A Feed-Forward Loop Consisting of the Response Regulator RpaB and the Small RNA PsrR1 Controls Light Acclimation of Photosystem I Gene Expression in the Cyanobacterium Synechocystis sp. PCC 6803

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Introduction

Photosynthetic organisms have to maintain a balance between the energy supply by the photosynthetic electron transport chain and its consumption by cellular metabolism. Regulation of these processes in response to changes in environmental factors, such as light intensity and nutrient availability, is particularly important for controlling the redox balance of the cell (Oelze et al. 2008). A decrease in the amount of photosynthetic pigments and photosystems, especially that of PSI, to avoid the absorption of excess light energy is one of the main strategies of high-light (HL) acclimation in cyanobacteria (Kawamura et al. 1979, Murakami and Fujita 1991, Muramatsu and Hihara 2012). Two mutants of Synechocystis sp. PCC 6803 (S.6803), disruptants of pmgA (sll1968) and sli1961, both of which are not able to maintain their PSI content at a low level, exhibit severe growth inhibition under prolonged HL conditions (Hihara et al. 1998, Fujimori et al. 2005). This suggests physiological significance of the selective decrease in PSI content during HL acclimation.

A whole-genome DNA microarray analysis suggested that a decrease in PSI content during acclimation to HL is regulated at the level of transcription (Hihara et al. 2001). Genes encoding subunits of PSI are actively transcribed under LL conditions, whereas their transcription is co-ordinately and greatly down-regulated immediately after a shift to HL conditions (Muramatsu and Hihara 2003). RpaB, a DNA-binding response regulator which is highly conserved in cyanobacteria and even in red algae plastid genomes, constitutes, together with the histidine kinase Hik33 (also named as NblS, DspA), a key regulatory system for HL acclimation (reviewed in Wilde and Hihara 2016). It has been proposed that RpaB is phosphorylated by Hik33 under LL and its phosphorylation level is lowered upon exposure to HL in Synechococcus elongatus PCC 7942 (Moronta-Barrios et al. 2012). We found that binding of RpaB to the high light regulatory 1 (HLR1) sequence located upstream of the core promoter region (position –70 to –46, relative to the transcription start site) enables the co-ordinated
HL response of PSI genes dispersed throughout the S.6803 genome (Seino et al. 2009). Binding of RpaB to the HLR1 sequence under LL conditions results in the activation of PSI genes, and simultaneously results in the repression of HL-inducible genes such as hliB (Kappell and van Waasbergen 2007), and hliA and rpoD3 in S. elongatus PCC 7942 (Seki et al. 2007).

However, the regulatory network that controls photosynthetic gene expression must integrate various environmental signals, such as the availability of inorganic carbon and other nutrients. In addition, differences in ambient light which may change in sudden and unpredictable ways under natural conditions. This may involve non-coding small RNAs (sRNAs) that control gene expression at the post-transcriptional level. Although usually only 80–140 nucleotides in length, these sRNAs can repress or activate gene expression and frequently control multiple mRNAs (Wright et al. 2013). In enterobacteria, sRNAs are known to modulate almost every aspect of bacterial physiology and behavior (Storz et al. 2011). Cyanobacteria were also shown to harbor many potential regulatory RNA molecules, which control different cellular functions (Hess et al. 2014, Kopf and Hess 2015). Indeed, the HL response of PSI genes in S.6803 has been found recently to be under additional control of the sRNA PsrR1 (photosynthesis regulatory RNA1) (Georg et al. 2014). PsrR1, widely conserved within the cyanobacterial phylum, is induced under HL conditions and interacts with the ribosome-binding site of transcripts of photosynthetic genes such as psaLI, psaj, chlN and cpcA to inhibit translation. Moreover, PsrR1 is likely to decrease the stability of target transcripts, judging from the observation that the psaLI mRNA was cleaved by the endonuclease RNase E upon the binding of PsrR1 to the 5′-untranslated region (UTR). The amounts of phycocyanin, Chl and the PSI complex all decrease upon overexpression of PsrR1, indicating that PsrR1 is critical for the post-transcriptional down-regulation of the photosynthetic apparatus under HL (Mitschke et al. 2011, Georg et al. 2014). In addition, overexpression of PsrR1 results in a decrease in the PSI trimer to monomer ratio (Georg et al. 2014). Trimeric PSI complexes were shown to have a larger cross-section than monomeric complexes and were suggested to be favored under LL (Baker et al. 2014). Under HL, a specific decrease in the amount of PSI trimer is observed (Kopečná et al. 2012). Thus, PsrR1 might be involved in balancing the excitation of both photosystems by down-regulation of psaL expression under HL, thereby inducing monomerization of PSI.

