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ORIGINAL ARTICLE

# Diversification of DnaA dependency for DNA replication in cyanobacterial evolution

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**Regulating DNA replication is essential for all living cells. The DNA replication initiation factor DnaA is highly conserved in prokaryotes and is required for accurate initiation of chromosomal replication at *oriC*. DnaA-independent free-living bacteria have not been identified. The *dnaA* gene is absent in plastids and some symbiotic bacteria, although it is not known when or how DnaA-independent mechanisms were acquired. Here, we show that the degree of dependency of DNA replication on DnaA varies among cyanobacterial species. Deletion of the *dnaA* gene in *Synechococcus elongatus* PCC 7942 shifted DNA replication from *oriC* to a different site as a result of the integration of an episomal plasmid. Moreover, viability during the stationary phase was higher in *dnaA* disruptants than in wild-type cells. Deletion of *dnaA* did not affect DNA replication or cell growth in *Synechocystis* sp. PCC 6803 or *Anabaena* sp. PCC 7120, indicating that functional dependency on DnaA was already lost in some nonsymbiotic cyanobacterial lineages during diversification. Therefore, we proposed that cyanobacteria acquired DnaA-independent replication mechanisms before symbiosis and such an ancestral cyanobacterium was the sole primary endosymbiont to form a plastid precursor.**
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## Introduction

Chromosomal DNA replication is a fundamental process required to inherit the genetic information to progeny in both prokaryotes and eukaryotes. Replication in most bacteria, which have single copy genome, is initiated at an *oriC* region, whereas in eukaryotes and some archaea, the process is initiated at multiple origins. Initiation of bacterial DNA replication is regulated by the DnaA initiator protein. DnaA binds to the DnaA box located at *oriC* to facilitate the unwinding of duplex strands (Katayama *et al.*, 2010; Scholefield *et al.*, 2011); consequently, replisome complexes are recruited to the unwinded *oriC*. As such, DnaA is essential for DNA replication initiation in most bacteria. Indeed, with the exception of certain symbiotic species, there are no known free-living, DnaA-independent bacteria (Akman *et al.*, 2002; Ran *et al.*, 2010; Nakayama *et al.*, 2014).

Cyanobacteria are prokaryotes that utilize an oxygen-producing photosynthetic system and share a common ancestor with chloroplasts. Cyanobacteria display morphological and ecological diversity relating to structural characteristics (for example, rod shaped, coccus and filamentous) and environmental habitat (for example, ocean, fresh water, desert) (Herrero and Flores, 2008). Among many cyanobacteria, three typical freshwater cyanobacteria, *Synechococcus elongatus* PCC 7942 (rod shape), *Synechocystis* sp. PCC 6803 (coccus) and *Anabaena* sp. PCC 7120 (filamentous) (hereafter *S. elongatus*, *Synechocystis* and *Anabaena*), have been used as model organisms for the study of cyanobacterial physiology such as photosynthesis, circadian rhythm, nitrate fixation and development (Herrero and Flores, 2008). Molecular phylogenetic analysis of DNA sequence converge to a monophyletic origin for plastid (Criscuolo and Gribaldo, 2011; Shih *et al.*, 2013; Li *et al.*, 2014; Ochoa de Alda *et al.*, 2014), meaning that the plastid originated from a single primary endosymbiosis in which a heterotrophic protist engulfed and retained a cyanobacterium in its cytoplasm. However, the identification of the nearest current cyanobacterial species remains controversial (Deusch *et al.*, 2008; Criscuolo and Gribaldo, 2011; Shih *et al.*, 2013; Li *et al.*, 2014; Ochoa de Alda *et al.*, 2014) and then characteristics of the ancestral cyanobacterium are largely unclear.

Few studies have focused on the mechanism of cyanobacterial DNA replication, as it was believed to

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be similar to the bacterial mechanism. Indeed, the *dnaA* gene and several replication-related factors are conserved in many cyanobacteria. Our recent studies in *S. elongatus* revealed that replication is initiated at the DnaA box-containing *oriC* region (Watanabe *et al*., 2012) by a light-dependent mechanism (Ohbayashi *et al*., 2013); this suggests that replication is DnaA dependent, as in typical bacteria. In contrast, the *dnaA* gene is not essential for cell viability in *Synechocystis* (Richter *et al*., 1998), although additional characteristics were not addressed. The *dnaA* gene is inactivated by a transposon insertion in the symbiotic cyanobacterium *Nostoc azolla* (Akman *et al*., 2002) and is not conserved in the diatom endosymbiont that is of cyanobacterial origin (termed a spheroid body) with a 2.7-Mb genome (Ran *et al*., 2010). Moreover, recent study suggested that most of the DNA replication enzymes in chloroplast originated from cyanobacteria and others (Moriyama and Sato, 2014), and the vestige of symbiotic evolution was observed. However, the only *dnaA* ortholog is not conserved in chloroplasts of plants and algae for which the genomes have been sequenced (Supplementary Table S1), except for *Cucumis sativus*. It is possible that the *dnaA* gene of *C. sativus* is derived from horizontal gene transfer or genome sequence data of *C. sativus* include some contaminations because this *dnaA* gene matches that of *Enterobacter cloacae* ECNIH5, ECR091 and ECNIH3 at 100% according to blast search. These suggest that dependence on DnaA varies among cyanobacteria and was lost before the emergence of symbiosis and when or how DnaA-independent mechanisms were acquired in chloroplast has been mysterious.

