

Timing the day: what makes bacterial clocks tick?

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Abstract | Chronobiological studies of prokaryotic organisms have generally lagged far behind the study of endogenous circadian clocks in eukaryotes, in which such systems are essentially ubiquitous. However, despite only being studied during the past 25 years, cyanobacteria have become important model organisms for the study of circadian rhythms and, presently, their timekeeping mechanism is the best understood of any system in terms of biochemistry, structural biology, biophysics and adaptive importance. Nevertheless, intrinsic daily rhythmicity among bacteria other than cyanobacteria is essentially unknown; some tantalizing information suggests widespread daily timekeeping among Eubacteria and Archaea through mechanisms that share common elements with the cyanobacterial clock but are distinct. Moreover, the recent surge of information about microbiome–host interactions has largely neglected the temporal dimension and yet daily cycles control important aspects of their relationship.

Diurnal

Active during the day.

Nocturnal

Active during the night.

Hourglass timer

A simple non-oscillatory timer that is set in motion and keeps track of time linearly, and thus does not self-sustain a cycle.

Photoautotrophic bacteria

Bacteria that derive their energy exclusively from light to drive photosynthetic carbon fixation and the synthesis of organic compounds.

Many bacteria experience substantial environmental changes during the day and night. Most free-living bacteria are exposed to daily cycles of light and darkness, and/or temperature. The gut microbiota is also exposed to daily changes in the intestinal environment, as most animal hosts eat on a daily schedule — usually during the day for diurnal animals and during the night for nocturnal animals — which creates a daily rhythm of feast and fast in the digestive system. Although endogenous circadian rhythms were once thought to be an exclusive characteristic of eukaryotic organisms, all defining properties of circadian clocks (BOX 1) were also found in cyanobacteria^{1–3}. Many processes are circadian in cyanobacteria^{4–12}; for example, nitrogen-fixing unicellular cyanobacteria rhythmically fix nitrogen during the night, approximately twelve hours out of phase from photosynthesis, which peaks at mid-day¹³. This is adaptive biochemistry, as nitrogenase, a nitrogen-fixing enzyme, in these cyanobacteria is inactivated by even tiny amounts of oxygen, and its activity is thus incompatible with photosynthesis, which releases oxygen. This is one simple example of why bacteria may have evolved timekeeping systems to coordinate metabolic events during the day to optimize performance and/or to temporally separate potentially conflicting metabolic processes. Whereas the timekeeper of cyanobacteria is well established as a prototypical circadian clock, other bacteria may have evolved alternative timekeeping systems, such as an hourglass timer, which confers a fitness advantage¹⁴.

What might other functions of a daily timekeeper in bacteria be? The most common hypothesis is that having a ~24 h timekeeper enables the cell to anticipate and/or prepare for the daily transformation of environmental conditions that affect many bacteria. Anticipation might be most easily discerned in the case of photoautotrophic bacteria, such as cyanobacteria (FIG. 1), owing to the fitness disadvantage of maintaining energetically expensive photosynthetic pathways during the night. Thus, bacteria that anticipate dawn by activating quiescent photosynthetic processes late in the night could have an advantage over bacteria that merely react to dark-to-light transitions. Anticipating daily changes might also facilitate protective responses in bacteria that are exposed to the damaging effects of sunlight (especially UV light; FIG. 1). Even visible sunlight can be absorbed by cytochromes in the electron-transport chain and affect metabolism¹⁵. The effects of sunlight inspired the ‘escape from light’ hypothesis, which proposes that circadian clocks evolved owing to the selective pressure of daily cycles of light and darkness, in which light impaired growth^{16,17}.

A gut bacterium that anticipates when fresh nutrients are entering the digestive tract could have an advantage in the competition for resources. Furthermore, gut bacteria may also benefit from daily timing when they attempt to survive outside of the host following excretion, in a similar manner to bacteria that are always exposed to the external world (FIG. 1). Therefore, bacteria that have a daily timekeeping mechanism, even if it is not a complete

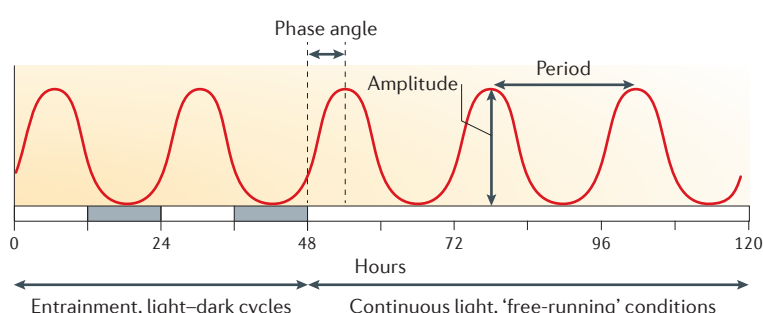
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Box 1 | What constitutes a circadian clock?

Circadian rhythms are defined by three well-established phenomenological criteria and not by their molecular mechanism⁹⁸ (see the figure). The first criterion is that they persist with a self-sustained oscillation of approximately 24 h under constant conditions (that is, under constant temperature and either constant light or constant darkness). The expression of the endogenous clock under constant conditions is termed a 'free-running' rhythm. The second criterion is that these endogenous rhythms of approximately 24 h can be entrained to exactly 24 h by daily cyclic cues in the environment, such as light and darkness, temperature, and feeding and fasting. Finally, these rhythms are temperature compensated, so that they proceed at almost the same rate and period in a physiological range of constant ambient temperatures. Another common property, but not a defining characteristic, is conditionality; that is, endogenous rhythmicity is robustly expressed under some environmental conditions but not others^{99,100}. The fascination of this phenomenon is to explain how its biochemical mechanism can robustly sustain a long period of oscillation (~24 h) with high precision, and how it enhances fitness in the natural environment.



circadian system such as those found in cyanobacteria, probably have adaptive advantages. In this Review, we highlight recent mechanistic insights into circadian oscillators in cyanobacteria and timekeepers in other bacteria^{14,18,19}. Moreover, we consider the potential adaptive value of both oscillating clocks and hourglass timers. We also examine recent research on the evolution of proto-circadian rhythms and how the relationship between a host and its microbiota is influenced by biological timekeeping^{14,20–25}.