Accumulating data suggest that RpaB and PsrR1 are the two most important critical factors for the regulation of PSI gene expression during HL acclimation. Therefore, we addressed the possibility of an interplay between these two regulators. In this study, we found that RpaB binds to the HLR1 sequence located within the core promoter region of psrR1 to repress its expression under LL conditions. Our findings suggest that RpaB and PsrR1 constitute a feed-forward loop for regulation of PSI gene expression to achieve rapid and strict repression upon the shift to HL conditions.

Results

HLR1 sequence is located in the core promoter region of the psrR1 gene

First, we carried out an in-depth search for PsrR1 homologs in the cyanobacterial phylum. We were able to extend the number of potential homologs of PsrR1 and furthermore detected up to three homologs in individual organisms (Supplementary Fig. S1). PsrR1 is widely conserved in the cyanobacterial phylum, especially in the more complex filamentous cyanobacteria, whereas we could not identify homologs in marine picocyanobacteria (Prochlorococcus and Synechococcus), thermophilic Synechococcus, S. elongatus, Gloeobacter and some other strains. This distribution differs from that of RpaB, which is ubiquitously present in cyanobacteria.

PsrR1 expression was shown to be strongly up-regulated upon the shift to HL in S.6803 (Georg et al. 2014). To elucidate the mechanism of transcriptional regulation of psrR1, we searched the proposed promoter regions upstream of the psrR1 homolog genes from several strains and carried out an initial motif search with MEME (Bailey et al. 2009). In a subset of the proposed psrR1 promoters, we detected a motif which resembled the previously characterized HLR1 motif. A ClustalW multiple sequence alignment of the promoter regions of 14 PsrR1 homologs revealed that the HLR1 sequence was found in the core promoter region, overlapping with its –10 box (Figs. 1A, 2A). It should be noted that the promoter region including the HLR1 sequence is more highly conserved than the 5′ portion of the psrR1 gene itself among different cyanobacteria (Fig. 1B).

RpaB binds to the HLR1 sequence of psrR1 in vitro

In order to test whether RpaB binds to the promoter region of psrR1 from S.6803, we overexpressed recombinant RpaB protein fused to a C-terminal 6× histidine-tag (His-RpaB) in Escherichia coli. His-RpaB was purified by nickel affinity chromatography from the soluble fraction (Supplementary Fig. S2). First, we examined DNA binding activity of the purified His-RpaB to the promoter fragment of psaE (–97 to +23, relative to the transcription start site, 120 bp) previously characterized as the target gene of RpaB (Seino et al. 2009) by gel mobility shift assay (Supplementary Fig. S3). In the previous study using N-terminal His-tagged RpaB purified from the insoluble fraction, the addition of 0.5 μg of His-RpaB was not sufficient for a band shift (Seino et al. 2009). In contrast, a distinct shifted band was observed with 0.5 μg of His-RpaB in the present study (Supplementary Fig. S3). When the psrR1 promoter fragment (–84 to +16, relative to the transcription start site, 100 bp) was used as a probe, 0.5 μg of His-RpaB was also sufficient for a band shift (Fig. 2B).

