

## Diel Expression of Cell Cycle-Related Genes in Synchronized Cultures of *Prochlorococcus* sp. Strain PCC 9511

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**The cell cycle of the chlorophyll *b*-possessing marine cyanobacterium *Prochlorococcus* is highly synchronized under natural conditions. To understand the underlying molecular mechanisms we cloned and sequenced *dnaA* and *ftsZ*, two key cell cycle-associated genes, and studied their expression. An axenic culture of *Prochlorococcus* sp. strain PCC 9511 was grown in a turbidostat with a 12 h–12 h light-dark cycle for 2 weeks. During the light periods, a dynamic light regimen was used in order to simulate the natural conditions found in the upper layers of the world's oceans. This treatment resulted in strong cell cycle synchronization that was monitored by flow cytometry. The steady-state mRNA levels of *dnaA* and *ftsZ* were monitored at 4-h intervals during four consecutive division cycles. Both genes exhibited clear diel expression patterns with mRNA maxima during the replication (S) phase. Western blot experiments indicated that the peak of FtsZ concentration occurred at night, i.e., at the time of cell division. Thus, the transcript accumulation of genes involved in replication and division is coordinated in *Prochlorococcus* sp. strain PCC 9511 and might be crucial for determining the timing of DNA replication and cell division.**

Most knowledge about the regulation of bacterial cell division and replication of DNA stems from the analysis of only three species, *Escherichia coli* (43, 44), *Caulobacter crescentus* (35, 37), and *Bacillus subtilis* (25, 34). In some cyanobacteria these processes are reported to be under the control of a circadian clock (1, 5, 15, 18, 24, 39). However, studies directly concerning the diel expression of cell cycle-relevant genes in cyanobacteria are scarce (21).

The cell cycle of the marine cyanobacterium *Prochlorococcus* is characterized by a well-defined and discrete DNA synthesis phase, S (42). In the field, the cell cycle is highly synchronized by the daily alternation of night and day. DNA replication occurs in late afternoon, and cell division occurs at night (15, 20, 41, 42). It is not known at which stage or by which regulatory mechanism the linkage between cell cycle and environmental conditions is achieved. Experiments in which the time of light onset was changed suggested that the passage from darkness to light (equivalent to sunrise) might be involved in timing of DNA synthesis in *Prochlorococcus* (16a).

To elucidate potential components involved in the synchronization and diel control of cell cycle progression in *Prochlorococcus*, the genes *dnaA* and *ftsZ* were cloned and analyzed. The GTP-binding protein FtsZ is widely distributed among eubacteria, archaea, and plastids and is usually considered the key factor in the initiation of cell division by the formation of a ring-shaped structure that recruits several other proteins (FtsA, FtsQ, and FtsW) to the division site (8). FtsZ has also recently been found in a mitochondrion (2), where it is normally replaced by Dynammin (reviewed in reference 9). DnaA is

a ubiquitous bacterial protein that acts as a helicase to initiate DNA replication in eubacteria. In *E. coli*, it recognizes asymmetric 9-bp AT-rich elements, called DnaA boxes, near the origin of replication, *oriC*. Furthermore, it acts as a repressor for its own expression and as a transcriptional regulator for other genes (23).

Laboratory cultures of *Prochlorococcus* are difficult to grow and to maintain axenically. Other obstacles are that average cell densities reached by *Prochlorococcus* in culture are lower than for most bacteria and that its cell size is particularly small (0.6  $\mu\text{m}$  on average), leading to low biomass yields (27). Here, this issue was resolved using a large-volume turbidostat exposed to a dynamic light regime, with irradiance progressively varying in a bell curve-like fashion between 0 and about 1,000 quanta (micromoles meter<sup>-2</sup> second<sup>-1</sup>) during the 12-h photoperiod (6). These conditions allowed us to simulate average light conditions found in the upper mixed layer of oceanic waters near the equator. Using this system, the expression of *ftsZ* and *dnaA* was monitored in synchronized cultures of *Prochlorococcus* sp. strain PCC 9511.

