Full Paper

Identification and analysis of a principal sigma factor interacting protein SinA, essential for growth at high temperatures in a cyanobacterium

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Introduction

Cyanobacteria are a diverged group of bacteria that perform oxygenic photosynthesis (Rozanov and Astafieva, 2009). They inhabit and survive in various environments (Schwarz and Grossman, 1998), which is made possible through sophisticated environmental stress-responsive mechanisms. One such mechanism, transcriptional regulation, mediated by sigma factors, plays a pivotal role (Imamura et al., 2003; Osanai et al., 2008).

Sigma factor is a subunit of RNAP that binds and determines promoter recognition specificity and the ability to initiate transcription in bacteria to the catalytic core RNAP complex (Gruber and Gross, 2003). Most bacteria encode multiple sigma factor species in their genome and differential use and the promoter recognition specificity of these sigma factors often explain differential gene expression from the genome depending on internal and external conditions (Gruber and Gross, 2003; Sharma and Chatterji, 2010). Sigma factors are categorized into several groups. The principle, or group 1, sigma factor is a unique and essential sigma factor in each bacterium and is responsible for the transcription of most housekeeping genes under physiological growth conditions. Group 2 sigma factors share a highly homologous structure with group 1 sigma factors but are not essential for cell viability. Other sigma factors are categorized as group 3 sigma factors, while group 4 sigma factors were proposed as extracytoplasmic function sigma factors (Helmann, 2002). Each alternative sigma factor is activated under various stressed conditions and is responsible for activating relevant stress responsive genes (Osanai et al., 2008).

Sigma factor binding proteins exist in various bacteria
and modulate sigma factor activity through their interactions (Guell et al., 2011; Hughes and Mathee, 1998; Paget, 2015). Well-known examples are anti-sigma factors that inactivate sigma factor functions; interaction between sigma and anti-sigma factors prevents sigma-core RNAP association and downstream gene expression (Hughes and Mathee, 1998; Paget, 2015). In this study, we identified a candidate for the principal sigma factor binding protein among cyanobacteria and confirmed the interaction in a cyanobacterium *Synechococcus elongatus* PCC 7942 (hereafter *S. elongatus*). Cyanobacteria harbor multiple alternative sigma factors, including several group 2 sigma factors, to help to respond to various environmental stresses (Osanai et al., 2008). A role of the newly-identified factor for the sigma factors substitution and the heat-stress responsive transcriptional regulation is discussed.

**Materials and Methods**

**Bacterial strains, culture conditions, and recombinant DNA techniques.** *S. elongatus* and mutants were grown in BG-11 (Rippka, 1988) liquid medium or on BG-11 plate containing 1.5% w/v agar at 30°C with 2% CO2 aeration under constitutive fluorescent light (30 µmol photons/m² s). BG-11 plates were supplemented with 40 µg/mL spectinomycin or 10 µg/mL kanamycin as required. *E. coli* DH5α was used for all cloning procedures using standard techniques. PCR reactions were performed using KOD-Plus-Neo (Toyobo Co., Ltd., Osaka, Japan). Oligonucleotide primers were commercially purchased from a supplier (Integrated DNA Technologies, Inc., Coralville, IA, USA). Detailed procedures for the construction of plasmids and strains are described in Supplemental Materials and Methods.

**Phylogenetic analysis.** The amino acid (aa) sequences of ten SinA homologs were identified based on Cyanobase (Nakamura et al., 1998) and GenBank (Benson et al., 2009) and analyzed using ClustalW (Thompson et al., 1994). The unrooted phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) with MEGA7 (Kumar et al., 2016) based on the multiple sequence alignments. The bootstrap procedure sampled 1,000 times with replacement with MEGA7 (Kumar et al., 2016) to estimate the reliability of the tree.

**Cell viability test.** The cell suspensions were serially diluted in 10-fold intervals with BG-11 medium, and a 7 µL aliquot from each dilution was spotted onto BG-11 plates. Plates were then incubated under illumination at a high temperature (40°C) for 7 days before being photographed.

