTGGTGGCG	
	psbAII	F	CGAACCTTGACAACCTAATACATAC	This study
		R	TTGTTGGTGCTGGTGATCCA	
	gnd	F	TCTGCACGGTCCTTCCCTGA	This study
		R	TCACATAATGGCCCGCACCC	
	gap1	F	CCACGGACGTTTTCAGGGTA	This study
		R	GCCCCAGCTTCTAGGTGTTT	
	rbcL	F	CTCCGCGGTGGTTTAGACTT	This study
		R	CATCTGCTCGCAGGTAGGAG	
	rnpB	F	GTGGGGAGCAAGGTGG	Yoshida et al. (2005)
		R	CTTTTACCTTTGTTGGAATAGAG	
Ma-LMM01				
	nblA	F	GTGAGTGCCATTCCTGC	This study
		R	TCTTCTTGATGATAGCCGC	
	g91	F	ACATCAGCGTTCGTTTCGG	Yoshida et al. (2008a)
		R	CAATCTGGTTAGGTAGGTCG	

F: forward, R: reverse

of phage particles increased from 7×10^7 particles/mL to 2×10^8 particles/mL (Fig. 2-1). Because the latent period of this phage is 6–12 h (Yoshida et al. 2006) and the infective process occurs mainly during the light period (Kimura et al. 2012), we calculated that based on the decrease in host cell number >90 % of the cells were infected by the phage. Ma-LMM01 has at least three different temporal classes of transcripts; i.e., early, middle (e.g., *nblA*) and late (e.g., *g91*) genes (our unpublished data). Transcripts of the phage *nblA* appeared at 1–6 h pi, peaked at 2 h pi, and remained at the

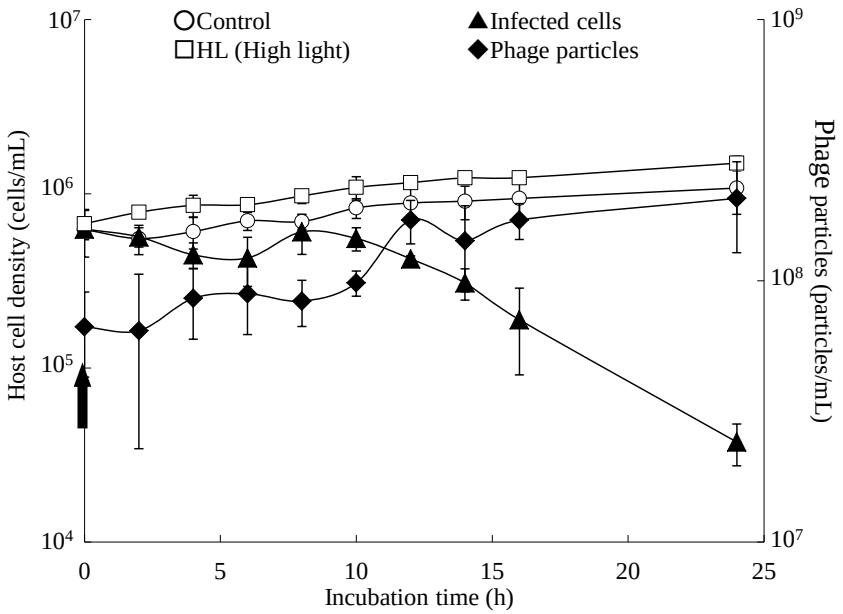


Fig. 2-1. Ma-LMM01 infection of *M. aeruginosa* strain NIES-298, showing decrease of cell density and expression of phage genes. Error bars represent SDs of three replicates. Host cell density and phage particles were determined by direct count using microscopy with DAPI and SYBR Gold, respectively. Addition of the virus is indicated by an arrow.

same level until lysis (Fig. 2-2); in comparison, transcripts of *g91* (tail sheath gene) progressively increased during the infection (Fig. 2-2).

Host gene expression was monitored from 0 h (beginning of the light period) up to 6 h. For the housekeeping genes, three genes were chosen: *sigA*, the primary σ factor (Lonetto et al. 1992); *gyrB*, the DNA gyrase subunit B (Mizuuchi et al. 1978); *recA*, the DNA recombination and repair protein (Radding 1981). These genes are distantly located on the genome (Kaneko et al. 2007). There was no significant difference between mRNA expression of *recA* (transcript level) in the control cells and that in the phage-infected cells during the experimental period (Fig. 2-3). In comparison, *sigA* transcript level in the phage-infected cells was 5.1-fold lower ($P < 0.05$) than that in the control cells at 0 h pi, but there was no significant difference at other time points

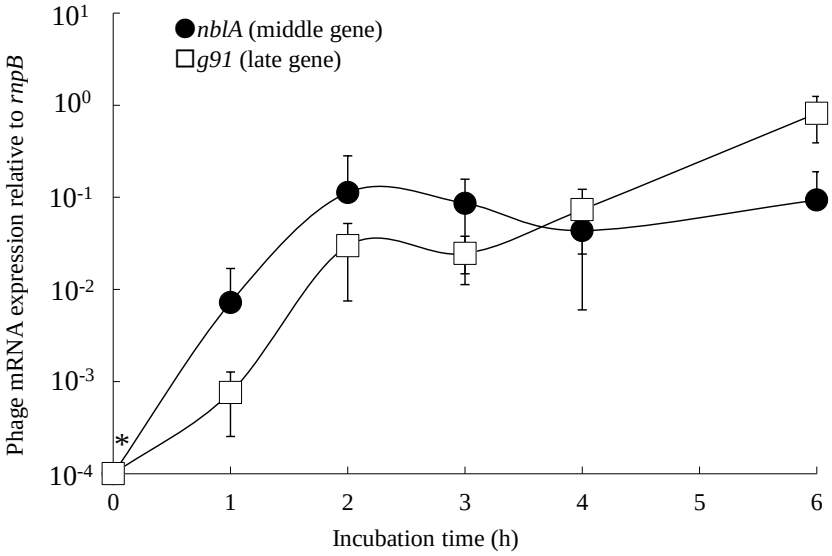


Fig. 2-2. mRNA levels of phage middle gene *nblA* (phycobilisome degradation small peptide) and late gene *g91* (tail sheath) were determined using real-time RT-PCR. An asterisk indicates detection limit. Error bars represent SDs of three replicates. Transcript levels were normalized to an internal control (*rnpB*).

(Fig. 2-3). *gyrB* transcript level in the phage-infected cells was different from that of the control cells at 0 h psi (2.8-fold lower; $P < 0.05$), 2 h pi (4.5-fold lower; $P < 0.01$), and at 4 h (3.6-fold higher; $P < 0.01$), but there was no statistically significant difference at 6 h pi (Fig. 2-3).

Five heat shock genes were chosen for study, namely, *groES*, a small subunit of co-chaperonin (Hohn et al. 1979), *hspA* and *hspA2*, 16.6-kDa small heat shock proteins (Vierling 1991), *htpG*, the molecular chaperone required for stabilization of phycobilisomes under HL and high temperatures (Sato et al. 2010; Tanaka and Nakamoto 1999), and *htrA*, a serine protease involved in the repair cycle of PSII D1 protein (Barker et al. 2006; Clausen et al. 2011). Compared to the control cells, transcripts of all these genes in the phage-infected cells were at least partially induced. In particular, transcripts of *hspA* (25.9-fold at 4 h pi; $P < 0.01$) and *groES* (16.1-fold at

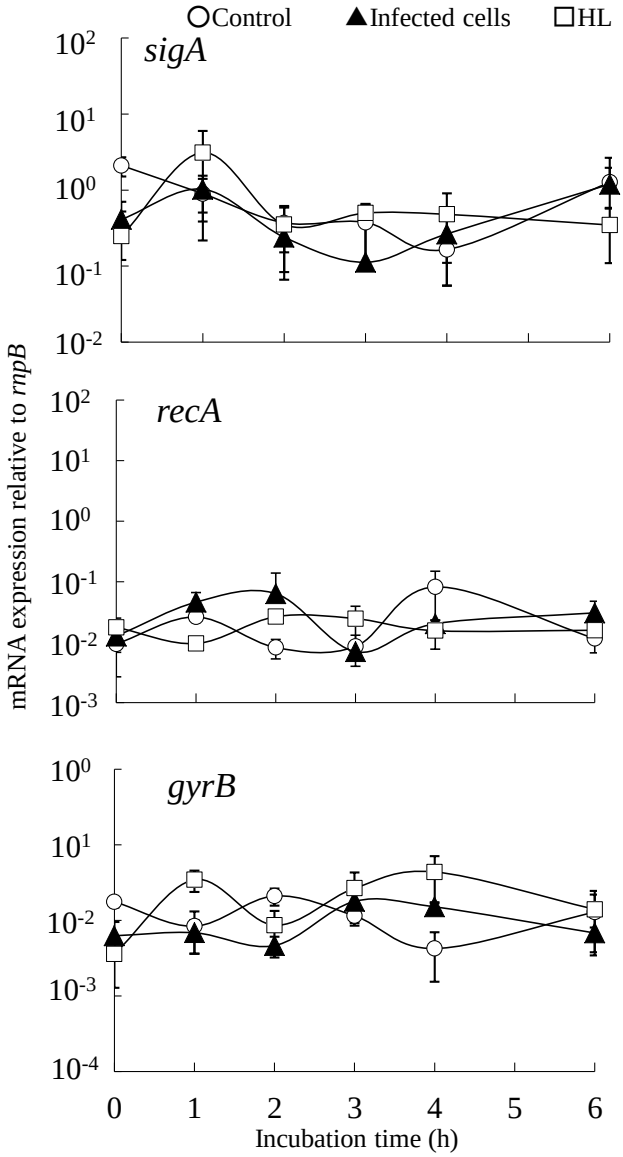


Fig. 2-3. Expression profiles of host housekeeping genes were determined using real-time RT-PCR. Error bars represent SDs of three replicates. Transcripts of *sigA* (primary s factor), *recA* (DNA recombinase), and *gyrB* (DNA gyrase subunit B) were normalized to an internal control (*rnpB*).

6 h pi; $P < 0.01$) was prominently induced (Fig. 2-4). The transcript levels of *hpsA2* and *htpG* were also 5.6–8.7-fold higher ($P < 0.05$) at 3–4 h pi and 4.0–4.7-fold higher ($P < 0.05$), respectively, in the phage-infected cells at 2–4 h pi than in the control cells (Fig. 2-4). While *htrA* transcripts were partially downregulated, they were still 9.9-fold higher ($P < 0.05$) than the *htrA* transcript level determined in control cells at 6 h pi (Fig. 2-4). There was no statistically significant difference between *sodB* transcript level in the control and that in phage-infected cells, except at 2 h pi at which time the *sodB* transcript level was 33.2-fold higher ($P < 0.05$) than that in the control cells (Fig. 2-4).

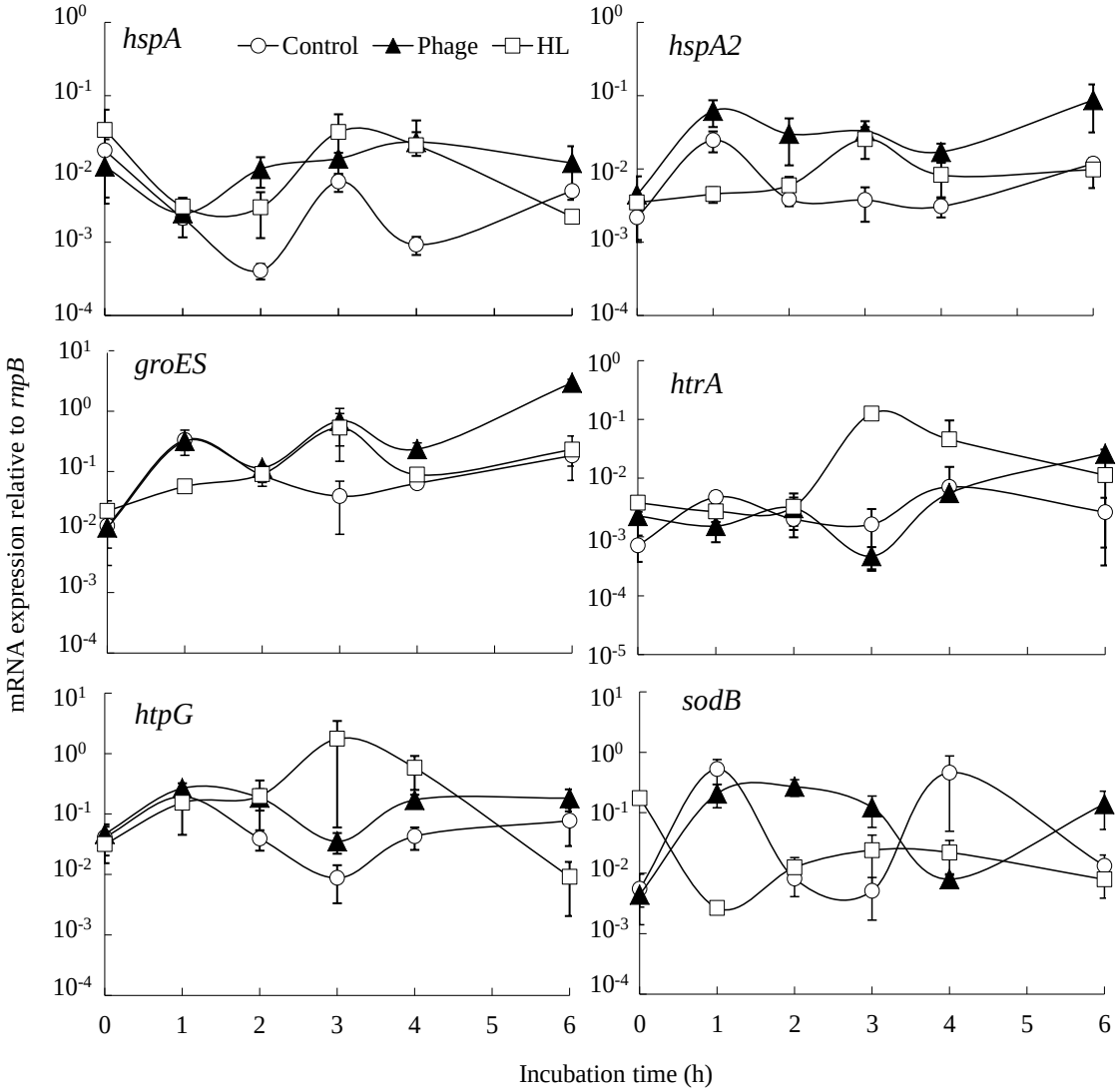


Fig. 2-4. Expression profiles of stress response genes were determined using real-time RT-PCR. Error bars represent SDs of three replicates. Transcripts of *hspA* and *hspA2* (16.6 k Da small heat shock proteins), *groES* (small subunit of co-chaperonin), *htrA* (serine protease), *htpG* (molecular chaperone), and *sodB* (superoxide dismutase) were normalized to *rnpB*.

Comparison of the phage-infected and HL cells revealed some differences in the transcript levels of *groES*, *sodB*, and *htrA*. *sodB* transcript level in the phage-infected cells was 39.3-fold lower ($P < 0.001$) at 0 h pi and 21.5-fold higher ($P < 0.05$) at 2 h pi than that in the HL cells. *htrA* transcript level was distinctly lower in the phage-infected cells than in the HL cells at 3 h pi (267.3-fold; $P < 0.05$). In particular, the abundance of

groES transcripts was significantly higher in the phage-infected cells than in the HL cells at 1 and 6 h [5.6-fold ($P < 0.05$) and 12.8-fold ($P < 0.001$), respectively]. As the *hspA* promoter is recognized by SigB in *Synechocystis* 6803 (Imamura et al. 2004), we monitored *sigB* transcripts and found that *sigB* transcripts in the phage-infected cells were significantly induced at 2, 3 and 6 h pi relative to the control cells [2 h pi: 25.9-fold ($P < 0.01$); 3 h pi: 2.8-fold ($P < 0.05$); 6 h pi: 9.5-fold ($P < 0.05$)] (Fig. 2-5). *sigB* transcripts in the HL cells were induced during the period 0–3 h pi and were 10.3-fold higher ($P < 0.05$) at 3 h, followed by a decline to the same level as the control cells.

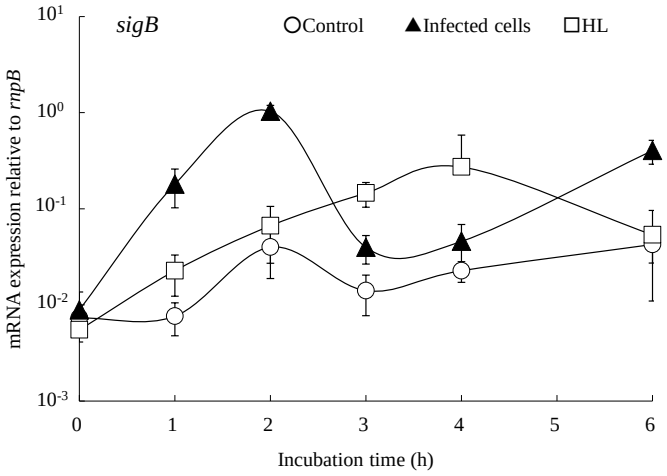


Fig. 2-5. Expression profiles of *sigB* (group 2 alternative sigma factor) were determined using real-time RT-PCR. Error bars represent SDs of three replicates.