### MATERIALS AND METHODS

**Culture conditions and sampling.** Two replicate 10-liter turbidostat cultures of the axenic *Prochlorococcus* sp. strain PCC 9511 (31) were grown in PCR S11 medium (27) in 20-liter polycarbonate flasks, placed in a thermoregulated bath at  $21 \pm 1^\circ\text{C}$  and under a cycle of 12 h of light and 12 h of dark (L/D) (light from 8:00 a.m. to 8:00 p.m.). In two related studies, these times were shifted by 2 h in order to have solar noon at 12:00 (6, 12). These papers report details about the turbidostat setup and light systems (6) as well as the expression of photosynthetic genes (12).

During the photoperiod, cells were illuminated by two symmetrical computer-controlled banks of light bulbs (OSRAM DuluxL 55 W daylight) providing a modulated irradiance varying in a sinusoidal way from 0 to 970 quanta or  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . After 15 days of acclimation to these conditions, the two turbidostat cultures were sampled during four consecutive photocycles. One of the two replicate turbidostats was used for measuring a variety of photosynthetic param-

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TABLE 1. Bacteria and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source or reference
<b>Strains</b>		
<i>E. coli</i> XL1-Blue	F <sup>-</sup> <i>recA1 end gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacIZΔM15 Tn10</i> (Tet <sup>r</sup> )]	Stratagene
<i>Prochlorococcus</i> sp. strain PCC 9511	Axenic strain	31
<b>Plasmids</b>		
pGEM-T	Ap <sup>r</sup> cloning vector, dT-tailed <i>EcoRV</i> site	Promega
pPCCdnaA540	pGEM-T with 540-bp <i>dnaA</i> PCR fragment cloned into the dT-tailed <i>EcoRV</i> site	This study
pPCCrnpB	pGEM-T with 300-bp <i>rnpB</i> fragment cloned into the dT-tailed <i>EcoRV</i> site	A. Schön
pMOSBlue	Ap <sup>r</sup> cloning vector, dT-tailed <i>EcoRV</i> site	Amersham
pPCCftsZ571	pMOSBlue with 571-bp <i>ftsZ</i> PCR fragment cloned into the <i>EcoRV</i> site	This study
pBluescript SK(+)	Ap <sup>r</sup> cloning vector	Stratagene
p4K13	4,790-bp <i>HindIII</i> fragment containing <i>ddlB</i> (partial), <i>ftsQ</i> , <i>ftsZ</i> , <i>panB</i> , and a part of <i>hemN</i> cloned in pBluescript	This study
pPCCZAP4372	pBluescript SK(-) 4,372-bp fragment containing ORF1 (partial), ORF2, ORF3, <i>dnaA</i> , and a part of ORF4	This study

eters every 2 h, as detailed elsewhere (6), while the second was sampled for RNA (400 ml) every 4 h. Cell concentration and DNA distributions were analyzed on SYBR green I-stained cells in both cultures using flow cytometry (22).

**Preparation and analysis of DNA.** Most DNA manipulations were carried out according to standard protocols (36). *Prochlorococcus* sp. strain PCC 9511 DNA was purified from freeze-dried cells as described previously (14). PCR was performed using 10 ng of DNA, 10 pmol of each primer, 250 μM concentrations of each deoxynucleoside triphosphate, 2 U of *Taq* DNA polymerase (Qiagen) or AmpliTaq Gold (Perkin-Elmer), and 1× *Taq* buffer supplemented with 2.5 mM MgCl<sub>2</sub>. After initial denaturation at 93°C for 5 min, the reaction mixtures were heated at 93°C for 45 s. Annealing was performed for 45 s at 54°C for a 571-bp *ftsZ* fragment or 62°C for the amplification of a 540-bp PCC 9511 *dnaA* fragment. Elongation occurred at 72°C for 1 min. After 35 cycles, the final step at 72°C was extended for 5 min. Plasmid and PCR product purification was done using commercial kits (Qiagen and Genomed). Products of cycle sequencing reactions (BigDye terminator cycle sequencing kit) were separated on an ABI 373 automatic sequencer (Applied Biosystems Inc., Perkin Elmer). The 571-bp PCR fragment of *ftsZ* obtained by primers FTF and TFR (3) was cloned, yielding pPCCftsZ571 (strains and plasmids are reported in Table 1). This plasmid served as a template to generate single-stranded RNA probes or as a probe in Southern hybridization to isolate the *ftsZ* coding region from a size-selected *HindIII* plasmid minibank in vector pBluescript SK(+). A genomic library of *Prochlorococcus* sp. strain PCC 9511 was established by the ligation of partially digested *EcoRI* fragments into λZAPII (Stratagene). This library was screened for *dnaA* by hybridization using the *Prochlorococcus marinus* SS120 *dnaA* gene as a probe (30). The RNase P probe was kindly provided by Astrid Schön, Würzburg, Germany. Primers for the generation of probes and sequencing are listed in Table 2. Preliminary sequence data for *Prochlorococcus* MED4 were obtained from the DOE Joint Genome Institute at [http://spider.jgi-psf.org/JGI\\_microbial/html/](http://spider.jgi-psf.org/JGI_microbial/html/).

