Full Paper

Overexpression of the response regulator rpaA causes an impaired cell division in the Cyanobacterium Synechocystis sp. PCC 6803

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In photosynthetic microorganisms, cell cycle progression depends on day and night cycles; however, how cell division is regulated in response to these environmental changes is poorly understood. RpaA has been implicated in the signal output from both circadian clocks and light/dark conditions in the unicellular spherical-celled cyanobacterium Synechocystis sp. PCC 6803. In the present study, we investigated the involvement of a two-component response regulator RpaA in cell division regulation. Firstly, we examined the effects of rpaA overexpression on cell morphology and the expression levels of cell division genes. We observed an increase in the volume of non-dividing cells and a high proportion of dividing cells in rpaA-overexpressing strains by light microscopy. The expression levels of selected cell division-related genes were higher in the rpaA-overexpressing strain than in the wild type, including minD of the Min system; cdv3 and zipN, which encode two divisome components; and murB, murC, and pbp2, which are involved in peptidoglycan (PG) synthesis. Moreover, in the rpaA-overexpressing strain, the outer membrane and cell wall PG layer were not smooth, and the outer membrane was not clearly visible by transmission electron microscopy. These results demonstrated that rpaA overexpression causes an impaired cell division, which is accompanied by transcriptional activation of cell division genes and morphological changes in the PG layer and outer membrane.

Key Words: cell division; circadian rhythm; cyanobacteria; cytokinesis; Synechocystis; transcription factor

Introduction

In photosynthetic eukaryotes and prokaryotes, cell cycle progression is dependent on daily light/dark cycles, and cell division is restricted to a specific time period. In photosynthetic eukaryotes, cell growth (or cell enlargement) occurs during the daytime, whereas cell division occurs during the night time, to prevent photodamage to DNA (Johnson, 2010). The regulatory mechanism of cell cycle progression in a unicellular photosynthetic eukaryote was recently elucidated (Miyagishima et al., 2014). In contrast, in some photosynthetic prokaryotes, photosynthesis and cell cycle progression both occur during the daytime (Mori et al., 1996; Yu et al., 2017). However, the regulation of cell cycle progression in photosynthetic prokaryotes is poorly understood.

Most prokaryotic cells divide by binary fission, in which a mother cell divides into two daughter cells (Eswara and Ramamurthi, 2017) (Fig. 1). In several model bacteria, cell division is initiated by polymerization of the tubulin homologue and GTPase protein FtsZ into a ring-like structure at midcell. The FtsZ ring at the division site serves as a scaffold for the recruitment of proteins required for cytokinesis. This macromolecular structure is known as the divisome (den Blaauwen et al., 2017). Eventually, new peptidoglycan (PG) is synthesized at the septum (and the

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outer membrane is invaginated in gram-negative bacteria) to separate the two daughter cells. There are two well-known molecular mechanisms that restrict FtsZ ring formation to midcell, the Min and nucleoid occlusion systems. The process of cytokinesis is based on studies of model bacteria, such as gram-negative *Escherichia coli* and gram-positive *Bacillus subtilis*, which are both rod-like (cylindrical) cells. Although the mechanisms controlling the division of cylindrical bacteria are relatively well characterized, those in non-cylindrical bacteria are incompletely understood, particularly in coccoid bacteria (Eswara and Ramamurthi, 2017; Pinho et al., 2013).

Cyanobacteria are gram-negative photoautotrophs and are regarded as the ancestor of the chloroplast (Gray, 1993). They have various cell morphologies, including cylindrical, spherical, and spiral. Most cell division studies in cyanobacteria have been focused on identifying the cytokinetic factors in the unicellular spherical and cylindrical cells of *Synechocystis* sp. PCC 6803 (*Synechocystis*) and *Synechococcus elongatus* PCC 7942 (*Synechococcus*), respectively. (Cassier-Chauvat and Chauvat, 2014; Miyagishima et al., 2005). Cyanobacteria share cytokinetic factor-encoding genes with not only gram-negative but also gram-positive bacteria, the chloroplast, and the nuclear genome of plants and algae (Cassier-Chauvat and Chauvat, 2014); thus, elucidation of the regulatory mechanisms of cell division in cyanobacteria will also be beneficial for understanding division in various other organisms and organelles. Furthermore, these findings will have important implications for the endosymbiotic event leading to chloroplasts, because although the chloroplast division complex resembles the cyanobacterial divisome, many components were lost after the endosymbiotic event (Miyagishima, 2011). Despite its importance, few studies have attempted to identify the transcriptional mechanisms involved in cyanobacterial cell division.