In this study, we examined DnaA dependency on DNA replication in three model freshwater cyanobacteria. We demonstrate the diversification of DnaA dependency in the cyanobacterial lineage. We observed that *S. elongatus* was DnaA dependent, but could adapt to *dnaA* deletion by plasmid integration into the genome; in addition, *dnaA* deletion conferred an advantage in long-term culturing conditions. Conversely, *Synechocystis* and *Anabaena* were DnaA independent, and replication-sequence analysis suggested multiple replication origins, unlike many other bacteria.

## Materials and methods

*Bacterial strains and culture conditions*

Wild-type (WT) and mutant strains of *S. elongatus* PCC 7942, *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 were grown at 30 °C in BG-11 liquid medium under white light (50 μmol $m^{-2}$ $s^{-1}$) with 2% $CO_2$ aeration. To synchronize cell proliferation, cells were cultured for 10 days until they reached the stationary phase, and were then diluted with fresh BG-11 medium, such that the optical density at 750 nm ($OD_{750}$) was 0.2. After culture for 18 h in the dark, the culture was transferred to the light condition (time 0) to restart cell growth; $OD_{750}$ and cell number were measured 3 h later. To assess cell growth, synchronized cells were labeled with bromodeoxyuridine (BrdU) for 1–4 h and examined by immunocytochemistry using an antibody against BrdU (Roche Diagnostics, Tokyo, Japan); BrdU incorporation was assessed using microscopy and western blot analysis (100 ng DNA). Cell viability was evaluated using SYTOX Green (Invitrogen, Carlsbad, CA, USA) staining.

*Antiserum against SyfDnaA*

Mouse anti-SyfDnaA antiserum was generated as follows: the full-length *dnaA* gene (Synpcc7942_1100) was PCR-amplified using primers dnaA-F-*Bam*HI and dnaA-R-*Sal*I (Supplementary Table S3) and the fragment was cloned into pDEST-cold-TF (Imamura *et al*., 2008). The resulting vector was transformed into *Escherichia coli* strain Rosetta 2(DE3)pLys (Takara Bio Inc., Otsu, Japan). Protein purification and polyclonal antibody generation (Japan Lamb Co., Ltd, Hiroshima, Japan) were performed as previously described (Watanabe *et al*., 2013).

*Replication-sequencing (Repli-seq) analysis*

BrdU-labeled DNA was isolated by immunoprecipitation, and a library was prepared for next-generation sequencing using an Illumina genome analyzer (San Diego, CA, USA), as previously described (Watanabe *et al*., 2012).

*Pulsed-field gel electrophoresis analysis and Southern blot hybridization*

Cells were embedded in agarose, lysed with lysozyme and proteinase K (Bio-Rad Laboratories, Inc., Hercules, CA, USA), digested with *Nde*I (Takara Bio Inc.) and subjected to pulsed-field gel electrophoresis under conditions recommended by the manufacturer (Bio-Rad: switch time, 1–10 s; run time, 20 h; angle, 120°; voltage gradient, 6 V $cm^{-1}$ in 0.5 × Tris-buffered EDTA at 14 °C). The gel was then transferred to a Hybond-$N^+$ nylon membrane (GE Healthcare Japan, Tokyo, Japan), hybridized with digoxigenin-labeled probes generated by PCR using primers specific to each region (Supplementary Table S3, Southern blotting), and detected using a digoxigenin High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions.

*RNA sequencing analysis*

Total RNA was extracted from each strain as previously described; rRNA was removed from 1 μg of total RNA using the Ribo-Zero Magnetic Kit for Gram-Negative Bacteria (Epicentre, Madison, WI, USA), and a library was constructed with the remaining RNA using the NEBNext mRNA Library Prep Master Mix Set for Illumina (New England

Biolabs, Herts, UK). In total, 17 791 908, 22 260 758 and 16 294 140 sequence reads were obtained for WT and *ΔdnaA*-1 and -2, respectively, that were mapped to the *S. elongatus* reference genome. Reads per kilobase (kb) per million mapped reads were calculated using CLC Genomics Workbench software version 7.0.4 (CLC Bio, Germantown, MD, USA).

