Transcriptional regulation of CmpR, the LysR family protein involved in CO$_2$-responsive gene regulation in the cyanobacterium *Synechococcus elongatus*

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**Abstract**

The *cmpR* gene of the cyanobacterium *Synechococcus elongatus* PCC 7942 encodes a LysR family protein that activates the *cmpABCD* bicarbonate transporter operon under the conditions of CO$_2$ limitation. Using the *luxAB* reporter genes, the promoter of the *cmpR* gene was shown to be transiently activated under the low-CO$_2$ conditions. Much higher levels of *∆cmpR* expression were observed in a *luxAB* reporter construct under the conditions of CO$_2$ limitation. Using the *PcmpR-luxAB* promoter, exposure of the *∆cmpR* strain to the low-CO$_2$ conditions further increased the *luxAB* expression level, showing the involvement of an additional transcriptional activator responding to CO$_2$ limitation. From the *ΔcmpR* strain, two mutant strains showing slower growth, lower contents of phycocyanin and even higher levels of *P$_{luxAB}$* expression than the parental strain were isolated and shown to have a partial deletion in the ORF of *cpcE*, encoding the alpha subunit of phycocyanobilin lyase. Expression of plasmid-borne *cpcE* in the mutants restored the growth and reduced the luciferase expression level to those observed in the parental strain, while partially restoring the phycocyanin content. These results indicated occurrence of multiple layers of regulation of *cmpR* expression in addition to the autoregulation by CmpR.

**Abbreviations:** ABC, ATP-binding cassette; CBB, Calvin-Benson-Bassham; CCD, Charge Coupled Device; CCMs, CO$_2$ concentrating mechanisms; HC, high-CO$_2$; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPTG, Isopropyl β-D-1-thiogalactopyranoside; LC, low-CO$_2$; LTTRs, LysR-type transcriptional regulators; NS1, neutral site 1; OD$_{730}$, Optical Density at 730 nm; ORF, open reading frame; PC, phycocyanin; PCR, Polymerase Chain Reaction; 2-PG, 2-phosphoglycerate; RT-PCR, Reverse Transcription Polymerase Chain Reaction; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; UVC, ultraviolet-C.

**Introduction**

Though the atmospheric CO$_2$ concentration is rising because of combustion of fossil fuels, it is not high enough to saturate ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), the enzyme responsible for photosynthetic CO$_2$ fixation, under ambient air in *vitro*. O$_2$ serves as the alternative substrate of Rubisco to CO$_2$ and hence interferes with the carboxylation of ribulose 1,5-bisphosphate (RuBP). To cope with this problem, photosynthetic organisms have developed various "CO$_2$ concentrating mechanisms" (CCMs) to raise the CO$_2$ concentration in the vicinity of Rubisco to enhance the carboxylation reaction.

Cyanobacteria are photoautotrophic prokaryotes capable of plant-type, oxygenic photosynthesis. They have an efficient CCM, which consists of two steps: (i) intracellular accumulation of high concentrations of HCO$_3$; and (ii) localized conversion of HCO$_3$ to CO$_2$ in the carboxysomes, the polyhedral inclusion bodies to which Rubisco is sequestered. Intracellular accumulation of HCO$_3^-$ is mediated by active HCO$_3^-$ transporters and specialized NADPH dehydrogenase complexes that convert CO$_2$ to HCO$_3^-$ in an energy-dependent manner [1-5]. The intracellular conversion of CO$_2$ to HCO$_3^-$ lowers the intracellular CO$_2$ level low, allowing for uptake of CO$_2$ from the external medium via passive diffusion. For both the HCO$_3^-$ and CO$_2$ uptake mechanisms, those having high-affinity for the respective substrates are distinguished from those having low-affinity for the substrates. The genes related to the high-affinity HCO$_3^-$ and CO$_2$ uptake mechanisms are generally repressed under high-CO$_2$ (HC) conditions, i.e., when cells are incubated under air supplemented with 1-5% (v/v) of CO$_2$. Since transcription of these genes are sharply induced by incubation of the HC-grown cells under low-CO$_2$ (LC) conditions, i.e., under air containing 0.04% or lower CO$_2$ concentrations, they are thought to be LC-inducible genes [6-8].