Like most bacteria, cyanobacteria possess two-component regulatory systems consisting of a histidine kinase (Hik) and a cognate response regulator (Rre), and the function of the Hik is to modify the phosphorylation state of the Rre in response to changing environmental cues (Ashby and Houmard, 2006; Ashby and Mullineaux, 1999). In *Synechococcus*, RpaA transduces the circadian signal from the core oscillator (consisting of the KaiA, KaiB, and KaiC proteins) in concert with two antagonistic Hiks (SasA and CikA) (Gutu and O’Shea, 2013; Takai et al., 2006). RpaA also plays a role in cell division in *Synechococcus*; it was shown that cell division after cell elongation was blocked by overexpression of phosphomimetic RpaA protein (RpaA~P) (Markson et al., 2013). Similarly, in *Synechocystis*, SasA and RpaA interact with the KaiAB1C1 core oscillator (Köbler et al., 2018). However, the putative role of RpaA in cell division in *Synechocystis* is unknown. To explore the involvement of RpaA in cell division in *Synechocystis*, we examined the effects of rpaA overexpression on cell morphology, the expression of cell division genes, and cell ultrastructure. Our data demonstrated that rpaA overexpression causes an impaired cell
division through transcriptional activation of cell division genes and morphological changes in the PG layer and outer membrane.

Materials and Methods

Strains and culture conditions. A glucose-tolerant (designated as GT) strain of *Synechocystis* 6803 isolated by Williams (1988) and a *rpaA*-overexpressing strain, called ROX310 (Iijima et al., 2015), were grown in BG-11 medium (Rippka, 1988) with some modification; 17.5 mM NaNO_3_ was replaced with 5 mM NH_4Cl and buffered with 20 mM HEPES-KOH, pH 7.8. Among the available GT substrains, the GT-I strain was used in this study (Kanesaki et al., 2012). Liquid cultures were bubbled with 1% (v/v) CO_2 in air and incubated at 30°C under continuous illumination (ca. 50–70 µmol photons m^{-2} s^{-1}). Cell density was estimated by measuring the optical density at 730 nm (OD_{730}) using a UV-VIS spectrophotometer (UV-2700, Shimadzu, Kyoto, Japan). Kanamycin (10 µg ml^{-1}) was added to ROX310 pre-cultures.

Immunoblot analysis. GT and ROX310 cells grown under normal conditions were harvested in mid-log phase (OD_{730} ~ 0.7). Cells were collected by centrifugation and re-centrifuged at 20,400 g and stored at -80°C. The final pellet was re-suspended in residual medium and re-centrifuged at 20,400 × g for 1 min. The supernatant was removed by pipetting, and the cells were suspended in 0.5 ml of lysis buffer (25 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, and 1 mM DTT). After the addition of glass beads (diameter, 15–212 µm; SIGMA), the cells were disrupted by vortexing for 10 min (ten rounds of 30 s each with 30 s rest on ice). The soluble protein fraction was obtained by centrifugation at 10,000 × g for 5 min. Total protein was separated by 12% (w/v) SDS-PAGE. Then, the separated proteins were immunoblotted as described previously (Osanai et al., 2014a), except that the blocking buffer was replaced with TBS-T (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20). For antibody production, the glutathione S-transferase (GST)-tagged RpaA protein was expressed in *Escherichia coli*, and purified by GST-affinity chromatography. Immunodetection was performed using rabbit polyclonal antibodies raised against GST-tagged RpaA proteins.

Microscopy and image analysis. GT and ROX310 cells were grown for 48 hours under normal conditions. After 24 h, 100 µl aliquots were removed and centrifuged at 5,800 × g for 2 min. The supernatant was removed, and the pellet was resuspended in residual medium. Then, 1 µl of the resuspended culture was spotted on a glass slide and covered with a thin glass coverslip. For samples grown for 48 h, 1 µl of the culture, without centrifugation, was spotted on a plate and covered with a glass coverslip. Cells were imaged using a light microscope (LEICA DM 500) equipped with a camera (LEICA MC120 HD). For image analysis, cell diameter measurements were obtained by using ImageJ software (Schneider et al., 2012). Statistical analysis was carried out using Microsoft Excel.

Quantitative real-time PCR. The samples for total RNA extraction were taken from GT and ROX310 cells inoculated at an initial OD_{730} of approximately 0.1 and grown for 24 h. Total RNA was isolated as previously described (Osanai et al., 2014b). cDNAs were generated with the SuperScript III First-Strand Synthesis System (Life Technologies Japan, Tokyo, Japan) by using the isolated RNA as a template. Quantitative real-time PCR was performed with the StepOnePlus Real-Time PCR System (Life Technologies Japan) according to the manufacturer’s protocol, using the primers listed in Supplementary Table S1. The *rpbB* gene (encoding RNase P subunit B) was used as a standard.