In order to address whether RpaB binds to the HLR1 sequence, gel mobility shift experiments were performed with probes having a base substitution within the HLR1 sequence of psrR1 (Fig. 2). RpaB could not bind to the ‘sub1’ fragment with four base substitutions within the half site of the HLR1
Fig. 1  (A) Multiple sequence alignment of the promoter regions of 14 psrR1 homolog genes from selected organisms. Position-wise nucleotide conservation is visualized with a weblogo (Crooks et al. 2004). The proposed HLR1 motif and –10 elements are highlighted. The first transcribed nucleotide is corroborated by transcriptome data from S.6803, Synechocystis sp. PCC 6714 (Kopf et al. 2014, Kopf et al. 2015) and Synechococcus sp. PCC 7002 (Ludwig and Bryant 2012). (B) Conservation of the promoter sequences and the psrR1 3’ parts is more pronounced than that of the psrR1 5’ parts. The conservation plot was based on the full-length sequences of the 14 psrR1 homologs including the promoter regions and drawn using the Emboss tool plotcon with a window size of 10. The sequence logos of the promoter regions and the 3’ parts are shown below the conservation plot.
sequence distal to the transcription start site, whereas it showed binding activity to the 'sub2' fragment with three base substitutions within the proximal half site of HLR1.

**PsR1 is induced within 5 min after the shift from LL to HL conditions**

The HL response of *psrR1* and the established target genes of RpaB, *hliB*, *psaA* and *psal*, was examined by RNA gel blot analysis in the wild-type (WT) strain ([Fig. 3](#), left). Georg et al. (2014) reported that the transcript level of *psrR1* increased dramatically within 30 min after the shift to HL and then slowly decreased. Even after 24 h of incubation under HL, the accumulation level of PsrR1 was 4-fold higher than that under LL conditions. In this study, we examined changes in transcript levels of RpaB target genes within the first 30 min after the shift to HL, since RpaB is involved in transcriptional regulation just after the shift to HL. Accumulation of PsrR1 was induced within 5 min after the shift from LL (20 μmol photons m⁻² s⁻¹) to HL (300 μmol photons m⁻² s⁻¹) conditions and continued to increase during 30 min. The amount of *hliB* transcript also increased within 5 min, but began to decrease after 15 min of HL exposure. In the case of *psaA* and *psal* genes encoding PSI subunits, transcript accumulation continued to decrease after the shift from LL to HL conditions as reported previously (Hihara et al. 2001, Muramatsu and Hihara 2003).

**DNA binding activity of RpaB to the promoter regions of psrR1 and other target genes decreased after the shift from LL to HL conditions**

The in vivo DNA binding activity of RpaB to the upstream regions of *psrR1* and other target genes was examined by chromatin affinity purification (ChAP) analysis. For ChAP analysis, the 12 x His-RpaB strain was generated by disruption of the
endogenous \textit{rpaB} gene and insertion of the C-terminal 12 × His-tagged \textit{rpaB} gene with its own promoter into a neutral site within the genome (Supplementary Fig. S4A). By using this strain, specific binding of RpaB to target promoters in vivo can be examined by affinity purification of cross-linked DNA–protein complexes. We confirmed the complete disruption of the endogenous \textit{rpaB} and introduction of 12 × His\textit{-}rpaB in the mutant cells by PCR (Supplementary Fig. S4B). Immunoblot analysis using anti-RpaB and anti-His-tag antisera revealed that only the His-tagged RpaB protein was expressed in the 12 × His-RpaB strain (Supplementary Fig S4C). We also confirmed that the HL response of the RpaB regulon was similar between WT and 12 × His-RpaB strains (Fig. 3).

ChAP analysis was performed using the 12 × His-RpaB strain and the WT strain as a negative control. After in vivo cross-linking of DNA–protein complexes by formaldehyde and affinity purification of 12 × His-RpaB by nickel chromatography, the amount of co-purified DNA was examined by quantitative PCR (qPCR) analysis and expressed as percentage recovery in relation to the total input DNA. As shown in Fig. 4A, in ChAP samples prepared from the 12 × His-RpaB strain grown under LL conditions, \textit{psaA}, \textit{psrR1}, \textit{psaL}, \textit{cyabrB2} and \textit{glnB} promoters exhibited a higher percentage recovery (0.015–0.06%) than \textit{cyabrB2} and \textit{glnB} promoters not having the HLR1 sequence (0.004–0.007%). In ChAP samples prepared from the WT strain grown under LL conditions, each promoter...
showed a percentage recovery of <0.004% irrespective of the presence or absence of the HLR1 sequence. These results indicate that RpaB specifically binds to the promoter region of psrR1 as well as to those of known target genes, psaA and psaL, under LL conditions.