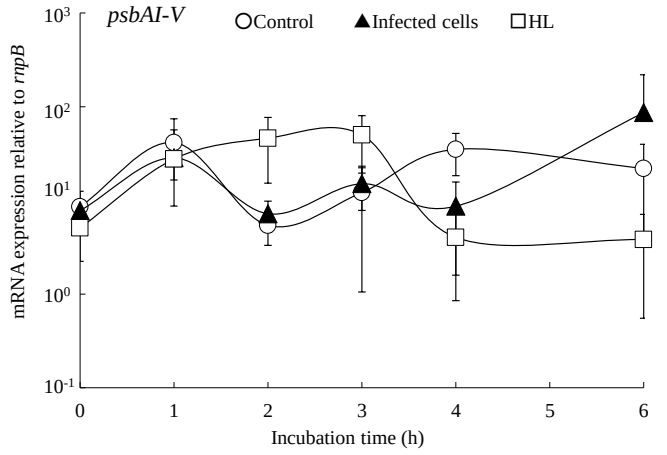


Fig. 2-6. Expression profiles of *psbA* genes (PSII D1 protein) were determined using real-time RT-PCR. Error bars represent SDs of three replicates. Transcripts of total *psbA (psbAI-V*) were normalized to *rnpB*.

Transcripts of *psbA* genes, which *M. aeruginosa* harbors five *psbA* genes (*psbAI–V*), were monitored to evaluate photosynthesis. The transcript levels of *psbAI–V* in the phage-infected cells and the HL cells did not differ significantly from those in the control cells, with the exception of 3 h in the HL cells they were 8.7-fold lower ($P < 0.05$) than those in the control cells (Fig. 2-6).

To evaluate carbon metabolism, we determined the transcript levels of the carbohydrate metabolic genes *gnd* (PPP), *gap1* (Calvin cycle), and *rbcL* (ribulose bisphosphate carboxylase large subunit, Calvin cycle). There was no statistically

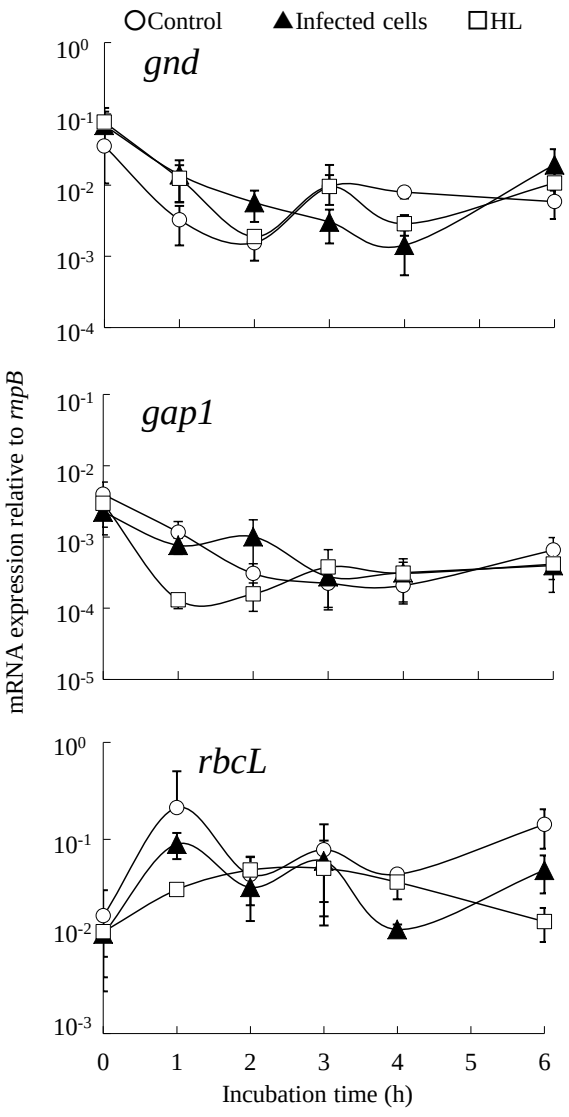


Fig. 2-7. Expression profiles of carbon metabolic genes were determined using real-time RT-PCR. Error bars represent SDs of three replicates. Transcripts of *gnd* (6-P-gluconate dehydrogenase, PPP), *rbcL* (RuBisCO large subunit, Calvin cycle), and *gap1* (glyceraldehydes 3-phosphate dehydrogenase, Calvin cycle) were normalized to an internal control (*rnpB*).

significant difference between the transcript levels of *gap1* in the phage-infected cells and HL cells and that in the control cells (Fig. 2-7). *gnd* transcription was partially downregulated in the phage-infected and HL cells and was 5.6-fold ($P < 0.01$) and 2.8-fold lower ($P = 0.01$) lower, respectively that in than the control cells at 4 h pi (Fig. 2-7). The transcript level of *rbcL* in the phage-infected cells was 3.7-fold lower ($P < 0.001$) than that in the control cells at 4 h pi, but there was no statistically significant difference at other times (Fig. 2-7). In the HL cells, *rbcL* transcription was not significantly different from that in the control cells.

Discussion

When *Escherichia coli* is infected by the phage T4, host gene transcription is rapidly shut down and the phage genes are transcribed (Koerner and Snustad 1979; Pineda et al. 2004). However, complete shutdown of host transcription has not been observed in the Cyanobacteria–cyanophage system. In the study on *Prochlorococcus* cells infected by podovirus P-SSP7, the transcription of 75 % of the host genes was reduced to about one-half of its normal level (Lindell et al. 2007). In another study on *Synechococcus* infected with S-PM2, the authors reported that there was no significant shutdown of host transcription and that genes involved in photosynthesis were partially upregulated (Clokie et al. 2006). It has been hypothesized that this maintenance of host transcription avoids the potential shortage of nucleic acids or amino acids that are required for progeny phage production because *Synechococcus* or *Prochlorococcus* inhabiting oligotrophic ocean environments often encounter nutrient limitations (Wikner et al. 1993; Wu et al. 2000). In my study, the transcription of *Microcystis* housekeeping genes did not show significant downregulation during Ma-LMM01

infection (Fig. 2-3), indicating that the maintenance of host transcription may also occur in the infection system for eutrophic freshwater organisms. Poranen et al. pointed out that drastic changes in host physiology during infection is disadvantageous for the phage due to induction of the host defense system. *M. aeruginosa* possesses the highest number of defense systems against foreign genetic elements among all prokaryotes sequenced to date (Makarova et al. 2011; Poranen et al. 2006), including CRISPR (clustered regularly interspaced short palindromic repeats)–Cas (CRISPR-associated genes) systems (Barrangou et al. 2007; Kuno et al. 2012), restriction–modification (RM) systems, and toxin-antitoxin (TA) systems (Fineran et al. 2009). Of these, cell death caused by TA systems can be robustly influenced by host transcription activity (Engelberg-Kulka et al. 2006), suggesting that Ma-LMM01 may maintain host transcription so as not to induce host defense systems.

In my study, transcripts of heat shock genes, which are involved in protecting the photosynthetic apparatus, and *sigB* were upregulated in the phage-infected cells. *sigB* is induced under various stress conditions, such as heat, salt, hyperosmotic stress, UV-irradiation, and oxidative stress (Imamura and Asayama 2009). These conditions cause inactivation of photosynthetic activity due to denaturation of PSII (Imamura and Asayama 2009). Based on my results, I speculate that *Microcystis* PSII were maintained through phycobilisome degradation by phage *nblA* as well as its own *nblA*, and by protecting it using the host's stress response genes. It would appear important for cyanophages to avoid photo-inhibition during their infection cycle so they can invade oxygenic phototrophs and effectively propagate themselves.

Transcription levels of the Calvin cycle genes and pentose phosphate pathway genes were slightly downregulated or did not change (Fig. 2-7). Therefore, it would

appear that sufficient amounts of NADPH and the nucleic acids required for the phage propagation may be provided without the AMGs involved in redirection of the host carbon flux or without modification of host gene expression.

To summarize, the potential infection strategy of Ma-LMM01 may be as follows: (1) no significant shutdown of host transcription, (2) PSII protection by phage-encoded *nblA* and host stress response genes including *sigB*, and (3) no significant alteration of carbon metabolism gene expression. This strategy may have benefits for the fitness of the viruses infecting cyanobacteria inhabiting surface waters. In addition, protection of the photosynthetic apparatus without extensive genetic manipulation may be advantageous for Ma-LMM01 to avoid the host defense system. As a result of such infection strategy, induction of *sigB* could be a sole signal for the phage infection in *Microcystis*.

Chapter 3

Analysis of upstream sequence of cell division gene *ftsZ* in *Microcystis aeruginosa*

In Chapter 2, I revealed that stress response genes involved in protection of PSII and the alternative sigma factor *sigB* involved in diverse stress response were induced during phage infection (Honda et al. 2014). These findings suggest that the protection of photosynthetic apparatus including PSII is directly or indirectly induced by phage infection in *Microcystis*, and some part of this induction may mediated by SigB. This is the first report of transcription response when *Microcystis* cells encountered with die of phage infection, suggesting that *sigB* may be a marker of bloom termination.

In Chapter3, I focused on bloom formation. To identify marker gene for normal cell growth, I searched gene which coordinates those two cell cycle events, called checkpoints, in *Microcystis* because bacterial cell growth demands both successful DNA replication and cell division. In Chapter 3-1, I investigated possible checkpoint factor which may coordinates DNA replication and cell division throughout transcriptional regulation of cell division gene *ftsZ* (Honda et al. 2014). In Chapter 3-2, I identified this protein and analyzed how *Microcystis*'s cell division is controlled in transcription level.

3-1 Investigation of transcriptional regulator of *ftsZ* which coordinates DNA replication and cell division

Introduction

The fundamental cell cycle events and the manner of its control of bacteria are similar to those of the eukaryotes: chromosome is replicated and replicated chromosome divided into daughter cells (Goley et al. 2007). In the bacteria, chromosome replication and cell division are coordinated with growth (Cambridge et al. 2014), so that cell division is arrested when DNA replication is unsuccessful, suggesting existence of the checkpoint between the C period and D periods. Studies on the checkpoint have been focused on coordination of chromosome replication/segregation and Z-ring formation. Z-ring is a circular polymer of the tubulin-like protein, FtsZ which is well studied cell division protein (Bi and Lutkenhaus 1991). In the D period, Z-ring is formed at circumferential on the cytoplasmic membrane inner surface at mid-cell, and its constriction lead cell divide into two daughter cells.

Studies on the coordination of DNA replication and cell division with different approaches now suggest different models. First is an SOS response dependent division block by well characterized division inhibiter protein SulA or YneA in *Echerichea coli* and *Bacillus subtilis*, respectively (Kawai et al. 2003; Mukherjee et al. 1998; Trusca et al. 1998). When DNA is damaged or replication folks are stalled, LexA regulated SOS genes which involved in DNA repair and rescue of stalled replication folk as well as SulA or YneA are induced and prevents polymerization of the Z-ring. Second, some observations indicate that timing of Z-ring formation is coordinated with the replication status: Z-ring formation start at termination or midway in *E. coli* (Den Blaauwen et al.

1999) (Wang et al. 2005); or it is exactly coordinated with replication status (Inoue et al. 2009). In *B. subtilis*, early stage of replication initiation and replisome assembly potentiate Z-ring formation but it does not polymerize FtsZ until the replication complete about 70% (Moriya et al. 2010; Wu et al. 1995). Some of these division arrests are caused by SlmA and Noc in *E. coli* and *B. subtilis*, respectively (Bernhardt and De Boer 2005; Wu and Errington 2004), so called nucleoid occlusion (Mulder and Woldringh 1989). They binds to specific DNA sequences disseminated on the genome except for *Ter* region, and inhibit elongation of immature short FtsZ polymers, whereas direct interaction of Noc and FtsZ protein is yet clearly certified (Tonthat et al. 2011; Wu et al. 2009). Moreover, incompletely replicated nucleoid or unsegregated chromosome inhibits Z-ring formation by SOS response and nucleoid occlusion independent manner (Cambridge et al. 2014). Third, SOS-independent checkpoint system is observed in *E.coli* and *Caulobacter crescentus*, coordinating cell division and chromosome replication by transcriptional regulation of *ftsZ* (Liu et al. 2001; Wortinger et al. 2000). *C. crescentus* is dimorphic α-proteobacteria and its response regulator CtrA globally controls cell cycle dependent gene expression and transcripts cell division genes, as well as *ftsZ*, in pre-divisional cells which correspond to the D phase (Kelly et al. 1998; McAdams and Shapiro 2003). However, CtrA is only found in α-proteobacteria which exhibit radically different lifestyles from each other, and *E. coli* checkpoint factor which regulates *ftsZ* transcription have been unknown. Thus, studies on bacterial checkpoint between chromosome replication and cell division have been established in only a few species. As for the cyanobacterial, very little is known about cell cycle (Holtzendorff et al. 2001; Sakr et al. 2006; Sandh et al. 2009; Wang and Xu 2005).

Unicellular cyanobacteria *Microcystis aeruginosa* frequently form bloom at the eutrophic fresh water throughout the world (Backer and McGillicuddy 2006; Guo 2007). Because some strains produce potent hepatotoxins, called microcystins (MacKintosh et al. 1990; Namikoshi et al. 1992; Nishiwaki-Matsushima et al. 1992; Yoshizawa et al. 1990), which is at risk for toxification or death of livestock (Oberholster et al. 2009; Pouria et al. 1998) and humans (Codd et al. 2005), it is issues in maintaining safe water supplies to control its toxic blooms (Backer and McGillicuddy 2006; Pearson and Neilan 2008).Yoshida et al. demonstrated that cell division was arrested and transcription of *ftsZ* was suppressed in *M. aeruginosa* when DNA replication was blocked by either nalidixic acid or hydroxyurea treatment. These indicate that DNA replication and cell division is coordinated by transcriptional regulation of *ftsZ* in *Microcystis* (Yoshida et al. 2005). In this section, to search transcription factor which regulates *ftsZ* transcription, I investigated upstream region of *ftsZ* gene using electrophoresis gel mobility shift assay (EMSA) (Revzin 1989).

Materials and Methods

Strains, growth conditions, synchronization, replication blocking and sampling

Microcystis aeruginosa NIES-298 was obtained from the National Institute for Environmental Studies (NIES), Environmental Agency. The cells were grown in CB medium (Kasai et al. 2004) under a 12:12 light/dark (L/D) cycle with a light intensity of 40 μmol photons/m^2/s at 30 ºC and aeration with 0.5% CO_2 (v/v) (Yoshida et al. 2005). Exponential growth cultures were synchronized by the block-release method (Yoshida et al. 2005). Exponentially growing cells were inoculated into a 3 L glass flask containing 1.8 L of the medium to initial cell density of 10^4 cells/mL and incubated

under the above conditions. After the 4th L/D cycle, cultures were left in darkness for 36 h followed by continuous illumination (Yoshida et al. 2005).

To block DNA replication, nalidixic acid dissolved in 50 mM NaOH was added to the synchronized culture to a final concentration of 3 μg/mL as previously described (Yoshida et al. 2005). As a control, the same volume of 50 mM NaOH without nalidixic acid was added (Yoshida et al. 2005).Twelve hour after addition of nalidixic acid , the cells were collected on the 3 μm TSTP membrane filter (MILLIPORE Billerica, MA, USA) and washed with 20 mM Tris-HCl (pH 8.0). Collected cells were resuspended in 20 mM Tris-HCl (pH 8.0) and dispensed into 1.5 ml tubes. Resuspended cells were centrifuged at 18,600 ×*g* for 15 min at 4 ºC, and stored at -80 ºC. For cell counts, cultures were collected and immediately fixed by adding 25% glutaraldehyde to a final concentration of 1%. To measure the cell concentration and division index (dividing cells/total cells), fixed cells were stained by 4', 6-diamidino-2-phenylindole (DAPI) at a final concentration of 1 μg/mL and observed with epifluorescent microscopy (BHS-RFCA, Olympus, Tokyo, Japan).

Gel electrophoresis mobility sift assay (EMSA)

For preparation of crude protein extract from *Microcystis* cells, equal volumes of 20 mM Tris-HCl (pH 8.0) were added to the cell pellets and sonicated (Handy Sonic UR-20P, Tomy Seiko, Tokyo, Japan) on ice. The resulting suspensions were centrifuged at 18,600 ×*g* for 15 min at 4 ºC, and the supernatants collected as crude protein extracts. Oligonucleotides corresponding to the upstream of *ftsZ* were synthesized as shown in Fig. 1A. Sequence data of this region obtained from the DDBJ/EMBLE/GenBank DNA data bases (accession no. AB205180). Oligonucleotide pairs were suspended in nuclease free water, incubated at 95 ºC for 10 min, and annealed by slowly cooling at room

temperature. Annealed oligos were digoxigenin (DIG)-labeled using the DIG Oligonucleotide 3' - End Labeling Kit (Roche, Basel, Schweiz). Point four μM DIG-labeled DNA fragments were mixed with 5μg of crude extracted protein, binding buffer [100 mM Tris-HCl (pH 8.0), 50m M dithiothreitol, 50m M EDTA, 100 mM KCl, 20 mM $MgCl_2$ and 30% glycerol] and 5μg of poly (dI-dC)/(dI-dC) (Sigma-Aldrich, MO, USA). After incubation for 15 min at 14 ºC, the mixtures were applied to non-denaturing PAGE with a 5% gel in 0.5×TBE. The probe signals were developed with a chemiluminescent substrate according to manufacturer's instructions and exposed to X-ray film (RX-U, Fuji Photo Film, Tokyo, Japan) for 30 min at 25 ºC.