**RNA extraction and Northern hybridization.** RNA was extracted from 400 ml of culture for each sampling point as described (10). Northern hybridization was carried out at 61.5°C for single-stranded RNA probes and 50°C for DNA probes in 120 mM sodium phosphate buffer (pH 7.2)—250 mM NaCl—7% sodium dodecyl sulfate (SDS)—50% formamide. RNA probes were produced with 1.85 MBq of [<sup>32</sup>P]UTP (Amersham) using the Maxiscript transcription kit (Ambion). Plasmids pPCCftsZ571 and pPCCdnaA540 served as templates. RNase protection assays were performed in accordance with the manufacturer's instructions (RPAIII kit; Boehringer).

**Immunology.** For protein extraction, cells were collected by centrifugation, disrupted by adding 0.1% SDS, sonicated three times for 10 s each time at 4°C with a Sonopuls HD 60 set at 50% of maximum power, and incubated twice at 95°C for 5 min. Protein concentrations were measured using the Bio-Rad protein assay. A polyclonal antiserum against recombinant *Anabaena* sp. strain PCC 7120 FtsZ (courtesy of C.-C. Zhang) was used for expression analysis (19). Western blots were prepared from total proteins separated on SDS—12% polyacrylamide gels (normalized to 1 μg per lane) and blotted on Hybond-C extra membranes (Amersham). Incubation with antisera was performed at titers of 1:1,000 (FtsZ antibody). Secondary antisera were conjugated with horseradish peroxidase, and blots were developed with the chemiluminescence substrate SuperSignal (Pierce). Signals were quantified using PCBAS 2.09 software.

**Nucleotide sequence accession numbers.** The sequences reported in this paper have been deposited in the EMBL database under the accession numbers AJ011025 and AF158628.

## RESULTS

**Organization of the genomic regions encoding DnaA and FtsQ to FtsZ in *Prochlorococcus* sp. strain PCC 9511.** The gene arrangement around *dnaA* (Fig. 1A) is very unusual compared to gene arrangements in other eubacteria. However, it is apparently highly conserved among different *Prochlorococcus* strains, since almost the same arrangement is found in *P. marinus* SS120 (30). ORF1 shows pronounced similarity to the gene encoding YCF25 (AAC08078), a protein encoded in the plastid genome of *Porphyra purpurea* (29). The other hypothetical proteins are similar to the products of several ORFs in *Synechocystis* sp. strain PCC 6803 (17). Over the whole length, the amino acid sequence of strain PCC 9511 DnaA (463 amino acids) is 85% identical to that of SS120 (461 amino acids) and 49% identical to that of *Synechocystis* sp. strain PCC 6803. The *ftsZ* gene of *Prochlorococcus* sp. strain PCC 9511 is preceded by two genes highly similar to

TABLE 2. Desoxyoligonucleotides used for the generation of probes and DNA sequence analysis

