In bacterial DNA replication, the initiator protein DnaA binds to the multiple DnaA box sequences located at oriC to facilitate the unwinding of duplex DNA strands. The cyanobacterium Synechococcus elongatus PCC 7942, which contains multiple chromosomal copies per cell, has DnaA box-like sequences around the oriC region, which is located upstream of dnaN. We previously observed the binding of DnaA around the oriC region; however, the DNA-binding specificity of DnaA to DnaA box sequences has not been examined. Here, we analyzed the binding specificity of DnaA protein to the DnaA box in S. elongatus by using bio-layer interferometry (BLI), a method for monitoring intermolecular interactions. We observed that recombinant DnaA protein recognized specifically the DnaA box sequence TTTTCCACA in vitro. In addition, DNA binding activity was significantly increased by R328H mutation of DnaA. This is the first report to characterize DnaA binding to the DnaA box sequence in cyanobacteria.

Key Words: BLI; cyanobacteria; DnaA; DnaA box

Introduction

Initiation of bacterial DNA replication is regulated by the DnaA initiator protein. DnaA binds to the multiple DnaA box sequences located at oriC to facilitate the unwinding of duplex strands (Katayama et al., 2010); consequently, replisome complexes are recruited to the unwound oriC. In Escherichia coli, the DnaA box has been identified as a non-palindromic 9-bp sequence (TTWTNCACA) (Schaper and Messer, 1995; Weigel et al., 1997). The sequence of the DnaA box is highly conserved among bacteria, although several variations have been noted (Luo and Gao, 2019). DnaA binds both ATP and ADP (hereafter shown as ATP- and ADP-DnaA), but only ATP-DnaA is capable of forming an oligomeric structure at the oriC region. In E. coli, the level of DnaA is constant throughout the cell cycle progression, whereas the ratio of ATP-DnaA to the total DnaA pool peaks just before chromosome replication during the cell cycle (Kurokawa et al., 1999).

Cyanobacteria are prokaryotic microorganisms; they rely on an oxygen-producing photosynthetic system similar to that in plant chloroplasts. The freshwater cyanobacterium Synechococcus elongatus PCC 7942 (hereafter referred to as S. elongatus) carries two to eight chromosomal copies per cell, which are equally distributed along the major axis of the cell (Griese et al., 2011; Watanabe et al., 2015, 2018). We previously identified the oriC region in S. elongatus, which is located upstream of dnaN (Watanabe et al., 2012) and includes putative multiple DnaA box-like sequences (consensus sequence: TTTTCCACA, Fig. 1) (Liu and Tsinoremas, 1996; Luo and Gao, 2019). We also showed that the regulation of DNA replication initiation is similar to the bacterial mechanism: S. elongatus DnaA binds to the oriC region (Ohbayashi et al., 2015), and DnaA regulates the number of replicating chromosome copies during the growth of the organism (Ohbayashi et al., 2019). The R328H mutation of DnaA (DnaAR328H), which inhibits ATPase activity, increases the binding activity to the
Predicted DnaA boxes (consensus: TTTTCCACA) are shown in bold.

DnaA box cluster region in *S. elongatus* (genomic region, 2695653–2695903) is highlighted in gray. The region used for the BLI assay (126 bp) is shown in italics.

**Materials and Methods**

**Plasmid construction.** Because *S. elongatus* DnaA protein (ORF ID: Synpc7942_1100, 52.0 kDa) is easily aggregated, 6×His-tagged trigger factor (TF, 53.2 kDa) was fused to the N terminus of *S. elongatus* DnaA<sup>WT</sup> and DnaA<sup>R328H</sup> DNA fragments amplified by PCR using primer sets DnaA-IF-F and DnaA-IF-R (Table 1) were cloned into pColdTF vector (TaKaRa, Shiga, Japan). For the introduction of the R328H mutation, site-directed mutagenesis was carried out with the KOD mutagenesis kit (TOYOBO, Osaka, Japan) using primer set R328H-F and R328H-R (Table 1) and pColdTF-syfDnaA plasmid as a PCR-template. After verification of the sequences of both dnaA genes in resulting plasmids pColdTF-syfDnaA<sup>WT</sup> and pColdTF-syfDnaA<sup>R328H</sup>, the two plasmids were independently introduced into *E. coli* Rosetta competent cells and used for the purification of TF-DnaA fusion proteins as described (Watanabe et al., 2007).