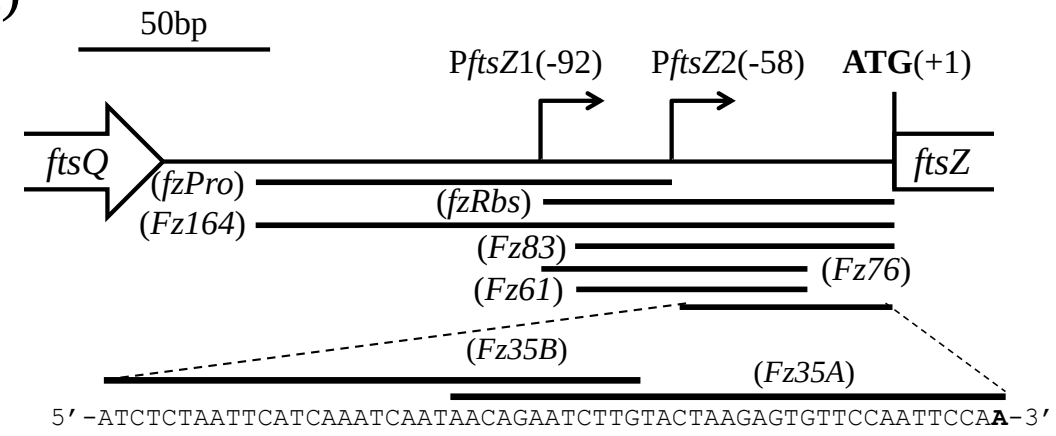
Sequence comparison

The multiple alignment of nucleotide sequences were performed by using CLUSTAL W (Thompson et al. 1994). Cyanobacterial nucleotide sequences used in sequence comparison were obtained from CyanoBase (http://genome.kazusa.or.jp/cyanobase).

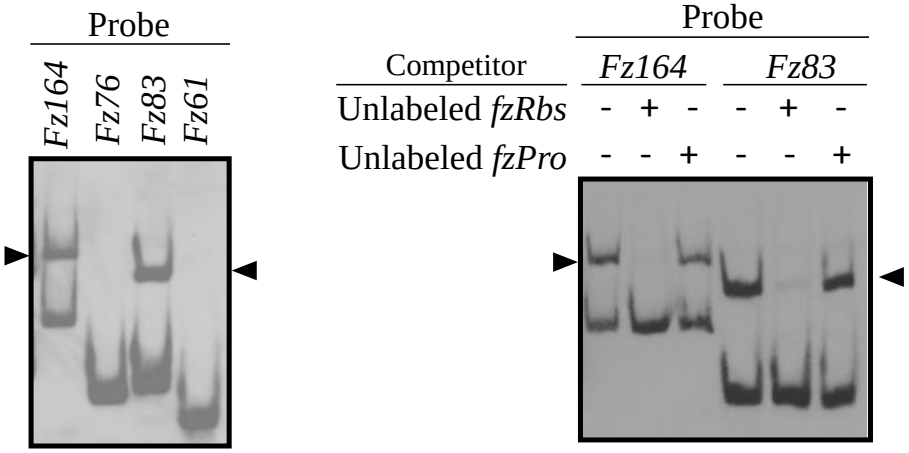
Results

To explore any factors binding to upstream regions of the *ftsZ* gene of *Microcystis* genome, we performed gel electrophoresis mobility shift assay (EMSA) analysis with crude protein extract of *M. aeruginosa* NIES-298. Initially, we synthesized two double-strand DNA probes corresponding to a region just upstream of *ftsZ* (98 bp, *fzRbs*) and an intergenic spacer region of the *ftsQ-Z* (110 bp, *fzPro*), respectively (Fig. 3-1-1a). The first EMSA analysis revealed that band shift was caused by only *fzRbs*. (data not shown). Then additional EMSA was performed using the following three DNA probes: *Fz83*, the PftsZ1 region removed from *fzRbs*; *Fz76*, 35 bp immediately

(a)


50bp
P*ftsZ*1(-92)
P*ftsZ*2(-58)
ATG(+1)
ftsQ
ftsZ
(*fzPro*)
(*fzRbs*)
(*Fz164*)
(*Fz83*)
(*Fz76*)
(*Fz61*)
(*Fz35B*)
(*Fz35A*)
5′-ATCTCTAATTCATCAAATCAATAACAGAATCTTGTACTAAGAGTGTTCCAATTCCA**A**-3′

(b)


Probe
Fz164
Fz76
Fz83
Fz61
Probe
Competitor
Fz164
Fz83
Unlabeled *fzRbs* - + - - + -
Unlabeled *fzPro* - - + - - +

(c)

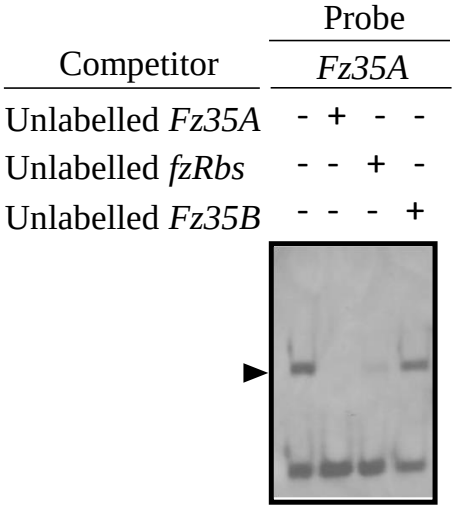

Probe
Competitor
Fz35A
Unlabelled *Fz35A* - + - -
Unlabelled *fzRbs* - - + -
Unlabelled *Fz35B* - - - +

Fig. 3-1-1. Schematic representation of the promoter region of the *ftsZ* of *M. aeruginosa* NIES-298 and searching of protein binding to *ftsZ* upstream by EMSA. (a) The limits shown in below the physical map indicate double strand oligonucleotide fragment used for the EMSA. Each fragment was labeled with DIG and incubated with crude protein extract from *M. aeruginosa* NIES-298. *fzRbs, fz164,* and *fz83* were positive in the band retardation assay, whereas *fzPro, fz76, fz61, Fz35B* did not result in a band shift. The location of the transcription start points of *ftsZ* , PftsZ1 and PftsZ2, are indicated with bended arrows. Bold face indicates start codon. (bLeft) Additional EMSA was performed using followed three DNA probes, *Fz83*: PftsZ1 region was removed from *fzRbs*, *Fz76*: 35 bp immediately upstream of *ftsZ* start codon was removed from *fzRbs* and *Fz61*: 35 bp immediately upstream of *ftsZ* start codon was removed from *Fz83*. A full length of *ftsQ-Z* intergenic spacer (*Fz164*) segment was used as a positive control. (bLight) Excess amount of non-labeled fragment of *fzRbs* were added to the reaction mixture containing *Fz164* or *Fz83*. Arrowheads indicate DNA-protein complexes. (c) The retarded band disappeared when non-labeled *Fz35A , Fz35B* or *fzRbs* was added reaction mixture containing DIG-labeled *Fz35A*. Arrowheads indicate DNA-protein complexes.

---

upstream of *ftsZ* start codon removed from *fzRbs*; and *Fz61,* 35 bp immediately upstream of *ftsZ* start codon removed from *Fz83* .A full length of *ftsQ-Z* intergenic spacer (*Fz164*) segment was used as a positive control. A retarded band was detected when *Fz164* or *Fz83* was used as probe (Fig. 3-1-1b left). Addition of excess non-labeled *fzRbs* to the reaction mixture containing *Fz164* or *Fz83* eliminated retarded bands (Fig.1 3-1-1b right). Moreover, a sequential EMSA using a 35 bp sequence just upstream from *ftsZ* start codon (*Fz35A*) resulted in band retardation. The retarded band disappeared when non-labeled *Fz35A* or *fzRbs* was added (Fig. 3-1-1 left).

Fz35A was incubated with crude protein extract prepared from a culture having DNA replication blocked by treatment with nalidixic acid (Nal+) or from a non-treated culture (Nal-), followed by EMSA analysis. Band retardation was observed with crude

protein from Nal- culture, but not with crude protein from Nal+ culture (Fig. 3-1-2).

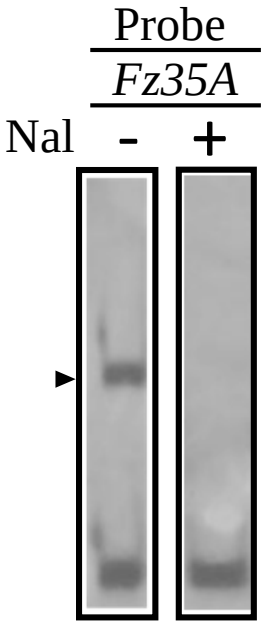


Fig. 3-1-2. EMSA performed with *Fz35A* and crude protein extract from cell division blocked cells by treated with nalidixic acid (+). Arrowhead indicates probe-protein complex.

Discussion

Yoshida et al. (2005) showed *ftsZ* was transcribed from 58 and 92 bp upstream of the initial codon of *ftsZ* located between *ftsQ-Z* spacer region (Yoshida et al. 2005). EMSA analysis using upstream sequences of *ftsZ* as probe revealed a protein binding 35bp upstream (Fz35A) from start codon. Addition of excess unlabeled Fz35A caused disappearance of the retarded band, clearly indicating that the protein binds to a site 35bp upstream of *ftsZ*. Almost half of σ^{70}-dependent operons are transcribed from more than one promoter and each has an activation or repression sites (Collado-Vides et al. 1991). In general, activators bind to upstream of -35 element, while repressor site are found between the -10 and -35 elements, just downstream from the -10 core element, or, rarely, at remote upstream sites (Collado-Vides et al. 1991). However, our EMSA analysis showed that BpFz bound to a just upstream of coding sequence, suggesting that BpFz is not conical activator or repressor. These might suggest that BpFz is a

CyAbrB-like protein homologue, CalA, recently identified by DNA affinity chromatography using DNA fragments from the upstream region of *ftsZ* in heterocyst-producing filamentous cyanobacteria *Anabaena* sp. PCC7120 may be involved in the expression of *ftsZ* in vegetative cells rather than in differentiation to heterocyst (He and Xu 2010; Ishii and Hihara 2008). As no DNA consensus sequence recognized by AbrB was found upstream of the target genes, AbrB-DNA binding interactions are hypothesized to entail the recognition of specific stretches of three-dimensional DNA helix configurations (Bobay et al. 2004; Ishii and Hihara 2008).

Blocking DNA replication by nalidixic acid treatment causes inhibition of cell division and repression of *ftsZ* expression in *Microcystis* cells. Thus we previously showed DNA replication and cell division are coordinated, and some part of this coordination is mediated by *ftsZ* transcription depending on DNA replication (Yoshida et al. 2005). When DIG labeled *Fz35A* was incubated with crude protein extract from *Microcystis* cells that had cell division blocked by treatment with nalidixic acid, band shift was not observed. From these data we hypothesize that BpFz is involved in coordination of DNA replication and cell division via transcription control of *ftsZ*. To contribute to understanding regulation of cyanobacterial transcription involved in coordination of DNA replication and cell division, further investigations concerning the purification and characterization of BpFz are required.

Chapter 3-2

Purification and characterization of the protein which binds to upstream of *ftsZ*

Introduction

Cell proliferation proceeds accompanying cell cycle progression. Because cell division, which is a final step of cell proliferation, requires complete chromosome replication (Clark 1968), these two cell cycle events need to be well coordinated (checkpoints) (Elledge 1996). Coordination of cell division and DNA replication has been reported in some bacteria, i.e., *Escherichia coli* (Liu et al. 2001), *Caulobacter crescentus* (Wortinger et al. 2000), *Bacillus subtilis* (Love and Yasbin 1984; Wu and Errington 2004; Wu et al. 2009) and *Mycobacterium smegmatis* (Santi et al. 2013). Studies on checkpoints in bacteria has been mostly focused on the coordination of chromosome replication and Z-ring formation which is circular polymer of cell division protein FtsZ and representative of cell division machinery (Bi and Lutkenhaus 1991). Some checkpoints associated with the FtsZ (or *ftsZ*) have been reported. 1) In *E. coli*, Z-ring formation is inhibited by SOS response which is induced by DNA damage or replication error DNA (Kawai et al. 2003; Mukherjee et al. 1998; Trusca et al. 1998); 2) In *E. coli* and *C. crescentus*, transcriptional regulation of *ftsZ* is downregulated when DNA is damaged (Liu et al. 2001; Wortinger et al. 2000). In *E. coli*, cell division is arrested and transcription of *ftsZ* is suppressed in SOS independent manner when DNA replication is blocked. However, transcription factor of *ftsZ* is still unknown. In *C. crescentus*, global response regulator CtrA controls cell cycle dependent gene expression, and *ftsZ* is timely transcribed in late stage of the D period (Kelly et al. 1998;

McAdams and Shapiro 2003; Wortinger et al. 2000). Although much of regulation process of bacterial metabolism is controlled at the transcription level (Seshasayee et al. 2006), little is known about bacterial checkpoint system which mediated by transcriptional control of cell division gene.

Microcystis aeruginosa produce potent hepatotoxins, called microcystins (MacKintosh et al. 1990; Namikoshi et al. 1992; Nishiwaki-Matsushima et al. 1992; Yoshizawa et al. 1990). In cyanobacteria *Synechococcus elongates* PCC7942, cell division is controlled by circadian rhythm of which oscillator protein KaiC and the response regulator RpaA (Dong et al. 2010; Markson et al. 2013), so that cyanobacterial cell division is thought not to be coordinated with DNA replication (Mori et al. 1996). In *Microcystis*, however, DNA replication and cell division is coupled by transcription of *ftsZ* (Yoshida et al. 2005). In the previous section, I found an unknown protein factor which specifically binds *ftsZ* upstream, BpFz (Honda et al. 2012). The final destination of this study is identification of maker genes for normal growing cells. In this section, I purified and characterize BpFz to gain insight for checkpoint.

Materials and methods

Strain and growth conditions

Microcystis aeruginosa strain NIES-298 was obtained from the National Institute for Environmental Studies (NIES), Environmental Agency. The cells were grown in CB medium (Kasai et al. 2004) under a 12:12 light/dark (L/D) cycle with a light intensity of 40 μmol photons/m^2/s at 30 ºC and aeration with 0.5% CO_2 (v/v) (Yoshida et al. 2005). Exponentially growing cells were collected on 3 μm TSTP membrane filter (Millipore, Billerica, USA) and washed with 20 mM Tris-HCl (pH 8.0). Obtained cells were

resuspended in 20 mM Tris-HCl (pH 8.0) and dispensed into 50-mL tubes. Resuspended cells were centrifuged at 18,600× *g* for 15 min at 4 ºC. The resultant pellets were stored at -80 ºC until use.

Purification of BpFz

For preparation of crude protein from *Microcystis* cells, equal volume of 20 mM Tris-HCl (pH 8.0) was added to the thawed pellets and sonicated (UD-211, Tomy Seiko, Tokyo, Japan) on ice. The suspension was centrifuged at 18,600 ×*g* for 15 min at 4 ºC, and the supernatant was collected as crude protein.

BpFz was enriched from crude protein using DNA affinity chromatography. For the ligand DNA, the 5' end of the *Fz35A* upper strand was biotinylated (5'-biotin-AACAGAATCTTGTACTAAGAGTGTTCCAATTCCAA-3') followed by annealed with lower strand of *Fz35A* (5'-TTGGAATTGGAACACTCTTAGTACAAGATTCTGTT-3') for 10 min at 95 ºC. The double stranded ligand DNA (Bio-*Fz35A*) was mixed with crude protein in the binding buffer [20 mM Tris-HCl (pH 8.0), 10m M dithiothreitol, 10m M EDTA, 20 mM KCl and 4 mM $MgCl_2$, final concentration] and incubated for 30 min at 14 ºC. The mixture was applied to streptavidin column (HiTraqp streptavidin HP, GE Healthcare Bio-Sciences, NJ, USA) equilibrated with 10 column volume (CV) of the binding buffer at 4 ºC. The column was washed with 10 CV of binding buffer to remove unbound proteins. To remove proteins which non-specifically interact with *Fz35A*, column was washed with the binding buffer containing 0.1 M KCl. Then, BpFz were successively eluted with the binding buffer containing 1.0 M KCl.

To isolate BpFz, the elutant were dialyzed against 20 mM Tris-HCl (pH 8.0) and subjected to gel electrophoresis mobility shift assay (EMSA) using *Fz35A*. Three

reaction mixtures were prepared as previously described (see Chapter 3-2) and applied to three wells. Of these, two reaction mixtures contain non-labelled *Fz35A* instead of DIG-labelled *Fz35A*. After electrophoresis, each lane was subjected to different treatment as follows. The signals of DIG-labelled *Fz35A* probe were detected with a chemiluminescent substrate according to manufacturer's instructions (Roche, Basel, Schweiz) and exposed to X-ray film (RX-U, Fuji Photo Film, Tokyo, Japan) for 15-30 min at 25 ºC. Protein signals of the gel containing non-labelled *Fz35A* were detected by silver staining (Bio-Rad, MO, USA). The gel segment corresponding to the signal of DIG probe-BpFz complex was sliced from non-treated gel. Then BpFz was electro eluted from the gel segment using a Model 422 electro-eluter (Bio-Rad, MO, USA) in 20 mM Tris-HCl (pH 8.0) at 4 ºC.