Name	Sequence
FTF	.....AATGC(CT)GTTAACCG(GC)ATGATT
TFR	.....GCC(CT)(GT)AC(AG)TC(AT)GCAAA(AG)
dnaAPCCSP12	.....CTTGGAGGAAGGAATATACACAAGAAG
dnaAPCCRT3	.....GCTTAGATCAGTTCCTGCCTC
SPFT 3	.....CCAATACCTGGATTCCCTCTGCTC
SPFT 4	.....TGCAGGAGCTCCACTTCAAGAAGC
SPFT 5	.....GATTGTTGGGGTCAATTAGGTCG
SPFT 6	.....ACAGGTAACCTGTTATTGCAACAGGTTT
SPFT 7	.....AGGCTCATCACTTGGTATTTGAA
SPFT 8	.....CATGTTAGGAATGAGACTTAAAGAGGG
SPFT 9	.....TTTAAACGCCAAACCTGGTCAG
SPFT 9b	.....CTGGTCAGGATCGGAAGTAG
SPFT 10	.....GCTGATCCAGAGGTACAGAATGTTATTT
SPFT 11	.....AAAAGGGAATATTTGCTTAAAAACTGAAAC
SPFT 12	.....TAAAGATATTGCTATTAGAAGTTGTAGAGCAT
SPFT 13	.....TGAATAAGAACTTCAACCGATTTGAG
SPFT 14	.....AGAGAGCTAGAGAGTGGATGGAT
SPFT 15	.....GAATGGGTAGCAGCCGAAAAG
SPFT 16	.....AACCACTTGCCCAATTCCTAAAAA

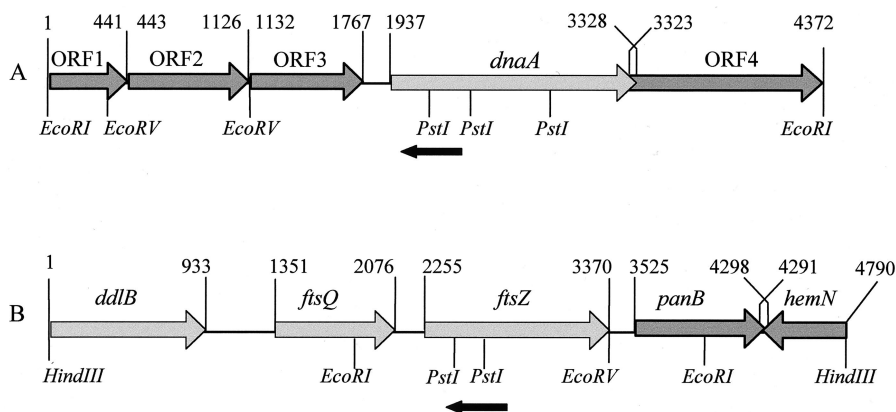


FIG. 1. Genomic region containing *ftsZ* and *dnaA* in *Prochlorococcus* sp. strain PCC 9511. (A) The *dnaA* gene is framed by four putative genes, ORF1 to ORF4, close homologues of which reside at corresponding sites in *P. marinus* sp. strain SS120 (30). (B) Organization of the *ftsZ* locus. The numbers designate putative gene start and stop codons (GenBank accession no. AJ011025 for *ftsZ* and AF158628 for *dnaA*). Genetic symbols are as follows: *ddlB*, D-alanine—D-alanine ligase; *ftsQ*, filamentous temperature-sensitive Q; *ftsZ*, filamentous temperature-sensitive Z; *panB*, 3-methyl-2-oxobutanoate hydroxymethyltransferase; and *hemN*, oxygen-independent coproporphyrinogen III oxidase. The locations of antisense RNA probes used in the expression analysis are indicated by arrows.

genes present in the cell wall and division gene cluster of *E. coli*. These genes encode a D-alanine—D-alanine ligase (8) and FtsQ, an intermediate recruit to the division site (7). PCC 9511 lacks a homologue of *ftsA*, which is located in *E. coli* between *ftsZ* and *ftsQ*. Scanning of the total genome sequence of *Prochlorococcus* sp. strain MED4 confirmed the absence of an *ftsA* homologue in the genome of that strain. The gene downstream of *ftsZ* shows similarity to *panB* and is followed by a putative homologue of *hemN*. Both genes overlap by 8 bp at their 3' ends. In *E. coli* the product of *hemN* catalyzes the oxidative decarboxylation of coproporphyrinogen III to form protoporphyrinogen IX (40). Database searches show that FtsZ of *Prochlorococcus* sp. strain PCC 9511 has the highest identity to FtsZ from the marine *Synechococcus* strain WH8103 and a significantly lower similarity to three other cyanobacterial FtsZ proteins (Table 3). Both nucleotide sequences determined in this study (4,372 nucleotides [nt] for *dnaA* and 4,790 nt for *ftsZ*) are 100% identical to the respective DNA segments in the total genome of *Prochlorococcus* sp. strain MED4, indicating that these two strains might be genetically the same organism.