Timekeeping mechanisms in cyanobacteria

In cyanobacteria, the clock system controls multiple output processes, including the transcriptome^{4–6}, the proteome⁷, the timing of cell division^{8,9}, nitrogen fixation^{1,13}, chromosomal topology^{6,10,11} and metabolism¹². In photoautotrophic cyanobacteria, there are changes in metabolite composition during the day and night, and, owing to the self-sustained function of the clock, these changes persist in continuous light¹². Even the redox status of *Synechococcus elongatus* cells oscillates in continuous light and may influence the oxidation status of peroxiredoxins^{26,27}. Interestingly, changes in metabolism that depend on the light and dark cycle, in particular redox status and/or energy levels, can directly entrain the cyanobacterial clockwork^{28–31}. Metabolic feedback might even provide mechanisms to compensate for temperature and other changes to enable the clock to maintain accurate timekeeping³².

The core mechanism of the circadian clock in eukaryotic cells is widely thought to be based on a transcription–translation feedback loop (TTFL)^{33,34}, although

there is evidence that this model may be incomplete or inaccurate^{26,35–37}. In cyanobacteria, initial experiments also supported a TTFL model³⁸. However, the remarkable discovery that three cyanobacterial clock proteins (KaiA, KaiB and KaiC) could reconstitute a circadian oscillator *in vitro* for many days³⁹ led to our current understanding of the clock system in cyanobacteria; a biochemical oscillator is the core pacemaker, which operates in (and regulates) a larger TTFL that, in turn, controls outputs and replenishes the essential proteins of the oscillator^{40–43} (FIG. 2a). At the present time, the cyanobacterial clock is the best understood of any circadian mechanism from a biochemical and biophysical perspective.

The key component of the cyanobacterial clock is KaiC^{38,44}. This protein has an astonishing range of biochemical activities, including autokinase, autophosphatase, phosphotransferase and ATPase, and is the core of a rhythmically associating and dissociating nanocomplex (FIG. 3). KaiC is a double hexamer that consists of two similarly sized rings, CI and CII, and a carboxy-terminal region in CII that interacts with KaiA through protruding peptide 'tentacles' (REF. 44). Both rings catalyse the hydrolysis of ATP⁴⁵. As described below, the ATPase activity of the CI ring provides the rate-limiting reaction of the timing circuit^{19,45}, whereas the ATP that is hydrolysed by the CII ring catalyses sequential phosphorylation and dephosphorylation reactions of serine and threonine residues^{44,46–50}. The level of KaiC phosphorylation is a convenient marker of circadian phase *in vivo* and *in vitro*, including when transcription and/or translation are suppressed^{51,52}. As neither transcription nor translation is required for KaiC phosphorylation to oscillate, it is a post-translational oscillator (PTO; FIGS 2a,3).

Phosphorylation and the nanocomplex cycle. The status of KaiC phosphorylation is not merely a marker of the circadian phase, it also regulates the association and dissociation of the KaiA–KaiB–KaiC nanocomplex, thereby switching KaiC between its autokinase and autophosphatase states (FIG. 3). The rhythm begins with unphosphorylated KaiC. KaiA stimulates the intrinsic autokinase activity of KaiC through interaction with the CII-terminal tentacles. This leads to the phosphorylation of two key residues in the CII domain, T432 followed by S431 (REFS 46,47,49,53–56). Dephosphorylation proceeds in the same order, phosphorylated T432 (pT432) followed by pS431, such that the overall process from the hypophosphorylated to the hyperphosphorylated and back to the hypophosphorylated state of KaiC follows the sequence: (T432/S431) to (pT432/S431) to (pT432/pS431) to (T432/pS431) to (T432/S431)^{46,47} (FIG. 3). The dephosphorylation phase proceeds, at least partially, by regenerating ATP from CII-bound ADP and the phosphates that are bound to T432 and S431 (REFS 57,58). This remarkable phosphotransferase activity limits the overall consumption of ATP, which possibly preserves clock function when cellular ATP concentrations fluctuate; the cyanobacterial clock accurately tracks time during extended exposure to the dark, when cells are not able to produce ATP by photosynthesis^{28,32,51,52,57}.

Proto-circadian

A broad term that includes hourglass timers, damped oscillators and other potential timekeeping mechanisms that may be ancestral systems that include some, but not necessarily all, of the canonical properties of circadian clocks. Proto-circadian systems may be a step along an evolutionary trajectory that might ultimately lead to a bona fide circadian system.

Peroxiredoxins

A class of antioxidant enzymes that control peroxide levels.

Entrain

The process whereby the period of a biological rhythm becomes equal to that of an environmental cycle (for example, of light and dark). Entrainment also establishes a stable phase relationship between the entraining cycle and the biological rhythm.

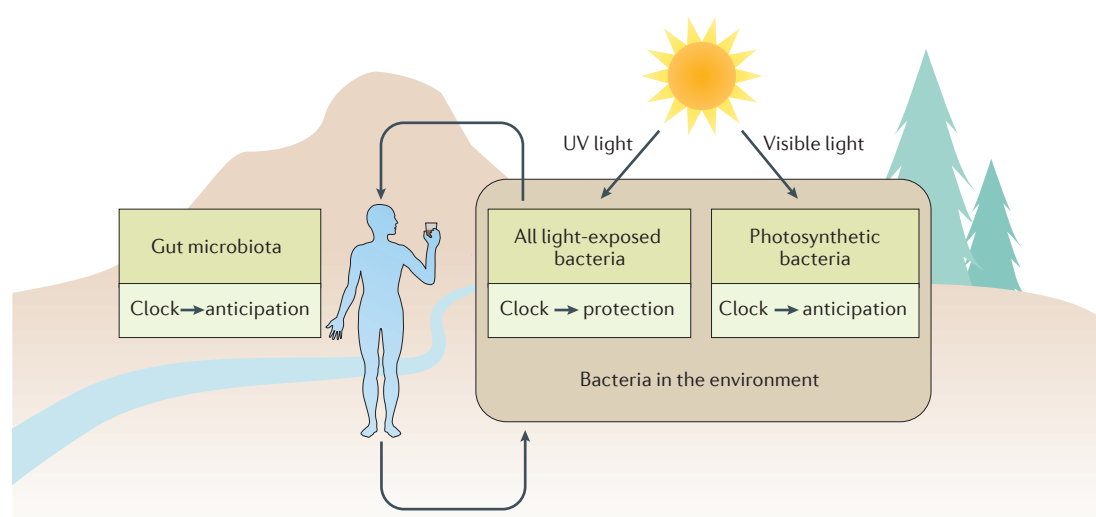


Figure 1 | Many bacteria are exposed to daily selective pressures. Free-living bacteria can be exposed to daily cycles of light and temperature that affect viability (for example, exposure to UV light) and/or provide energy (for example, through photosynthesis). Even the gut microbiota is often exposed to daily cycles of nutrients, owing to the rhythmic eating habits of the host, and temperature, as most animal hosts have daily rhythms of body temperature that are metabolically controlled in endotherms and behaviourally controlled in ectotherms. These same bacteria may be exposed to daily environmental cycles of light and temperature following excretion from the gut.