Next, we examined the dynamic change in the RpaB binding activity to the target promoters upon the shift from LL to HL conditions. For promoters of psaA (Fig. 4B), psrR1 (Fig. 4C) and psaL (Fig. 4D), 5 min of exposure to HL resulted in a decrease of binding activity of RpaB to <35% of the LL level, and the low binding activity was maintained for at least 15 min. After 30 min, recovery of binding activity to the LL level was observed. On the other hand, the change in binding activity of RpaB after HL shift was insignificant in the case of the cyabrB2 (Fig. 4E) and glnB promoters (Fig. 4F). These results indicate that RpaB binds to the promoter regions of psrR1 and other target genes under LL conditions and that HL exposure causes the prompt and transient decline of the binding activity.

**Discussion**

The recent progress in the functional characterization of sRNAs demonstrated that sRNA-based post-transcriptional regulation, as well as transcriptional regulation, is important for bacteria to acclimate to changing environmental conditions, including cyanobacteria (Georg et al. 2014, Klähn et al. 2015). In various bacterial species, it has been reported that sRNAs participate in global regulatory networks together with protein regulators by forming regulatory circuits (Beisel and Storz 2010).

In the present study, we propose that RpaB, a key regulator for HL acclimation, and the sRNA PsrR1 constitute a feed-forward loop to regulate expression of PSI genes upon exposure to HL conditions in the cyanobacterium S.6803.

The HLR1 sequence, the recognition sequence for RpaB, is highly conserved in the upstream region of the psrR1 gene among cyanobacterial species (Fig. 1). Gel mobility shift assays revealed that RpaB binds to the HLR1 sequence overlapping with the −10 box of the psrR1 promoter in S.6803 (Fig. 2). It should be noted that RpaB can bind to the 'sub2'
fragment with three substituted bases within the proximal half site of HLR1, but not to the 'sub1' fragment with four base substitutions within the distal half site. Previously, we reported that RpaB cannot bind to the HLR1 sequence of the psaE promoter when base pair substitutions were introduced in one of the HLR1 half sites (Seino et al. 2009). Since the recombinant RpaB protein used for gel mobility shift assay was different mainly in the location of the His-tag between the previous and present studies, we re-examined the effect of base substitutions in HLR1 within the psaE promoter using the RpaB protein purified in this study. As shown in Supplementary Fig. S3, RpaB bound to the 'sub2' fragment and a slight band shift was observed in the case of the 'sub1' fragment of the psaE promoter. The recombinant protein used in this study showed a higher DNA binding activity than that in the previous study, and this can be explained by differences between these proteins such as the location of the His-tag (N-terminus or C-terminus), purification method (from the insoluble fraction or the soluble fraction) and location of the start codon leading to a difference in protein length (234 or 243 amino acids). The fact that RpaB can bind to the 'sub2' fragment with the mutated proximal half site is consistent with the report by Hanaoka et al. (2012). They observed that RpaB binds to the HLR1-like sequence containing only one half site in the case of the circadian clock-regulated kaiBC promoter in S. elongatus PCC 7942. Although these results suggest that one half site is sufficient for RpaB binding, our previous reporter assay examining the effect of base substitution within HLR1 revealed that RpaB requires both half sites to activate the psaE promoter under LL (Seino et al. 2009). Further studies are needed to determine the minimal sequence requirements for binding and promoter activation by RpaB.

RNA gel blot analysis (Fig. 3) together with ChAP analysis (Fig. 4) suggested that RpaB binds to the psrR1 promoter to act as a repressor under LL conditions, and its release from the promoter results in induction of the sRNA PsrR1 within 5 min after the shift to HL. This is consistent with the results of chromatin immunoprecipitation (ChIP) analysis in S. elongatus PCC 7942 examining the binding activity of RpaB to the upstream region of HL-inducible genes, hliA and rpoD3, upon a shift to HL conditions (Hanaoka and Tanaka 2008). RpaB dissociated already after 1 min of HL exposure from target promoters in S. elongatus PCC 7942. Our ChAP analysis also suggested that co-ordinated down-regulation of PSI genes under HL is regulated by the light-dependent change in binding activity of RpaB: binding of the activator RpaB under LL and its release upon the shift to HL. It has been proposed that the effect of RpaB binding to target promoters is determined by the location of the HLR1 sequence. In the case of PSI genes, binding of RpaB to the HLR1 sequence located upstream of the core promoter region may facilitate binding of RNA polymerase and increase the rate of transcription initiation. On the other hand, HL-inducible genes possess the HLR1 sequence in the 5’-UTR like rpoD3 in S. elongatus PCC 7942 (Seki et al. 2007) or in the core promoter regions like hliB (Kappel and van Waasbergen 2007) and psrR1 in S.6803. Binding of RpaB to the HLR1 sequence of psrR1 may prevent the interaction between RNA polymerase and the –10 element, leading to the repression of promoter activity under LL.