Transmission electron microscopy. Cultures (15 ml) of the GT and ROX310 strains were sampled at 48 h of growth under normal conditions. The samples were centrifuged at 5,800 × g for 3 min, and the supernatant was removed. Then, the cell pellet was washed with 0.1 M phosphate buffer (pH 7.2), fixed in 2% glutaraldehyde, and post-fixed in 2% osmium tetroxide. The cells were washed and embedded in 1.5% agarose. The samples were dehydrated in a graded ethanol series and propylene oxide, and then embedded in Epon resin (Nishshin EM, Tokyo, Japan). Ultrathin sections were cut with a diamond knife and stained with uranium and lead citrate. Transmission electron microscopy was carried out at 140 kV on a JEM-2010 (JEOL, Tokyo, Japan).

Results

Heterogeneity in cell size in an *rpaA*-overexpressing strain

Firstly, we estimated the amount of RpaA protein in an *rpaA*-overexpressing strain of *Synechocystis*, called ROX310, in which *rpaA* overexpression was driven by the
Immunoblot analyses revealed that RpaA protein was detectable as a single band of approximately 25 kDa in ROX310 cells but was not detectable in the glucose-tolerant (GT) strain under normal growth conditions (Fig. 2A). RpaA proteins were also not detected by immunoblotting with increased total protein (24 µg). Under normal growth conditions, the growth of GT and ROX310 was similar (Fig. 2B). To examine the effect of rpaA overexpression on cell morphology in *Synechocystis*, the morphology of GT and ROX310 cells grown for 24 and 48 h (at mid- and late-log phases, respectively) were observed by light microscopy. The ROX310 cells were heterogeneous, and most of these cells were larger than the GT cells at mid-log phase (Fig. 3A). Single cells and actively dividing cells of both strains were visually distinguished by light microscopy. The diameter of a single cell was measured from microscopic images of the cells, and cell volume was calculated for spherical-shaped cells. The average single cell volume of ROX310 was larger than that of GT at both mid-log phase (GT, 7.81 ± 0.24 µm³; ROX310, 10.7 ± 0.61 µm³) and late-log phase (GT, 6.25 ± 0.18 µm³; ROX310, 6.63 ± 0.42 µm³), but the difference between the strains was smaller at late-log phase (Fig. 3B). In addition, the ratio of dividing cells for ROX310 was higher than that for GT at both mid-log phase (GT, 65.6%; ROX310, 78.5%) and late-log phase (GT, 55.3%; ROX310, 63.3%), but the difference between the strains was smaller at late-log phase than at mid-log phase (Fig. 3C).

Transcriptional activation of cell division genes in the rpaA-overexpressing strain

To determine the cause of the observed cell enlargement and the higher ratio of dividing cells in ROX310, the GT and ROX310 strains were analysed by quantitative RT-PCR (Fig. 4). Genes encoding cell division factors in *Synechocystis* have been previously characterized by Dr. Chauvat's group (Marbouty et al., 2009a, b, c; Mazouni et al., 2004). Here, we focused on genes encoding Min system, divisome, and peptidoglycan synthesis in the glucose-tolerant (GT) and rpaA-overexpressing (ROX310) strains.

**Fig. 3.** Morphology of glucose-tolerant (GT) and rpaA-overexpressing (ROX310) strains.

A. Light microphotographs of GT and ROX310 cells. B. Volume of non-dividing single cells of GT and ROX310 strains was quantified by measuring the diameter of cells in microscopy images. The mean values ± SD of three biological replicates are shown. C. Percentage of non-dividing single cells versus dividing cells. The percentages indicate the ratio of the dividing cells. Statistical significance was determined by Student’s *t*-test. Asterisks indicate *P* < 0.05.