Concomitant with the phosphorylation and dephosphorylation cycle is a rhythm of association and dissociation of the KaiA–KaiB–KaiC nanocomplex (FIG. 3). At the beginning of the nanocomplex cycle, KaiA rapidly interacts intermittently with the CII tentacles of KaiC^{59–62}. This transient interaction stimulates the phosphorylation of KaiC (or inhibits dephosphorylation, thereby shifting the reaction towards phosphorylation)^{57,58,63}, and when S431 of KaiC achieves the (T432/pS431) state in the phosphorylation sequence, KaiB binds to KaiC. A recent study reported that KaiB exists in two diametrically different conformations: a ground state (gsKaiB) that does not interact readily with KaiC, and a substantially fold-shifted state (fsKaiB) that binds to the CI domain¹⁸ (FIG. 3). The fsKaiB state is conformationally similar to a structural motif of SasA, which is a histidine kinase that interacts with KaiC and transmits its oscillatory information. fsKaiB competes with SasA for binding to KaiC and thus regulates the downstream transcriptional cascade of SasA and its response regulator RpaA^{30,64–69} (FIG. 2a). In addition to displacing SasA from CI of KaiC, KaiB forms a complex with CI domains that also stably sequesters and inactivates KaiA, so that KaiA can no longer stimulate the phosphorylation of KaiC, and KaiC switches to its autophosphatase mode^{18,41,47,70} (FIG. 3). When KaiC fully dephosphorylates, KaiB and KaiA are released, which restarts the nanocomplex and phosphorylation cycles (FIG. 3).

The slow pace of ATPase activity. How can this biochemical oscillator maintain such a long time constant (for example, a period of 24h)? Are the phosphorylation and nanocomplex cycles the core pacemakers of the cyanobacterial circadian clock? Similar to the TTFL, there is nothing intrinsic about these cycles that makes them

oscillate with a 24h period. Certainly, phosphorylation events and protein associations and dissociations can occur very rapidly; these biochemical reactions do not dictate a slow 24h timekeeping. Kondo and co-workers have championed the hypothesis that the ATPase activity of KaiC is the core rate-limiting reaction^{19,45} (FIG. 2b). This reaction is very slow (only 15–16 ATP molecules are hydrolysed by one KaiC monomer per day) and is temperature compensated⁴⁵. Moreover, an analysis of KaiC mutants *in vivo* and *in vitro* showed a strict correlation between the ATPase activity of KaiC and circadian frequency, which implies that the circadian period depends directly on the energy that is provided by ATP hydrolysis^{19,45}. There is even evidence for a highly damped oscillation of the ATPase activity of KaiC in the absence of KaiA and KaiB¹⁹.

However, is the ATPase hypothesis as the core pacemaker just an ‘infinite regress’, with another process underlying the slow rate? Given that many ATP-hydrolysing reactions have very fast rates, can we explain the sedate tempo of the KaiC ATPase? A structural study identified features in the CI domain of KaiC that might explain its slow ATPase activity¹⁹. The lytic water molecule that is involved in nucleophilic attack on the γ -phosphate of ATP is positioned away from the optimal position, so that hydrolysis is less favourable than in the catalytic sites of more typical ATPases, such as kinesin and F₁-ATPase. A second source of the slow activity is slow *cis*–*trans* isomerization of a crucial peptide bond, which increases the activation energy barrier of the ATPase reaction. These two features decrease the probability of ATP hydrolysis and thereby establish a remarkably slow but stable ATPase cycle in the CI domains of the KaiC hexamer¹⁹. This landmark study provided the first biochemical and biophysical explanations of how a circadian oscillator could have such a long time constant.

Ectotherms

‘Cold-blooded’ animals in which the body temperature depends on the environmental temperature but is often modulated behaviourally.