*Phylogenetic analysis*
Phylogenetic relationship between cyanobacteria was analyzed by 16S ribosomal RNA sequences using MEGA ver. 6.06 (Tamura *et al*., 2013). The sequence acquired from Cyanobase (http://genome.microbedb.jp/cyanobase) were aligned by MUSCLE, and phylogenetic tree was generated by the neighbor-joining method based on the alignment file, from which probability was confirmed by the 1000-time trial using the bootstrap method. The graphics of phylogenetic tree was arranged by Fig Tree version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

## Results

*Functional role of DnaA in DNA replication and effect of dnaA deletion in S. elongatus*
We analyzed DnaA protein expression and evaluated the protein's ability to bind *oriC* in *S. elongatus*. DnaA was constitutively expressed (Figure 1a and Supplementary Figure S1a) and bound to *oriC* but not to the opposite region (*ter*) (Figure 1b), indicating that it acts as an initiator protein in *S. elongatus*. To determine whether replication initiation can occur in a DnaA-independent manner as in *Synechocystis* (Richter *et al*., 1998), we constructed a *dnaA* deletion mutant. After two rounds of selection, 18 colonies were isolated during the stationary growth phase with complete disruption of the *dnaA* gene (Supplementary Figures S1b and c), from which two mutants (*ΔdnaA*-1 and -2) were selected. Expression of the DnaA protein was undetectable in these disruptants (Figure 1a), but cell growth was comparable to that of the WT strain (Figure 1c). Replication was assayed by pulse-labeling cells with BrdU using thymidine kinase transductants (Watanabe *et al*., 2012), followed by immunocytochemical analysis of BrdU incorporation (Figure 1d). The ratio of BrdU-positive cells was reduced by $>50\%$ in *dnaA* disruptants compared with that of the WT strain at each time point (Figure 1e). These results suggest that *dnaA* deficiency decreases the replication initiation frequency and that DnaA plays an important role in the initiation of DNA replication in *S. elongatus*.

*Elucidation of the origin of DNA replication in dnaA disruptants*
Given that *ΔdnaA*-1 and -2 exhibited DNA replication activity, we performed Repli-seq analysis to identify the replication initiation site. BrdU-labeled DNA was immunoprecipitated using an anti-BrdU antibody and analyzed using next-generation sequencing. In the WT strain, a large number of sequencing reads was mapped only at *oriC*, as shown in our previous study (Watanabe *et al*., 2012) (Figure 2a). Interestingly, in *dnaA* disruptants, the peak was observed in two different regions (Figure 2a), indicating that DNA replication was initiated from sites other than *oriC*.

To test whether a DnaA-independent replication mechanism was acquired by a suppressor mutation, we performed whole-genome sequencing of *dnaA* disruptants. Surprisingly, the 46 kb pANL plasmid, which is extra-chromosomal in WT *S. elongatus* (Chen *et al*., 2008), was integrated into the *ΔdnaA*-1 and -2 chromosome at different sites, although there were no other mutations. Moreover, Southern blotting and capillary sequencing revealed that all pANL copies were integrated in the middle of the *Syn7942_0826* gene in *ΔdnaA*-1 and in the intergenic region between *Syn7942_1297* and *Syn7942_1298* in *ΔdnaA*-2 (Figure 3 and Supplementary Figure S2), respectively. These integration sites were identified as new replication origins by Repli-seq analysis (Figures 2a and 3, and Supplementary Figure S2). Interestingly, capillary sequencing also revealed that the chromosome sequence was shifted to the plasmid sequence at 7 or 3 bp (GAAAATC or ACC) homologous regions in *ΔdnaA*-1 and -2, respectively (Supplementary Figure S2). These findings indicate that plasmids were integrated by homologous recombination via single crossovers at short homologous sequences. We also investigated the involvement of the DNA helicase DnaB, which forms the pre-priming complex with DnaA at *oriC*, and then DnaB binds to the *oriC* region related to the other region. Preferential binding at this site was not detected in *dnaA* disruptants, although DnaB was found to bind the *oriC* region rather than other regions in WT (Supplementary Figure S3), indicating that *dnaA* disruptants could not recruit DnaB to the *oriC* region. These results revealed that the DNA replication origin in *dnaA* disruptants was shifted from *oriC* to a plasmid integration site, strongly suggesting that the DnaA/*oriC* system shifted to a plasmid-based DNA replication initiation system.