LysR-type transcriptional regulators (LTTRs) of CbbB subfamily were first identified in chemoautotrophic bacteria and phototropic bacteria as the regulator of the genes encoding the enzymes of the Calvin-Benson-Bassham (CBB) cycle [9]. In cyanobacteria, CbbR homologs were shown to be the major player in regulation of the LC-induced expression of the CCM-related genes; *Synechocystis* sp. PCC 6803 has two closely-related CbbR homologs, one of which (CmpR) was identified as the activator of the *cmp* operon, which encodes the...
subunits of an ABC-type high-affinity HCO$_3^-$ transporter [10], while the other (NdhR or CcmR) was shown to serve as a repressor under HC conditions, inhibiting expression of several CCM-related genes including its own gene, the sbtA gene encoding the Na$^+$-dependent high-affinity HCO$_3^-$ transporter, and the ndhF3 operon encoding the components of the high-affinity CO$_2$ uptake mechanism [6,7,11]. *Synechococcus elongatus* PCC 7942, on the other hand, has only one CbbR homolog designated CmpR, which was shown to be the activator of the cmpABCD operon [10]. The CmpR protein of *S. elongatus* PCC 7942 was shown in *in vitro* experiments to bind to the promoter of the cmpABCD operon in the presence of 2-phosphoglycolate (2-PG), one of the products of the RuBP oxygenation reaction [12]. It is therefore supposed that 2-PG serves as the signal of CO$_2$ deficiency in vivo to activate transcription of the cmpABCD operon. It has remained unclear whether the *S. elongatus* CmpR protein regulates its own expression as many of the LTTR proteins do. Using a *luxAB* reporter protein transcriptionally fused to the *cmpR* promoter, it is shown in this study that the protein not only activates the cmp operon under LC conditions, but represses its own expression under HC conditions. Studies on the reporter strain reveal an additional layer(s) of regulation of the LC conditions. Genetic studies on the strain further point to a novel relationship between the integrity of the light harvesting system and the activity of the promoter.

### Materials and methods

#### Strains and growth conditions

A derivative of *S.elongatus* strain PCC7942 (SPc), which is cured of the resident pUH24 plasmid [13], was used as the parental strain of all the mutants used in this study. For construction of a ΔcmpR mutant, a 0.8-kbp DNA region upstream of *cmpR* (nucleotides -798 to +3 with respect to the translation start site) and a 0.9-kbp region downstream of *cmpR* (nucleotides +1 to +888 with respect to the termination codon) were amplified by PCR using the primer pairs P1/P2 and P3/P4, respectively (Table 1). The chloramphenicol resistance gene was included the sequences complementary to the primers P2 and P3. The resulting DNA fragment was used to transform the SPc strain to construct a chloramphenicol-resistant mutant. The chloramphenicol resistance gene was amplified by PCR using the primers P5 and P6, which respectively amplified the DNA regions (-XbaIdownR GTGCTCATCTAGAAGGTGCCTACAGCAAACC and BamHIR TGCAGCGTTAGATTTTTGGATCCAACTCAAATCACCATTC) that were used for construction of a ΔccmR mutant of the SPc strain (Figure 1B). The PccmR::luxAB fusion thus constructed was transferred into the neutral site 1 (NS1) locus on the chromosome of the SPc and MR5 strains to construct the CW and CT strains, respectively.

The liquid medium used for cultivation of the cyanobacterial strains was a modification of the BG11 medium containing 15 mM KNO$_3$ and 20 mM HEPES-KOH (pH 8.2) [15]. Cells were grown at 30°C under continuous illumination provided by fluorescent lamps at a light intensity of 50 μE m$^{-2}$ s$^{-1}$. Cultures were aerated with ambient air (low-CO$_2$ conditions) or air supplemented with 2% (v/v) CO$_2$ (high-CO$_2$ conditions). Solid medium was prepared by addition of 1.5% (w/v) Bactoagar (Difco) to the liquid medium. Where appropriate, chloramphenicol (5 μg ml$^{-1}$), spectinomycin (20 μg ml$^{-1}$) and kanamycin (15 μg ml$^{-1}$) were added to the media.