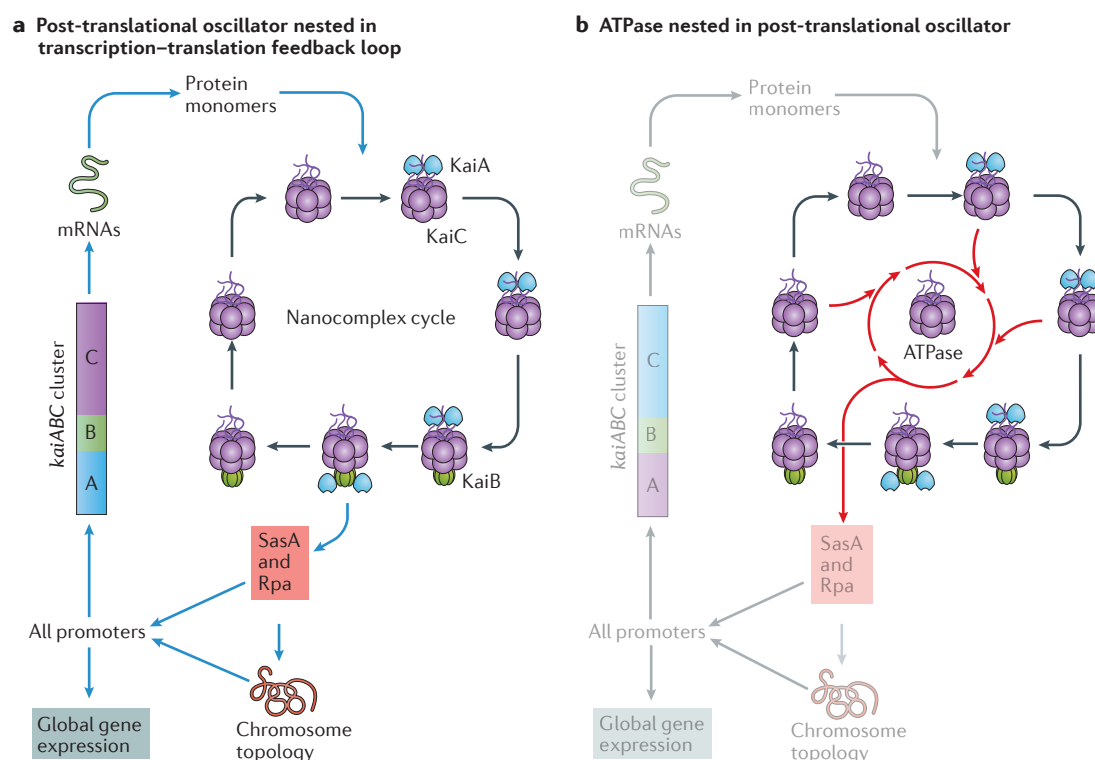


Figure 2 | Circadian molecular and genetic oscillators in cyanobacteria. a | A self-sustained post-translational oscillator (PTO) is embedded in a transcription-translation feedback loop (TTFL). The synthesis of non-phosphorylated KaiC monomers feeds into the molecular oscillator, which undergoes a cycle of association into, and disassociation from, the KaiA–KaiB–KaiC nanocomplex⁷¹. KaiC interacts with competing KaiB and SasA proteins to mediate the activity of transcriptional factors (such as RpaA) and rhythmic DNA torsion to control global transcription levels, including those that drive the expression of the essential clock genes *kaiA*, *kaiB* and *kaiC*. **b** | The core clock reaction that is embedded in the PTO is ATP hydrolysis, which is primarily catalysed by the ATPase in the CI domain of KaiC. In this view, the phosphorylation and nanocomplex cycles enhance and regulate the ATPase activity to promote robustness and temperature compensation. Part **a** is adapted with permission from REF. 42, AAAS.

Cycles within cycles: robustness and synchrony. The aforementioned studies reveal TTFL, phosphorylation–nanocomplex and ATPase cycles in the cyanobacterial clockwork (FIGS 2b,3). Why are there so many cycles? An analysis of the relationship between the PTO and the TTFL found that they have a definite hierarchical interdependency; the PTO is the core pacemaker, whereas the TTFL is a ‘slave oscillator’, which damps when the PTO stops⁷¹ and is no longer temperature-compensated under some metabolic conditions. The TTFL feeds back into the PTO through the synthesis of new clock proteins, which can reinforce and/or fine-tune the core PTO pacemaker. This coupled oscillator system is more resilient to intracellular noise⁷¹. Moreover, an analysis of cells in a microfluidic device found that the clocks of individual cells were less stable and synchrony was not maintained among cells in the population in the absence of the TTFL⁷². The advantage of coupled oscillators can not only be demonstrated mathematically^{71,73}, it also makes sense biologically. The TTFL controls transcription and translation, whereas the clock-specific nanocomplex, which is composed of KaiA–KaiB–KaiC, is a mass-action biochemical oscillator; these two types of oscillator are likely to be sensitive to different kinds of intrinsic and extrinsic cellular noise

(cell division, transcriptional noise, fluctuating metabolites, and so on). Moreover, it is very likely that the core ATPase reaction^{19,45} is stabilized and temperature-compensated through its interactions with the phosphorylation–nanocomplex and TTFL cycles. Therefore, the three nested and interacting cycles (that is, the TTFL, phosphorylation–nanocomplex and ATPase cycles; FIG. 2b) provide a resilient circadian system^{71,72,74}.

A separate but related issue regarding robustness is synchrony among the KaiC hexamers in the PTO. Remarkably, *in vitro*, KaiA–KaiB–KaiC reactions can oscillate for up to ten days without detectable dampening⁷⁵. Why is the period of each KaiC hexamer not sufficiently different from every other hexamer in the reaction so that the phasing in the population of hexamers becomes asynchronous after a few cycles? There must be a mechanism, or mechanisms, to keep the hexamers synchronized; indeed, at least two such mechanisms are known. First, a KaiC hexamer can exchange monomeric subunits with neighbouring hexamers^{59,60,76}, and this monomer exchange maintains synchrony of the phosphorylation status among the KaiC hexamers in the population^{60,75}. Another important factor for maintaining synchrony among KaiC hexamers is the sequestration and inactivation of KaiA activity during the

Halophilic

Adapted to high concentrations of salt.

dephosphorylation phase^{41,47}. Therefore, KaiC monomer exchange and KaiA sequestration ensure that KaiC hexamers remain synchronized in the PTO.

The sun also rises for other bacteria

In cyanobacteria, all three Kai proteins are required for circadian oscillations *in vivo* and *in vitro*^{38,39}. Homologues of *kaiB* and *kaiC* are distributed widely among both photosynthetic and non-photosynthetic members of the Eubacteria and the Archaea, but *kaiA* is found only in cyanobacteria^{77,78}. Even among cyanobacteria, the *kaiA* gene was lost during evolution in the important cyanobacterial genus *Prochlorococcus*, which retained *kaiB* and *kaiC* and has daily rhythms, but not a sustained circadian rhythm, under constant conditions^{79,80}. The loss of the *kaiA* gene from *Prochlorococcus* may have 'de-evolved' an original KaiA–KaiB–KaiC circadian clock to a simpler timer that is based on only KaiB–KaiC and keeps track of time similarly to an hourglass^{81,82}. Another example of a potential hourglass timer is found in the halophilic archaeal genus *Halobacterium*, which has a single-domain *kaiC* but neither *kaiA* nor *kaiB*; *Halobacterium* spp. exhibit light–dark-entrained daily transcription but not sustained oscillations under constant conditions⁸³.

Does this mean that all three Kai proteins are required for self-sustained circadian oscillations, or is it possible that a circadian clock could be composed of KaiB and KaiC, or even just KaiC alone?