*Increased viability of dnaA disruptants under long culture conditions*
The reason why diversification of DnaA dependency occurred in cyanobacteria is unclear. The functional significance of *dnaA* deficiency was investigated by assessing cell growth in *dnaA* disruptants. The loss of DnaA function could have been advantageous on survival in certain environmental conditions if cyanobacteria had evolved a variety of DnaA dependence mechanisms. Although we tested the growth and viability under some stress conditions, including high light, low and high temperature and oxidative stress, significant differences were not observed. However, after long culture

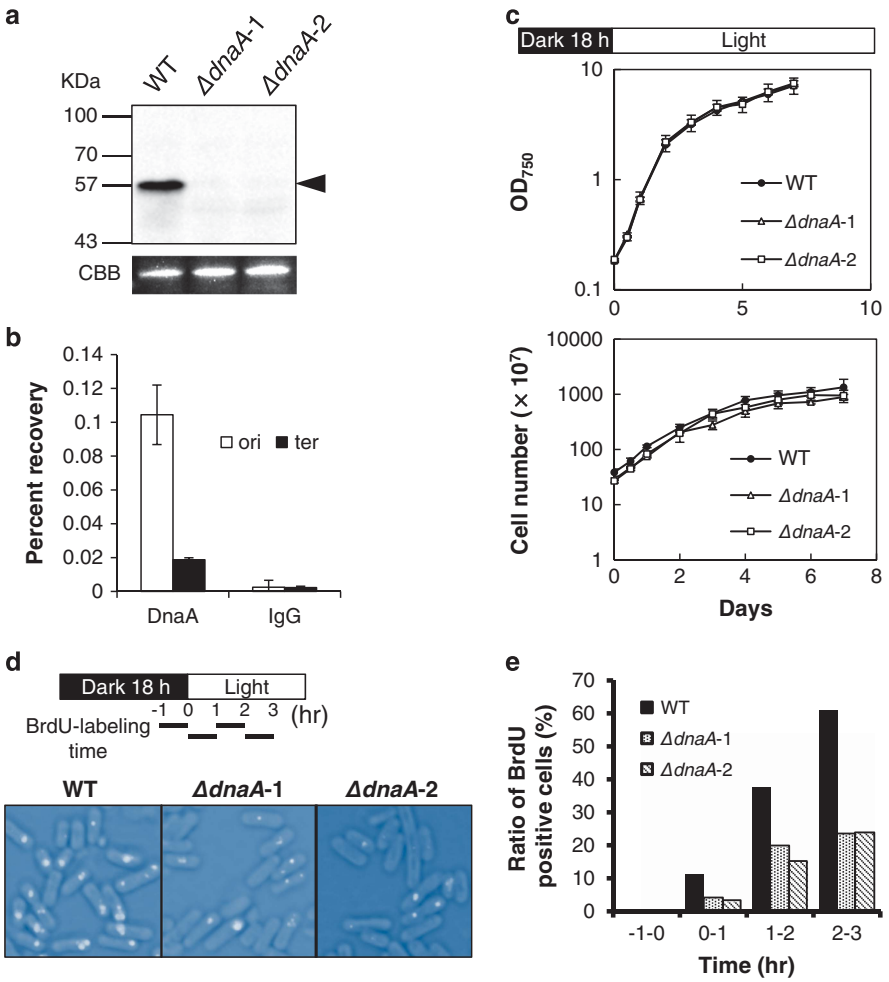


**Figure 1** Deletion of *dnaA* reduces DNA replication initiation frequency but not growth in *S. elongatus*. (**a**) Analysis of DnaA expression by western blotting. (**b**) DnaA binding at *oriC* and *ter* was evaluated by chromatin immunoprecipitation using an anti-DnaA antibody and quantitative PCR to amplify *oriC* and *ter* regions. (**c**) Cell growth at the stationary phase; $OD_{750}$ (upper) and cell number (lower) were measured. (**d**) Schematic representation of culture conditions and the BrdU labeling period. Cells were labeled with BrdU for 1 h at each time point, as indicated by bars (BrdU labeling time). BrdU-labeled cells at 2–3 h after synchronization are shown in merged bright-field and immunofluorescence images. (**e**) Quantitative analysis of BrdU-positive cells. Total and BrdU-positive cell numbers are as follows: WT: −1 to 0 h = 376, 0 to 1 h = 451, 1 to 2 h = 362, 2 to 3 h = 471; *dnaA1*: −1 to 0 h = 453, 0 to 1 h = 441, 1 to 2 h = 429, 2 to 3 h = 422; *dnaA2*: −1 to 0 h = 482, 0 to 1 h = 391, 1 to 2 h = 423, 2 to 3 h = 438, respectively.

periods, viability was notably higher in *ΔdnaA*-1 and -2 compared with that of WT cells (Figures 4a and c). A viability assay was performed using SYTOX Green that exclusively stains dead cells. At 4 and 5 weeks after inoculation, ~60% viability was observed in both *ΔdnaA*-1 or -2 disruptants, in contrast with respective viabilities of 32% and 20% in WT cells. DnaA protein was expressed until 6 weeks (Figure 4b) in WT cells, indicating that DnaA was very stable in long culture conditions. These results indicate that deletion of the *dnaA* gene conferred a survival advantage to *S. elongatus*; indeed, the disruptants were isolated from stationary phase cultures (Supplementary Figure S1b). Thus, these culture conditions could be necessary to obtain complete *dnaA* disruptants. The mechanistic basis for the enhanced survivability of *ΔdnaA*-1 and -2 was investigated by performing a transcriptome analysis. In stationary phase cultures of *dnaA* disruptants, transcription of many metabolism-related genes, including the gene encoding adenosine triphosphate synthase and genes involved in photosynthesis and carbon metabolism, was downregulated compared with that of the WT strain (Supplementary Figure S5 and Supplementary Table S2). These data suggest that disruptants maintained a low level of metabolic activity during the stationary phase to preserve cellular energy and enhance viability. DnaA could also directly regulate expression of these genes, given its function as a transcription factor in many bacteria (Messer and Weigel, 1997; Ishikawa *et al*., 2007; McAdams and Shapiro, 2009).