**Synchronization by modulated L/D cycles.** The turbidostat culture of PCC 9511 was maintained in exponential growth at an average density of  $9.76 \times 10^7 \pm 3.1 \times 10^7$  cells ml<sup>-1</sup> during the 4 days of sampling (data not shown). Flow cytometric

analyses indicated that the cell cycle was highly synchronized and that the daily alternation of cell cycle phases was very similar every day throughout the experiment (Fig. 2). From the beginning to the middle of the light period, almost all cells of the population were in the G<sub>1</sub> phase. At the end of the day about 70% of the cell population had entered the S phase. Two hours after virtual sunset, this population proceeded through G<sub>2</sub>, and in the middle of the night (6 h after the end of the light period), most cells had divided and were back in G<sub>1</sub>.

**Transcript accumulation in synchronized *Prochlorococcus* sp. strain PCC 9511 cultures follows a diel rhythm.** The steady-state level of *ftsZ* and *dnaA* mRNA showed considerable temporal variation and oscillated during the course of each cell cycle in a periodic way (Fig. 3). To detect minor differences in the total amount of RNA per lane, the RNA component of RNase P was used as an internal standard. The expression of *ftsZ* and *dnaA* peaked at the end of the light period (i.e., 10 h after light onset). This time corresponded to the S-phase maximum. In a parallel study using the same material, diel expression of several photosynthetic genes was shown but with maxima at time points very different from those for *ftsZ* and *dnaA* (12).

**Immunodetection of FtsZ.** Antibodies raised against the recombinant FtsZ of *Anabaena* sp. strain PCC 7120 detected a single protein band of about 50 kDa in the *Prochlorococcus* sp.

TABLE 3. Sequence identity between the FtsZ protein of *Prochlorococcus* sp. strain PCC 9511 and that of other cyanobacteria and chloroplasts<sup>a</sup>

Strain	Identity (%) to:						Accession no.
	1	2	3	4	5	6	
1. <i>Prochlorococcus</i> sp. strain PCC 9511	100	84	70	74	70	64	AJ011025
2. <i>Synechococcus</i> sp. strain WH 8103		100	67	73	68	62	AAC72389
3. <i>Synechocystis</i> sp. strain PCC 6803			100	78	80	62	S77393
4. <i>Synechococcus</i> sp. strain PCC 7942				100	79	68	AAC26227
5. <i>Anabaena</i> sp. strain PCC 7120					100	69	P45482
6. <i>Physcomitrella patens</i> sp. strain FtsZ1						100	CAA04845

<sup>a</sup> The alignment is available upon request.

