

Biochemical Evidence for a Timing Mechanism in *Prochlorococcus*^{∇†}

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Organisms coordinate biological activities into daily cycles using an internal circadian clock. The circadian oscillator proteins KaiA, KaiB, and KaiC are widely believed to underlie 24-h oscillations of gene expression in cyanobacteria. However, a group of very abundant cyanobacteria, namely, marine *Prochlorococcus* species, lost the third oscillator component, KaiA, during evolution. We demonstrate here that the remaining Kai proteins fulfill their known biochemical functions, although KaiC is hyperphosphorylated by default in this system. These data provide biochemical support for the observed evolutionary reduction of the clock locus in *Prochlorococcus* and are consistent with a model in which a mechanism that is less robust than the well-characterized KaiABC protein clock of *Synechococcus* is sufficient for biological timing in the very stable environment that *Prochlorococcus* inhabits.

Cyanobacteria are photosynthetic prokaryotes that are known to possess a true circadian clock. Gene expression and other biological activities follow rhythmic cycles with a circa 24-h period. Rhythmic behavior is maintained even in the absence of environmental stimuli such as light and temperature. The underlying core oscillator consisting of the clock proteins KaiA, KaiB, and KaiC is the only characterized prokaryotic circadian oscillator. It was previously demonstrated that these three proteins, together with ATP, can produce 24-h oscillations of KaiC phosphorylation *in vitro* (17). The essential roles of KaiA and KaiB in oppositely influencing KaiC phosphorylation are well documented for the oscillator of “*Synechococcus elongatus*” PCC 7942 (hereafter *S. elongatus*), the species for which most bacterial circadian research has been conducted. Thus, it is puzzling that marine cyanobacteria of the genus *Prochlorococcus*, probably the most abundant photosynthetic organisms on Earth (5, 28), contain homologs of only two of these clock proteins, KaiC and KaiB (3, 11, 20). Laboratory cultures (10) as well as natural *Prochlorococcus* populations (24) display a rhythmic cell cycle together with a daily periodicity of gene expression that can be explained by the functioning of a circadian clock. Alternatively, these rhythms could be controlled directly by the daylight (10). The functional role of the Kai proteins from *Prochlorococcus* has remained entirely unclear and has not been experimentally addressed thus far.

In the well-studied protein clock of *S. elongatus*, KaiC hexamers are at the center of the circadian oscillator, combining three intrinsic enzymatic activities: autokinase, autophosphatase, and ATPase. KaiA and KaiB modulate KaiC's activ-

ities in opposite manners. KaiA seems to be essential for the shift between autophosphatase and autokinase, and for generating KaiC phosphorylation rhythms, by stabilizing C-terminal residues of KaiC, the A-loops (12). Thus, the absence of KaiA should have consequences for the enzymatic activities of the remaining Kai proteins of *Prochlorococcus*. In this study, the previously unknown functions of the *Prochlorococcus* sp. strain MED4 protein KaiB (ProKaiB) and ProKaiC are examined. In our *in vitro* experiments, we analyzed the recombinant proteins ProKaiB and ProKaiC in direct comparison to the core oscillator of *S. elongatus*, which consists of *S. elongatus* KaiA (SynKaiA), SynKaiB, and SynKaiC. We show here that both clock proteins from *Prochlorococcus* sp. strain MED4 independently exhibit their known biochemical functions, although the influence of ProKaiB on ProKaiC dephosphorylation is different certainly due to the absence of KaiA, the third protein of the oscillator. For ProKaiC, we demonstrate ATPase activity as well as the phosphorylation of serine 427 (S427) and threonine 428 (T428) using mass spectrometry and high-resolution sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Moreover, we suggest that the deletion of *kaiA* is compensated by the enhanced autophosphorylation activity of ProKaiC. Our results might have further implications for the analysis of a possible timing mechanism in other bacterial species, such as purple bacteria that encode KaiB and KaiC homologs but that lack the KaiA component.

MATERIALS AND METHODS

Bacterial strains and culture. Plasmids pMED4KaiC and pMED4KaiB were constructed by PCR amplification of the *kaiC* and *kaiB* sequences (CyanoBase accession numbers IPR013503 and IPR013474, respectively) from *Prochlorococcus* sp. strain MED4 using primers MED4_kaiC_fw (5'-GGATCCAAAGATAAAAAATTAGTAAATC-3'), Med_kaiC_rev (5'-GCGGCCGCTAATTTTTTCAATTCCT-3'), MED4_kaiB_fw (5'-GGATCCGTGGCAAGAAAAAC-3'), and MED4_kaiB_rev (5'-GCGGCCGCTAATTTTTTGTACCTC-3'). PCR fragments were subcloned into the pGEM-T vector (Promega), digested with BamHI and NotI, and cloned into the respective restriction endonuclease sites of vector pGEX-6P-1.