The N-terminal sequence of the first 8 residues of the BpFz was determined with the Edman degradation by APRO life Science Institute, Inc (Tokushima, Japan).

Determination of the binding sequence of BpFz

To determine the binding sequence of BpFz, EMSA analysis was performed using crude protein and DIG-labelled mutant probes. The mutant probes were configured by introducing transversion to some nucleotides of *Fz35A* (see Fig. 3-2-5b).

Prediction of the regulon and functional annotation of BpFz

To predict BpFz regulated genes, which contain DNA binding sequence of BpFz in their transcription regulatory site (TRS) (-100 to +100 bp as start codon +1), I searched for predicted binding sequence of BpFz in the genome sequence of *M. aeruginosa* strain NIES-843 (GenBank accession no. NC_010296) using Regulatory Sequence Analysis Tools (http://www.rsat.edu) (Thomas-Chollier et al. 2011). In addition, I searched their downstream genes which were predicted to compose an operon (or transcription units,

TUs). TUs were predicted using FGENESB (http://www.softberry.com/berry) (Solovyev and Salamov 2011).

Functional annotation of the genes was according to their COGs (Clusters of orthologous groups of proteins) obtained from The Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.kegg.jp/) (Nakaya et al. 2013).

Phylogenetic analysis

For species tree based on sequences of 16SrRNA and BpFz homologues, the sequences of fully sequenced cyanobacteria (the status of NCBI genome sequencing project is “Chromosomes”) were obtained from NCBI and were aligned using MEGA6 (Kumar et al. 2008). A maximum likelihood tree was generated by PhyML (http://www.atgc-montpellier.fr/) (Guindon and Gascuel 2003), using GTR model with fixed gamma-distributed rate variation (four categories) and an estimation of proportion of invariable sites for DNA sequence, and Dayhoff model (Schwartz and Dayhoff 1978) for amino acid sequence. Five hundred of bootstrap sampling was performed for each analysis.

Results

Isolation and identification of BpFz

In the DNA affinity chromatography, DNA binding activity was obtained in a fraction of elution buffer containing 1.0 M KCl. (Fig. 3-2-1). To isolate BpFz, the active fraction was applied to EMSA. BpFz activity was recovered from non-treated EMSA gel according to DIG probe-BpFz signal (Fig. 3-2-2).The protein electro-eluted from the gel segment was subjected to SDS-PAGE. I detected a single band (27 kDa) with CBB

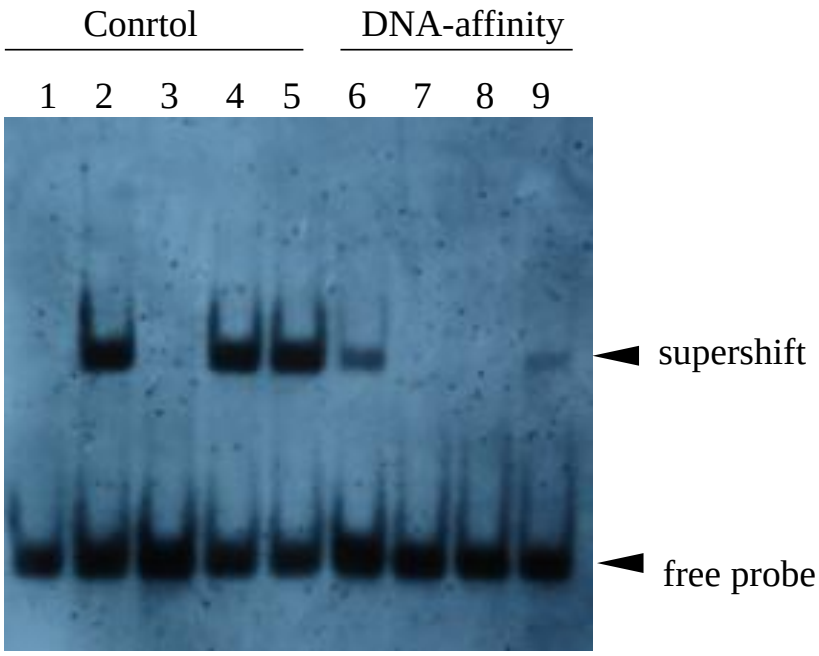


Fig. 3-2-1. EMSA analysis using *Fz35A* and BpFz partially purified from *Microcystis* cells by the DNA affinity chromatography. Lanes: 1, *Fz35A* only; 2 and 5, crude protein with *Fz35A*; 3, excess amounts of unlabeled *Fz35A* was added to the reaction mixture of lane 2; 4, excess amounts of unlabeled unrelated sequence was added to the reaction mixture of lane 2; 6, *Fz35A* with flow through fraction of DNA affinity chromatography; 7, wash fraction; 8, 0.1 M KCl eluted fraction with *Fz35A*; 9, 1.0 M KCl eluted fraction with *Fz35A.*

staining (Fig. 3-2-3a). The EMSA showed that this protein specifically bound to*Fz35A* (Fig. 3-2-3b).

N-terminus amino acid sequence analysis of BpFz revealed the first eight residues as TNLESLTQ. This sequence identical to 2nd to 9th residues of the N-terminus of transcription repressor LexA of *M. aeruginosa* strain NIES-843 (Fig. 3-2-4).

Analysis of LexA recognition sequence

Cyanobacterial LexA recognition sequence (LexA box) is reported in only two organisms, *Synechocystis* sp. PCC6803 (CTAN$_9$CTA) (Patterson-Fortin and Owttrim 2008) and *Anabaena* sp. PCC7120 (RGTACN$_3$DGTWCB) (Mazón et al. 2004). The

Fz35A partially matched those of *Synechocystis* sp. PCC6803 and *Anabaena* sp. PCC7120, respectively (Fig. 3-2-5a). I predicted that potential *Microcystis* LexA box was GTACTAN$_3$GTGTTC (Fig. 3-2-5c). Then, EMSA analysis was performed using the mutant probes and crude protein of *Microcystis*. For further definition of *Microcystis* LexA box, the GTACTAN$_3$GTGTTC was compared with LexA box consensus sequences of other bacterial phyla (Erill et al. 2007). Seventeen sequences from 12 bacterial phyla and *Anabaena* sp. PCC7120 were aligned (Fig. 3-2-6a). Of these, ten sequences contained GTACN$_7$GTTC (Fig. 3-2-6b). In the *Anabaena* sp. PCC7120 LexA box, the last three nucleotides is TWC. In addition, *Synechosystis* sp. PCC6803 LexA recognizes direct repeat motif (i.e., CTAN$_9$CTA) (Patterson-Fortin and Owttrim 2008). Therefore, most probable *Microcystis* LexA box is predicted as GTWCN$_7$GTWC. The genes, *lexA*, *recA*, *ssb* encoding single strand-binding protein (Glassberg et al. 1979) and *uvrA* encoding A subunit of DNA repair enzyme ABC excinuclease (Grossman et al. 1975) are shared among *Anabaena* sp. PCC7120 and *Synechosystis* sp. PCC6803 LexA regulon (Mazón et al. 2004). I found complete or partial sequences of this predicted LexA box in upstream regions of these genes in *Microcystis*, except for *uvrA* (Fig. 3-2-7 and summarized in Table3-2-1).

Prediction of *Microcystis* LexA regulon

To predict LexA regulon and its function in *Microcystis*, I searched for putative LexA box GTWCN$_7$GTWC in TRS of *M. aeruginosa* strain NIES-843 genome. I detected 75 putative LexA box in 73 TUs in *Microcystis*, and 87 genes (here after I call these LexA genes) were estimated to be included in *Microcystis* LexA regulon (Table 3-2-2). *ftsZ* and *ssb* were included in the predicted LexA regulon. However, *lexA* and

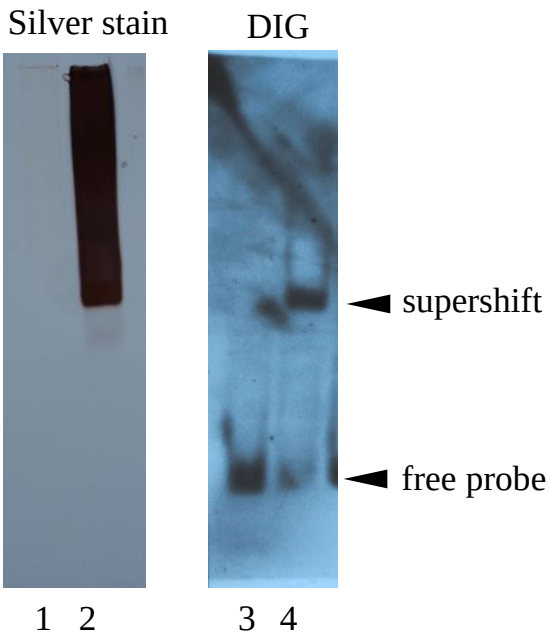


Fig. 3-2-2. Purification of BpFz by EMSA. BpFz partially purified by the DNA affinity chromatography was subjected to EMSA using *Fz35A*. The signal of BpFz-probe was detected by DIG detection system (right) and silver staining (left). Lanes: 1 and 3, *Fz35A* only; 2 and 4, protein with *Fz35A*. After the signal detection, the gel segment corresponding to *Fz35A*-BpFz signal was sliced from non-treated third gel.

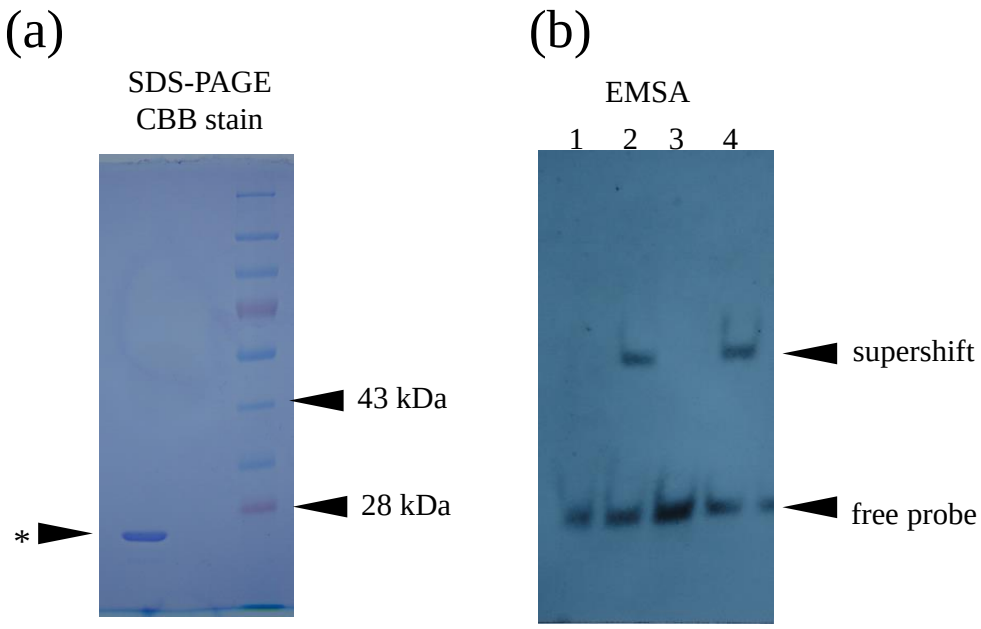


Fig. 3-2-3. SDS-PAGE of purified BpFz. Electro-eluted protein from active region of EMSA gel was subjected to SDS-PAGE followed by CBB staining (a), and EMSA(b). An asterisk indicates single 27 kDa band of BpFz.

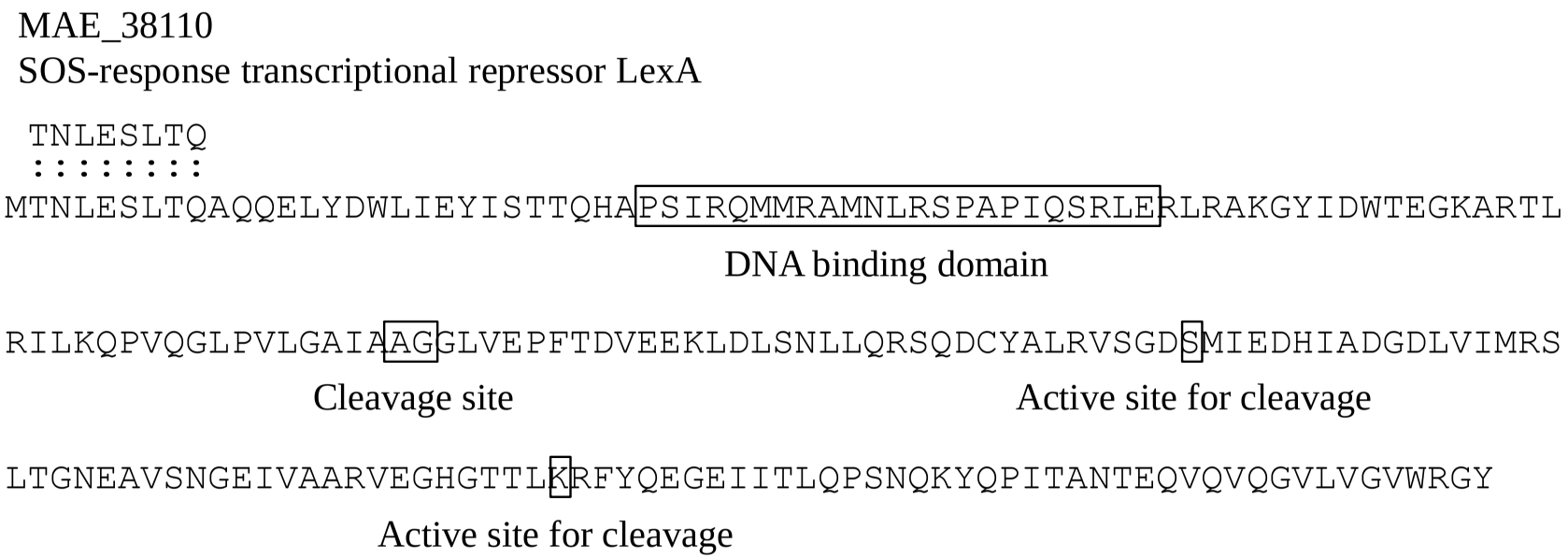


Fig. 3-2-4. Eight residue of N-terminal amino acid sequence of the purified BpFz identical to LexA N-terminus of *M. aeruginosa* NIES-843 (GenBank accession no. NC_010296). Boxes indicate DNA binding domain, RecA dependent self-cleavage site and catalytic residues, respectively.

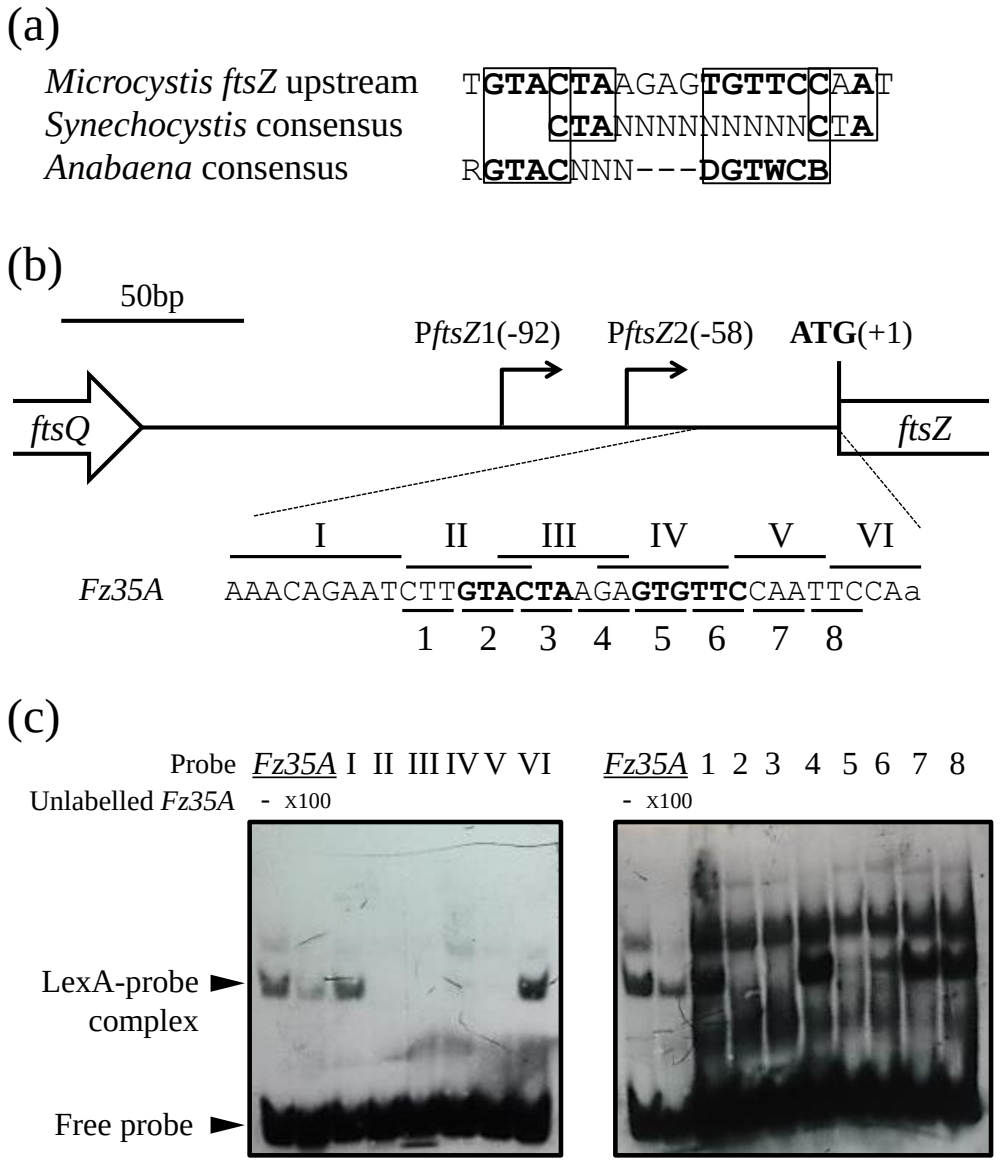


Fig. 3-2-5. Identification of core sequences of the LexA recognition site in *Microcystis*. (a) The sequence of *Microcystis ftsZ* upstream was compared with LexA box consensus sequence of *Synechocystis* sp. PCC6803 (Patterson-Fortin et al. 2008) and *Anabaena/Nostoc* sp. PCC7120 (Mażon et al. 2004). Bold face indicates conserved nucleotides. (b) Schematic representation of the upstream region of the *ftsZ* in *Microcystis*. *Fz35A* was mutagenized by generating transversion in lined bases (I-VI, 1-8), followed by probed with DIG. Promoters, PftsZ1 and PftsZ2, are indicated with bent arrows. Small character indicates the first nucleotide of start codon.(c) EMSA analysis using the mutant probes and crude protein extract from *M. aeruginosa*. For the control of LexA binding signal, 100-fold excess amounts of unlabelled *Fz35A* were added.