For random mutagenesis in the CT strain, cells in liquid medium were irradiated with a 15W UVC lamp (254 nm) for 4 min (survival rate ~ 0.2%) and then treated for 24 h with 40 μg ml$^{-1}$ of ampicillin in the light under LC conditions. The cells were then spread onto solid medium prepared in rectangular plates of 128 mm × 86 mm in size and incubated under the HC conditions for 10 days. The agar plates, which contained a total of 17,000 colonies, were subsequently incubated under low CO$_2$ conditions and screened for mutant colonies showing altered expression levels of bioluminescence.

#### Bioluminescence measurements

Time-course measurements of bioluminescence from colonies of the P$_{cmpR}$::luxAB reporter strain were performed essentially as described previously [16,17] using an automated bioluminescence

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**Table 1. Primers used in this study.**

| No. | Name | Sequence (5'-3') |
|-----|------|-----------------|
| P1  | ccmRup978F | AACAGGGGAGTTAAACGATCTGC |
| P2  | ccmRup17R  | CACAATCTAAAATCCATTCT |
| P3  | ccmRdown1F | GCAGATGGATAGGCACTCCACTCC |
| P4  | ccmRdown888R | CCGGGTCTCTTATCGGTGCCTCCG |
| P5  | ccmRint1F | GAATTGTGGAATGTGAGTGTTGGGACAGTCGTCAGAGGACAGTCAGAGTC |
| P6  | ccmRint888R | GAGGATGAGCCATCAACGTGCCGATGGGTAAAGGGCA |
| P7  | procmR115NotIF | CGTACGAGAACGATGGCAAGGCGAAACCCGCGCAGATGG |
| P8  | procmR150NotI | TGGACGGGAGTATTTTTGTGAGC |
| P9  | cmpRup30F | ACTTAAATCTTGGTGAAGTTGAGTTGTT |
| P10 | cmpRdown28R | TTTGATGGGATGTGATGTGAGCTACATCAGTCAGTC |
| P11 | cpcE-NcoIP | TGAGCCCCGACTTCCATGATGAGTGAAGGC |
| P12 | cpcE-XbaIdownR | GTGCTCATCAAGAGGGGATGGTACAGGAC |
| P13 | cpcFF | ATGACGACGAGTGGATACGCTGCATGG |
| P14 | cpcFR | TTAATACGCCGATGGGATGGTACGCTGCATGG |
| P15 | rnpBR | GAGGAAAGTCCGGGCTCCCA |
| P16 | cmpR | TAAAGCCCCGTGTCGTCGTCGTCG |
| P17 | luxAF | CAGCGGCGGAGTATGAGG |
| P18 | luxAR | ATCCGCGGATGGTTCGTCGTCG |

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monitoring apparatus (model LL04-1, Churitsu Electric Co., Nagoya, Japan); Cells of CT and CW strains were plated on solid medium and incubated under HC conditions for 7 days. Agar pieces carrying 10 to 20 colonies were sealed in 35-mm petri dishes together with 30 μl of 3% n-decanal solution in cooking oil and 100 μl NaHCO₃ (10%, w/v), and incubated under illumination for 16 h. Expression of P_{cmpR::luxAB} was induced by removing the NaHCO₃ solution from the petri dish, and bioluminescence was measured at 30-min intervals. The data were analyzed using the SL00-01 bioluminescence-analyzing software (SL00-01, Churitsu Electric Co.) [18]. Bioluminescence intensity was expressed as cps per colony.

Identification of the mutant colonies derived from the UVC-irradiated population of the luxAB reporter strain CT was carried out using a high-throughput real-time bioluminescence monitoring system equipped with a CCD image sensor (Churitsu Electric Corp., Nagoya, Japan; http://www.churitsu.co.jp/products/bio/highthroughput.html) essentially as described by Kondo and Ishiura [19].