### *DnaA is not essential for DNA replication and cell growth in Synechocystis and Anabaena*

To determine whether *dnaA* is universally required for DNA replication, we constructed *dnaA* deletion

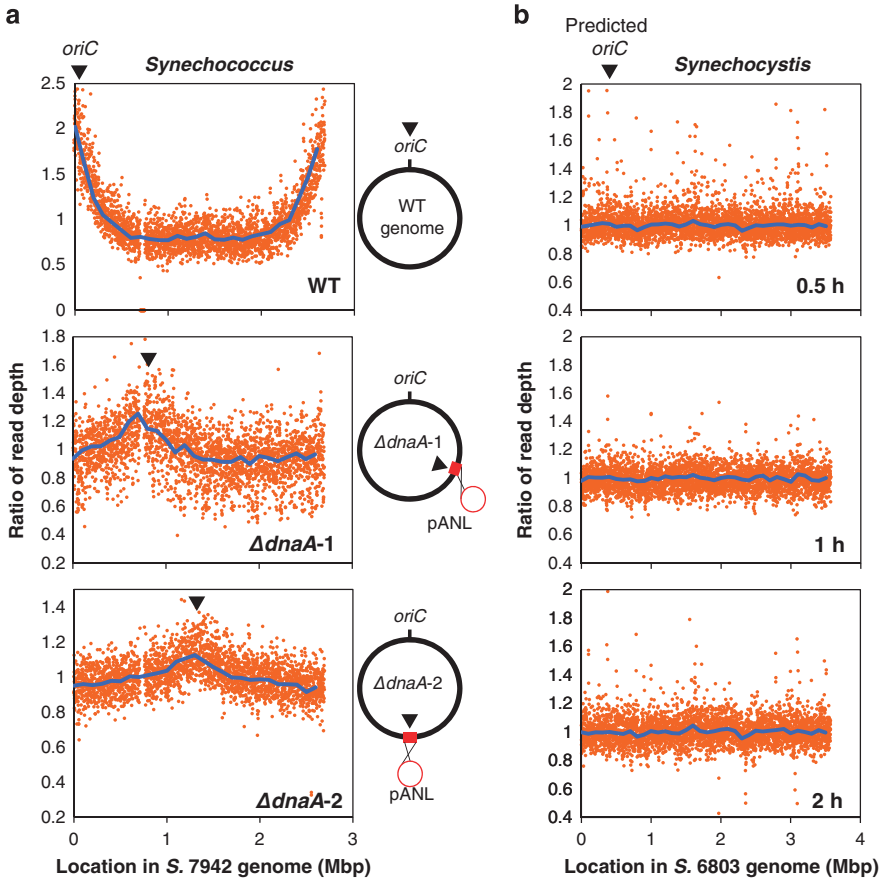


**Figure 2** Replication origin in *S. elongatus* and *Synechocystis*. Ratios of read depth at each genomic position analyzed by Repli-seq in WT and two *dnaA* disruptants of *S. elongatus* (**a**) and WT *Synechocystis* (**b**). (**a**) Synchronized cells were labeled with BrdU for 4 h; libraries of BrdU-labeled DNA were analyzed by next-generation sequencing and mapped using the *S. elongatus* (*S.* 7942) genome as a reference. Genome maps are shown to the right of the depth plot. Inverted triangles denote replication initiation points. (**b**) Synchronized *Synechocystis* cultures were labeled with BrdU for 0.5, 1 and 2 h. Sequence reads were mapped using the *Synechocystis* (*S.* 6803) genome as a reference.

mutants in model cyanobacteria *Synechocystis* and *Anabaena* that are more closely related to chloroplasts than *S. elongatus*, as shown in a phylogenetic tree (Turner *et al.*, 1999; Falcon *et al.*, 2010; Shih *et al.*, 2013; Ochoa de Alda *et al.*, 2014) (Figure 5a and Supplementary Figure S6). We readily obtained *dnaA* deletion mutants using *Synechocystis* and *Anabaena*, in contrast to *S. elongatus*. In these genera, growth and DNA replication in the disruptants were indistinguishable from those in WT cells (Figures 5b and c). Deletion of the *dnaA* box-containing predicted *oriC* region (POR) could also be obtained in *Synechocystis*, and replication and cell growth were unaffected in the *Synechocystis* POR-deletion mutant. We verified whether these mutants possessed a suppressor mutation such as plasmid integration, as detected in *S. elongatus* using a next-generation sequencing approach. No mutations or plasmid integrations were detected in *Synechocystis* and *Anabaena*, indicating that these utilized a DnaA/*oriC*-independent system of DNA replication.