(a)

M. aeruginosa	:TTGTACTANNNGTGTTCCA-
Anabaena sp. PCC7120	:-RGTACNNN---DGTWCB--
Firmicutes	:-CGAACRNR---YGTTCG--
Thermotoga	:TCGAACNNN---NGTTCGA-
Green non-sulfur bacteria	:--GAACNNN---NGTTC---
Fibrobacteres	:-TGNHCNNN---NGHYCA--
Alphaproteobacteria	:--GTTCNNNNNNNGTTC---
*Deltaproteobacteria*_1	:CTRHAMRYB---YGTTCAGS
*Deltaproteobacteria*_2	:-GGTTNNNNNNNNNWACC--
*Gammaproteobacteria*_1	:CTGTA-TAT--ATATACAG-
*Gammaproteobacteria*_2	:--GTACNNN---NGTRC---

(b)

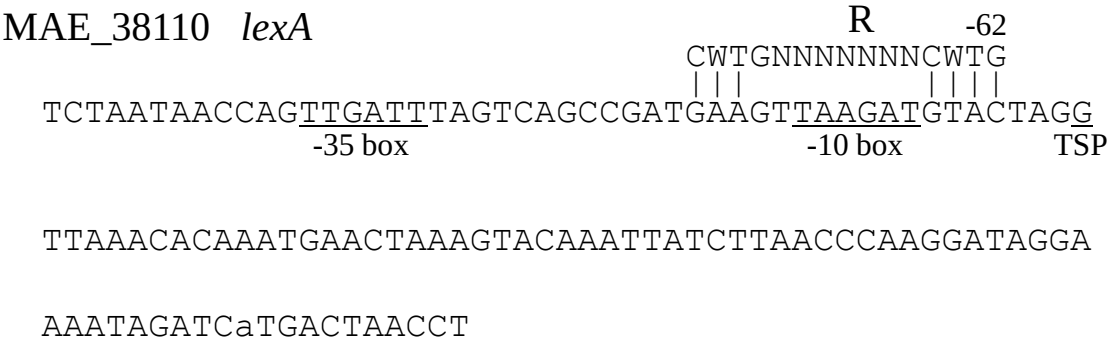
Fig. 3-2-6. (a) An alignment of consensus sequences of LexA box in *Cyanobactera* (*Microcystis* and *Anabaena* sp. PCC7120) and other bacterial phylum (Erill et al. 2007). Identity is indicated by shade: black, >80%; dark gray, >60%; light gray, >40%. (b) Consensus sequence was generated using WebLogo (www.weblogo.berkley.edu) (Crooks et al. 2004).

Table 3-2-1. Summaries of putative LexA box found in TRS of core gene of cyanobacterial LexA regulon.

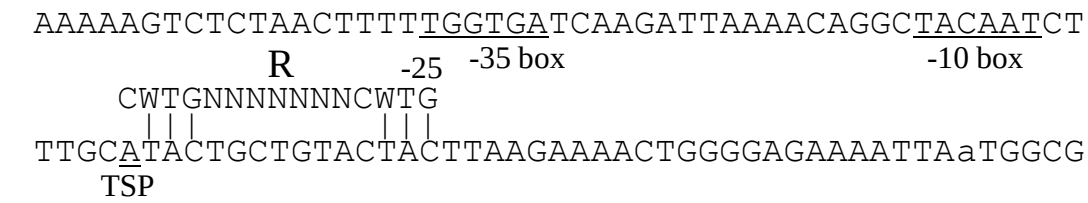
Locus tag[a)]	Name	Sequence (5' - 3')	Direction[b)]	Matching bases	Position[c)]
-	LexA box	GTWCNNNNNNNGTWC	-	-	-
MAE_38110	*lexA*	**GTAC**ATCTTAAC**TTC**	Reverse	7	-62
MAE_16070	*recA*	**GTA**GTACAGCA**GTA**T	Reverse	6	-25
MAE_37950	*ssb*	**GTAC**TTTTTCA**GTAC**	Forward	8	-38

[a)] Identification number in CyanoBase (http://genome.microbedb.jp/cyanobase) is given.
[b)] Direction with respect to coding strand is shown.
[c)] Position indicates upstream (-) or downstream (+) from start codon as +1.

MAE_38110 *lexA*



MAE_16070 *recA*



MAE_37950 *ssb*

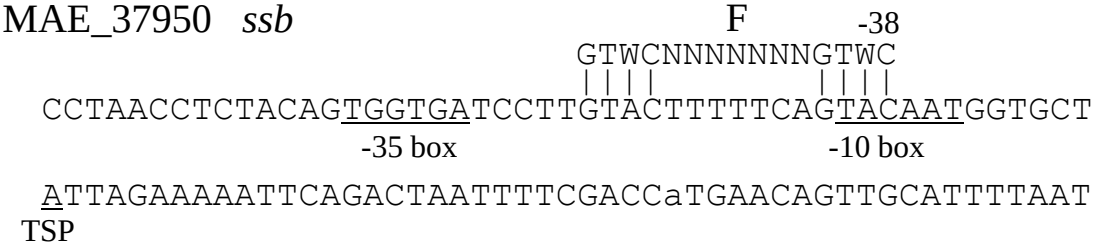


Fig. 3-2-7. Putative LexA boxes were searched in TRS of LexA core genes of *Microcystis*. LexA box (F, forward; R, reverse) and its position is indicated upon the sequences. Small character indicates the first nucleotide of start codon as +1. Under bars indicate promoter sequence -35 or -10 box, and putative transcription start site (TSP).

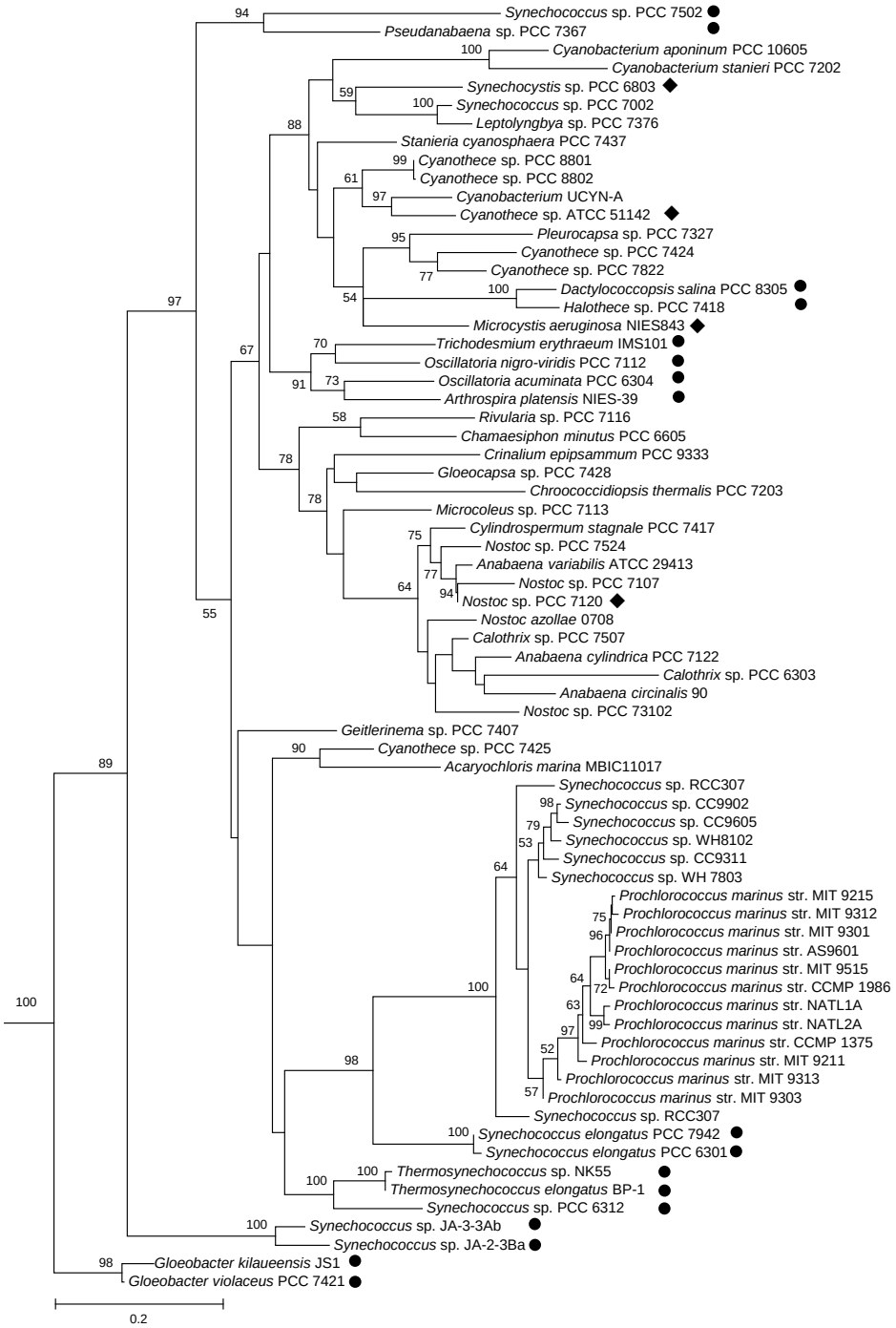


Fig. 3-2-8. Maximum likelihood phylogenetic tree of cyanobacterial 16S rRNA gene with bootstrap support. *Agrobacterium* sp. K-Ag-3 and *Rhodospirillum rubrum* ATCC 11170 were used as outgroup. Bootstrap values of > 50 % for 500 replicates are indicated at respective nodes. Dots indicate absence of LexA homologue. Diamonds indicates the species whose LexA regulon were predicted in this study.

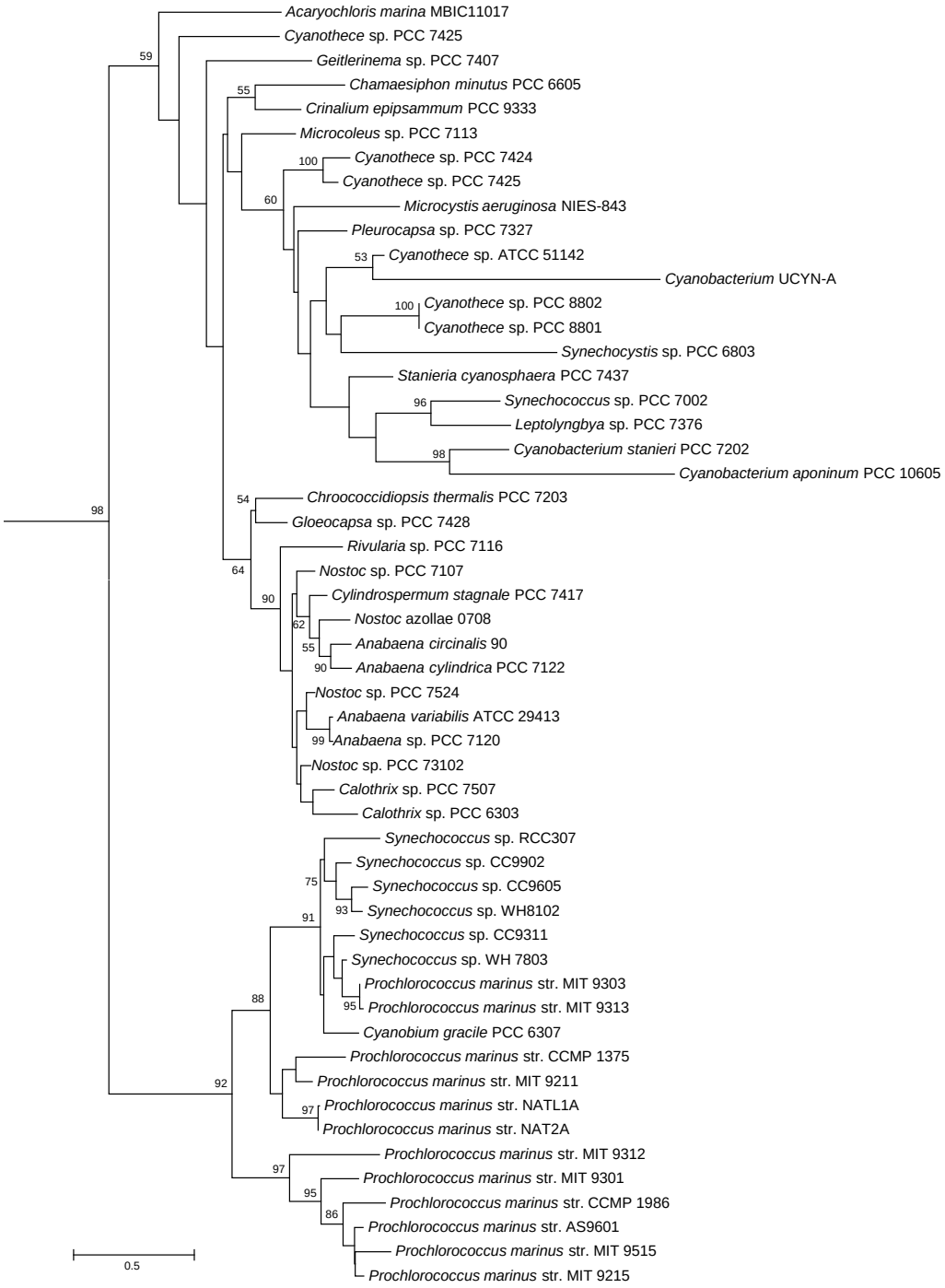


Fig. 3-2-9. Maximum likelihood phylogeny for cyanobacterial LexA. LexA proteins of *Rhodospirillum rubrum* ATCC 11170 and *E. coli* str. K-12 substr. MG1655 were used as outgroup. Bootstrap values of > 50 % for 500 replicates are indicated at respective nodes.

recA was not detected because this approach could not detect incomplete motif (Fig. 3-2-7). The 55 of 87 LexA genes were assigned to 13 COG categories. Eleven of these genes (20.0%) were assigned to COG-R (general function prediction only) or COG-S (function unknown) (Fig. 3-2-10). The most abundant category of the *Microcystis* LexA regulon was COG-L (replication, recombination and repair) (5.0%, 27 genes). Almost all COG-L genes were composed of transposase (92.6%, the 25 of 27 genes), and the rest were *uvrC* encoding C subunit of DNA repair enzyme ABC excinuclease (Thomas et al. 1985) and *ssb* (Table 3-2-2). The secondly abundant COG category was COG-P (inorganic ion transport and its metabolism) (3.1%, 5 genes) (Fig. 3-2-10): MAE_09260 and MAE_09270 both encoding subunit of phosphate ABC transporter permease protein (Pitt et al. 2010); NADPH production as the last electron receptor of PSI; *petH* encoding ferredoxin-NADP oxidoreductase (FNR) (DeRuyter and Fromme 2008); MAE_59870 encoding potassium-transporting ATPase subunit C (Matsuda and Uozumi 2006), MAE_38710 encoding probable chloride channel protein (http://genome.microbedb.jp/cyanobase). The thirdly abundant was COG-D (cell cycle control, cell division, chromosome partitioning) (1.7%, 1 gene) and COG-G (carbohydrate transport and metabolism) (1.7%, 2 genes). In addition, as Li et al. (2010) and Domain et al. (2004) reported that cyanobacterial LexA regulates some photosynthetic genes. The putative *Microcystis* LexA regulon contained photosynthesis related genes: MAE_02520 (*nblA*, phycobilisome degradation protein) (Bienert et al. 2006) and MAE_12570 (*petH*, FNR).