For measurements of bioluminescence from the E3 and E11 mutants, which form only tiny colonies on solid medium, cells grown in liquid medium under HC conditions were collected by centrifugation, re-suspended in fresh medium to give an OD730 value of 0.5, and incubated under illumination with aeration. 0.5 ml aliquots of the cell suspension were occasionally withdrawn from the culture, mixed with 20 μl of 3% n-decanal solution, incubated for 5 min and then subjected to photon counting for 30 s using an AQUACOSMOS/VIM system (Hamamatsu Photonics, Hamamatsu, Japan).

**Re-sequencing analysis of genomic DNA**

Genomic DNA was extracted from Synechococcus cells using a Wizard Genomic DNA Purification Kit (Promega), after treatment of the cells with lysozyme as follows; Cells were collected and re-suspended in 450 μl of a buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 5 mM EDTA and after mixing with 50 μl of a lysozyme solution (50 mg ml⁻¹), incubated at 37°C for 1 h. The cells were subsequently collected by centrifugation at 15,000 g at RT for 2 min and lysed by mixing with 0.6 ml of the Nuclei Lysis Solution of the Promega kit. Re-sequencing of the genomic DNA was performed using a SOLID 5500 system (Life Technologies) as described previously [20].

**Expression of the plasmid-borne cpeE in Synechococcus.**

An 859-bp DNA fragment carrying the coding region of cpeE was amplified from the genomic DNA of S. elongatus PCC 7942 by PCR using the primers P11 and P12 carrying added NcoI and XbaI recognition sequences, respectively (Table 1). The amplified DNA

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**Figure 1.** Analysis of the effects of CmpR deficiency on regulation of the cmpR promoter, using the transcriptional fusion of the cmpR promoter and the luxAB ORFs encoding a bacterial luciferase. A. The map of the cmpR genomic region of the SPc and CW strains, as compared with that of the ΔcmpR strains MR5 and CT. Arrows show the primers used for the analysis shown in panel B. B. PCR analysis of the structure of the cmpR genomic region in SPc and MR5. C. Organization of the genes inserted into the NS1 genomic region of the reporter strains CW and CT. D. Induction of bioluminescence from the CW and CT strains after transfer of the colonies grown under HC conditions to LC conditions. Data shown are the means ± SE from biological triplicates.
RNA extraction and semi-quantitative RT-PCR

25 ml aliquots of the cultures of *S. elongatus* (OD$_{730}$ ~0.5) were used for RNA extraction, following the protocol reported by Ruffing [22]. From the lysozyme/ethanol mixture thus obtained, RNA was purified using a SV Total RNA Isolation System (Promega). The isolated total RNA was used for the synthesis of cDNA using a SuperScript III First-Strand Synthesis System (Life Technologies). The obtained cDNA was used as the template for semi-quantitative RT-PCR analysis using the primer pairs specific to *luxA*, *cpcF* and *rnpB* (P13-P18; Table 1). The cycle numbers for PCR amplification was 30 for *luxA*, 30 for *cpcF*, and 26 for *rnpB*.

Results

CmpR-dependent and independent mechanisms of *cmpR* regulation

As previously reported for the *cmpR* insertional mutant MR4 [10], cells of the *cmpR* deletion mutant MR5 grew as fast as the parental SPC strain both under LC and HC conditions (data not shown). Accordingly, the *P$_{cmpR}$::luxAB* strains CW and CT, which were constructed from SPC and MR5 respectively, formed colonies of essentially the same size (data not shown). Low CO$_2$ response of the CW and CT strains were therefore compared by measuring the bioluminescence emitted from colonies on the surface of agar plates, using a real-time bioluminescence monitoring system (Figure 1D). When HC-grown cells of the CW strain, which carries the wild-type *cmpR* gene, was transferred to LC conditions, there was a sharp induction of bioluminescence (Figure 1D, CW, open symbols). After reaching a peak at $t \approx 4$ h, the intensity of bioluminescence declined to a lower level. These results indicated that transcription of *cmpR* is activated by CO$_2$ limitation. Unlike the CW strain, the reporter strain deficient in *cmpR* (CT) showed high level of bioluminescence even when grown under the HC conditions (Figure 1D, CT, $t = 0$), showing that CmpR autoregulates its own expression as many other LTTRs do. After transfer of CT cells to LC conditions, further increase in the luciferase expression level was observed (Figure 1D, CT, open symbols); The bioluminescence level increased for about 15 h after the transfer and remained at the high level thereafter. The results indicated the presence of a CmpR-independent mechanism(s) for LC-responsive activation of *cmpR*.