**Fig. 4.** Transcript levels of genes involved in the Min system, divisome, and peptidoglycan synthesis in the glucose-tolerant (GT) and rpaA-overexpressing (ROX310) strains. Ct-values were normalized to *rnpB* levels. The mean values ± SD of three biological replicates are shown. Asterisks indicate *P* < 0.05.
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tem components and presumed divisome components. We also examined genes encoding penicillin-binding protein (\textit{pbp}) that physically interact with the divisome (Marbouty et al., 2009a), and the murein biosynthetic genes (\textit{mur}) which RpaA binds to their promoters in \textit{Synechococcus in vivo}, which is involved in PG synthesis (Markson et al., 2013). We measured the expression levels of 18 genes related to cell division, \textit{minC} (sll0288), \textit{minD} (sll0289), and \textit{minE} (sll0546) of the Min system; \textit{ftsQ} (sll1632), \textit{ftsI} (sll1833), \textit{ftsW} (sll1267), \textit{ftsZ} (sll1633), \textit{zipS} (sll1939), \textit{sepF} (sll0270), \textit{cdv3} (sll0848), \textit{ylmD} (sll1593), and \textit{zipN} (sll0169) of the divisome; and \textit{murB} (sll1424), \textit{murC} (sll1423), \textit{pbp1} (sll0002), \textit{pbp2} (sll1710), and \textit{pbp3} (sll1434) for PG synthesis. Of the Min system genes, the expression of \textit{minD} was 1.8-fold higher in ROX310 cells than in GT cells, whereas the expression levels of \textit{minC} and \textit{minE} were similar between the two strains. Of the cell divisome-related genes, the expression levels of \textit{cdv3} and \textit{zipN} were 3.2-fold and 20-fold higher, respectively, in ROX310 cells than in the GT cells, whereas the expression levels of the other cell divisome-related genes, \textit{ftsI}, \textit{ftsQ}, \textit{ftsW}, \textit{ftsZ}, \textit{sepF}, \textit{zipS}, and \textit{ylmD}, were similar between the strains. Of the PG synthesis-related genes, the expression levels of \textit{murB}, \textit{murC}, and \textit{pbp2} were higher in ROX310 cells than in GT cells, whereas the levels of \textit{pbp1} and \textit{pbp3} were similar between the two strains.

\textbf{Morphological changes in the outer membrane and cell wall peptidoglycan layer in \textit{rpaA}-overexpressing cells}

The cell envelope of \textit{Synechocystis} comprises the plasma membrane, PG layer, and outer membrane (Liberton et al., 2006). To examine whether the impaired cell division in ROX310 is accompanied by changes in the surface and intracellular structures, we compared the ultrastructure of

\textbf{Fig. 5.} Electron microphotographs of glucose-tolerant (GT) and \textit{rpaA}-overexpressing (ROX310) cells.

Whole cell images of GT (A–D) and ROX310 (E–H) at different cell cycle phases. High-magnification images of the septal peptidoglycan at midcell of GT (I and J) and ROX310 (K and L). High-magnification images of the cell envelope of GT (M) and ROX310 (N). M and N are the enlarged cell images of A and E, respectively. Open arrowheads, outer membrane; filled arrowheads, peptidoglycan layer; black arrows, plasma membrane.
ROX310 cells with that of GT cells by transmission electron microscopy (TEM). Each of the 10 cells was observed, respectively. The intracellular structures of single and dividing GT cells (Figs. 5A–D, Supplementary Figs. S1A–C) and ROX310 cells (Figs. 5E–H, Supplementary Figs. S1D–F) were similar. The electron densities of the cell wall PG layer and septal PG at midcell in ROX310 cells (Figs. 5K and L) were comparable to those in GT cells (Figs. 5I and J). However, the outer membrane and cell wall PG layer of ROX310 cells were less smooth than those of GT, and the outer membrane of ROX310 was less clearly visible than that of GT (Figs. 5M and N).

Discussion

In order to determine the involvement of RpaA in cell division in a spherical cyanobacterium, we examined the effects of rpaA overexpression on cell morphology and the expression levels of cell division-related genes in *Synechocystis*. Light microscopic observation revealed an increase in the volume of single cells (Fig. 3B) and an increase in the ratio of dividing cells in the ROX310 strain at mid-log and late-log phases (Fig. 3C), indicating a cell enlargement, which is caused by a delay in daughter cell separation, in ROX310. However, the growth of ROX310 was similar to GT (Fig. 2B). Different from the results in this study, a previous study of our group shows that ROX310 strain grows slightly slower than GT strain (Iijima et al., 2015), possibly caused by the changes in growth conditions. In any case, the results demonstrate that ROX310 cells are not defective in cell division. Consistent with this implication, we found no unusually shaped cells in the ROX310 culture, such as cloverleaf-like clusters of four unseparated cells, resulting from initiating of a second round of cell division before the completion of the first round, at either phase (Fig. 3A) (Marbouty et al., 2009a). In addition, the proportion of enlarged cells (volume range, 12–21 μm^3; Fig. 3B) in ROX310 was lower at late-log phase (1.72%) than at mid-log phase (24.3%), suggesting that the enlarged cells are capable of cell division.