Table 3-2-3. Potential LexA Boxes and LexA regulated genes in *Microcystis* genome

Locus tag[a)]	Name	TU[b)]	Strand	Function[c)]	Sequence[d)]	Position[e)]	Direction[f)]
MAE_00230	*uvrC*	12_Tu1	+	excinuclease ABC subunit C	ggaaGTACACCCCCGGTTCgagg	-78	R
MAE_00340	-	undefined	+	hypothetical protein	agagGTTCCTTCTAGGTTCaaaa	+21	D
MAE_00390	-	23_Op1	-	hypothetical protein			
MAE_00400	-	23_Op2	-	transposase	acagGTTCCTTTTAGGTTCaaaa	+34	R
MAE_01560	-	96_Tu1	-	transposase	acagGTTCCTTTTAGGTTCaaaa	+34	R
MAE_01700	-	105_Tu1	-	transposase	acagGTTCCTTTTAGGTTCaaaa	+34	R
MAE_02520	*nblA*	156_Tu1	+	phycobilisome degradation protein	aacaGTTCAACATTCGTTCtttt	-35	D
MAE_02620	-	163_Tu1	+	phosphoenolpyruvate synthase	gcttGTTCTCTAGAGGTTCtcgc	-25	R
MAE_02670	-	168_Tu1	+	transposase	acagGTTCCTTTTAGGTTCaaaa	-20	R
MAE_04880	-	307_Tu1	-	hypothetical protein	cagaGTACAGATGATGTACccag	-83	R
MAE_06840	-	421_Op1	+	methionine aminopeptidase	gtagGTTCGCCGCGAGTACagaa	-55	D
MAE_06850	-	421_Op2	+	universal stress protein UspA-like protein			
MAE_07250	-	444_Op1	-	hypothetical protein			
MAE_07260	-	444_Op2	-	hypothetical protein	ttgaGTTCTTATCTCGTTCccag	-67	R
MAE_08290	-	512_Tu1	-	transposase	acagGTTCCTTTTAGGTTCaaaa	+34	R
MAE_09260	-	568_Op1	-	phosphate transport system permease protein			
MAE_09270	-	568_Op2	-	phosphate transport system permease protein	taaaGTTCGTGATCGGTTCtccc	-30	R
MAE_10330	-	639_Op2	+	hypothetical protein	gaacGTACCACACCGGTTCtgct	-72	R
MAE_10550	-	655_Tu1	-	hypothetical protein	tatgGTACAGTCTTAGTTCcgtt	-48	D
MAE_10850	-	677_Op1	-	hypothetical protein	taaaGTTCGGTACGAGTTCaagt	-17	R
MAE_11600	-	726_Op1	+	hypothetical protein	agaaGTTCCTCCCAAGTTCaatt	-62	R
MAE_11610	-	726_Op2	+	hypothetical protein			
MAE_11670	-	730_Tu1	-	transposase	acagGTTCCTTTTAGGTTCaaaa	+34	R
MAE_12570	*petH*	791_Tu1	+	ferredoxin-NADP oxidoreductase	taatGTACAGTCCGAGTACacta	-2	D
MAE_13540	-	850_Op1	-	hypothetical protein	atgaGTACCATCCAAGTTCaaat	+18	D
MAE_13750	-	863_Op1	-	hypothetical protein			
MAE_13760	-	863_Op2	-	transposase	acagGTTCCTTTTAGGTTCaaaa	+34	R
MAE_13970	-	874_Tu1	+	transposase	acagGTTCCTTTTAGGTTCaaaa	-20	R
MAE_17820	-	undefined	+	hypothetical protein	tagaGTTCGTCCCATGTACcttt	+4	R
MAE_17850	-	1120_Op1	-	hypothetical protein	cccaGTTCTCCAATAGTTCagat	-8	R
MAE_20960	-	1308_Tu1	+	transposase	acagGTTCCTTTTAGGTTCaaaa	-20	R

Table 3-2-3. Continued

Locus tag[a)]	Name	TU[b)]	Strand	Function[c)]	Sequence[d)]	Position[e)]	Direction[f)]
MAE_21540	-	1350_Op1	-	hypothetical protein			
MAE_21550	-	1350_Op2	-	Na-Ca exchanger/integrin-beta	ggcgGTTCAATAATCGTTCctgt	-75	R
MAE_22200	-	1387_Tu1	+	transposase	acagGTTCCTTTTAGGTTCaaaa	-20	R
MAE_22310	-	undefined	-	hypothetical protein	acagGTTCCTTTTAGGTTCaaaa	-7	D
MAE_22390	-	undefined	+	hypothetical protein	gtcgGTTCTCGCTATGTTCgccg	-21	R
MAE_23840	-	1492_Tu1	-	hypothetical protein	aatgGTTCGCATAAGGTTCagcc	+7	R
MAE_24070	-	1507_Tu1	+	ABC transporter ATP-binding protein	ggcaGTTCTTTTACTGTTCtctt	+31	R
MAE_24710	-	undefined	+	hypothetical protein	acagGTTCCTTCTAGGTTCaaaa	+21	D
MAE_26420	*ftsZ*	1655_Op1	+	cell division protein FtsZ	tcttGTACTAAGAGTGTTCcaat	+23	D
MAE_26430	-	1655_Op2	+	phosphomethylpyrimidine kinase			
MAE_26970	-	1693_Tu1	-	transposase	acagGTTCCTTTTAGGTTCaaaa	+34	R
MAE_27030	-	undefined	-	hypothetical protein	actgGTTCTCGTCAAGTTCgtcg	-19	R
MAE_27320	-	undefined	-	hypothetical protein	tcctGTTCGCAAGAGGTACacta	+7	R
MAE_29730	-	undefined	+	hypothetical protein	tgagGTTCGATTGGAGTACccct	-81	D
MAE_30660	-	1912_Op2	+	hypothetical protein	aaccGTACTCGCTAAGTTCggcg	-18	R
					tgacGTTCTCTTAATGTACgata	-58	R
MAE_34410	-	2130_Tu1	-	transposase	acagGTTCCTTTTAGGTTCaaaa	+34	R
MAE_36350	-	2250_Tu1	+	transposase	acagGTTCCTTTTAGGTTCaaaa	-20	R
MAE_37820	-	2344_Tu1	+	beta-lactamase-like protein	ccagGTTCGGAAACCGTTCgtta	-61	D
MAE_37860	-	2347_Tu1	-	hypothetical protein	atctGTTCTACTTGCGTTCttaa	+81	R
MAE_37940	-	2352_Tu1	-	amino acid ABC-transporter permease	cattGTACTGAAAAAGTACaagg	-33	D
MAE_37950	*ssb*	2353_Tu1	+	single-stranded DNA-binding protein	cattGTACTGAAAAAGTACaagg	+53	R
MAE_38710	-	2401_Tu1	-	chloride channel protein	ttaaGTTCTGTCACCGTTCacct	-86	R
MAE_39200	-	2435_Tu1	-	transposase	acagGTTCCTTTTAGGTTCaaaa	+34	R
MAE_40170	-	2502_Tu1	+	transposase	acagGTTCCTTTTAGGTTCaaaa	-20	R
MAE_41070	-	2551_Tu1	-	transposase	acagGTTCCTTTTAGGTTCaaaa	+34	R
MAE_42120	-	undefined	-	hypothetical protein	aggtGTTCGCACTATGTTCtaaa	+69	D
MAE_44460	-	2760_Tu1	-	transposase	acagGTTCCTTTTAGGTTCaaaa	+34	R
MAE_44680	-	undefined	-	hypothetical protein	caagGTTCTACCTTGGTACgaga	+22	D
MAE_45420	-	2821_Tu1	-	transposase	acagGTTCCTTTTAGGTTCaaaa	+34	R
MAE_45550	-	2830_Tu1	+	transposase	acagGTTCCTTTTAGGTTCaaaa	-20	R

Table 3-2-3. Continued

Locus tag[a)]	Name	TU[b)]	Strand	Function[c)]	Sequence[d)]	Position[e)]	Direction[f)]
MAE_46390	-	2892_Op1	-	NAD-dependent epimerase/dehydratase			
MAE_46400	-	2892_Op2	-	glycosyl transferase family protein			
MAE_46410	-	2892_Op3	-	transposase	acagGTTCCTTTTAGGTTCaaaa	+34	R
MAE_46900	-	2927_Tu1	-	transposase	acagGTTCCTTTTAGGTTCaaaa	+34	R
MAE_47060	-	undefined	+	hypothetical protein	acagGTTCCTTCTAGGTTCaaaa	+21	D
MAE_47650	-	2970_Tu1	+	transposase	acagGTTCCTTCTAGGTTCaaaa	-20	R
MAE_48580	-	undefined	+	hypothetical protein	agaaGTTCATAAGGTGTACaaag	+31	R
MAE_48840	-	3049_Tu1	-	hypothetical protein	atctGTTCGGACATAGTTCggtg	+9	R
MAE_49410	-	3088_Tu1	+	hypothetical protein	ttaaGTACCTTATTGGTTCgcta	-70	D
MAE_50390	-	3148_Op1	+	hypothetical protein	ttggGTTCCCGTCACGTTCccat	+41	D
MAE_50400	-	3148_Op2	+	hypothetical protein			
MAE_50470	-	3154_Tu1	-	hypothetical protein	aattGTACTAGCAAAGTACaagt	-38	R
MAE_51270	-	3207_Op1	-	OstA-like protein			
MAE_51280	-	3207_Op2	-	hypothetical protein			
MAE_51290	*ftrC*	3207_Op3	-	ferredoxin-thioredoxin reductase catalytic chain	aacaGTACGCTAGACGTACaaat	+73	D
MAE_52230	-	3263_Tu1	-	transposase	acagGTTCCTTTTAGGTTCaaaa	+34	R
MAE_54200	-	3392_Op1	+	hypothetical protein	tcctGTTCGCAGGAGGTACatta	+71	R
MAE_54210	-	undefined	-	hypothetical protein			
MAE_54460	-	3409_Tu1	+	2-isopropylmalate synthase	ccaaGTTCGTTAACGGTACaaaa	+24	D
MAE_59420	-	undefined	-	hypothetical protein	aaatGTACGTTGATAGTTCtagc	-67	R
					caacGTACATTTTCGGTTCgaca	-56	D
MAE_59430	-	3701_Tu1	+	CAB/ELIP/HLIP superfamily protein	aaatGTACGTTGATAGTTCtagc	-68	D
MAE_59870	-	3726_Tu1	+	potassium-transporting ATPase subunit C	atccGTTCTACCCTAGTTCtctg	-34	D
MAE_60350	-	3753_Tu1	+	transposase	acagGTTCCTTGTAGGTTCaaaa	-20	R
MAE_61500	-	3824_Op3	+	hypothetical protein	cgccGTACATATAGTGTTCaaaa	+41	R
MAE_62020	-	3861_Tu1	+	transposase	acagGTTCCTTTTAGGTTCaaaa	-20	R
MAE_62450	-	3891_Tu1	-	UDP-glucose 4-epimerase	attgGTTCCCATGCGGTTCtagc	66	D

[a] Identification number in CyanoBase (http://genome.microbedb.jp/cyanobase) is given.
[b] TU was predicted using FgeneB (http://www.softberry.com/berry) (Kumar 2013 Proc Nat Acad Sci USA). The number of front indicates number of TU. Tu1 indicates single transcription unit. Op indicates operon. The number of the next to Op indicates the operon compsition.
[c] Description of function according to Cyanobase is given.
[d] Upper case and small case indicates Lex box and franking bases, respectively.
[e] Position indicates upstream (-) or downstream (+) from start codon as +1.
[f] Direction with respect to coding strand is given.

Comparing *Microcystis* LexA regulon with other cyanobacteria

To verify whether the content of *Microcystis* LexA regulon is common in cyanobacteria, I predicted LexA regulon in additional three cyanobacterial species: *Cyanothece* sp. ATCC51142, *Anabaena* sp. PCC7120 and *Synechocystis* sp. PCC6803. *Cyanothece* sp. ATCC51142 belongs to phylogenetically same clade to *Microcystis* (Fig. 3-2-8) and its LexA box is uncharacterized, *Microcystis* LexA box was used for the prediction of the regulon.

The size of LexA regulon (LexA genes/genome-encoded total ORFs) was 1.4 % (87 genes) in *M. aeruginosa* NIES-843, 1.3 % (68 genes) in *Cyanothece* sp. ATCC51142, 13.8 % (494 genes) in *Synechocystis* sp. PCC6803 and 0.3 % (17 genes) in *Anabaena* sp. PCC7120, indicating that the size of LexA regulon differed with species. Genes of LexA regulon assigned to COGs, except for COGs-S and COG-R, were 80 % (44 genes) in *M. aeruginosa* NIES-843, 75 % (33 genes) in *Cyanothece* sp. ATCC51142, 79 % (330 genes) in *Synechocystis* sp. PCC6803, and 90 % (9 genes) in *Anabaena* sp. PCC7120. Distribution of COG contents was different among each organism (Fig. 3-2-11). Among these species, COG-L and COG-P were frequently found. When compared with COG content of *M. aeruginosa* NIES-843 and *Cyanothece* sp. ATCC51142, 10 of the 11 categories of *Microcystis* COGs were shared. However, COG-D was found only in

Microcystis LexA regulon.

Table 3-2-2. Genetic character for each species.

Species [a)]	MIC843	CYA51142	SYN6803	ANA7120
Genome size (bp) [b)]	5,842,796	5,460,087	3,947,019	7,311,789
Number of TUs	73	60	355	11
Number of LexA genes	87	68	494	17
Legulon size (%) [c)]	1.4	1.3	13.8	0.3
Number of genes assigned to COGs (%) [d)]	80	79	75	90

[a)] MIC843: *M. aeruginosa* NIES-843; CYA51142: *Cyanothece* sp. ATCC51142; SYS6803: *Synechocystis* sp. PCC6803; ANA7120: *Anabaena* sp. PCC7120.
[b)] Including plasmids.
[c)] The value was calculated as the number of LexA genes divided by the number of total ORFs.
[d)] The value was calculated as the number of LexA genes which assigned to COGs except for COG-S and COG-R divided by the number of LexA genes.

Discussion

BpFz was specifically bind to upstream sequence of *ftsZ* and this binding was disappeared in the cells cultivated with addition of replication inhibitor nalidixic acid (Fig. 3-1-2) (Honda et al. 2012). I revealed that purified BpFz was LexA. This is the first report that LexA binds to upstream of *ftsZ*. LexA is a repressor of DNA damage-inducible SOS genes. *E. coli* SOS response is the well characterized; global and relatively large network (~40 genes) include DNA damage repair enzyme, translesion synthesis polymerase and cell division inhibitors (Fernández de Henestrosa et al. 2000; Wade et al. 2005). These SOS genes are derepressed by RecA mediated LexA self-cleavage when DNA damage or stalling of replication folk occur (Horii et al. 1981; Slilaty and Little 1987). *Microcystis* LexA also possesses three important sites for RecA-mediated self-cleavage (Ala-Gly dyad site, and Ser and Lys active sites). However, *Microcystis* LexA binds to upstream of *ftsZ* when chromosome was normally replicated as well as *ftsZ* was probably transcribed (Fig. 3-2-1). This implies that LexA

may play a function as transcriptional activator of *ftsZ* in *Microcystis* (Fig. 3-2-4). Although it is unclear that how the *Microcystis* LexA functions as an activator through RecA-LexA system, LexA may function as an activator in *Synechocystis* sp. PCC6803(Gutekunst et al. 2005).

SOS response governed by RecA-LexA system had been thought to be an universal system in bacteria (Erill et al. 2007), although there are some exception in *Epsilonproteobacteriasea* , *Bacteroidetes*-Green sulfur bacteria group and *Firmicutes Streptococcus pneumoniae* (Prudhomme et al. 2006). In these organisms, DNA damage response system is thought to be able to substituted by other stress response (Erill et al. 2007). Cyanobacterial LexA does not regulate classical SOS genes. In *Synechocystis* sp. PCC6803, LexA regulates inorganic carbon dependent expression of carbon assimilation gene (Domain et al. 2004), the redox-sensitive RNA helicase (Patterson-Fortin et al. 2006) and bidirectional Ni-Fe-hydrogenase operon *hoxEFUYH* (Gutekunst et al. 2005).