Characterization of the mutants with altered $P_{cmpR}$ regulation

To gain insight into the CmpR-independent mechanism(s) of *cmpR* regulation, CT cells were treated with UVC irradiation, subjected to an ampicillin enrichment procedure under LC conditions, plated on solid medium and screened for colonies showing altered levels of bioluminescence, using a high-throughput bioluminescence monitoring system equipped with a CCD image sensor. Among the mutant candidates, two (designated E3 and E11) were found to reproducibly show slow growth compared to the parental CT strain using cells grown in liquid cultures and re-suspended in fresh growth medium to give an OD$_{730}$ value of 0.5 (Figure 4). The results confirmed that the expression level of luciferase was much higher in the E3 and E11 strains than in the parental CT strain (Figure 4).

To identify the mutations that caused activation of the *cmpR* promoter in the E3 and E11 mutants, genome re-sequencing analysis of the CT, E3 and E11 mutants was performed (Table 2). The CT strain had no mutation as compared with the parental SPC strain except for the replacement of the *cmpR* gene with the chloramphenicol resistance gene and the insertion of a spectinomycin resistance gene and the *PempR::luxAB* reporter construct into the NS1 site. E3 and E11 each harbored the same insertion, and the mutations that caused activation were located in the promoter region of the *cmpR* gene.
**Table 2. List of mutations found in the E3 and E11 strains compared to the CT genomic sequence.**

| Position | GENE ID | CT\(a\) | E3\(b\) | E11\(c\) | Amino acid change | Gene product |
|----------|---------|---------|---------|---------|------------------|--------------|
| 463280-466710 | 0479-0481 | - | 3431-bp deletion (100%) | - | - | GTP-binding protein LepA, GAF sensor signal transduction histidine kinase, ProtEase |
| 924881 | 0918 | G | T (100%) | G | G268V | Acyl-ACP synthetase |
| 1061288 | 1048 | C | C | T (100%) | A133V | Phycocyanin α subunit CpaA |
| 1065043 | 1053 | C | C | T (100%) | A133V | Phycocyanin α subunit CpaA2 |
| 1065485-1065605 | 1054 | - | 121-bp deletion (100%) | 121-bp deletion, frame shift | 41-AA deletion, frame shift | Phycocyanin alpha subunit phycocyanobilin lyase CpeE subunit |
| 2660395 | Upstream of R0052 | G | A (50%) | G | - | 16S ribosomal RNA |

*This column shows the nucleotide in the genome sequence of the CT strain.

*These columns show the mutations and their frequency in E3 and E11.

E11 respectively had several mutations on the genome, but they had a common deletion of 121-bp in the cpeE gene, which forms an operon with the cpeF gene located downstream. The cpeE-cpcF operon encodes the subunits of the heterodimeric phycocyanin-α-subunit phycocyanobilin lyase, which mediates attachment of phycocyanobilin to the α subunit (CpcA) of phycocyanin (PC) [23], a blue-colored light-harvesting protein consisting of the α and β subunits encoded by the highly conserved cpeA and cpeB genes. In accordance with the previously reported properties of the cpeE deletion mutant of *S. elongatus* [24], both E3 and E11 showed significantly reduced amounts of PC as revealed by their absorption spectra, showing lower absorption peak at 625 nm as compared with the parental CT strain (Figure 3).

To determine whether the CpeE deficiency was the cause of the growth defect and the enhanced activation of *PcmpR* under LC condition, a plasmid carrying the *cpeE* ORF downstream of the *PcmpR* promoter (see methods) was introduced into E3 and E11 to construct the E3R and E11R strains. Expression of *cpeE* from the *PcmpR* promoter could only partially restore the PC content (Figure 3), presumably due to the polar effect of the 121-bp deletion in the chromosomal *cpeE* gene on the expression of the *cpeF* gene. Nevertheless, growth of the E3R and E11R strains were comparable to that of CT, indicating that the amount of PC was sufficient to support normal photosynthetic growth under the given conditions (Figure 2). The level of bioluminescence was also reduced to that observed in the parental CT strain by expression of *cpeE*.