In this context, the example of purple bacteria may be instructive. Purple bacteria are globally distributed members of the Proteobacteria that frequently inhabit soil and water environments together with cyanobacteria. Tests on circadian rhythmicity have been reported for two species of purple bacteria, *Rhodobacter sphaeroides*⁸⁴ and *Rhodospseudomonas palustris*¹⁴, which both have homologues of *kaiB* and *kaiC*. *R. palustris* does not sustain rhythmicity, as monitored by nitrogen fixation and the phosphorylation of KaiC, in constant conditions¹⁴ (FIG. 4), which is a defining characteristic of circadian rhythms (BOX 1). In *R. sphaeroides*, rhythms of gene expression were detected, 21 h under aerobic conditions and 11 h under anaerobic conditions, but other defining circadian characteristics, such as temperature compensation and entrainment, were not reported⁸⁴.

Recently, the daily profiles of KaiC phosphorylation during light–dark cycles, continuous darkness and continuous light were compared among *S. elongatus*, *Prochlorococcus marinus* and *R. palustris*^{14,51,71} (FIG. 4). *S. elongatus*, which contains *kaiA*, *kaiB* and *kaiC*, exhibits

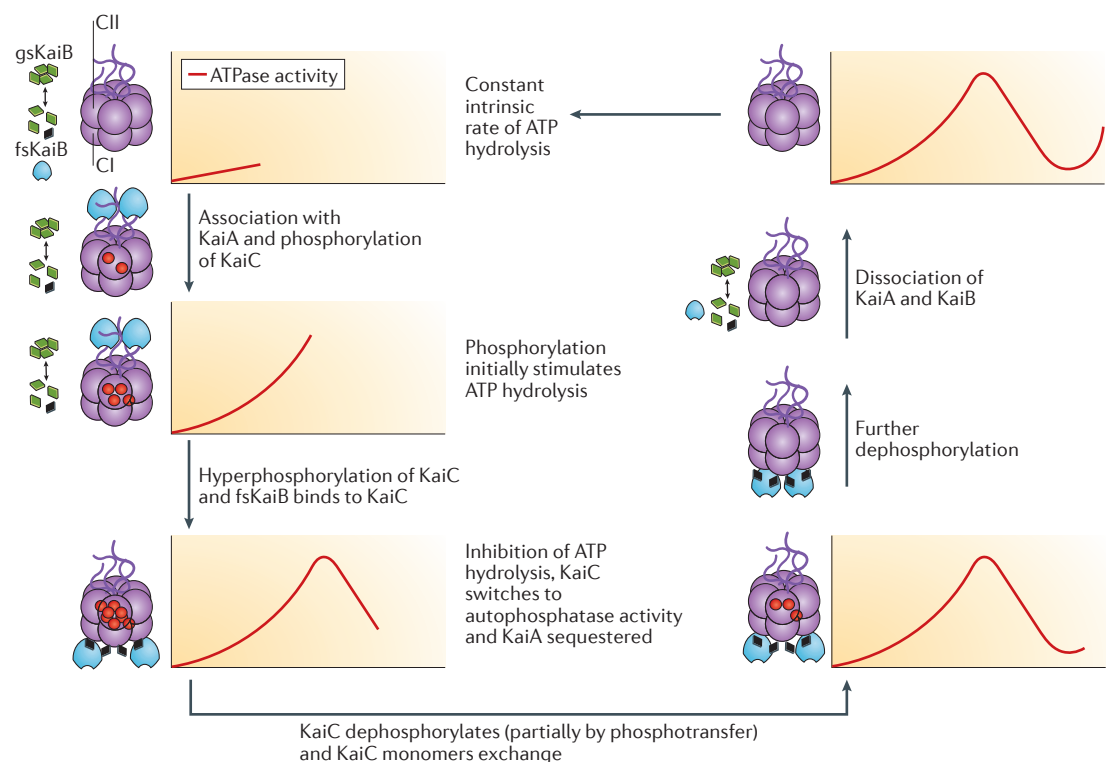


Figure 3 | The post-translational oscillator (PTO). The post-translational oscillator (PTO) is composed of rhythmic associations between KaiC (purple), KaiA (blue) and KaiB (green or black) to form a nanocomplex that regulates the ATPase and phosphorylation activities of KaiC. KaiC forms two hexameric rings, CI and CII. KaiB monomers undergo a slow transition from ground-state KaiB (gsKaiB; green diamonds) to the fold-shifted state (fsKaiB; black diamonds), which binds to the CI domain of KaiC hexamers. KaiA associates with hypophosphorylated KaiC and stimulates the autophosphorylation of KaiC (red circles indicate the added phosphates), which accelerates the rate of ATP hydrolysis. The association and dissociation of the nanocomplex coincide with changes in the phosphorylation state of KaiC; residue T432 is phosphorylated first, followed by S431, and dephosphorylation occurs in the opposite order. Adapted with permission from REF. 37, Elsevier.

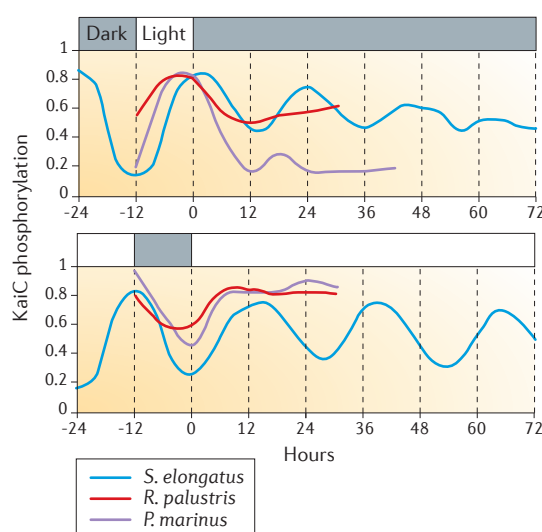


Figure 4 | KaiC phosphorylation temporal patterns differ between complete circadian clocks that consist of KaiA–KaiB–KaiC and proto-circadian KaiB–KaiC systems. The phosphorylation of KaiC in light and dark cycles, constant darkness and constant light is shown for three different species of bacteria: the two cyanobacteria *Synechococcus elongatus* and *Prochlorococcus marinus* and the purple bacterium *Rhodospseudomonas palustris*. *S. elongatus* harbours *kaiABC* and has a complete circadian system, whereas *P. marinus* and *R. palustris* only harbour *kaiBC*.

daily rhythms during light–dark cycles that are sustained in both continuous darkness and continuous light. By contrast, *P. marinus* and *R. palustris*, which contain *kaiB* and *kaiC*, exhibit daily rhythms of KaiC phosphorylation during light–dark cycles that dampen rapidly under constant conditions (FIG. 4). We previously proposed that the timekeeping system in *R. palustris* is a ‘proto-circadian’ oscillator that does not persist under constant conditions¹⁴. This hypothesis is consistent with the observation that *P. marinus* — which ‘lost’ *kaiA*^{77–79} — also does not sustain robust rhythms of gene expression or KaiC phosphorylation under constant conditions^{79–82} (FIG. 4). Taken together, the data support the hypothesis that all three Kai proteins are necessary for sustained circadian oscillations in bacteria, and that *kaiBC*-based and *kaiC*-based systems might be capable of hourglass timekeeping but not a persistent circadian oscillation^{81,82}. Given that the sequestration of KaiA promotes synchrony in *S. elongatus*^{41,47}, KaiA was perhaps an evolutionary innovation of cyanobacteria to sustain oscillations, which resulted in their robust persistent rhythms².