We investigated the possibility that rpaA overexpression may affect the expression of cytokinetic genes, leading to the impaired cell division. The expression levels of some cytokinetic genes were higher in ROX310 than in GT, including minD of the Min system; the divisome components cdv3 and zipN; and murB, murC, and pbp2, which are involved in PG synthesis (Fig. 4). These results indicate that rpaA overexpression widely affects the transcript levels of genes involved in different stages of cytokinesis, including the gene expression of proteins related to morphological features in the cell wall PG layer (Fig. 5). Interestingly, zipN was induced higher than other genes in the rpaA-overexpressing strain. ZipN (Z-ring interacting protein N, alternative name Ftn2) plays a central role in the assembly of the *Synechocystis* divisome (Marbouty et al., 2009b), as it functions to link the Z-ring to the membrane, alternative assembly of downstream cytokinetic factors at the divisome (Marbouty et al., 2009b; Mazouni et al., 2004). The hypothetical cyanobacterial divisome is illustrated in Fig. 1, which was modelled based on protein-protein interactions detected using bacterial two-hybrid assays and co-immunoprecipitation assays (Marbouty et al., 2009a, b). Among the divisome components, CdV3 (a DivIVA-like protein), which is presumably involved in destabilization and positioning of the Z-ring, was shown to interact only with ZipN (Marbouty et al., 2009b). Consistent with this finding, we observed that the mRNA expression of both cdv3 and zipN was induced by rpaA overexpression (Fig. 4). In *Synechocystis*, CdV3-depleted mutant results in giant cells and ZipN-depleted mutant exhibits spiralled cell shape (Marbouty et al., 2009b). The effects of overexpression of these genes on cell division have not been examined. In *Synechococcus*, the overexpression of minC, minD, minE, cdv3 and zipN genes inhibits the cell division (Ducat et al., 2018). Taken together, it is possible that an optimal ratio of these divisome components is required to achieve cell division, as has been shown for the cytokinetic factors FtsZ, ZipA, and FtsA in *E. coli* (Rueda et al., 2003). Here, we could not find any consensus sequences in the promoter regions of the target genes. Therefore, it is obscure whether RpaA directly regulates the expression of the target genes or not. To investigate whether RpaA directly or indirectly regulates the gene expression in vivo, chromatin immunoprecipitation (ChIP) assay should be performed in the future.

In this study, the cellular surface structures of ROX310 and GT cells were apparently different by transmission electron microscopic observation (Figs. 5M and N). In order to reveal the morphological changes in detail, further studies are needed. However, it is possible that such morphological changes in ROX310 cells may weaken the contractile force required for cytokinesis, thus interfering with cytokinesis. Yet, the mechanism of force generation in bacterial cell division remains controversial (Erickson and Osawa, 2017).

According to Köbler et al. (2018), the transcript levels of cytokinetics-related genes were not altered in rpaA deletion mutant compared with wild-type, except for ylmD level is higher by DNA microarray analysis. Thus, it is possible that the cytokinesis may not be transcriptionally regulated only by RpaA. Interestingly, using co-immunoprecipitation-coupled mass spectrometry analysis, they also indicated FtsZ, ZipN, MinD and MurC proteins are potential RpaA interaction partners (Köbler et al., 2018). Therefore, RpaA might regulate cell division by not only transcriptionally, but also posttranslationally.

*Synechocystis* possesses *hik8* (sasA) and *hik24* (cikA), which are orthologs of *Synechococcus* Hiks that are coupled to RpaA and are presumably involved in the circadian signal transduction pathways and day/night cycles. Previous experiments by our group showed that the Hik8-RpaA system regulates primary metabolism, including sugar and amino acid metabolism, in response to the light/dark transition (Iijima et al., 2015; Osanai et al., 2015). Thus, RpaA may fine tune the rate of cell division during light/dark transition. It is possible that the cell division regulation by RpaA is needed to resolve discrepancy between circadian rhythms and environmental light cycle such as seasonal variations in the length of the daytime period. To demonstrate the involvement of RpaA in light/
dark cycle, the protein levels of RpaA and the phosphorylated form of RpaA in cells should be examined by higher resolution over light/dark cycles. Unlike Synechococcus, Synechocystis can survive light-activated heterotrophic growth conditions (Anderson and McIntosh, 1991). Therefore, despite the presence of RpaA and Hiks orthologs, the day/night cycles and cell division cycles in Synechocystis may be connected via a different mechanism from the one in Synechococcus. To determine this putative mechanism, further studies are needed to explore the signalling pathways that integrate environmental and temporal information by carefully monitoring cell division dynamics under several growth conditions.

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Conflicts of interest: No conflicts of interest declared.

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Supplementary Materials
Supplementary figure is available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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