**Purification of the TF-DnaA proteins.** *E. coli* Rosetta harboring pColdTF-syfDnaA<sup>WT</sup> or pColdTF-syfDnaA<sup>R328H</sup> were grown in 100 ml of LB medium at 30°C. When the culture reached an OD<sub>600</sub> of 0.5, IPTG was added to the medium at a final concentration of 0.1 mM and the growing temperature was shifted to 15°C. After 24 h, cells were harvested by centrifugation, washed with a purification buffer (20 mM Tris-HCl [pH 8.0 at 4°C] and 250 mM NaCl), and stored at −80°C until use. For protein purification, frozen cells were suspended in 5 ml of purification buffer containing 100 mM PMSF. The cells were disrupted by sonication and centrifugated at 15,000 × g for 30 min at 4°C, the resulting supernatant was mixed with 1 ml of Ni<sup>2+</sup>-NTA agarose resin (QIAGEN) equilibrated with purification buffer and loaded onto a column. After washing by 10 ml of washing buffer 1 [20 mM Tris-HCl (pH 8.0), 250 mM NaCl, and 5 mM imidazole] and 50 ml of washing buffer 2 [20 mM Tris-HCl (pH 8.0 at 4°C), 250 mM NaCl, and 20 mM imidazole], proteins were then eluted with 10 ml of an elution buffer [20 mM Tris-HCl (pH 8.0 at 4°C), 250 mM NaCl, and 200 mM imidazole], and dialyzed against the dialysis buffer [20 mM HEPES (pH 7.5), 5 mM MgSO<sub>4</sub>, 1 mM EDTA, 0.001% (w/v) BSA, 0.05% Tween 20, 150 mM NaCl, and 30% Glycerol]. 15 µg of purified TF-DnaA fusion proteins (TF-DnaA<sup>WT</sup> and TF-DnaA<sup>R328H</sup>, 107.2 kDa) were subjected to SDS-PAGE (Fig. 2A).

**BLI analysis.** The interactions between DnaA protein and the DnaA box were examined using the BLItz system from Pall ForteBio LLC (Fremont, CA, USA). The binding buffer (20 mM HEPES [pH 7.5], 5 mM MgSO<sub>4</sub>, 1 mM EDTA, 0.0001% [w/v] BSA, 0.05% Tween 20, 150 mM NaCl, 1 mM DTT and 100 µM ATP) was prepared as described (Nozaki et al., 2009) with minor modification and used for the dilution of ligand DNA (biotinylated ssDNA and complementary ssDNA) and analyte proteins (TF-DnaA<sup>WT</sup> and DnaA<sup>R328H</sup> and BSA). All experiments were carried out at room temperature. The ligand DNAs (0.1 µM of biotinylated ssDNA and complementary ssDNA; Table 1), obtained from Eurofins Genomics (Ebersberg, Germany), were captured on a streptavidin biosensor that had been equilibrated with binding buffer. The analyte proteins (0.1 µM TF-DnaA<sup>WT</sup>, TF-DnaA<sup>R328H</sup> and BSA) were bound to the ligand DNA-reacted biosensor, and the association was then analyzed. The binding buffer was also used for the dissociation step.

**Results and Discussion**

To study the interaction between DnaA protein and the DnaA box in *S. elongatus*, we obtained soluble DnaA protein (Fig. 2A). TF-tagged DnaA proteins (TF-DnaA) were used for the following assay, because *S. elongatus* DnaA aggregated when the TF-tag was removed by digestion with protease (data not shown). For the BLI assay, we used a 126-bp region upstream of dnaN, which contains five putative DnaA boxes (consensus sequence: TTTTCCACA). Baseline was determined by using the binding buffer after each step. In the steps for loading ligand DNA, the 5′-biotinylated ssDNA (126 bp-D5-Biotin, Table 1) was first allowed to bind to a streptavidin biosensor, and then complementary ssDNA (126 bp-D5-Complement, Table 1) was added. The binding signal increased with each addition (Fig. 2B), indicating the formation of dsDNA on the biosensor. A clear increase in the binding signal during the association step was observed when 0.1 µM TF-DnaA<sup>WT</sup> was used as an analyte (Fig. 2B), indicating the formation of a complex consisting of the 126-bp ligand DNA and TF-DnaA<sup>WT</sup>. No such increase was detected when BSA was used, and thus we used BSA.
signal should go down (Sultana and Lee, 2015). However, in the case of TF-DnaA WT (Fig. 2B), a decrease in the signal was not observed, indicating the irreversible binding on the biosensor between TF-DnaA WT and the DnaA box.