Based on the results obtained in this study, we suggest that RpaB and PsrR1 constitute a dual repression system of PSI gene expression under HL at both the transcriptional and post-transcriptional level (Fig. 5). RpaB activates the expression of PSI genes under LL, whereas the regulation is lost under HL, leading to the decline of transcription activity of PSI genes. In addition to this transcriptional effect, there is post-transcriptional regulation through PsrR1 under HL. Transcription of psrR1 is repressed by RpaB under LL, but release of RpaB from the promoter upon the shift to HL results in de-repression of psrR1. Transcribed PsrR1 interacts with the ribosome-binding site of PSI transcripts to inhibit translation (Georg et al. 2014). Although transcription activity of PSI genes is down-regulated within 5 min after the shift to HL, it takes >30 min for complete degradation of the pre-existing PSI transcripts (Fig. 3). PsrR1 is likely to be highly induced within 5 min and prevents translation from these transcripts under HL. Furthermore, it was shown that binding of PsrR1 to the psaL mRNA induces cleavage of the transcript by RNome E (Georg et al. 2014), thereby enhancing RNA degradation upon HL stress. This dual repression at the transcriptional and post-transcriptional level, rapid and strict down-regulation of PSI genes under HL is attained.

Recent discoveries concerning how sRNAs participate in global regulatory networks in bacteria revealed the existence of several types of regulatory circuits, such as single-input modules, dense overlapping regulons, positive and negative feedback loops and feed-forward loops (Beisel and Storz 2010). Dual repression of PSI genes achieved by RpaB and PsrR1 can be categorized into the ‘type-4 coherent feed-forward loop’ (Mangan and Alon, 2003), where regulator ‘A’ activates target ‘C’ and represses regulator ‘B’, and regulator ‘B’ represses target ‘C’ (Fig. 5). Pairs of transcription factor ‘A’ and sRNA ‘B’ making up the type-4 feed-forward loop have been reported in E. coli, e.g. involving the transcription factor CRP and the sRNA Spot42 (Beisel and Storz 2011) or the response regulator OmpR and the sRNA MicC (Chen et al. 2004). They were proposed to decrease leaky expression of target ‘C’ under steady-state repressing conditions to allow cells to adapt rapidly to the new environment. Based on the concept of the feed-forward loop, we can speculate about the benefits of dual repression of PSI genes by RpaB and PsrR1 upon the shift to HL. By inhibiting translation and fast degradation of the remaining mRNAs, cells can divert metabolic resources and energy toward HL acclimation responses and therefore can speed up the HL response. After 30 min of incubation under HL, we observed the recovery of binding activity of RpaB (Fig. 4B–D). Resumption of regulation by RpaB may be required for some target genes at this stage of HL acclimation, but expression of PSI genes should still be kept at a low level. Then, highly accumulating PsrR1 molecules, together with RNase E, function in the inactivation and degradation of PSI transcripts. It is notable that binding activity of RpaB remained at a low level after 30 min in S. elongatus PCC 7942 (Hanaoka and Tanaka 2008) which has no PsrR1 homologs.
Table 1 Oligonucleotides used in this study

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<th>Name</th>
<th>Sequence (5’ to 3’)</th>
<th>Purpose*</th>
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<tr>
<td>pET21-NdeI-rpaB-F</td>
<td>AAGGAGATATACATATGGAGAACAAATAAAGAAAG</td>
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<tr>
<td>pET21-XhoI-rpaB-R</td>
<td>GGTGGTGGTGCTCGAGCGGTTCTTCCCCC</td>
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<tr>
<td>rpaB-TTG-F</td>
<td>CATATGTTGAAGAACAATAAAAGAGAG</td>
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<td>rpaB-TTG21-R</td>
<td>TGGTTTCCAACATATGTATAGTGATGATGATG</td>
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<td>GTTAACCTCACATTGGATTC</td>
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<td>RT-psrR1-R</td>
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*These oligonucleotides were used for C, cloning; S, segregation check; G, gel mobility shift assay; R, RNA gel blot analysis; Q, quantitative real-time PCR analysis.