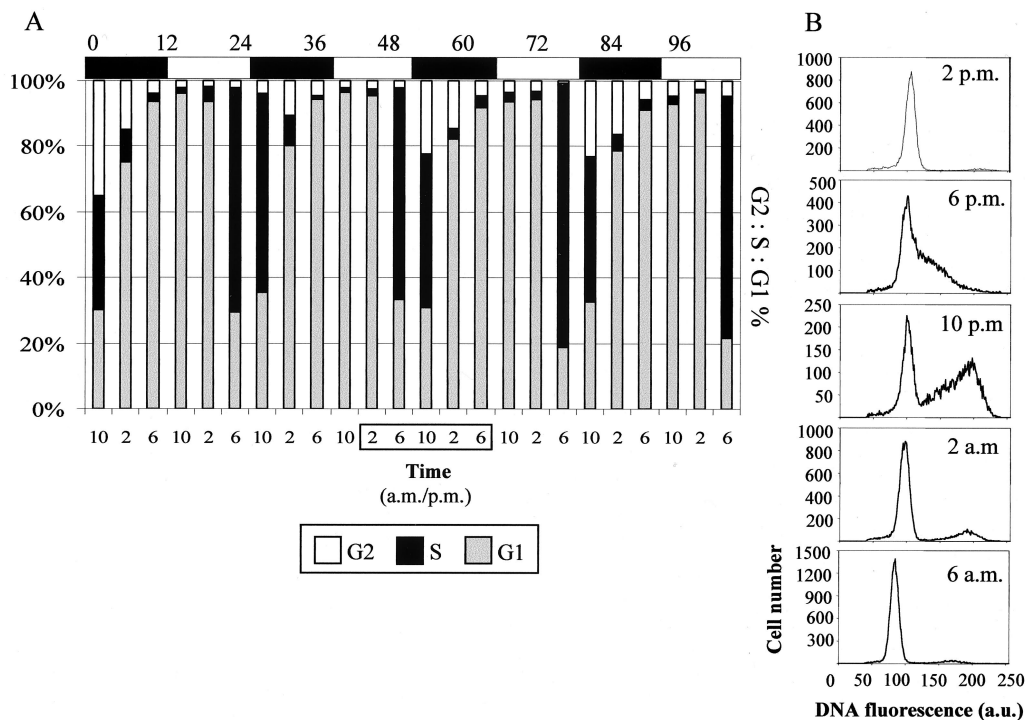


FIG. 2. Synchronization of *Prochlorococcus* sp. strain PCC 9511 by modulated L/D cycles. (A) Distribution of the cell population over the different cell cycle phases at each sampling point during four consecutive L/D cycles; (B) Flow-cytometric DNA fluorescence distributions for five representative time points (boxed). a.u., arbitrary units.

strain PCC 9511 lysate (Fig. 4A). This size corresponds well to that obtained for *Anabaena* (19). To analyze FtsZ abundance at different cell cycle stages, total cell proteins from three consecutive days were immunoblotted using this serum. Although expression changed slightly from day to day, the overall pattern was similar during the three consecutive photocycles. FtsZ concentration reached a minimum during the light period (when the number of cells in G<sub>1</sub> is maximum) and increased during the S and the division phases (Fig. 4B). Two- and fourfold dilutions of the sample taken 26 h after the beginning of the experiment indicated that the amount of FtsZ varied by

a factor of 2 to 4 during a 24-h L/D cycle (Fig. 4C). This variation did not result from loading variability, since immunostaining of the same membrane using an antiserum against the photosynthesis protein PsbO did not reveal a comparable drop during the light period (data not shown).

## DISCUSSION

The genome region surrounding *ftsZ* in *Prochlorococcus* sp. strain PCC 9511 is partially conserved compared to that in *E. coli*, where the genes are clustered in the order *ddlB-ftsQ-ftsA-*

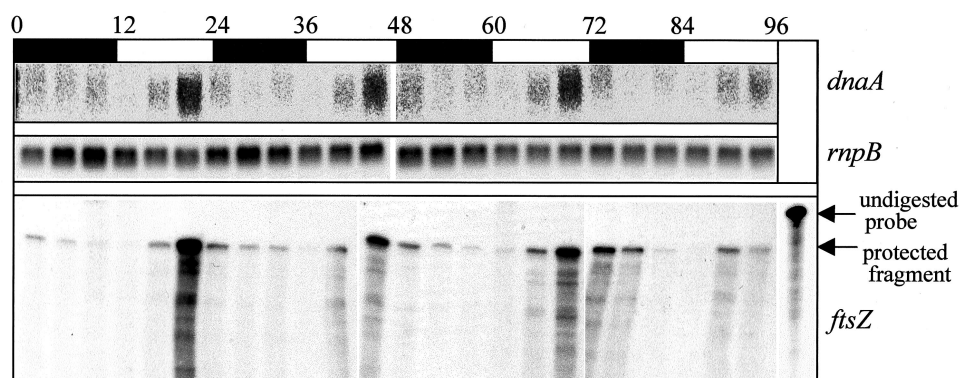


FIG. 3. Transcript levels of *dnaA* (top) and *ftsZ* (bottom) in the turbidostat culture of *Prochlorococcus* sp. strain PCC 9511. Light (8 a.m. to 8 p.m.) and dark (8 p.m. to 8 a.m.) phases are shown by the black and white bars, respectively. Samples were taken at 4-h intervals. The RNA levels were determined by Northern hybridization (*dnaA*) or RNase protection assays (*ftsZ*). The size of the undigested *ftsZ* RNA probe was 621 nt, and that of the full-length *ftsZ* protected fragment was 571 nt. A DNA probe for *rnpB* was used to assess the amounts of RNA loaded per lane.