### Table 1. Oligonucleotides used in this study.

| Primer         | Sequence (5′ to 3′)* |
|----------------|----------------------|
| Cloning of pColdIF-DnaA | TACCCCTGAGGGAGTCCATGGCAGCCGGCAAAAAG |
| Dnaa-IF-F      | GCTTGAATTGGATGCCGCTAGCTAC |
| Dnaa-IF-R      | CATGAGCTCGAGGGCGCTTGAC |
| Dnaa-IF-R      | GATGTTGGAGGTGACGGCAGTGC |

**Introduction of the R328H mutation**

- R328H-F: CATGAGCTCGAGGGCGCTTGAC
- R328H-R: GATGTTGGAGGTGACGGCAGTGC

**BLI analysis**

- 126bp-DB5-Biotin: CCCCCCTTGATCCCCCCTGTGGAAAACCCTCTTAGTTTTTCCACAGCTTTTCCACA
- 126bp-DB5-Complement: TTTTCCACAGCTTTTCCACA
- 60bp-DB3-Biotin: CCCCCCTTGATCCCCCCTGTGGAAAACCCTCTTAGTTTTTCCACAGCTTTTCCACA
- 60bp-DB3-Complement: TTTTCCACAGCTTTTCCACA
- 30bp-DB2-Biotin: CCCCCCTTGATCCCCCCTGTGGAAAACCCTCTTAGTTTTTCCACAGCTTTTCCACA
- 30bp-DB2-Complement: TTTTCCACAGCTTTTCCACA
- 20bp-DB1-Biotin: CCCCCCTTGATCCCCCCTGTGGAAAACCCTCTTAGTTTTTCCACAGCTTTTCCACA
- 20bp-DB1-Complement: TTTTCCACAGCTTTTCCACA
- 20bp-DBM1-Biotin: CCCCCCTTGATCCCCCCTGTGGAAAACCCTCTTAGTTTTTCCACAGCTTTTCCACA
- 20bp-DBM1-Complement: TTTTCCACAGCTTTTCCACA
- 20bp-DBM2-Biotin: CCCCCCTTGATCCCCCCTGTGGAAAACCCTCTTAGTTTTTCCACAGCTTTTCCACA
- 20bp-DBM2-Complement: TTTTCCACAGCTTTTCCACA
- 20bp-DBM3-Biotin: CCCCCCTTGATCCCCCCTGTGGAAAACCCTCTTAGTTTTTCCACAGCTTTTCCACA
- 20bp-DBM3-Complement: TTTTCCACAGCTTTTCCACA
- 20bp-DBM4-Biotin: CCCCCCTTGATCCCCCCTGTGGAAAACCCTCTTAGTTTTTCCACAGCTTTTCCACA
- 20bp-DBM4-Complement: TTTTCCACAGCTTTTCCACA

*DnaA box-like sequences (consensus: TTTTCCACA) are underlined.

**Fig. 2.** BLI assay using BLItz. A. SDS-polyacrylamide gel electrophoresis of purified TF-DnaA WT and -DnaA R328H. B. Summary of BLI assay. We used 0.1 µM of biotinylated ssDNA (126 bp) and 0.1 µM TF-DnaA WT (bold line) or BSA (broken line, control) for the BLI assay. The timing of the steps is as follows: initial baseline, 0–30 sec; loading of biotinylated ssDNA, 31–150 sec; baseline, 151–180 sec; loading of complementary ssDNA, 181–300 sec; baseline, 301–600 sec; association with TF-DnaA or BSA, 601–720 sec; dissociation in buffer, 721–800 sec.

As the control in this assay, for the dissociation step, the binding buffer was added to the sensor in which the complex of ligand DNA and TF-DnaA WT had formed. When the analyte protein dissociates from the ligand DNA, the signal should go down (Sultana and Lee, 2015). However, in the case of TF-DnaA WT (Fig. 2B), a decrease in the signal was not observed, indicating the irreversible binding on the biosensor between TF-DnaA WT and the DnaA box.
Therefore, we focused on the only step of the association in following assay.

To simplify the analysis of the interaction between TF-DnaA and the DnaA box, a 20-bp DNA ligand that contained only one DnaA box was used for a second BLI assay. This shorter ligand was sufficient for detecting binding of TF-DnaA\textsuperscript{WT} (Fig. 3, DB1), although the binding signal increased depending on the number of DnaA boxes included. We also assayed using a 20-bp ligand DNA in which the DnaA box consensus sequence had been replaced (DB-, TTTTCCACA to CCCCTTGTG) as a negative control, and disappearance of the binding signal was confirmed with this ligand DNA (Fig. 3).

Several differences among DnaA box sequences around the \textit{oriC} region in \textit{S. elongatus} have been noted (Fig. 1) (Liu and Tsinoremas, 1996; Luo and Gao, 2019). To study the sequence specificity of the DnaA box, mutated DNA ligands that correspond to these sequence differences were assayed. As in \textit{E. coli}, the adenines at positions seven and nine of the \textit{S. elongatus} DnaA box sequences were consistently present, and thus we prepared ligand DNAs in which the adenines at positions seven and nine were changed to guanine and cytosine, respectively (M1, TTTTCCACC; M2, TTTTCCGCA). As expected, the binding signals disappeared when either of these mutant sequences was used (Fig. 4). In addition, we analyzed the effects of mutating the thymines at the 5′-end of the DnaA box sequence, because these changes are present in DnaA box sequences around the \textit{oriC} region in \textit{S. elongatus} (M3, TTCTCCACA, located at 2695747-2695755; M4, GCTTCCACA, 2695191-2695199). Both mutations also strongly inhibited the signal of DnaA binding (Fig. 4), suggesting that TF-DnaA\textsuperscript{WT} strictly recognizes the DnaA sequence TTTTCCACA in \textit{S. elongatus}.