To identify alternative replication initiation sites, a Repli-seq analysis was performed using a WT *Synechocystis* strain. There were no peaks observed at either POR or other genomic regions (Figure 2b), although a replication origin could be detected in the case of a single origin. This finding suggests that there are multiple replication origins that fire asynchronously, as in eukaryotic nuclear chromosomal replication. Leading and lagging strand replication are subjected to different mutational pressures, resulting in an asymmetric genomic composition (Lobry and Sueoka, 2002; Lobry and Louarn, 2003), including GC skew, that has been used to predict the DNA replication origin at the shift point of GC skew (Frank and Lobry, 1999; Arakawa and Tomita, 2007). However, in *Synechocystis*, the replication origin was not predicted from GC skew, as there are many shift points (Watanabe *et al.*, 2012). Moreover, these genomic compositions suggest the multiple replication origin in some prokaryote (Gao, 2014, 2015). These also support our hypothesis that *Synechocystis* has multiple replication origins.

## Discussion

Freshwater cyanobacteria such as *S. elongatus* have evolved and diversified while maintaining a dependence on DnaA for the initiation of DNA replication,

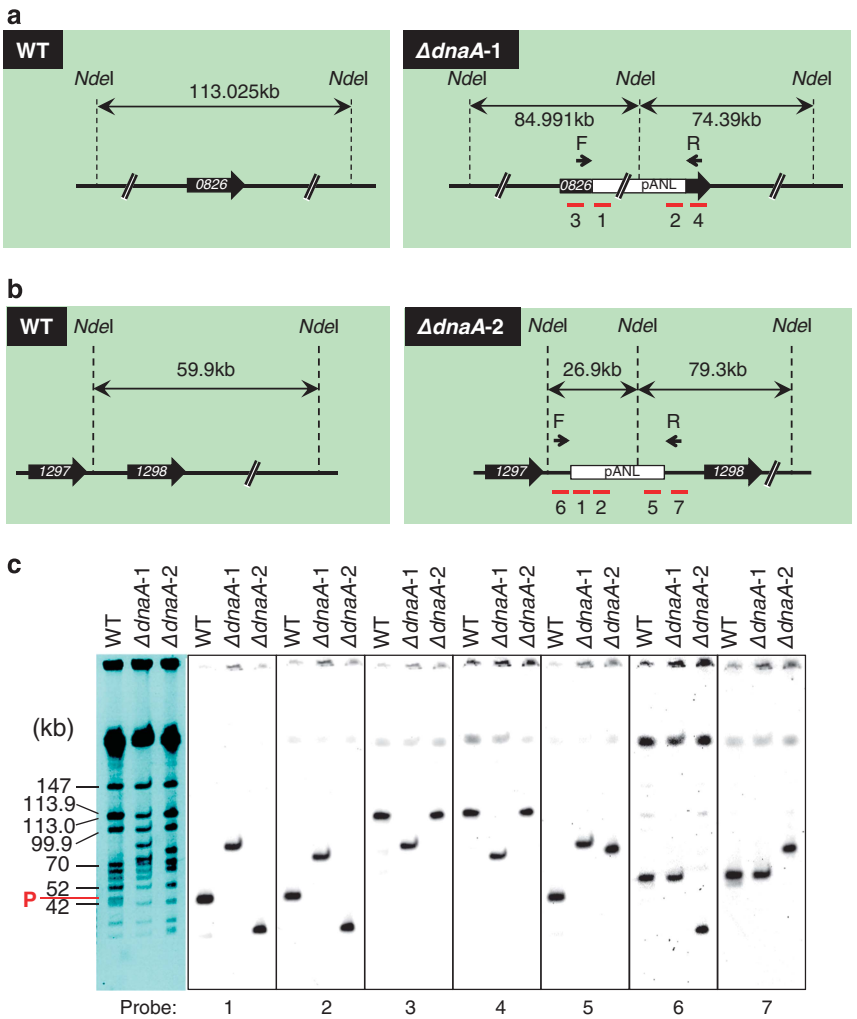


**Figure 3** Chromosomal insertion of plasmid pANL in *dnaA* disruptants of *S. elongatus*. Schematic representation of the region surrounding the plasmid insertion site in *ΔdnaA*-1 (**a**) and *ΔdnaA*-2 (**b**). Sizes of fragments digested with *Nde*I are indicated for WT (left) and *dnaA* disruptants (right) strains. Red bars with numbers indicate probes used for Southern blot hybridization in (**c**). The pANL plasmid was integrated into the middle of the *Synpcc7942_0826* gene and downstream of the *Synpcc7942_1297* gene in *ΔdnaA*-1 and *ΔdnaA*-2, respectively, and corresponded to new replication origins, as determined by Repli-seq. (**c**) Pulsed-field gel electrophoresis (PFGE) pattern (left) and results of Southern hybridization with DNA probes specific to the plasmid (1, 2 and 5), *Synpcc7942_0826* gene (3 and 4) and intergenic region between *Synpcc7942_1297* and *Synpcc7942_1298* (6 and 7), as shown in (**a**) and (**b**). Chromosome fragment sizes and plasmid fragments (P; 46 kb) are indicated to the left of PFGE image.