LexA box of *Microcystis* ($GTACN_7GTTC$) was similar to that of *Anabaena* sp. PCC7120 (10 of 11 nucleotides were shared) (Fig. 3-2-5a). However, *Microcystis* LexA box was not similar to that of *Synechocystis* sp. PCC6803, even if they are phylogenetically closely related (Fig. 2-2-8). *Synechocystis* sp. PCC6803 LexA protein lacks two of the three residues which are required for RecA mediated self-cleavage activity in *E. coli* (Ala of the Ala-Glya self-cleavage site and Ser of Ser-Lys active site) (Slilaty and Little 1987). Therefore, its activity as well as the regulation system is incompatible with RecA-LexA system (Patterson-Fortin et al. 2006). When I compared COG content of the LexA regulon, COG-L, COG-P and COG-H were shared among

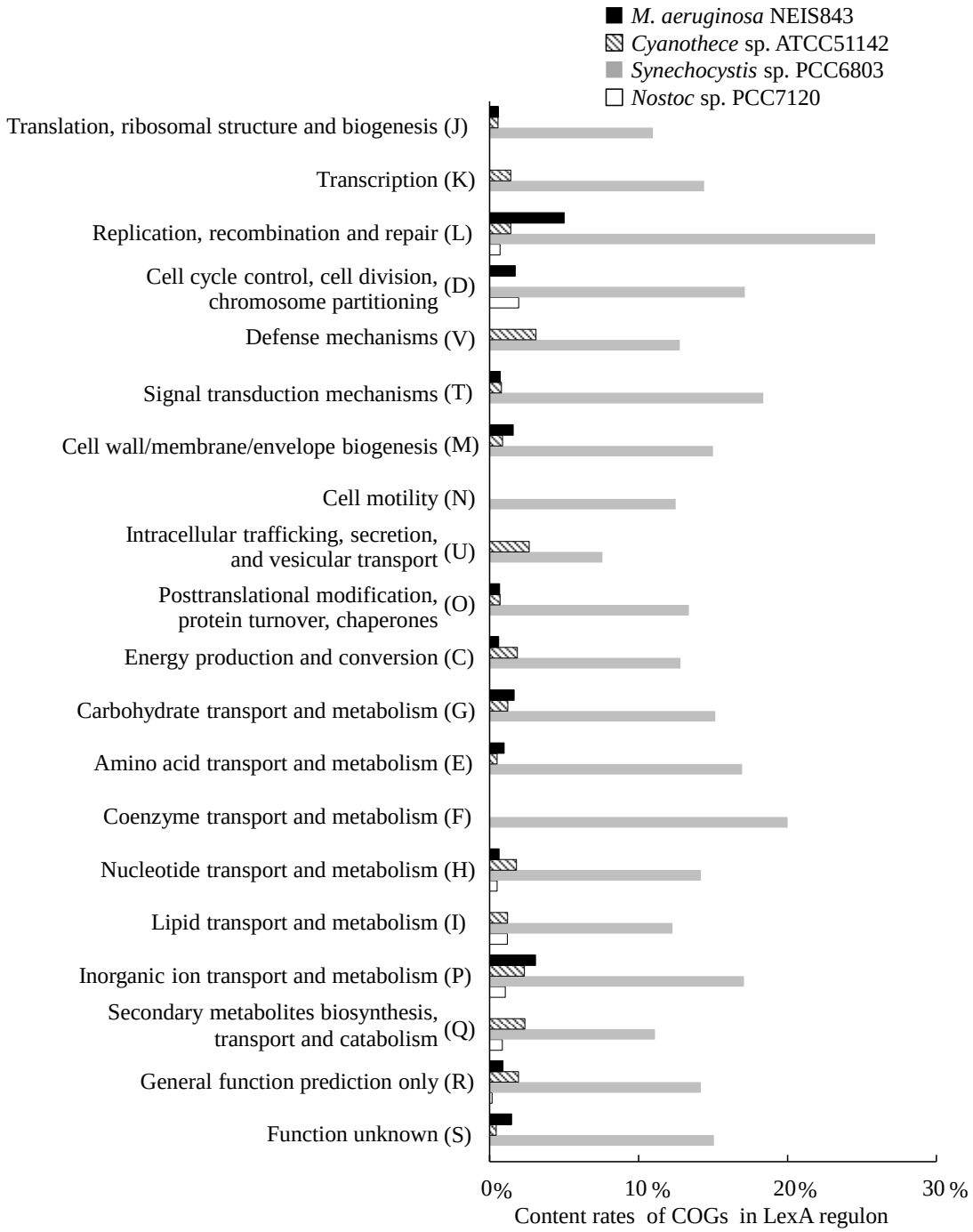


Fig. 3-2-10. Normalized number of COGs in LexA regulon per genome-encoded all COGs.

cyanobacteria, which is comparable to previous reports (Domain et al. 2004; Gutekunst et al. 2005; Patterson-Fortin et al. 2006).

When I focused on the genes involved in cell division, only *Microcystis* LexA regulon contained *ftsZ*. However, *Synechocystis* sp. PCC6803 LexA regulon contained *minD, E* which are components of *minCDE* system determining correct division site (de Boer et al. 1989). Therefore, this regulatory pathway may be unique to *Microcystis*, suggesting that expression monitoring of *lexA* gene may be applicable to evaluation of proliferation of *Microcystis*.

It is assumed that diversity of LexA regulon and LexA box may be derived from evolutional process that enable the regulatory circuits of inhabiting organisms to suit environmental niches (Patterson-Fortin et al. 2006). In photosynthetic cyanobacteria, LexA regulates genes which function as maintenance of photosynthetic activity (Domain et al. 2004; Patterson-Fortin et al. 2006). *Microcystis* floats near the surface water using a gas vacuole (Mlouka et al. 2004). In this environment, *Microcystis* is exposed to more intensively and frequently light than non-floating organisms, which may rapidly lead the cells to oxidative condition as well as photoinhibition. In addition, presence of photosynthesis related genes *nblA* and *petH* in *Microcystis* LexA regulon supported the idea that function of cyanobacterial LexA is to maintain maximal photosynthetic abilities respond to cellular redox status (Patterson-Fortin et al. 2006).

Chapter 4

Integration and outlook

Forecasting of occurrence and termination of *Microcystis* bloom is an important issue for water quality management throughout the world. However, how environmental factors (e.g., nutrient supply, light and temperature) effect on the dynamics of cyanobacterial blooms have not been elucidated. Therefore, none of strategy which perfectly and definitively forecast the toxic cyanobacterial blooms is available. Alternatively, I attempted to identify genes related with bloom formation and termination of *Microcystis* to predict its blooms by monitoring those genes. Therefore, I investigated *Microcystis* gene expression during phage infection, because phage infection is estimated to be major cause of death in natural environment. For the prediction of bloom formation, I searched for a gene marker of normally growing *Microcystis*, checkpoint factor which coordinates chromosome replication and cell division throughout the transcriptional regulation of major cell division gene, *ftsZ*.

In Chapter 2, I revealed that stress response genes involved in protection of photosystem II (PSII) are highly transcribed during phage infection. In addition, alternative sigma factor, *sigB*, which is induced under various stress conditions and may regulate transcription of some of stress response genes, was highly induced. These suggest that phage infection directly or indirectly induces protection of PSII via transcriptional regulation of stress response genes, and this regulation, at least partially, may be mediated by SigB.

In Chapter 3, I explored DNA binding protein bond to upstream sequence of cell division gene *ftsZ* using electrophoresis mobility shift assay (EMSA), and showed that

the DNA binding protein was the bacterial universal repressor LexA. To estimate regulatory system, the recognition sequence GTWCN$_7$GTWC determined by EMSA using mutant probes were scanned on the whole *Microcystis* genome. This analysis revealed that *Microcystis* LexA regulon does not contain classical SOS genes. When compared with predicted LexA regulon among four cyanobacterial species, the regulon composition was different among each organism. In addition, *ftsZ* was found only in *Microcystis*. These data support the idea that cyanobacterial LexA regulons are diversified within the phyla and thus, bring fitness advantage for inhabitant in various environments. Considering that *Microcystis* lives in the water surface floating with gas vesicle, this organism frequently exposed to oxidized conditions (i.e., UV, photooxidation and oxygen supersaturation) than non-floating cyanobacteria. Therefore, coordination of chromosome replication and cell division via transcriptional regulation of *ftsZ*, which is meditated by DNA damage-inducible LexA, may be beneficial to arrest sterile division sequence when DNA is damaged.

As described above, this study provided fundamental knowledge about gene regulation during termination and formation of *Microcystis* bloom. Further experiments, i.e., long-term expression monitoring, studies on linkage of gene expression and termination or formation of *Microcystis* bloom in natural environment, may enable to apply expression monitoring of marker genes for bloom forecasting.

Acknowledgements

I am grateful to Professor Yoshihiko Sako who entirely supported my studies and encouraged me with graceful words. I am also grateful to Professor Shigeki Sawayama and Professor Tatsuya Sugawara for reviewing this dissertation.

I would like to show my grateful appreciate to Associate Professor Takashi Yoshida. He gave me his precious time and great effort to support not only my work but also my life. He always encouraged and patiently admonished me even if I behaved rudely.

I want to say thanks to my (erstwhile) colleagues in the Laboratory of Marine Microbiology for their support and encouragement. Especially, I give a special thanks to Haruna Takahashi, PhD. Naohiko Hosoda, PhD. Takahiro Inoue, PhD. Satoshi Kawaichi, PhD. Sotaro Kuno, PhD. Shigeko Kimura and PhD. Teruki Amano. They are as my brother as my teacher.

Finally, I would like to thank my family and my prospective wife for giving me grate support over a long period of time.

References

Backer L and McGillicuddy D (2006) Harmful algal blooms. Oceanography 19:94-106

Barker M, de Vries R, Nield J, Komenda J and Nixon PJ (2006) The deg proteases protect *Synechocystis* sp. PCC 6803 during heat and light stresses but are not essential for removal of damaged D1 protein during the photosystem two repair cycle. J Biol Chem 281:30347-30355

Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA and Horvath P (2007) CRISPR provides acquired resistance against viruses in prokaryotes. Science 315:1709-1712

Bergh Ø, BØrsheim KY, Bratbak G and Heldal M (1989) High abundance of viruses found in aquatic environments. Nature 340:467-468

Bernhardt TG and De Boer PA (2005) SlmA, a nucleoid-associated, FtsZ binding protein required for blocking septal ring assembly over chromosomes in *E. coli*. Mol Cell 18:555-564

Bi E and Lutkenhaus J (1991) FtsZ ring structure associated with division in *Escherichia coli*. Nature 354:161-164

Bienert R, Baier K, Volkmer R, Lockau W and Heinemann U (2006) Crystal structure of NblA from *Anabaena* sp. PCC 7120, a small protein playing a key role in phycobilisome degradation. J Biol Chem 281:5216-5223

Bobay BG, Benson L, Naylor S, Feeney B, Clark AC, Goshe MB, Strauch MA, Thompson R and Cavanagh J (2004) Evaluation of the DNA binding tendencies of the transition state regulator AbrB. Biochemistry (Mosc) 43:16106-16118

Cambridge J, Blinkova A, Magnan D, Bates D and Walker JR (2014) A replication-inhibited unsegregated nucleoid at mid-cell blocks Z-ring formation

and cell division independently of SOS and the SlmA nucleoid occlusion protein in *Escherichia coli*. J Bacteriol 196:36-49

Carstens EB and Ball LA (2009) Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2008). Arch Virol 154:1181-1188

Clark DJ (1968) Regulation of deoxyribonucleic acid replication and cell division in *Escherichia coli* B/r. J Bacteriol 96:1214-1224

Clausen T, Kaiser M, Huber R and Ehrmann M (2011) HTRA proteases: regulated proteolysis in protein quality control. Nat Rev Mol Cell Biol 12:152-162

Clokie MR, Shan J, Bailey S, Jia Y, Krisch HM, West S and Mann NH (2006) Transcription of a 'photosynthetic' T4-type phage during infection of a marine cyanobacterium. Environ Microbiol 8:827-835

Codd GA, Lindsay J, Young FM, Morrison LF and Metcalf JS (2005) From mass mortalities to management measures. In: Huisman J, Matthijs HCP, Visser PM (eds) Harmful cyanobacteria. Springer, Dordrecht, The Netherland, pp 1-23

Collado-Vides J, Magasanik B and Gralla JD (1991) Control site location and transcriptional regulation in *Escherichia coli*. Microbiol Rev 55:371-394

Crooks GE, Hon G, Chandonia JM and Brenner SE (2004) WebLogo: a sequence logo generator. Genome Res 14:1188-1190

de Boer PA, Crossley RE and Rothfield LI (1989) A division inhibitor and a topological specificity factor coded for by the minicell locus determine proper placement of the division septum in *E. coli*. Cell 56:641-649

Den Blaauwen T, Buddelmeijer N, Aarsman ME, Hameete CM and Nanninga N (1999) Timing of FtsZ assembly in *Escherichia coli*. J Bacteriol 181:5167-5175

DeRuyter Y and Fromme P (2008) Molecular structure of the photosynthetic apparatus. In: Herrero A, Flores E (eds) The Cyanobacteria: Molecular Biology, Genomics and Evolution. Horizon Scientific Press, Poole, U.K.,

Domain F, Houot L, Chauvat F and Cassier-Chauvat C (2004) Function and regulation of the cyanobacterial genes *lexA*, *recA* and *ruvB*: LexA is critical to the survival of cells facing inorganic carbon starvation. Mol Microbiol 53:65-80

Dong G, Yang Q, Wang Q, Kim YI, Wood TL, Osteryoung KW, van Oudenaarden A and Golden SS (2010) Elevated ATPase activity of KaiC applies a circadian checkpoint on cell division in *Synechococcus elongatus*. Cell 140:529-539

Elledge SJ (1996) Cell cycle checkpoints: preventing an identity crisis. Science 274:1664-1672

Engelberg-Kulka H, Amitai S, Kolodkin-Gal I and Hazan R (2006) Bacterial programmed cell death and multicellular behavior in bacteria. PLoS Genet 2:e135

Erill I, Campoy S and Barbé J (2007) Aeons of distress: an evolutionary perspective on the bacterial SOS response. FEMS Microbiol Rev 31:637-656

Fernández de Henestrosa AR, Ogi T, Aoyagi S, Chafin D, Hayes JJ, Ohmori H and Woodgate R (2000) Identification of additional genes belonging to the LexA regulon in *Escherichia coli*. Mol Microbiol 35:1560-1572

Fineran PC, Blower TR, Foulds IJ, Humphreys DP, Lilley KS and Salmond GP (2009) The phage abortive infection system, ToxIN, functions as a protein-RNA toxin-antitoxin pair. Proc Natl Acad Sci USA 106:894-899

Gao E, Gui J and Zhang Q (2012) A novel cyanophage with a cyanobacterial nonbleaching protein A gene in the genome. J Virol 86:236-245

Gao EB, Yuan XP, Li RH and Zhang QY (2009) Isolation of a novel cyanophage infectious to the filamentous cyanobacterium *Planktothrix agardhii* (*Cyanophyceae*) from Lake Donghu, China. Aquat Microb Ecol 54:163-170

Glassberg J, Meyer R and Kornberg A (1979) Mutant single-strand binding protein of *Escherichia coli*: genetic and physiological characterization. J Bacteriol 140:14-19

Goley ED, Iniesta AA and Shapiro L (2007) Cell cycle regulation in *Caulobacter*: location, location, location. J Cell Sci 120:3501-3507

Grossman L, Braun A, Feldberg R and Mahler I (1975) Enzymatic repair of DNA. Annu Rev Biochem 44:19-43

Guindon S and Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst biol 52:696-704

Guo L (2007) Doing battle with the green monster of Taihu Lake. Science 317:1166

Gutekunst K, Phunpruch S, Schwarz C, Schuchardt S, Schulz-Friedrich R and Appel J (2005) LexA regulates the bidirectional hydrogenase in the cyanobacterium *Synechocystis* sp. PCC 6803 as a transcription activator. Mol Microbiol 58:810-823

He D and Xu X (2010) CalA, a cyAbrB protein, binds to the upstream region of *ftsZ* and is down-regulated in heterocysts in *Anabaena* sp. PCC 7120. Arch Microbiol 192:461-469

Hohn T, Hohn B, Engel A, Wurtz M and Smith PR (1979) Isolation and characterization of the host protein *groE* involved in bacteriophage lambda assembly. J Mol Biol 129:359-373

Holtzendorff J, Partensky F, Jacquet S, Bruyant F, Marie D, Garczarek L, Mary I, Vaulot

D and Hess W (2001) Diel expression of cell cycle-related genes in synchronized cultures of *Prochlorococcus* sp. strain PCC 9511. J Bacteriol 183:915-920

Honda T, Yoshida T, Hiroishi S and Sako Y (2012) A protein binding to an upstream sequence of *ftsZ* involved in coordination of DNA replication and cell division in *Microcystis aeruginosa*. Fish Sci 78:375-379

Honda T, Takahashi H, Sako Y and Yoshida T (2014) Gene expression of *Microcystis aeruginosa* during infection of cyanomyovirus Ma-LMM01. Fish Sci 80:83-91

Horii T, Ogawa T, Nakatani T, Hase T, Matsubara H and Ogawa H (1981) Regulation of SOS functions: purification of *E. coli* LexA protein and determination of its specific site cleaved by the RecA protein. Cell 27:515-522

Huisman J, Matthijs HCP and Visser PM (eds) (2005) Harmful cyanobacteria. Springer, Dordrecht, The Netherland

Imamura S, Asayama M and Shirai M (2004) *In vitro* transcription analysis by reconstituted cyanobacterial RNA polymerase: roles of group 1 and 2 sigma factors and a core subunit, RpoC2. Genes Cells 9:1175-1187

Imamura S and Asayama M (2009) Sigma factors for cyanobacterial transcription. Gene Regul Syst Bio 3:65-87

Inoue I, Ino R and Nishimura A (2009) New model for assembly dynamics of bacterial tubulin in relation to the stages of DNA replication. Genes Cells 14:435-444

Ishii A and Hihara Y (2008) An AbrB-like transcriptional regulator, Sll0822, is essential for the activation of nitrogen-regulated genes in *Synechocystis* sp. PCC 6803. Plant Physiol 148:660-670

Kaneko T, Nakajima N, Okamoto S, Suzuki I, Tanabe Y, Tamaoki M, Nakamura Y,

Kasai F, Watanabe A, Kawashima K, Kishida Y, Ono A, Shimizu Y, Takahashi C, Minami C, Fujishiro T, Kohara M, Katoh M, Nakazaki N, Nakayama S, Yamada M, Tabata S and Watanabe MM (2007) Complete genomic structure of the bloom-forming toxic cyanobacterium *Microcystis aeruginosa* NIES-843. DNA Res 14:247-256