(Continued)

Table 1 Continued

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<td>RT-glnB-R</td>
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Bacterial strains and growth conditions

Escherichia coli XL1-blue cells were used for routine cloning, and BL21 pLyS (DE3) cells (Novagen) for protein expression. Cultures of E. coli were grown in TB at 37°C. Glucose-tolerant WT and mutant strains of S6803 were grown at 32°C in BG-11 medium containing 20 mM HEPES-NaOH, pH 7.0, under continuous illumination at 20 μmol photons m⁻² s⁻¹ with bubbling of air. Cell density was estimated by measuring optical density at 730 nm (OD 730) using a spectrophotometer (model UV-160A, Shimadzu). When necessary, antibiotics were added at the following concentrations: ampicillin, 100 μg ml⁻¹; kanamycin, 20 μg ml⁻¹; and chloramphenicol, 25 μg ml⁻¹.

Generation of the E. coli strain expressing C-terminal 6 × His-tagged RpaB

pET 21a vector was linearized by digestion with NdeI and Xhol. The coding region of the rpaB gene (from nucleotide 2 012 754 to 2 013 485 according to the numbering in Cyanobase) was amplified by PCR using the primer pair, pET21-NdeI-rpaB-F and pET21-XhoI-rpaB-R (Table 1), whose 5’ sequence is complementary to the 3’ ends of the linearized pET21a vector. The linearized vector and PCR product were fused using the In-Fusion HD Cloning Kit (Clontech) to generate the pET21a-RpaB construct. A TTG start codon was additionally introduced just upstream of the ATG start codon of the rpaB coding sequence using the PrimeSTAR Mutagenesis Basal Kit (TAKARA BIO INC.) and the primer pair, rpaB-TTG-F and rpaB-TTG21-R (Table 1), according to the reported successful production of recombinant RpaB protein (Kato et al. 2011). BL21 pLyS (DE3) competent cells (Novagen) were transformed with the pET21a-RpaB construct using the heat shock method to obtain the His-RpaB strain.

Purification of His-RpaB from E. coli cells

His-tagged RpaB protein was purified from the His-RpaB strain. The pre-culture was seeded into 1 liter of 2 × YT medium. Isopropyl-β-d-thiogalactopyranoside (IPTG) at a concentration of 100 μM was added to the mid-log culture to induce the expression of His-RpaB. After cultivating for 3 h at 37°C, cells...
were harvested and resuspended with 20 ml of the purification buffer (50 mM Hepes-KOH, pH 7.5, 150 mM NaCl, 10 mM MgCl2) with 20 mM imidazole. All protein purification procedures were performed at 4°C. Resuspended cells were disrupted by sonication on ice and then centrifuged at 16,000 × g for 20 min. The resulting supernatant was loaded onto a HiTrap chelating HP column (GE Healthcare) pre-equilibrated with the purification buffer, washed with the same buffer with 40 mM imidazole and eluted with the same buffer with 300 mM imidazole. Eluates containing purified His-RpaB were desalted by a HiTrap desalting column (GE Healthcare) equilibrated with purification buffer. After the addition of 20% (v/v) glycerol, the eluate was frozen in liquid N2 and stored at −80°C before use.

Protein concentration was determined using a Bio-Rad Protein Assay Kit (Bio-Rad) with bovine serum albumin as the standard. The purity of the proteins was assessed by fractionating an aliquot on an SDS–PAGE gel and staining with Coomassie Brilliant Blue (CBB).