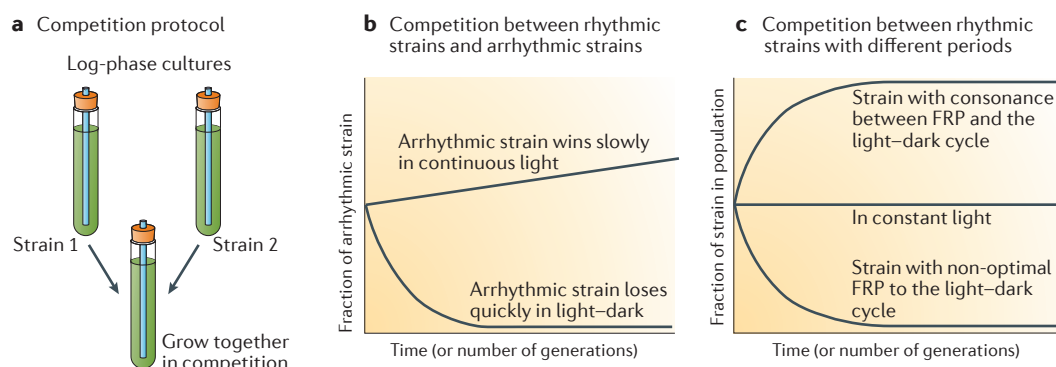
Timekeeper evolution and new criteria for testing adaptive fitness. Speculations about the potential adaptive values of timekeeping in bacteria are thought-provoking, but have there been any rigorous tests of a fitness advantage? In cyanobacteria, strains that have a functioning biological clock out-competed arrhythmic strains in rhythmic environments (for example, light–dark cycles), whereas in constant environments (for example, continuous light), rhythmic and arrhythmic strains grew at

comparable rates⁸⁵ (FIG. 5a,b). Moreover, among rhythmic strains, those that had endogenous free-running periods that closely matched the period of the environmental cycle had the greatest fitness advantage⁸⁶ (FIG. 5c). Competition experiments are a sensitive test of fitness and they clearly indicated that the cyanobacterial circadian system interacts with the environment to mediate an adaptive advantage, especially as clock mutants could out-compete wild-type cells under appropriate conditions⁸⁶.

More recently, the fitness benefits of a timekeeping programme have been assessed in *R. palustris*¹⁴. As mentioned above, *R. palustris* does not exhibit sustained oscillations under constant conditions¹⁴ (FIG. 4). Nonetheless, does KaiC carry out an adaptive time-keeping function in this purple bacterium? To address this question, the growth rates of wild-type *R. palustris* and a *kaiC*-knockout strain were compared in single-strain cultures¹⁴. Both strains grew at the same rate under non-selective continuous light conditions, but in the 24 h cycle (12 h of light and 12 h of darkness), the *kaiC*-knockout cells grew slower than wild-type cells¹⁴ (FIG. 5d,e). Therefore, KaiC enhanced growth in a 24 h light–dark cycle, but not under continuous conditions. Importantly, additional experiments with a 1 h light and 1 h dark cycle (FIG. 5f) ruled out the reduced amount of light exposure during cycles of 12 h of light and 12 h of darkness as a confounding factor. Therefore, the *kaiC* gene of *R. palustris* has an important effect on growth and fitness in 24 h cyclic environments, but not in non-24 h environments (continuous conditions or cycles of 1 h of light and 1 h of darkness); this result strongly implies that the fitness advantage of *kaiC* in *R. palustris* is related to adaptation to environments that have a 24 h periodicity¹⁴. Growth experiments similar to the ones described above can be considered as new criteria to assess whether KaiC is involved in an adaptive 24 h timekeeping mechanism in any bacterium¹⁴.

What might be the selective pressures that led to the evolution of timers versus oscillators? In a sufficiently regular environment, a damped oscillator or even an hourglass timer might enable adequate anticipation of daily changes in the environment (FIG. 6). Some chronobiologists have questioned the necessity of a sustained circadian rhythm; as circadian clocks evolved in a cyclic world (not continuous conditions), it is the mechanism of entrainment that evolved and not self-sustained oscillations⁸⁷. Nevertheless, among eukaryotes, daily biological clocks are almost universally self-sustained circadian systems. How and why is this the case? Perhaps studying the evolution of daily timekeepers in bacteria can help to address these questions, especially in bacteria that have *kai* homologues that can be identified and targeted for fitness tests, as shown in FIG. 5. For example, a bacterial species that is always exposed to a regular light–dark cycle with a consistent photoperiod and consistent temperature might effectively adapt to the cyclic world with an hourglass timer (FIG. 6). However, an oscillator enables several outputs and/or flexible phasing, even if the oscillator is damped and requires a relatively consistent light–dark cycle, such as the *kaiBC*-based system of *R. palustris* (FIG. 6). Finally,