with *dnaA* disruptants acquiring a survival advantage in the stationary phase. In contrast, DNA replication in cyanobacteria that are closely related to chloroplasts, such as *Synechocystis* and *Anabaena*, is DnaA independent. Thus, DnaA dependency corresponds to the phylogenetic relationship and then we propose that ancestral cyanobacteria acquired DnaA-independent mechanism before symbiosis as a fundamental biological phenomenon and such a cyanobacterium facilitated the evolution of symbiosis.

In the *S. elongatus dnaA* disruptants, an episomal plasmid was integrated into the chromosome, and chromosomal replication was initiated using the plasmid initiation system. In *E. coli*, it is known that the replication origin of a plasmid or phage is integrated into the chromosome in *oriC*- and *dnaA*-defective mutants (Lindahl *et al.*, 1971; Nishimura *et al.*, 1971; Louarn *et al.*, 1982; Gowrishankar, 2015). These strains, which depend on exogenous elements for their replication, displayed no advantageous compared with WT in both *E. coli* and *Bacillus subtilis*. In contrast, the *S. elongatus dnaA* disruptant not only exhibited normal growth, but also displayed increasing cell viability in long culturing conditions. Interestingly, polyploid archaea tolerate the deletion of all replication origins and mutants exhibit a faster growth rate compared with WT counterparts (Hawkins *et al.*, 2013). Freshwater cyanobacteria are also polyploid and their chromosomes are replicated asynchronously; that is, they may not initiate replication only once per chromosome per cell cycle (Chen *et al.*, 2012; Jain *et al.*, 2012; Watanabe *et al.*, 2012) in contrast to bacteria that have a single chromosome copy and therefore tightly regulate replication initiation via the DnaA/*oriC* system. Thus, polyploidy and asynchronous replication could have enabled the acquisition of

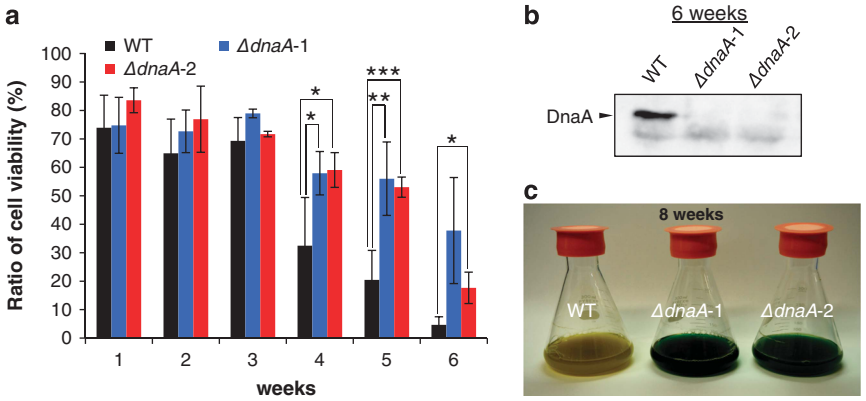


**Figure 4** DnaA deficiency confers a long-term survival advantage. (**a**) Cell viability in WT *S. elongatus* and *dnaA* disruptants. Total (bright-field) and viable (with autofluorescence and lacking green fluorescence) cells were counted by fluorescence microscopy (Supplementary Figure S4), and the percentage of viable cells (%) was calculated. Data represent the mean of three biological replicates; error bars indicate s.d. $*0.05<P<0.1$, $**0.01<P<0.05$, $***P<0.01$ (Student's *t*-test). (**b**) DnaA expression after 6 weeks of culture. (**c**) WT and *dnaA* disruptant cultures after 8 weeks of culture.