**Temperature upshift experiment.** Cultures were grown to OD750 = 0.5 in BG-11 liquid medium and the temperature upshifts were performed by moving the cultures to a water bath (45°C or 48°C). Cells were collected using centrifugation (7,000 × g, 2 min, 4°C), at 0, 5, 15 and 60 min, respectively, after temperature upshifts.

**RNA analysis.** The total RNA was extracted from cells using the hot phenol method and 5 µg RNA was subjected to a Northern hybridization analysis (Seki et al., 2007). A DNA probe for hspA was digoxigenin-labeled using PCR amplification with the primers [hspA-Fw and hspA-Rv].

**Protein analysis.** Soluble protein was isolated from the cells as described previously (Hanaoka and Tanaka, 2008). For the pull-down assay, soluble protein mixed with binding buffer [50 mM HEPES-KOH (pH 7.5), 140 mM NaCl, 0.2% NP-40] was bound to anti-FLAG-IsG-conjugated beads (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) at 4°C for 2 h. The beads were washed three times with 500 µL of binding buffer and eluted with 100 µL FLAG peptide mixture [30 ng/µL FLAG peptide mixture with the binding buffer]. Immunoblot analysis was conducted as described previously (Seki et al., 2007) using an anti-FLAG antibody (FUJIFILM Wako Pure Chemical Corporation), an anti-His-tag antibody (Medical & Biological Laboratories Co., Ltd.) and an anti-RpoD1 rabbit antiserum (Seki et al., 2007).

**Results and Discussion**

**Identification and phylogenetic analysis of SinA**

Protein-protein interaction is a basis of biological function in a cell, and comprehensive analyses of such interactions have been performed in various model organisms (Arifuzzaman et al., 2006; Ito et al., 2001; Uetz et al., 2000). A large-scale yeast two-hybrid screening analysis was also performed in the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) and the results have been used to identify and understand various molecular mechanisms (Sato et al., 2007). We have been interested in transcriptional regulation in cyanobacteria and especially focused on the responses to environmental changes involved in RNAP sigma factors (Imamura et al., 2003; Osanai et al., 2008). By examining the results for *Synechocystis*, we noticed that the principal sigma factor SigA was repeatedly identified from the yeast two-hybrid analysis using a hypothetical protein Smi0011 as a bait for the screening (Fig. 1). Because repetitive identification strongly indicated the interaction, we decided to further analyze the identified protein function in cyanobacteria. It was suggested that SinA interacts with the regions 2 and 3 of SigA. Because these regions are responsible for the promoter recognition and the interaction with core RNAP (Paget, 2015), we hypothesized that
Fig. 2. Structure and phylogenetic analysis of SinA.

A. Alignment of SinA homologs from represented cyanobacteria. The aa sequences of 10 SinA homologs were identified based on Cyanobase (Nakamura et al., 1998) and GenBank (Benson et al., 2009) and analyzed using ClustalW (Thompson et al., 1994). Synpcc7942_0452 was from Synechococcus sp. TETX 2973, Asl3981 was from Nostoc 7120, Tery_3913 was from Trichodesmium erythraeum IMS101, Sml0011 was from Synechocystis, SinA in SYNPCC7002 was from Synechococcus sp. WH7002, SYNPCC7002 was from Synechococcus sp. WH7002, P9301_02911 was from Prochlorococcus marinus strain MIT9301, and Gll0064 was from Gloeobacter violaceus PCC7421. SinA harbors a highly conserved domain as indicated, DUF3155, whose function was thus far unidentified. The N-terminal positions of Synpcc7942_0452, Gll0064 and Asl3981 annotated in Cyanobase (Nakamura et al., 1998) were revised and the originally annotated N-terminal positions were not used for the alignment. The extended N-terminal part of Asl3981 is indicated by shading. Perfectly conserved or similar aa positions are shown by “*” or “:”, respectively, below the alignment. Conserved basic or acidic residues are shown by black or white circles, respectively. The calculated molecular weight (kDa) and the aa identity (%) with Synpcc7942_0452 in DUF3155 are indicated in the figure.