Tests by competition of clock strains



Single-strain growth tests under timing-specific conditions

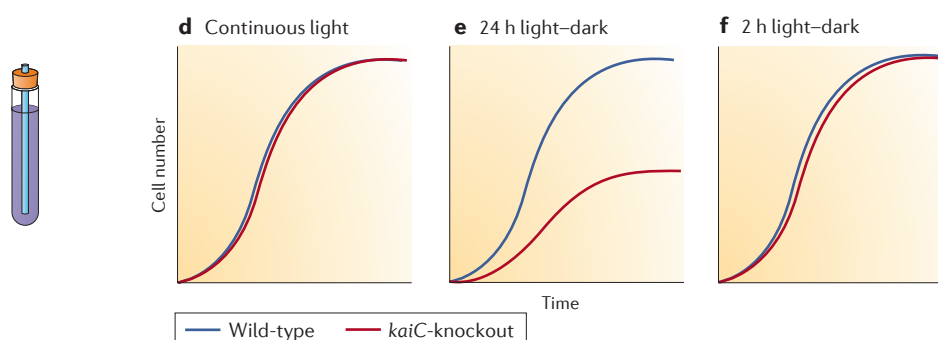


Figure 5 | Criteria for testing the adaptive fitness of daily timekeeping mechanisms. **a** | The fitness advantages of timekeeping can be assessed by competition between strains in mixed cultures under different environmental conditions. **b** | In competition between rhythmic and arrhythmic strains of *Synechococcus elongatus*, the arrhythmic strain is rapidly out-competed by the rhythmic wild-type strain in light-dark cycles, but slowly outgrows the wild-type strain in constant light, which provides no selective pressure for circadian timekeeping. **c** | In competition among strains that are rhythmic, a strain that has an endogenous rhythm (free-running period (FRP)) that closely matches the environmental light-dark cycle out-competes strains with non-optimal free-running periods. Under continuous light, all strains are maintained in the population. **d** | Fitness can also be inferred from single-strain growth rates of cultures of wild-type or *kaiC*-knockout cells in the purple bacterium *Rhodospseudomonas palustris* growing phototrophically. Under continuous light, *KaiC*-dependent timekeeping confers no advantage to the growth of *R. palustris*. **e** | A cycle of 12 h of light and 12 h of darkness, which matches the environmental conditions to which *R. palustris* has adapted, provides wild-type cells with a fitness advantage over the *kaiC*-knockout strain. **f** | Under a cycle of 1 h of light and 1 h of darkness, both the wild-type and *kaiC*-knockout cells grow at the same rate. Parts **a–c** are adapted from REF. 101, Elsevier. Parts **d–f** are adapted from REF. 14.

in bacteria that inhabit temporally irregular environments (for example, environments with unreliable exposure to light and darkness, inconsistent light intensities, annually varying photoperiods or irregularly available energy sources), a fully developed circadian oscillator provides the maximum temporal buffering to accurately track the solar day⁸⁸ (FIG. 6). As an example, seasonality may create very different selective pressures for free-living bacteria compared with gut bacteria. For a free-living phototroph, the differences in average temperature and photoperiod between winter and summer affect growth rates. Gut bacteria may enjoy a more consistent annual environment, but the diet of the host can differ substantially between winter and summer, which leads to drastic annual nutrition cycles that are known to affect the composition of the microbiome⁸⁹. Currently, the model shown in FIG. 6 is speculative. However, the widespread distribution of *kai* homologues in various

bacteria, which inhabit vastly different environmental niches, presents a unique set of test-cases with which to analyse the evolution of daily biological timekeeping.

Daily timing and the microbiome

The relationship between the host and its microbiome has become a central topic in biology and medicine. The temporal dimension of this relationship warrants serious investigation. At the very least, because most animal hosts eat on a daily schedule, the environment that is experienced by the gut microbiota undergoes a ~24 h transformation in nutrient availability. A bacterium that can anticipate the arrival of fresh nutrients might readily metabolize these resources and thus gain an advantage over time-ignorant competitors. Our knowledge that at least some free-living bacteria have daily timekeepers^{1,2,14} opens the possibility that bacteria of the microbiota have endogenous timekeepers

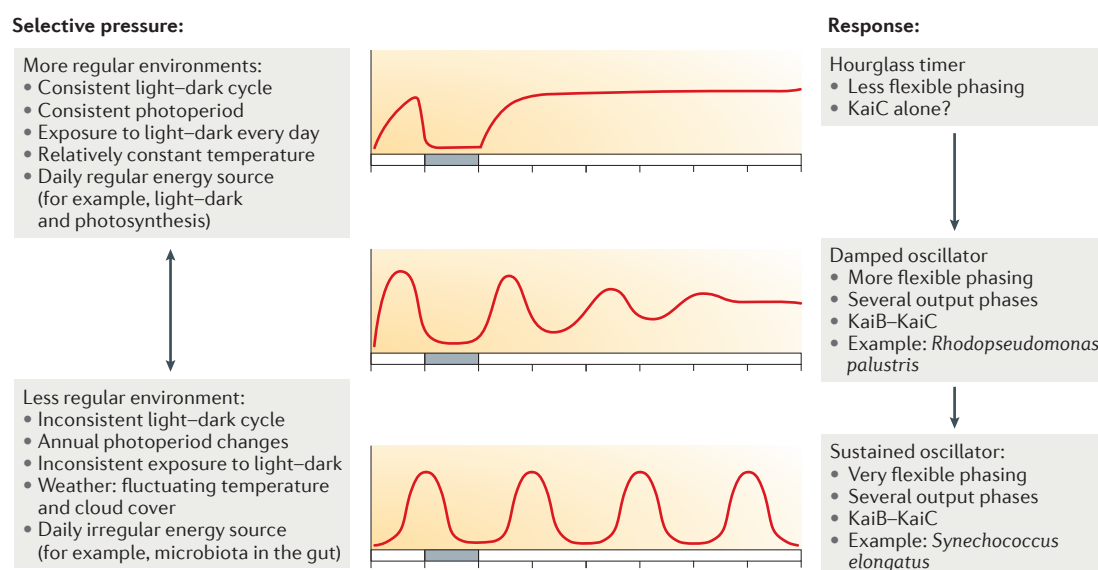


Figure 6 | A hypothesis for the evolution of Kai-based timekeepers in bacteria. The type of timekeeper (that is, hourglass timer, damped oscillator or self-sustained circadian oscillator) that evolves is primarily determined by the regularity of the environmental daily cycle of the bacterium, as this is the dominant selective pressure. In very regular environments, an hourglass timer that is based on KaiC alone is sufficient. In fluctuating environments, which require more flexibility of the timer, a sustained oscillator based on KaiA–KaiB–KaiC is advantageous. In intermediate environments, a damped oscillator that is based on KaiB–KaiC is sufficient.

that interact with the biological clock of the host. This blossoming area of enquiry has already identified a gut bacterium, *Enterobacter aerogenes*, that seems to express circadian rhythms when isolated outside of the host²⁰. Interestingly, the robustness of its rhythm is enhanced if melatonin is included in the culture medium. Melatonin is a circulating hormone that is rhythmically controlled by the circadian clock in vertebrates, and these results imply that melatonin could stimulate rhythmicity in members of the microbiota *in vivo*. In addition to melatonin, other candidates for daily time cues include diurnal cycles of nutrients and circadian body temperature rhythms (core body temperature oscillates diurnally in most endothermic animals).