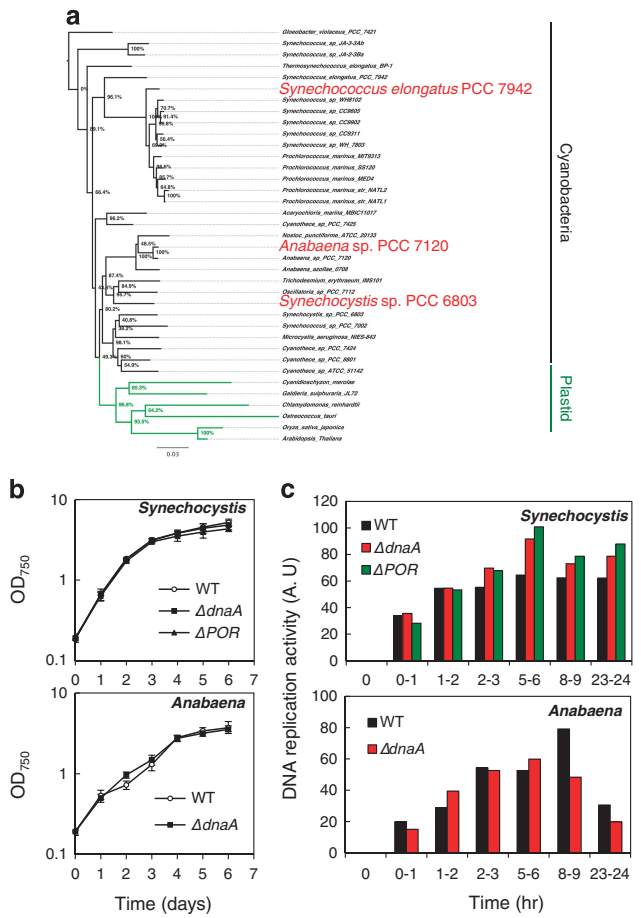


**Figure 5** DnaA dependence is variable among cyanobacteria. (**a**) Phylogenetic tree of the phylum Cyanobacteria (black bar) and plastids (green bar) based on *16S* rRNA alignment. (**b**) Cell growth in *dnaA* and *POR* disruptants of *Synechocystis* (upper) and a *dnaA* disruptant of *Anabaena* (lower). Data represent the mean of three biological replicates; error bars indicate s.d. (**c**) DNA replication activity as determined by western blot analysis of BrdU-labeled DNA in WT cells and *Synechocystis* (upper) and *Anabaena* (lower) deletion mutants. Signal intensity was quantified as arbitrary units (AU). The x axis shows periods of BrdU pulse labeling.

DnaA-independent replication control and facilitated DnaA-independent growth under various environmental conditions in freshwater cyanobacteria.

The *S. elongatus dnaA* disruptants were viable under conditions of nutrient starvation (Figure 4), suggesting that DnaA dependence not only affected DNA replication, but also environmental adaptation during cyanobacterial evolution. In fresh water, changes in environmental conditions such as nutrient availability are common. Therefore, freshwater cyanobacteria can survive for long periods without nutrients. In contrast, nutrients are constant in the ocean owing to the occurrence of waves. Thus, considering DnaA dependence with respect to the natural habitat of cyanobacteria, it can be concluded that marine and freshwater species are DnaA dependent and DnaA independent, respectively. Marine cyanobacterial *dnaA* gene expression oscillates with the cell cycle (Holtzendorff *et al*., 2001), and a GC skew pattern is consistent with that of the *oriC* region (Watanabe *et al*., 2012), suggesting that these species use DnaA for DNA replication, similar to other well-characterized bacteria. In this respect, the freshwater cyanobacterium *S. elongatus* is closely related phylogenetically to marine cyanobacteria (Figure 5a).

Among photosynthetic microorganisms, cyanobacteria are the only primary symbionts that have evolved into plastids (Reyes-Prieto *et al*., 2007). Subsequently, the reorganization of genetic material and regulatory mechanisms, including those pertaining to DNA replication, occurred between the nucleus and the symbiont (Kleine *et al*., 2009; Moriyama *et al*., 2014). Twinkle, an organelle DNA helicase and primase, is shared by plastids and mitochondria in green plants, whereas cyanobacterial *dnaB* helicase and *dnaG* primase are conserved in red algae. Thus, the evolutionary history of DNA replication from a cyanobacterial to a plastid system can be discerned from genetic evidence. Interestingly, *dnaA* is the only gene that is not conserved between red algae, the cyanobacterial symbiont

*Nostoc azollae* (Ran *et al.*, 2010) and the spheroid bodies (Nakayama *et al.*, 2014) of diatoms. We have shown that cyanobacteria have the capacity to shift the DNA replication initiation system from chromosomal to plasmid type by *dnaA* deletion. In red algae plastids, the substitution of cyanobacteria-type DNA polymerase (Pol III) with a plasmid-type (Pol I-like) (Moriyama *et al.*, 2011, 2014) suggests that polymerase reorganization occurred at early stages of symbiosis. We propose that the DnaA-independent replication initiation system in cyanobacteria accelerated the reorganization of replication components and that this flexibility in DNA replication yielded a preadaptive genotype that enabled symbiosis.

## Conflict of Interest

The authors declare no conflict of interest.

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## Author Contributions

RO, SW and HY designed the experiments. RO performed the experiments. SE constructed *Anabaena* mutant strains. RO and YK analyzed Repli-seq and RNA sequencing data. RO, SW, YK, SE, TC and HY wrote the paper.

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