**Gel mobility shift assay**

The psrR1 promoter fragment (−84 to +16, relative to the transcription start site, 100 bp) and the psaE promoter fragment (−97 to +23, relative to the transcription start site, 120 bp) were PCR amplified from genomic DNA using primer pairs PpsrR1-F and PpsrR1-R, and PpsaE-F and PpsaE-R, respectively (Table 1). PpsrR1sub1 and PpsrR1sub2 fragments were obtained using mutated reverse primers: PpsrR1sub1-R and PpsrR1sub2-R (Table 1), respectively. PpsaEsub1 and PpsaEsub2 fragments were obtained using mutated forward primers: PpsaEsub1-F and PpsaEsub2-F (Table 1), respectively. The 3′ end of the DNA fragment for each probe was labeled with digoxigenin (DIG)-ddUTP by the terminal transferase method according to the manufacturer’s instructions (DIG gel shift kit; Roche). Assays were performed by using a DIG gel shift kit as previously described (Seino et al. 2009).

**Generation of S.6803 strains expressing 12 x His-tagged RpaB**

For expression of C-terminal 12 × His-tagged RpaB from the neutral site of the genome, the pTKP2031-12 × His-RpaB construct was generated. Vector pTKP2031 contains a kanamycin resistance cassette and the psaA2 (slr1311) promoter flanked by a part of the coding regions of slr2030 and slr2031 as a platform for homologous recombination (Yamauchi et al. 2011). The psaA2 promoter was removed from pTKP2031 by linearization of the vector by inverse PCR using the primer pair Hpal-2031-F and Km-R (Table 1). The promoter and coding region of the rpaB gene (from nucleotide 2 012 627 to 2 013 485 according to the numbering in Cyanobase) was amplified by PCR using the primer pair Km-PpaB-F and slr2031-pab-R (Table 1), whose 5′ sequence is complementary to the 3′ ends of the linearized pTKP2031 vector. The linearized vector and PCR product were fused using the In-Fusion HD Cloning Kit (Clontech) to generate the pTKP2031-RpaB construct. The 12 × His-tag sequence was added just upstream of the stop codon of rpaB using the PrimeSTAR Mutagenesis Basal Kit (TAKARA BIO INC.) and the primer pair Hpal-2031-F and slr2031-12 × His-rpaB-R (Table 1) to generate the pTKP2031-12 × His-RpaB construct.

For disruption of the endogenous rpaB gene, the previously reported cosmid clone having an insertion of a chloramphenicol resistance cassette into the coding region of rpaB (Seino et al. 2009) was used. pTKP2031-12 × His-RpaB and the rpaB::Cm’ constructs were introduced into WT cells by natural transformation. Transformants were selected by the addition of kanamycin (20 μg ml−1) and chloramphenicol (25 μg ml−1).

**Immunoblot analysis**

Cell cultures (50 ml at OD730 = 0.5) were collected by centrifugation and resuspended with 200 μl of lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl). The cell suspension was mixed with 280 μl of glass beads (diameter 0.1 mm; AS ONE) in a 2 ml tube, disrupted with a Mini-Bead Beater (BioSpec) by three pulses of 10 s at 4°C, and the cell lysate was collected by centrifugation. Protein concentration was determined using a Bio-Rad Protein Assay Kit (Bio-Rad) with bovine serum albumin as the standard. Cell lysate was separated by 12% SDS–PAGE, blotted onto a polyvinylidenefluoride (PVDF) membrane (Immobilion-P; Millipore) and probed with the polyclonal antibody raised against His-RpaB recombinant protein or that against the His-tag (Bethyl). The bound antibodies were detected with goat anti-rabbit IgG secondary antibodies conjugated to horseradish peroxidase (Bio-Rad) using the chemiluminescence detection agent, ECL-WestLumi plus (Atto), as previously described (Kadowaki et al. 2015).

**RNA gel blot analysis**

Isolation of RNA by the hot-phenol method and RNA gel blot analyses using the DIG RNA labeling and detection kit (Roche) were performed as described previously (Muramatsu and Hihara 2003). To generate RNA probes by in vitro transcription, template DNA fragments for psrR1, hibB, psaA and psaL were amplified from the genomic DNA using the primers listed in Table 1. To use PCR products directly as templates for in vitro transcription, the T7 polymerase recognition site (TAATACGACTCACTATAGGGCGCA) was added to the reverse primers at their 5′ termini.