Kasai F, Kawachi M, Erata M and Watanabe MM (2004) List of strains, Microalgae and Protozoa. 7 edn. National Institute for Environmental Studies, Tsukuba, Japan

Kawai Y, Moriya S and Ogasawara N (2003) Identification of a protein, YneA, responsible for cell division suppression during the SOS response in *Bacillus subtilis*. Mol Microbiol 47:1113-1122

Kelly AJ, Sackett MJ, Din N, Quardokus E and Brun YV (1998) Cell cycle-dependent transcriptional and proteolytic regulation of FtsZ in *Caulobacter*. Genes Dev 12:880-893

Kimura S, Yoshida T, Hosoda N, Honda T, Kuno S, Kamiji R, Hashimoto R and Sako Y (2012) Diurnal infection patterns and impact of *Microcystis* cyanophages in a Japanese pond. Appl Environ Microbiol 78:5805-5811

Koerner JF and Snustad DP (1979) Shutoff of host macromolecular synthesis after T-even bacteriophage infection. Microbiol Rev 43:199-223

Kumar S, Nei M, Dudley J and Tamura K (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 9:299-306

Kuno S, Yoshida T, Kaneko T and Sako Y (2012) Intricate interactions between the bloom-forming cyanobacterium *Microcystis aeruginosa* and foreign genetic elements, revealed by diversified clustered regularly interspaced short

palindromic repeat (CRISPR) signatures. Appl Environ Microbiol 78:5353-5360

Lavigne R, Darius P, Summer EJ, Seto D, Mahadevan P, Nilsson AS, Ackermann HW and Kropinski AM (2009) Classification of *Myoviridae* bacteriophages using protein sequence similarity. BMC Microbiol 9:224

Lindell D and Post AF (2001) Ecological Aspects of *ntcA* Gene Expression and Its Use as an Indicator of the Nitrogen Status of Marine *Synechococcus* spp. Appl Environ Microbiol 67:3340-3349

Lindell D, Jaffe JD, Coleman ML, Futschik ME, Axmann IM, Rector T, Kettler G, Sullivan MB, Steen R, Hess WR, Church GM and Chisholm SW (2007) Genome-wide expression dynamics of a marine virus and host reveal features of co-evolution. Nature 449:83-86

Liu G, Begg K, Geddes A and Donachie WD (2001) Transcription of essential cell division genes is linked to chromosome replication in *Escherichia coli*. Mol Microbiol 40:909-916

Lonetto M, Gribskov M and Gross CA (1992) The sigma 70 family: sequence conservation and evolutionary relationships. J Bacteriol 174:3843-3849

Love PE and Yasbin RE (1984) Genetic characterization of the inducible SOS-like system of *Bacillus subtilis*. J Bacteriol 160:910-920

MacKintosh C, Beattie KA, Klumpp S, Cohen P and Codd GA (1990) Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. FEBS Lett 264:187-192

Maestrinp SY, Balode M and Bechemin C (1997) Nutrients limiting the algal growth potential (AGP) in the Gulf of. La mer 35

Makarova KS, Wolf YI, Snir S and Koonin EV (2011) Defense islands in bacterial and

archaeal genomes and prediction of novel defense systems. J Bacteriol 193:6039-6056

Markson JS, Piechura JR, Puszynska AM and O'Shea EK (2013) Circadian control of global gene expression by the cyanobacterial master regulator RpaA. Cell 155:1396-1408

Matsuda N and Uozumi N (2006) Ktr-mediated potassium transport, a major pathway for potassium uptake, is coupled to a proton gradient across the membrane in *Synechocystis* sp. PCC 6803. Biosci Biotechnol Biochem 70:273-275

Mazón G, Lucena J, Campoy S, de Henestrosa ARFdH, Candau P and Barbé J (2004) LexA-binding sequences in Gram-positive and cyanobacteria are closely related. Mol Genet Genomics 271:40-49

McAdams HH and Shapiro L (2003) A bacterial cell-cycle regulatory network operating in time and space. Science 301:1874-1877

McCarthy MJ, James RT, Chen Y, East TL and Gardner WS (2009) Nutrient ratios and phytoplankton community structure in the large, shallow, eutrophic, subtropical Lakes Okeechobee (Florida, USA) and Taihu (China). Limnology 10:215-227

Mizuuchi K, O'Dea MH and Gellert M (1978) DNA gyrase: subunit structure and ATPase activity of the purified enzyme. Proc Natl Acad Sci USA 75:5960-5963

Mlouka A, Comte K, Castets AM, Bouchier C and Tandeau de Marsac N (2004) The gas vesicle gene cluster from *Microcystis aeruginosa* and DNA rearrangements that lead to loss of cell buoyancy. J Bacteriol 186:2355-2365

Mori T, Binder B and Johnson CH (1996) Circadian gating of cell division in cyanobacteria growing with average doubling times of less than 24 hours. Proc Natl Acad Sci USA 93:10183-10188

Moriya S, Rashid R, Rodrigues C and Harry E (2010) Influence of the nucleoid and the early stages of DNA replication on positioning the division site in *Bacillus subtilis*. Mol Microbiol 76:634-647

Mukherjee A, Cao C and Lutkenhaus J (1998) Inhibition of FtsZ polymerization by SulA, an inhibitor of septation in *Escherichia coli*. Proc Natl Acad Sci USA 95:2885-2890

Mulder E and Woldringh CL (1989) Actively replicating nucleoids influence positioning of division sites in *Escherichia coli* filaments forming cells lacking DNA. J Bacteriol 171:4303-4314

Nakaya A, Katayama T, Itoh M, Hiranuka K, Kawashima S, Moriya Y, Okuda S, Tanaka M, Tokimatsu T and Yamanishi Y (2013) KEGG OC: a large-scale automatic construction of taxonomy-based ortholog clusters. Nucleic Acid Res 41:D353-D357

Namikoshi M, Rinehart KL, Sakai R, Stotts RR, Dahlem AM, Beasley VR, Carmichael WW and Evans WR (1992) Identification of 12 hepatotoxins from a Homer Lake bloom of the cyanobacteria *Microcystis aeruginosa*, *Microcystis viridis*, and *Microcystis wesenbergii*: nine new microcystins. J Org Chem 57:866-872

Nishiwaki-Matsushima R, Ohta T, Nishiwaki S, Suganuma M, Kohyama K, Ishikawa T, Carmichael WW and Fujiki H (1992) Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. J Cancer Res Clin Oncol 118:420-424

Oberholster PJ, Myburgh JG, Govender D, Bengis R and Botha AM (2009) Identification of toxigenic *Microcystis* strains after incidents of wild animal mortalities in the Kruger National Park, South Africa. Ecotoxicol Environ Saf

72:1177-1182

Oliver R, Hamilton D, Brooks J and Ganf G (2012) Physiology, blooms and prediction of planktonic cyanobacteria. In: Witton B (ed) Ecology of Cyanobacteria II. Springer, New York London,

Paerl HW and Huisman J (2008) Blooms like it hot. Science 320:57-58

Patterson-Fortin LM, Colvin KR and Owttrim GW (2006) A LexA-related protein regulates redox-sensitive expression of the cyanobacterial RNA helicase, *crhR*. Nucleic Acid Res 34:3446-3454

Patterson-Fortin LM and Owttrim GW (2008) A *Synechocystis* LexA-orthologue binds direct repeats in target genes. FEBS Lett 582:2424-2430

Pearson LA and Neilan BA (2008) The molecular genetics of cyanobacterial toxicity as a basis for monitoring water quality and public health risk. Curr Opin Biotechnol 19:281-288

Pineda M, Gregory BD, Szczypinski B, Baxter KR, Hochschild A, Miller ES and Hinton DM (2004) A family of anti-sigma70 proteins in T4-type phages and bacteria that are similar to AsiA, a transcription inhibitor and co-activator of bacteriophage T4. J Mol Biol 344:1183-1197

Pitt FD, Mazard S, Humphreys L and Scanlan DJ (2010) Functional characterization of *Synechocystis* sp. strain PCC 6803 *pst1* and *pst2* gene clusters reveals a novel strategy for phosphate uptake in a freshwater cyanobacterium. J Bacteriol 192:3512-3523

Poranen MM, Ravantti JJ, Grahn AM, Gupta R, Auvinen P and Bamford DH (2006) Global changes in cellular gene expression during bacteriophage PRD1 infection. J Virol 80:8081-8088

Pouria S, De Andrade A, Barbosa J, Cavalcanti R, Barreto V, Ward C, Preiser W, Poon GK, Neild G and Codd G (1998) Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. The Lancet 352:21-26

Prudhomme M, Attaiech L, Sanchez G, Martin B and Claverys JP (2006) Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. Science 313:89-92

Radding CM (1981) Recombination activities of *E. coli* RecA protein. Cell 25:3-4

Revzin A (1989) Gel electrophoresis assays for DNA-protein interactions. Biotechniques 7:346-355

Sakr S, Jeanjean R, Zhang CC and Arcondeguy T (2006) Inhibition of cell division suppresses heterocyst development in *Anabaena* sp. strain PCC 7120. J Bacteriol 188:1396-1404

Sandh G, El-Shehawy R, Díez B and Bergman B (2009) Temporal separation of cell division and diazotrophy in the marine diazotrophic cyanobacterium *Trichodesmium erythraeum* IMS101. FEMS Microbiol Lett 295:281-288

Santi I, Dhar N, Bousbaine D, Wakamoto Y and McKinney JD (2013) Single-cell dynamics of the chromosome replication and cell division cycles in mycobacteria. Nature communications 4:2470

Sato N (1995) A family of cold-regulated RNA-binding protein genes in the cyanobacterium *Anabaena variabilis* M3. Nucleic Acid Res 23:2161-2167

Sato T, Minagawa S, Kojima E, Okamoto N and Nakamoto H (2010) HtpG, the prokaryotic homologue of Hsp90, stabilizes a phycobilisome protein in the cyanobacterium *Synechococcus elongatus* PCC 7942. Mol Microbiol 76:576-589

Schwartz R and Dayhoff M (eds) (1978) Atlas of protein sequence and structure, vol 5. National Biomedical Research Foundation, Washington, D.C., USA

Seshasayee AS, Bertone P, Fraser GM and Luscombe NM (2006) Transcriptional regulatory networks in bacteria: from input signals to output responses. Curr Opin Microbiol 9:511-519

Slilaty SN and Little JW (1987) Lysine-156 and serine-119 are required for LexA repressor cleavage: a possible mechanism. Proc Natl Acad Sci USA 84:3987-3991

Smith VH (1983) Low nitrogen to phosphorus ratios favor dominance by blue-green algae in lake phytoplankton. Science 221:669-671

Solovyev V and Salamov A (2011) Automatic annotation of microbial genomes and metagenomic sequences. In: Li RW (ed) Metagenomics and its applications in agriculture, biomedicine and environmental studies. Nova Science Publishers, NY, USA, pp 61-78

Suttle CA (1994) The significance of viruses to mortality in aquatic microbial communities. Microb Ecol 28:237-243

Suttle CA (2005) Viruses in the sea. Nature 437:356-361

Suttle CA (2007) Marine viruses: major players in the global ecosystem. Nat Rev Microbiol 5:801-812

Tanaka N and Nakamoto H (1999) HtpG is essential for the thermal stress management in cyanobacteria. FEBS Lett 458:117-123

Thomas-Chollier M, Defrance M, Medina-Rivera A, Sand O, Herrmann C, Thieffry D and van Helden J (2011) RSAT 2011: regulatory sequence analysis tools. Nucleic Acid Res 39:W86-W91

Thomas DC, Levy M and Sancar A (1985) Amplification and purification of UvrA, UvrB, and UvrC proteins of *Escherichia coli*. J Biol Chem 260:9875-9883

Thompson JD, Higgins DG and Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acid Res 22:4673-4680

Thompson LR, Zeng Q, Kelly L, Huang KH, Singer AU, Stubbe J and Chisholm SW (2011) Phage auxiliary metabolic genes and the redirection of cyanobacterial host carbon metabolism. Proc Natl Acad Sci USA 108:E757-E764

Tonthat NK, Arold ST, Pickering BF, Van Dyke MW, Liang S, Lu Y, Beuria TK, Margolin W and Schumacher MA (2011) Molecular mechanism by which the nucleoid occlusion factor, SlmA, keeps cytokinesis in check. EMBO J 30:154-164

Trusca D, Scott S, Thompson C and Bramhill D (1998) Bacterial SOS checkpoint protein SulA inhibits polymerization of purified FtsZ cell division protein. J Bacteriol 180:3946-3953

Vierling E (1991) The roles of heat shock proteins in plants. Annu Rev Plant Physiol Plant Mol Biol 42:579-620

Wade JT, Reppas NB, Church GM and Struhl K (2005) Genomic analysis of LexA binding reveals the permissive nature of the *Escherichia coli* genome and identifies unconventional target sites. Genes Dev 19:2619-2630

Wang JD and Levin PA (2009) Metabolism, cell growth and the bacterial cell cycle. Nat Rev Microbiol 7:822-827

Wang X, Possoz C and Sherratt DJ (2005) Dancing around the divisome: asymmetric

chromosome segregation in *Escherichia coli*. Genes Dev 19:2367-2377

Wang Y and Xu X (2005) Regulation by *hetC* of genes required for heterocyst differentiation and cell division in *Anabaena* sp. strain PCC 7120. J Bacteriol 187:8489-8493

WHO (ed) (2011) Guidelines for drinking-water quality, fourth edition. WHO press, Geneva, Switzerland

Wikner J, Vallino JJ, Steward GF, Smith DC and Azam F (1993) Nucleic acids from the host bacterium as a major source of nucleotides for three marine bacteriophages. FEMS Microbio Ecol 12:237-248

Wortinger M, Sackett MJ and Brun YV (2000) CtrA mediates a DNA replication checkpoint that prevents cell division in *Caulobacter crescentus*. EMBO J 19:4503-4512

Wu J, Sunda W, Boyle EA and Karl DM (2000) Phosphate depletion in the western north Atlantic Ocean. Science 289:759-762

Wu L, Franks A and Wake R (1995) Replication through the terminus region of the *Bacillus subtilis* chromosome is not essential for the formation of a division septum that partitions the DNA. J Bacteriol 177:5711-5715

Wu LJ and Errington J (2004) Coordination of Cell Division and Chromosome Segregation by a Nucleoid Occlusion Protein in *Bacillus subtilis*. Cell 117:915-925

Wu LJ, Ishikawa S, Kawai Y, Oshima T, Ogasawara N and Errington J (2009) Noc protein binds to specific DNA sequences to coordinate cell division with chromosome segregation. EMBO J 28:1940-1952

Yagi O, Hagiwara T, Takamura Y and Sudo R (1984) Growth characteristics of axenic

and unialgal *Microcystis* isolated from Lake Kasumigaura. Jpn J Water Poll Res 7:496-503

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S and Madden TL (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13:134

Yoshida-Takashima Y, Yoshida M, Ogata H, Nagasaki K, Hiroishi S and Yoshida T (2012) Cyanophage infection in the bloom-forming cyanobacteria *Microcystis aeruginosa* in surface freshwater. Microbes Environ 27:350-355

Yoshida M, Yoshida T, Kashima A, Takashima Y, Hosoda N, Nagasaki K and Hiroishi S (2008a) Ecological dynamics of the toxic bloom-forming cyanobacterium *Microcystis aeruginosa* and its cyanophages in freshwater. Appl Environ Microbiol 74:3269-3273

Yoshida M, Yoshida T, Yoshida-Takashima Y, Kashima A and Hiroishi S (2010) Real-time PCR detection of host-mediated cyanophage gene transcripts during infection of a natural *Microcystis aeruginosa* population. Microbes Environ 25:211-215

Yoshida T, Maki M, Okamoto H and Hiroishi S (2005) Coordination of DNA replication and cell division in cyanobacteria *Microcystis aeruginosa*. FEMS Microbiol Lett 251:149-154

Yoshida T, Takashima Y, Tomaru Y, Shirai Y, Takao Y, Hiroishi S and Nagasaki K (2006) Isolation and characterization of a cyanophage infecting the toxic cyanobacterium *Microcystis aeruginosa*. Appl Environ Microbiol 72:1239-1247

Yoshida T, Nagasaki K, Takashima Y, Shirai Y, Tomaru Y, Takao Y, Sakamoto S, Hiroishi S and Ogata H (2008b) Ma-LMM01 infecting toxic *Microcystis*

aeruginosa illuminates diverse cyanophage genome strategies. J Bacteriol 190:1762-1772

Yoshizawa S, Matsushima R, Watanabe MF, Harada K-i, Ichihara A, Carmichael WW and Fujiki H (1990) Inhibition of protein phosphatases by microcystins and nodularin associated with hepatotoxicity. J Cancer Res Clin Oncol 116:609-614

Publication list

Honda T, Yoshida T, Hiroishi S and Sako Y (2012) A protein binding to an upstream sequence of *ftsZ* involved in coordination of DNA replication and cell division in *Microcystis aeruginosa*. Fish Sci 78:375–379

Honda T, Takahashi H, Sako Y and Yoshida T (2014) Gene expression of *Microcystis aeruginosa* during infection of cyanomyovirus Ma-LMM01. Fish Sci 80: 83-91

Kimura S, Yoshida T, Hosoda N, Honda T, Kuno S, Kamiji R, Hashimoto R and Sako Y (2012) Diurnal infection patterns and impact of *Microcystis* cyanophages in a Japanese pond. Appl Environ Microbiol 78: 5805-5811