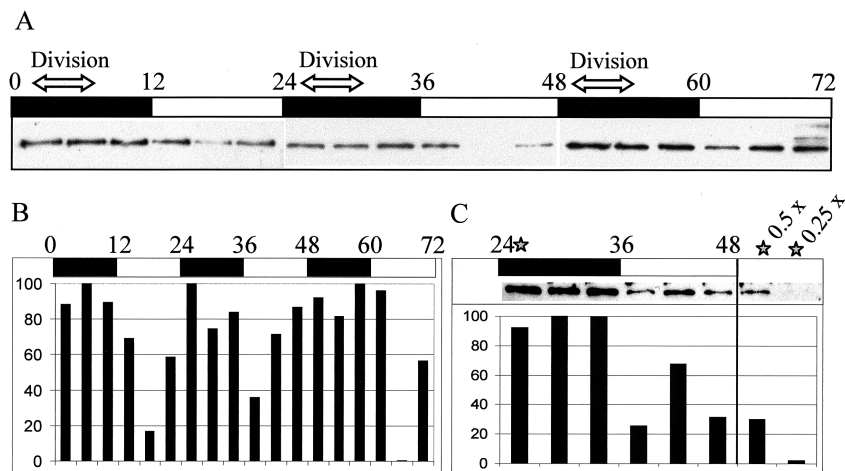


FIG. 4. Detection of FtsZ by immunoblotting. (A) FtsZ level in *Prochlorococcus* sp. strain PCC 9511 during three L/D cycles. Light and dark phases are displayed by black and white bars, respectively. (B) Graphic representation of FtsZ expression. (C) Semiquantitative assessment of the relative amount of FtsZ. Samples of the second L/D cycle were blotted together with two- and fourfold dilutions of the first sample of that day (taken at 26 h [stars]).

*ftsZ*. The apparent lack of *ftsA*, which is essential in *E. coli* (33), might be interpreted as an example of how genome minimization has been achieved during the evolution of the small genome of *Prochlorococcus* (38). The gene arrangement around *dnaA* is even less conserved than in *E. coli*, but it is similar to that previously found in *Prochlorococcus* SS120 (30). DnaA of the latter strain, expressed in vitro, recognized the *oriC* of *E. coli* and *B. subtilis*, suggesting a similar molecular basis for the initiation of replication in these eubacteria (30). The synteny of this genome region between the two *Prochlorococcus* strains is seemingly trivial. However, other markers are very different between these strains, e.g., multiple *pcb* genes (11) and a phycoerythrin gene cluster are present in strain SS120 (14), but not in strains MED4 and PCC 9511 (28, 31). The degree of 16S rRNA identity between the strains lies in the same range (98%) as that between members of different genera of enterobacteria.

We show here that *ftsZ* and *dnaA* mRNA levels covary and are maximally expressed during the S phase. A simultaneous expression of genes like *dnaA* and *ftsZ* might well constitute the molecular basis for coordinated timing between DNA synthesis and cell division. For *E. coli*, cell cycle-related variations in the amount of *ftsZ* mRNA have previously been demonstrated (13, 16, 32). However, the method used to achieve synchronization in our study (simulation of a natural light regimen) is completely different from those used for heterotrophic bacteria. The oscillation of *ftsZ* mRNA abundance is partially matched by changes at the protein level. Synthesis of FtsZ starts during the S phase, and the concentration reached a maximum at night, i.e., at a time at which the mRNA level has clearly dropped again. Although we only roughly determined the timing of FtsZ expression, the maximum FtsZ level seems to correlate well with the onset of cell division. For *E. coli*, a titration mechanism that triggers cell division once a certain amount of FtsZ per cell is reached has been postulated (26). As far as we know, the amount of FtsZ actually required has never been assessed. As shown here, such changes in FtsZ concentration might be rather small, given that in *Prochloro-*

*coccus* the decrease in FtsZ amount per unit of total cellular protein during the day was on the order of about 50 to 75% only.

The factors that coordinate the synchronous expression of genes such as the cell cycle genes *dnaA* and *ftsZ* will have to be further investigated. They could involve a circadian clock, as in the case of *Synechococcus* sp. strain PCC 7942 (18). Alternatively, they could be under the direct control of light through photoreceptors. Finally, these genes could be expressed when a specific cell constituent or cell property, such as size (4), reaches a critical threshold. The total genome sequences of three different *Prochlorococcus* strains to be available within the near future will become a powerful tool to elucidate these mechanisms in detail.

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