**Preparation of whole-cell extracts for ChAP analysis**

Preparation of whole-cell extracts for ChAP analysis was performed as described previously (Ishikawa et al. 2007, Hanaoka and Tanaka 2008) with some modifications. Cultures of S.6803 at OD730 = 0.2 incubated under LL or HL were treated with formaldehyde as a cross-linker to a final concentration of 1% at room temperature under room light conditions for 15 min with occasional shaking. To stop the fixation, glycine was added to a final concentration of 125 mM and cultures were incubated at room temperature for 5 min. Cells were then harvested by centrifugation at 8,000 r.p.m. for 3 min at 4°C, and washed twice with cold Tris-buffered saline (TBS; 20 mM Tris–HCl, pH 7.5, 500 mM NaCl). The resulting pellets were resuspended in 500 μl of UT buffer (50 mM Hepes-KOH, pH 7.5, 140 mM NaCl, 4 M urea, 1% Triton X-100, 5 mM imidazole, supplemented with Complete EDTA-free protease inhibitor cocktail (Roche)) at 4°C. After addition of 500 μg of zircon beads (diameter 0.1 mm; BioSpec), cells were broken by vigorous vortexing at 4°C for 45 min in a micro tube mixer (MT-360, TOMY). The cell lysate was sonicated for fragmentation of genomic DNA to an average size of 0.6–0.7 kb with a setting of 20% output, 90% duty cycle, 5 s sonication 15 times at 1 min intervals (Sonifer 450, Branson). After centrifugation twice at 14,000 × g for 15 min at 4°C to remove cell debris, the whole-cell extract was collected for affinity purification. Protein concentration was determined using a Bio-Rad Protein Assay Kit (Bio-Rad) with bovine serum albumin as the standard. These extracts were stored at −80°C until use.

**Affinity purification of His-RpaB and DNA purification**

A 1 mg aliquot of protein of the whole-cell extract was mixed with the UT buffer up to 500 μl and subsequently added to 100 μl of Ni2+ -Sepharose resin (COSMOGET His-Accept; Nacalai tesque) pre-equilibrated with UT buffer. After fractionation overnight at 4°C, the sample was centrifuged at 1,500 × g for 5 min and the pellet was resuspended in 1.5 ml of UT buffer. This washing step was repeated five times. Proteins were then eluted from the resin with 100 μl of the elution buffer (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 1% SDS, 500 mM imidazole) by incubation at 4°C for 20 min. The supernatant was collected and the elution step was repeated once. Then 8.3 μl of 5 M NaCl was added to the 200 μl of pooled eluate, and the mixture was incubated at 65°C for 5 h to revert cross-linking. To obtain input DNA required to calculate the original DNA quantity, 1/10th of the volume of whole-cell extract was subjected to reversion. After ethanol precipitation, washing with cold 70% ethanol and drying, the pellet was dissolved in 100 μl of TE and 100 μl of 2 × proteinase K buffer (20 mM Tris–HCl, pH 7.8, 10 mM EDTA, 1% SDS). Incubation at 50°C for 30 min was followed by extraction twice using phenol/chloroform/isoamylalcohol (25 : 24 : 1). Ethanol-precipitated DNA was finally dissolved in 100 μl of TE, and subjected to quantitative real-time PCR analysis.

**Quantitative real-time PCR analysis**

Quantitative real-time PCR analysis was performed using the Opticon 2 real-time qPCR monitoring system (MJ Research) according to its SYBR Green protocol. A 12 μl reaction mixture contains 5 pmol each of forward and reverse primers, 1 μl of purified DNA and 6 μl of Thunderbird SYBR qPCR Mix (TOYOBO). The primers used for amplification of the promoter region of...
each gene are listed in Table 1. PCR amplification was performed according to the manufacturer’s instructions. For quantification, standard curves were plotted using several dilutions of input DNA, and the percentage recovery for each purified DNA by ChAP relative to the input DNA was calculated as described previously (Hanaoka and Tanaka 2008). The percentage recovery is shown as the ratio of the quantity of purified DNA against that of input DNA contained in the same volume of whole-cell extract before affinity purification. As a negative control, the PCR without template DNA was performed.

### Supplementary data

Supplementary data are available at PCP online.

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### Disclosures

The authors have no conflicts of interest to declare.

### References