Fig. 3. BLI assays using different lengths of ligand DNAs.

The lengths of the 0.1 µM biotinylated ssDNA and complementary ssDNA are indicated. TF-DnaA\textsuperscript{WT} (0.1 µM) or BSA was used for the assay. The background BSA values were subtracted from the TF-DnaA\textsuperscript{WT} binding during the association step, with the results shown here. The number of DnaA box-like sequences (i.e., one to five) in each ligand (Fig. 1) are indicated (DB1–5) in addition to the length of each ligand DNA. A ligand with replacement of the DB sequence (DB-, CCCCTTGTG) was used as a negative control. Although a reproducible result has been obtained among several tests, only a representative data set is shown.

There are two forms of DnaA in \textit{E. coli}, ADP-DnaA and ATP-DnaA. As compared with ADP-DnaA, ATP-DnaA shows strong DNA-binding activity by forming an oligomeric structure (Speck et al., 1999). In \textit{E. coli}, the amino acid substitution R334H (arginine to histidine at amino acid position 334) in DnaA inactivates this intrinsic ATPase activity, which leads to the accumulation of ATP-DnaA (Su’etsugu et al., 2001). In a previous study, we showed that the R328H mutation of DnaA (DnaA\textsuperscript{R328H}, which corresponds to R334H of \textit{E. coli} DnaA) increases DnaA activity with respect to \textit{oriC} affinity as compared with wild-type DnaA (DnaA\textsuperscript{WT}) in \textit{S. elongatus}, suggesting that DnaA\textsuperscript{R328H} is in fact an ATP-DnaA form (Ohbayashi et al., 2019). To examine the effect of the R328H mutation on binding to the DnaA box, we compared the binding activity between DnaA\textsuperscript{WT} and DnaA\textsuperscript{R328H}. In both cases, the BLI signal almost reached a plateau at the end of the association step and TF-DnaA\textsuperscript{R328H} showed an ~2.5-fold increase in the binding signal compared with that of DnaA\textsuperscript{WT} in the BLI analysis (Fig. 5). In the BLI assay, the thickness at the biosensor tip is detected as a signal (Sultana and Lee, 2015), thus the increased BLI signal by R328H mutation is due to the promotion of DNA-protein and/or protein-protein inter-

Fig. 4. The effects of point mutations in the DnaA box sequence.

Assays were set up as described in Fig. 2. Data are presented as described in Fig. 3. The consensus sequence of the \textit{S. elongatus} DnaA box (WT) and its derivatives (M1–M4). Mutations are highlighted in gray and shown in bold.
activation. It has been reported that the oligomerization activity in ATP-DnaA is higher than that of ADP-DnaA in *Bacillus subtilis* (Scholefield et al., 2012), while ATP-DnaA and ADP-DnaA bind to DnaA boxes with equal affinity in *E. coli* (Speck et al., 1999). The oligomerization might be promoted in DnaA<sup>R328H</sup>, which inactivates the intrinsic ATPase activity, although there is no direct evidence at this time. To characterize the R328H mutation in cyanobacterial DnaA, further studies are necessary.

In this study, we observed the interactions between DnaA protein and the DnaA box sequences at the oriC region in *S. elongatus*. This is the first report to show binding of DnaA to the DnaA box in cyanobacteria. *In vitro*, recombinant TF-DnaA<sup>WT</sup> recognized the DnaA box sequence TTTTCCACA with strict specificity. The activity-promoting effect due to the R328H mutation is consistent with our *in vivo* analysis (Ohbayashi et al., 2019). In the future, to assess the formation of oligomeric structures at DnaA box sequences, it will be necessary to analyze the involvement between the number of DnaA box sequences and the R328H mutation of DnaA. The BLI method enabled analysis of the *S. elongatus* DnaA protein. This technique has extensive possibilities for analyzing proteins that tend to aggregate.

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**Fig. 5.** The DNA-binding activity of TF-DnaA<sup>R328H</sup>. The effects of the R328H mutation was assayed. We used 0.1 µM of biotinylated ssDNA, complementary ssDNA and 0.1 µM TF-DnaA<sup>WT</sup> or TF-DnaA<sup>R328H</sup> for the BLI assay. The results from the association step, which included subtraction of the BSA values, are shown.

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