B. Phylogenetic analysis of SinA. Based on the multiple sequence alignments (A), the unrooted phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) with MEGA7 (Kumar et al., 2016). A total of 104 positions were considered; the bar indicates the distances corresponding to 50 changes per 1,000 aa positions. The bootstrap procedure sampled 1,000 times with replacement by MEGA7 (Kumar et al., 2016) to estimate the reliability of the tree; and the number at each node indicates the percentage of trees supporting the same branching pattern in the bootstrap analysis.

SinA may modulate the RNAP activity through the principal sigma factor function.

Presently, 376 cyanobacterial genome sequences have been completely determined and are available in Cyanobase (Nakamura et al., 1998) and GenBank (Benson et al., 2009) and analyzed using ClustalW (Thompson et al., 1994). Synpcc7942_0452 was from Synechococcus sp. TETX 2973, Asl3981 was from Nostoc 7120, Tery_3913 was from Trichodesmium erythraeum IMS101, Sml0011 was from Synechocystis, SinA in SYNPCC7002 was from Synechococcus sp. WH7002, SynWH7002_0291 was from Synechococcus sp. WH7003, P9301_02911 was from Prochlorococcus marinus strain MIT9301, and Gll0064 was from Gloeobacter violaceus PCC7421. SinA harbors a highly conserved domain as indicated, DUF3155, whose function was thus far unidentified. The N-terminal positions of Synpcc7942_0452, Gll0064 and Asl3981 annotated in Cyanobase (Nakamura et al., 1998) were revised and the originally annotated N-terminal positions were not used for the alignment. The extended N-terminal part of Asl3981 is indicated by shading. Perfectly conserved or similar aa positions are shown by “*” or “:”, respectively, below the alignment. Conserved basic or acidic residues are shown by black or white circles, respectively. The calculated molecular weight (kDa) and the aa identity (%) with Synpcc7942_0452 in DUF3155 are indicated in the figure. B. Phylogenetic analysis of SinA. Based on the multiple sequence alignments (A), the unrooted phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) with MEGA7 (Kumar et al., 2016). A total of 104 positions were considered; the bar indicates the distances corresponding to 50 changes per 1,000 aa positions. The bootstrap procedure sampled 1,000 times with replacement by MEGA7 (Kumar et al., 2016) to estimate the reliability of the tree; and the number at each node indicates the percentage of trees supporting the same branching pattern in the bootstrap analysis.
cyanobacterial protein that interacts with the principal sigma factor, and we named the gene/protein sinA/SinA after the principal sigma factor interacting protein A. Interestingly, almost all sinA genes are found next to the conserved sensory histidine kinase gene hik2 in a head-to-head manner (Fig. S1), which may indicate the functional relationship.

SinA aa sequences from several cyanobacterial strains were aligned and their characteristics were compared (Fig. 2A). A conserved Pfam domain (Finn et al., 2006) of unknown function, DUF3155, was the main part of these proteins. SinA of S. elongatus (Synpcc7942_0452) and Gloeobacter violaceus PCC 7421 (Gll0064) were annotated in Cyanobase (Nakamura et al., 1998), however, the alignment analysis revealed the N-terminal 36 aa and 39 aa regions of these SinA proteins, respectively, do not show any similarity to each other, as well as with other homologs. Therefore, we re-examined the nucleotide sequences in detail. GTG at the annotated 37th codon, and TTG at the annotated 40th codon in Synpcc7942_0452 and Gll0064, respectively, are more likely to be the actual initiation codons based on the 5′-preceding ribosome binding site sequence (Figs. S2A and B). Similarly, the annotated SinA from Nostoc sp. PCC 7120 (Asl3981) lacks the amino-terminal conserved portion found in other SinA homologs, but detailed nucleotide sequence analysis identified a more probable initiation codon TTG in the 5′-upstream (Fig. S2C). Thus, the revised aa sequences were used for the alignment (Fig. 2A, grey shaded part). In the case of Synechococcus PCC 7002, SinA homologous sequence was not annotated in databases. However, a highly homologous SinA open reading frame (Fig. 2A) initiated from a TTG codon (Fig. S2D) was found by a detailed analysis of the nucleotide sequence around the conserved hik2 (SYNPCC7002_A1954) gene. GTG and TTG sequences can function as initiation codons as well as ATG in cyanobacteria (Sazuka et al., 2003), which is consistent with these ORF predictions. A constructed phylogenetic tree is consistent with those constructed based on the 16S rRNA sequence (Honda et al., 1999) (Fig. 2B), which indicates that the sinA gene has been conserved throughout the cyanobacterial evolution.