The recombinant *Prochlorococcus* glutathione *S*-transferase (GST)-KaiB and

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GST-KaiC fusion proteins were produced in *Escherichia coli* BL21 cells. For the production of the recombinant ProKaiC and ProKaiB proteins, cells were cultured overnight at 37°C in 100 ml of LB medium (1) containing 75 µg ml⁻¹ of ampicillin. The ProKaiC expression culture was diluted in 1 liter of LB medium and incubated for 5 h at 18°C. After the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), cells were incubated at 18°C for 60 h before harvest. The expression of ProKaiB was carried out for 21 h at 37°C. IPTG (1 mM) was added after the first 3 h. Cell pellets were resuspended in 20 ml of cold extraction buffer (50 mM Tris HCl [pH 8.0], 150 mM NaCl, 1 mM ATP, 5 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM EDTA) and lysed by the addition of 17,000 U lysozyme (BioChemika) and 125 U of Benzonase nuclease (Novagen) and sonication. The recombinant circadian clock proteins from *Synechococcus elongatus* PCC 7942 were produced in *E. coli* BL21 strains kindly provided by T. Kondo (Nagoya University, Japan). The recombinant GST fusion proteins ProKaiB, ProKaiC, SynKaiA, SynKaiB, and SynKaiC were purified using glutathione-Sepharose 4B and PreScission protease (GE Healthcare, Uppsala, Sweden) as described previously (9, 19). Protein concentrations were estimated according to methods described previously by Lowry et al. (15) as well as by a visual comparison of the different protein amounts on Coomassie-stained SDS gels.

Phosphosite mapping of KaiC from *Prochlorococcus* sp. strain MED4. In order to generate nonphosphorylated forms of ProKaiC, samples of 10 µg ProKaiC from two different preparations (prepared in the presence of 0.2 mM ATP or 0.5 mM ATP) were treated with 2 U of λ protein phosphatase (New England Biolabs) for 6 h. As a control, samples were incubated without phosphatase for 6 h. After phosphatase treatment, phosphorylated and nonphosphorylated forms of ProKaiC were separated by SDS-PAGE (11% T with 0.67% C) according to methods described previously (18).

Mass spectrometric analysis of ProKaiC phosphorylation sites was performed essentially as described previously (22, 23). Briefly, Coomassie-stained protein bands were excised from SDS-PAGE gels, minced, reduced with Tris(2-carboxyethyl)phosphine, and alkylated with iodoacetamide prior to proteolytic digestion. In four parallel in-gel reactions, KaiC was digested with the proteases proteinase K, thermolysin, elastase, and trypsin. The resulting peptides were extracted from the gel, and phosphopeptides were enriched from the complex peptide mixture on a TiO₂ column. Mass spectrometry was performed using a reversed-phase nanoflow liquid chromatograph coupled to a tandem mass spectrometer (CapLC and Q-ToF Micro; Micromass, Manchester, United Kingdom). Peak lists from nanoflow liquid chromatography (NanoLC)-tandem mass spectrometry (MS/MS) raw data were generated by Mascot Distiller 2.0 (Matrix Science, London, United Kingdom). Mascot Server 2.1 (Matrix Science) was used for database searching against the SwissProt database. ProKaiC phosphorylation sites were mapped using Phosm software (22).

In vitro assay of KaiC phosphorylation and dephosphorylation. Phosphorylation and dephosphorylation assays were performed as described previously (17), with [γ-³²P]ATP, but were carried out at 18°C when ProKaiC was used. Images were analyzed by Personal Molecular Imager FX (Bio-Rad) and Quantity One software (Bio-Rad).

ATPase assay. The analysis of ATPase activity is based on measuring the concentration of the released orthophosphate colorimetrically (14, 27). The assay was carried out with 0.3 µM KaiC (and 0.3 µM KaiB) of *Prochlorococcus* sp. strain MED4 (at 18°C) or KaiC of *Synechococcus* (at 30°C) as a control. Aliquots were sampled every 2 h. Assay buffer served as a negative control. The final buffer composition in the incubation mixtures was 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 mM ATP, 1 mM dithiothreitol, and 1 mM EDTA. Data were corrected for negative control and zero-time values.