Semi-quantitative RT-PCR analysis of the transcripts from the *luxA* gene showed that the gene is expressed at a much higher level in E3 than in CT under the LC conditions. Also, expression of plasmid-borne *cpeE* reduced the transcript level to a level comparable to that in CT. These results confirmed that it was the activity of the *PcmpR* promoter that responded to the presence or absence of the *cpeE* gene (Figure 5).

**Figure 5. Semi-quantitative RT-PCR analysis of expression of the cpeF, luxA, and rpmB genes in the CT, E3 and E3R strains under the HC and LC conditions.** HC-grown cells collected by centrifugation and re-suspended into fresh liquid medium were separated into two portions and incubated under HC or LC conditions. RNA samples extracted from the cells after 25 min of incubation were used for the analysis.

**Discussion**

The CmpR protein of *S. elongatus* PCC 7942 activates transcription of the *cmpAB* operon, which encodes the subunits of a high-affinity ABC HCO\(_3^-\) transporter, under the conditions of CO\(_2\) limitation [10]. Micromolar concentrations of 2-PG were shown to enhance the binding of the protein to the promoter of the *cmp operon in vitro*, suggesting that the product of the RuBP oxygenation reaction acts as a co-inducer of CmpR under the LC conditions [12]. In addition to its role as an activator of gene expression, the present results show that CmpR acts as a repressor of its own expression under the HC conditions (Figure 1). Thus, CmpR has two distinct modes of interaction with DNA; the one involving 2-PG as a co-activator and the other independent of 2-PG. The CcmR (NdhR) protein of *Synechocystis* sp. PCC 6803, the homolog of CmpR, is involved in repression of various CCM related genes under HC conditions [6,7,11]. By comparison of the promoters of these genes, a sequence ATAG-N\(_8^a\)-CTAT has been proposed as the consensus binding motif for CcmR [6]. Since the *cmpR* promoter of *S. elongatus* has two of this sequence (Figure 1C), CmpR presumably binds to these sites under HC in a 2-PG independent manner to repress transcription.

Since the *PcmpR-luxAB* fusion is activated under LC conditions even in the CT strain deficient in CmpR (Figure 1D, CT), it is deduced that *S. elongatus* has a CmpR-independent mechanism for LC-responsive activation of *PcmpR*. In an attempt to identify the genes relevant to this mechanism, we aimed at isolation of mutants that are defective in growth under LC conditions because of impaired CmpR response. The UVC-treated cells were hence subjected to an ampicillin enrichment procedure under LC conditions. The two mutant strains that showed a slow-growth phenotype were, however, found to grow slowly irrespective of the CO\(_2\) conditions (Figure 2) and to show stronger *PcmpR* induction than the parental CT strain under LC conditions (Figures 4 and 5). The *cpeE* mutation in the E3 and E11 strains is clearly responsible for the slow-growth phenotype and hyper-induction of *PcmpR* (Figures 2 and 5). The slow growth is presumed to be due to the reduced capacity of the cells to absorb light energy (Figure 3), but the underlying molecular mechanism that links CpeE and *PcmpR* activity...
is currently unclear. It is commonly supposed that the function of CCM becomes more important under high-light conditions, where the energy supply tends to exceed the supply of CO$_2$ to Rubisco. The activation of $P_{\text{cmpR}}$ in the cpcE mutant cells having smaller capacity of light absorption than the parental strain is therefore novel. Possible involvement of CpcE, a heat repeat protein, in direct interaction with $P_{\text{cmpR}}$ cannot be excluded, but it seems more likely that the reduced photosynthetic electron transport and the resulting metabolic change have relevance to the activation of $P_{\text{cmpR}}$ in the CmpR-deficient cpcE mutants. Although further study is required, the present results reveal a novel link between the light-harvesting capacity of the cells and regulation of the key regulator of CCM.

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