**Interaction of SinA with the principal sigma factor RpoD1 in S. elongatus**

SinA was predicted as a principal sigma factor interacting protein in Synechocystis. To demonstrate the conserved interaction among cyanobacteria, we performed pull-down analysis using S. elongatus cell lysate. The chromosomal sinA gene was first modified to express an epitope 3×FLAG-tag fused SinA protein at the C-terminus. Using this strain, named SF, the SinA-FLAG protein was pulled down with anti-FLAG-IgG-conjugated beads from the cell lysate and the eluate was analyzed using immunoblot analysis with the anti-RpoD1 (the principal sigma factor of S. elongatus) antiserum (Seki et al., 2007) and an anti-FLAG antibody (Fig. 3). The results showed the presence of RpoD1 in the eluate, which indicates the specific SinA-RpoD1 interaction in vivo. In addition, a unique ~20-kDa signal was detected, indicating the expression of SinA-FLAG in S. elongatus, the size of which is consistent with the revised translational initiation site (the calculated protein size is 17 kDa as the FLAG-fused protein).

**SinA mutation results in growth defects under high temperature (40°C)**

We next constructed a chromosomal sinA-deletion strain (DS) in S. elongatus and found that the mutant grew a little slower than the parental strain (WT) even under usual growth conditions (30°C) (Fig. 4A; left). We then checked the growth under various stressed conditions and found that the mutant showed few significant growth defects (data not shown). However, the mutant showed apparent growth defects under higher temperatures (40°C) in which the WT grew well (Fig. 4A; right). These growth defects were not observed for a complemented strain (DSC), which indicates that the phenotype results from the sinA deficiency. Thus, sinA is essential for the high-temperature growth under the examined conditions.

**SinA was required for the second-step heat induction of the hspA transcription**

The growth defects under a higher temperature suggested that SinA is required for heat-inducible gene expression. To examine this, expression of a typical heat responsive gene, hspA, in S. elongatus was monitored by a Northern hybridization analysis after a temperature upshift (from 30°C to 48°C). As shown in Figs. 4B and C, induction of the hspA transcript at 5 min was similar irrespective of the sinA allele; however, further increases of the transcript after 15 min were significantly alleviated or abolished by the sinA defect. In a previous study, we proposed a two-step model for heat-inducible transcriptional activation, where the second-step activation depends on alternative sigma factor expression and the substitution of the principal sigma factor in the RNAP holoenzyme (Kobayashi et al., 2017). Providing that the second-step activation of hspA occurs dependent on SinA, it was suggested that the second-step activation is dependent on alternative sigma factor(s) and that the substitution of the principal sigma factor RpoD1 was deficient in the sinA mutant. In a previ-
ous study, we also found that a group 2 sigma factor RpoD2 is induced by a temperature upshift and may be involved in the second-step activation (Kobayashi et al., 2017). Thus, we examined the hspA expression after a temperature upshift (from 30°C to 48°C) in the rpoD2 mutant (D2SP). However, the second-step activation was not affected by the rpoD2 mutation (Figs. 4B and C). This result suggests the involvement of some other alternative sigma factor(s) for the second-step activation.