Genome data and software tools. Cyanobacterial genomes were downloaded from the NCBI GenBank server (<ftp://ftp.ncbi.nih.gov/GenBank/genomes/Bacteria/>). For visualization of genomes and gene arrangements as well as for extraction of gene and intergenic sequences, Artemis software (22) was used. Sequence similarities and E values were calculated using NCBI BLASTp (23, 24). Multiple alignments were constructed with CLUSTALW 1.83 (25), and the phylogenetic 16S rRNA tree was constructed using the neighbor-joining method (26).

RESULTS

kaiA gene deletion during evolution of the kai operon. Most of the research on the cyanobacterial clock has been done on the model freshwater unicellular cyanobacterium *S. elongatus*. The genome of this organism contains single copies of the three *kai* genes, which are located together in a single gene

cluster. However, the set of core clock genes differs among the various groups of cyanobacteria. Many cyanobacterial genomes contain more than just one copy per *kai* gene, possibly indicating additional layers of complexity within the clock. In contrast, cyanobacteria belonging to the *Prochlorococcus* group do not even harbor one complete set of genes for the three-protein oscillator. Comparative analyses revealed that all *Prochlorococcus* genomes sequenced so far contain the genes for the KaiB and KaiC proteins but lack a *kaiA* gene. Moreover, it can be demonstrated at the sequence level that the *kaiA* clock component of the *kai* operon was deleted by genome reduction within the genus *Prochlorococcus* (for further details, see the supplemental material) (6). From this fact, the question arose of whether the remaining genes still encode functional ProKaiC and ProKaiB proteins and, therefore, could be assumed to be involved in a timing mechanism of the living *Prochlorococcus* cell.

Conservation of KaiC phosphorylation sites S427 and T428 in *Prochlorococcus* sp. strain MED4. To drive the well-studied KaiC phosphorylation cycle, sequential phosphorylation reactions of S431 and T432 are important (18, 21). The phosphorylation state of these two amino acid residues regulates autokinase and autophosphatase activities of KaiC from *S. elongatus*. Indeed, within the amino acid sequence of KaiC from *Prochlorococcus* sp. strain MED4, we found a serine (S427) next to a threonine (T428) residue aligning to the known phosphorylation sites S431 and T432 in KaiC of *S. elongatus* (Fig. 1A).

Using high-resolution SDS-PAGE for the optimal separation of different KaiC phosphorylation forms, we obtained mainly one band for recombinant ProKaiC after purification (Fig. 1B). After incubation of ProKaiC with λ protein phosphatase, faster-migrating protein bands appeared (Fig. 1C), possibly representing dephosphorylated ProKaiC forms. In order to identify the phosphorylation state of ProKaiC in the three different migrating bands, we mapped phosphorylation sites by NanoLC-MS/MS (see Fig. S4 in the supplemental material for MS/MS spectra). In the mass spectrometric analysis, we detected peptides representing three phosphorylation states in ProKaiC (see Table S1 in the supplemental material). The main fraction (Fig. 1B, top band) consisted of the double-phosphorylated (pS427 and pT428) form. Only relatively long periods of incubation (4 to 6 h) with a phosphatase resulted in significant amounts of singly phosphorylated (pS427 or pT428) and nonphosphorylated KaiC forms as found in the middle and bottom bands, respectively, in Fig. 1B. Thus, high-resolution SDS-PAGE and mass spectrometric analyses of *Prochlorococcus* sp. strain MED4 KaiC revealed that the two amino acid residues S427 and T428 can be phosphorylated in this species (Fig. 1A), suggesting that sequential phosphorylation reactions might also underlie the daily timing mechanism in *Prochlorococcus* sp. strain MED4.

KaiC from *Prochlorococcus* sp. strain MED4 shows autophosphorylation without KaiA. Although KaiC sequences of *S. elongatus* and *Prochlorococcus* sp. strain MED4 are very similar (identities of 373/497 [75%]) (see Fig. S3 in the supplemental material), several residues differ within the C tail and the adjacent region, termed the A-loop (12) (Fig. 1A). These A-loops were previously proposed to possess an important function, as they likely determine the steady-state level of phosphorylation of KaiC (12). In that model for *S. elongatus*, KaiA

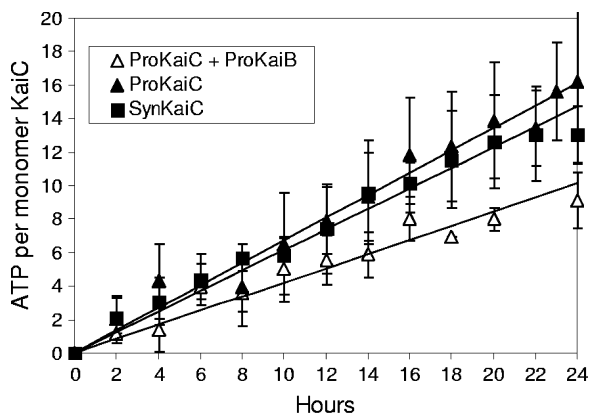


FIG. 3. ProKaiB reduces the ATPase activity of ProKaiC. ATPase assays were performed using the malachite green colorimetric method. Aliquots were sampled every 2 h. Assay buffer served as a negative control.