Moreover, if members of the gut microbiota have timekeeping capability, might they release signals that influence the circadian system of the host? The microbiota secretes a vast range of compounds, many of which are bioactive⁹⁰. For example, some bacteria that inhabit or infect mammals can release cytokine-like factors⁹¹, which, if released rhythmically, could influence the circadian clock of the host. For example, microbial metabolites (in particular, short-chain fatty acids, such as butyrate) can modulate circadian gene expression in hepatic organoids²². Conversely, perturbations of the biological clock of the host affect the composition of the gut microbiome. Several studies have reported diurnal changes in the relative abundance of bacterial phyla in the gut microbiome^{21–25}. Disruption of the biological clock of the host either by jet-lag or by mutations in the clock genes of the host alters the proportion of these phyla that exhibit daily changes^{23–25}. Moreover, alterations to diet, such as time-restricted feeding and

transition between a regular and a high-fat diet, are known to affect circadian clocks in mice and they also modify the composition of the gut microbiome^{21–24}.

At this time, it seems clear that the composition of the mammalian gut microbiome is temporally changing, and that changes to the circadian clock of the host affect the viability and relative growth of some members of the microbiota. Whether there is feedback from the microbiota to the circadian clock of the host is less well characterized, but this is a fascinating possibility to investigate²². Along these lines, Thaïs and co-workers reported a remarkable interplay between host and microbiota⁹². Antibiotic treatment of the host affects rhythmic localization and adherence of bacteria to the intestinal epithelium and this, in turn, reprogrammes chromatin and transcription oscillations in the host. Remarkably, these effects occur not only in host intestinal cells but also extend to the liver, which modulates hepatic circadian gene expression and detoxification⁹². Finally, at least some members of the gut microbiota may have endogenous timekeepers²⁰ that promote their adaptation to the intestinal environment and to the external environment after they are excreted (FIG. 1).

Beyond the gut environment, there are other fascinating examples of host–bacteria relationships that have temporal dimensions, such as the symbiosis between the squid *Euprymna scolopes* and the luminous bacterium *Aliivibrio fischeri* (formerly known as *Vibrio fischeri*)⁹³. *E. scolopes* has a specialized light organ that is colonized by the bacteria. The luminescence that is produced by *A. fischeri* is used by the squid as a camouflage strategy, in which the animal emits light from its ventral surface to mimic the nocturnal moonlight

Endothermic
‘Warm-blooded’, that is, the maintenance of body temperature metabolically so that it is independent of the environment and behaviour.

and starlight that shine from above. The luminescence varies during the day–night cycle, with the highest levels of luminescence in the early evening when the animal begins to forage^{94,95}. An examination of the transcriptome of the resident *A. fischeri* revealed day–night oscillations of metabolism between anaerobic respiration and fermentation⁹⁵. This adaptive temporal interaction between host and symbiont illustrates diurnal reciprocity between hosts and microbial partners⁹³.

Conclusions and future perspectives

Although cyanobacteria are an archetypal model system for endogenous self-sustained timekeeping mechanisms and fitness, we have barely glimpsed the panoply of likely timing mechanisms that have evolved among bacteria in response to various environments and selective pressures (FIGS 1,6). Homologues of the *kai* genes are widely distributed among Eubacteria and Archaea. Although the cyanobacterial ‘cycles within cycles’ design (FIG. 2) is optimized for resilience to internal and external noise, other bacteria might use Kai proteins in different configurations to adjust to the daily patterns of their particular environment (FIG. 6). There is every reason to believe that new types of timekeeper other than circadian clocks exist and are ready to be discovered. As we expand this research to other bacterial species, new tools will be required to monitor daily rhythms. Luminescence reporters have been crucial for optimally monitoring rhythmic gene expression in aerobic eukaryotes and cyanobacteria^{2,96,97}, but luciferase reporters require oxygen. Therefore, to monitor rhythms from bacteria that operate in various metabolic modes, the development of automated technologies for microaerobic and anaerobic environments will facilitate the assessment of the generality of timekeeping mechanisms among Eubacteria and Archaea.

Understanding that host–microbiota relationships have a temporal dimension is an exciting new research area. It is clear that the feeding rhythms and diet of the

host affect which bacterial phyla thrive in the gut. Signals that emanate from the microbiota reciprocally modulating the circadian clock of the host is a tantalizing possibility, and, if found to be true, the implications for chronobiology are profound. As many members of the microbiota are not restricted to the gut but can also exist in a larger ecosystem that is also rhythmic, a daily timekeeper is potentially useful in both environments (FIG. 1). Bacteria that alternate between internal and external environments might adjust the timekeeping mechanism if they relocate; for example, after excretion. For a timekeeper in the regular 24 h external environment, the precision of cadence may be essential, whereas in the more variable environment of a host gut, flexibility of tempo may be more valuable.

From the broader perspective of chronobiology as an example of evolutionary adaptation, testing simple hypotheses about fitness can be challenging in higher organisms, in which many processes become interdependent over evolutionary time so that the adaptive value of a specific trait independent of the background is difficult to ascertain. Bacteria may provide clearer insights into the relative roles of various environmental factors in the natural selection of biological timekeepers. This is not to say that bacteria are ‘simple’, but that their small and generally streamlined genomes may enable more precise manipulation and measurement of the fitness consequences of variation in traits that underlie daily timekeepers. These factors lead to greater confidence about the functional basis of interactions between trait variation and fitness because of greater experimental control over both the genomic background and the environmental conditions. Therefore, the adaptive role of daily timekeepers, and the selective pressures that led to the evolution of temporal precision, temperature compensation and entrainment, may be best studied in bacteria. It is time (and timely) for microbiology and chronobiology to contribute more to each other.

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Competing interests statement

The authors declare no competing interests.