The SinA and RpoD1 complex was dissociated from RNAP after temperature upshift

While the interaction between SinA and RpoD1 was demonstrated in Fig. 3, it remained unclear whether this interaction and the association of these proteins with RNAP remain unchanged after the temperature upshift. To analyze this point, we introduced a hexa-histidine tag (6 × His) to the C-terminus of the RNAP RpoC2 subunit of the SF strain for the immunodetection and performed a pull-down experiment using the FLAG-tagged SinA protein. At first, immunoblot analysis using an anti-FLAG antibody revealed that the amount of the FLAG-tagged SinA remained almost constant before and after the temperature upshift (Fig. 5A). After the pull-down experiment, the FLAG-tagged SinA was co-precipitated with RNAP core enzyme and RpoD1, as revealed by immunoblot analyses using the specific antibodies for each, while it was found that co-precipitated RNAP core enzyme decreased after the temperature upshift (Figs. 5B and C). It should be also noted that RpoD1 was similarly co-precipitated before and after the temperature upshift, which suggests that from the RNAP-RpoD1-SinA complex, RpoD1 and SinA are dissociated from RNAP as a stable complex after the temperature upshift. Meanwhile, the bands of RpoC2-6 × His and RpoD1 were observed in all flow-through lanes. This indicates that a major fraction of RpoD1 was not interacting with SinA before and after the temperature upshift. Further study is required to clarify the functional assignment of these RpoD1 fractions for the heat shock response.

Role of SinA for the heat sensing and acclimation in cyanobacteria

Based on the present study, a tentative scheme for the heat-inducible transcriptional activation is presented in Fig. 6. Temperature upshift is sensed by the sensory kinases Hik2 and Hik34 to increase phosphorylated Rre1, which activates the first step transcription of target genes including hspA (Kobayashi et al., 2017). This response is presumably dependent on RNAP holoenzyme containing RpoD1. SinA makes a complex with RNAP and RpoD1 and, while the upstream sensing mechanism is unclear, SinA-RpoD1 binary complex is dissociated from RNAP after the temperature upshift. This facilitates the RNAP holoenzyme formation with alternative sigma factor(s), and the target gene transcription is activated as the second step response.

Providing that SinA supports the sigma factor substitution, the function of SinA is similar to known anti-sigma factors (Hughes and Mathie, 1998). However, the SinA-RpoD1 interaction remained constant before and after the temperature upshift and the underlying mechanism may differ from those in other anti-sigma factors. Protein interaction analysis has indicated that SinA binds to the conserved regions 2 and 3 of the principal sigma factors (Fig. 1), where interaction with core RNAP and promoter DNA was suggested (Helmann and Chamberlin, 1988). Thus,
we suggest that the RNAP-RpoD1-SinA complex may not be directly engaged in initiating transcription, but functions as a reservoir for the sigma factor substitution in response to stressful conditions. This speculation is well consistent with the observation that only a small fraction of RpoD1, as well as RNAP, was co-precipitated with the SinA-FLAG protein (Fig. 5B). In this line, a slower growth of the sinA mutant under the usual growth condition (Fig. 4A) may result from the inability of the activation of alternative sigma factor(s)-dependent transcription required for the optimal growth.

It is interesting to note that the sinA gene is located next to the heat-sensing histidine kinase hik2 gene, and elucidation of the hypothetical interaction may reveal an unexpectedly intricate heat-sensing mechanism in cyanobacteria. Physiologically, the sinA function is required for the growth under the high temperature (40°C). This may result from the deficient second step transcriptional activation, but these short-term responses cannot be directly relevant to longer term phenotypes as the colony formation occurs in a scale of days. It is apparent that future study should connect these gaps and clarify the overall heat acclimation processes in cyanobacteria.

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Supplementary Materials

Supplementary figures and table are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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