DISCUSSION

For the model organism *S. elongatus*, all three *kai* genes, *kaiA*, *kaiB*, and *kaiC*, are essential for circadian timing, and an inactivation of any of them abolishes it (7). The three Kai proteins have been demonstrated to constitute the *S. elongatus* core oscillator (17). KaiA stimulates the autophosphorylation activity of KaiC, whereas KaiB enhances its intrinsic dephosphorylation activity. In contrast, cyanobac-

teria belonging to the *Prochlorococcus* group do not harbor the complete set of *kai* genes. Here, *kaiA* became deleted during an evolutionary reduction process streamlining the *Prochlorococcus* genome. However, genes encoding KaiB and KaiC remained at the genome locus and exhibit a high level of sequence similarity to the homologous sequences of *S. elongatus*. Thus, we analyzed biochemically if KaiC as well as KaiB from *kaiA*-lacking *Prochlorococcus* sp. strain MED4 are functional proteins. Indeed, we found residues S427 and T428, homologous to S431 and T432, the sites of KaiC phosphorylation in the *S. elongatus* oscillator, to also be phosphorylated in KaiC of *Prochlorococcus* sp. strain MED4 (Fig. 1A). Thus, sequential phosphorylation reactions at S427 and T428 might also underlie the daily timing mechanism of *Prochlorococcus* sp. strain MED4 (21). However, KaiC from *Prochlorococcus* sp. strain MED4 showed pronounced autokinase activity without KaiA (Fig. 2A) and remained hyperphosphorylated even in the presence of KaiB (Fig. 2B); this finding is in contrast to *S. elongatus* KaiC, which is an autophosphatase in the absence of KaiA.

Recently, it was demonstrated for *S. elongatus* that KaiA is critical for the shift between the autophosphatase and autokinase activities of KaiC resulting in the phosphorylation-dephosphorylation rhythms of the 24-h period (12). KaiA stabilizes the C-terminal residues of KaiC, termed the A-loops. For *Prochlorococcus* species, which lack *kaiA* homologs, a different mechanism has been suggested. Here, ProKaiC has been predicted to possess a high steady-state

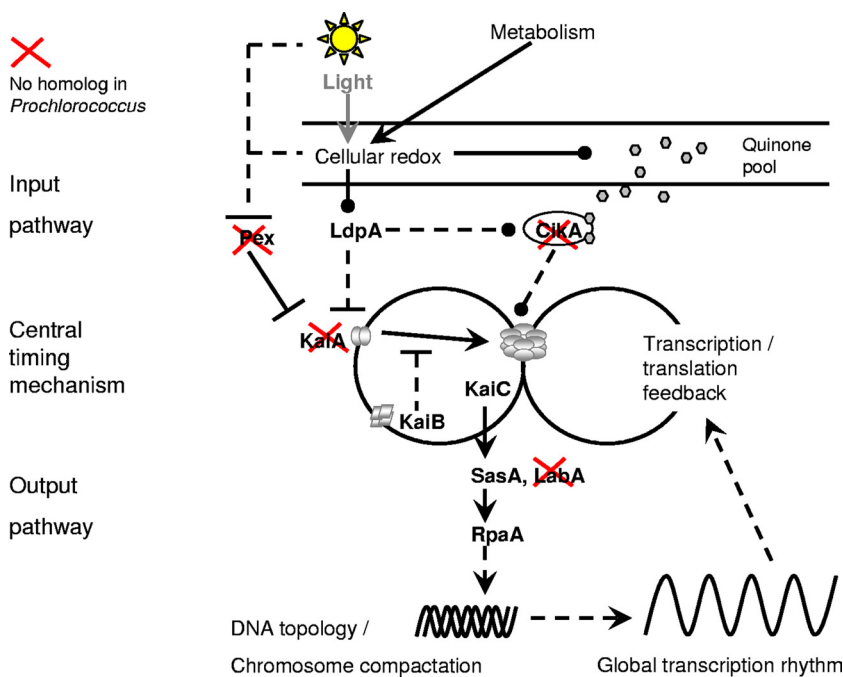


FIG. 4. Scheme illustrating a putative molecular mechanism for the circadian clock in *Prochlorococcus* sp. strain MED4 in comparison to *S. elongatus*. The scheme is adapted from data described previously by Dong and Golden (2), with permission of the publisher. The homologous components that are present or absent (red crosses) in *Prochlorococcus* sp. strain MED4 are indicated. In the input pathway, CikA and Pex are missing in *Prochlorococcus* sp. strain MED4, whereas a gene, PMM1560, homologous to LdpA was found by a BLASTp search against the genome. The central timing mechanism of *Prochlorococcus* sp. strain MED4 lacks the KaiA component. In the output pathway, SasA (PMM1077) and RpaA (PMM0128) might influence DNA topology by underlying global gene expression rhythms. An alternative pathway via LabA was recently suggested for *S. elongatus* (25) but is missing in *Prochlorococcus* sp. strain MED4. Solid lines indicate direct effects, and dotted lines indicate indirect or unknown mechanisms. Arrows indicate the direction of signaling. Blunt ends represent inhibition, and circle ends indicate an unspecific direction.

level of phosphorylation caused by a dynamic equilibrium that favors the exposed state of the A-loop (12). Thus, KaiC of *Prochlorococcus* seems to be a natural variant that favors autokinase activity and, thereby, compensates for the genome deletion of *kaiA*. In *S. elongatus*, constitutive KaiC hyperphosphorylation (13) does not abolish in vivo circadian gene expression, although the KaiC phosphorylation rhythm is disrupted. This finding suggests that an alternative pace-maker exists in living cells. A transcriptional/translational feedback loop has been proposed to lead to independent oscillations in the cell (13), a situation that might be relevant for *Prochlorococcus* species as well.

In our experiments, ProKaiB was able to stimulate the dephosphorylation of the SynKaiC protein, as does KaiB from *S. elongatus* (Fig. 2B); however, it had no effect on ProKaiC autokinase or autophosphatase activities, raising the question of the likely function of KaiB in *Prochlorococcus*. We reasoned that KaiB may modulate the ATPase, rather than autokinase or autophosphatase, activities of KaiC in *Prochlorococcus*. It was previously proposed that ATP hydrolysis might be the most fundamental reaction that defines the 24-h period of the clock (26). We measured a weak ATPase activity of ProKaiC (16 molecules of ATP per day) (Fig. 3), comparable to that of *S. elongatus* (26), which was reduced after the addition of ProKaiB (Fig. 3). Thus, in the *Prochlorococcus* system, the main mechanism of KaiBC function might be related to the regulation of ATPase activity.

In experiments with synchronized *Prochlorococcus* cultures, it was previously shown that robust 24-h rhythms of DNA replication and gene expression found under alternating 12-h-light and 12-h-dark periods rapidly damp under continuous light (6). Thus, it was previously proposed that a core timing mechanism and output apparatus are functional in *Prochlorococcus* but that they work in an hourglass-like fashion, requiring a daily resetting, rather than as a self-sustained oscillator (6). The biochemical properties that we have observed are consistent with such a daily timing mechanism for *Prochlorococcus* and might be important for other prokaryotes, e.g., species of proteobacteria and archaeobacteria, possessing *kaiB* and *kaiC* genes but also lacking the KaiA component (4).

The reduced timing mechanism of *Prochlorococcus* might be part of a similarly reduced network combining input and output pathways. Compared to the molecular mechanism suggested previously for *S. elongatus* (2), only one input pathway and one output pathway would remain, taking into account the homologous components present in *Prochlorococcus* sp. strain MED4 only (Fig. 4). All other genes have been deleted during evolution. In the reduced network of *Prochlorococcus*, an input signal might be transmitted via the LdpA homolog, PMM1560, which is likely sensitive to the redox state of the cell (8), into the central timer consisting of KaiC and KaiB. The central timing mechanism might be stabilized by an outer transcription/translation feedback loop, where the amount of phosphorylated KaiC regulates its own transcription (16). The output pathway via homologs to SasA, PMM1077, and RpaA, PMM0128, could forward the timing signal to the DNA and/or influence global gene expression.

The temperature-compensated posttranslational oscillator made of KaiA, KaiB, and KaiC might provide additional ro-

business when conditions are changing. Natural *Prochlorococcus* populations are highly synchronized to a stable environment in specific niches of the Earth's oceans; they divide exactly once a day and at the same time (6). Thus, an extremely robust oscillator like that in *S. elongatus* might not be required for the growth of marine cyanobacteria and, thus, was reduced during evolution.

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