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Low temperature nullifies the circadian clock in cyanobacteria through Hopf bifurcation

Short title: Nullification of circadian clock in cyanobacteria

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Abstract

Cold temperatures lead to nullification of circadian rhythms in many organisms. Two typical scenarios explain the disappearance of rhythmicity. The first is oscillation death, which is the transition from self-sustained oscillation to damped oscillation that occurs at a critical temperature. The second is oscillation arrest, in which oscillation terminates at a certain phase. In the field of nonlinear dynamics, these mechanisms are called the Hopf bifurcation and the saddle-node on an invariant circle bifurcation, respectively. Although these mechanisms lead to distinct dynamical properties near the critical temperature, it is unclear to which scenario the circadian clock belongs. Here we reduced the temperature to dampen the reconstituted circadian rhythm of phosphorylation of the recombinant cyanobacterial clock protein KaiC. The data led us to conclude that Hopf bifurcation occurred at approximately 19°C. Below this critical temperature, the self-sustained rhythms of KaiC phosphorylation transformed to damped oscillations, which are predicted by the Hopf bifurcation theory. Moreover, we detected resonant oscillations below the critical temperature when temperature was periodically varied, which was reproduced by numerical simulations. Our findings suggest that the transition to a damped oscillation through Hopf bifurcation contributes to maintaining the circadian rhythm of cyanobacteria through resonance at cold temperatures.

Keywords: circadian rhythms, low temperature, cyanobacteria, Hopf bifurcation, in vitro

Significance Statement

Loss of circadian rhythms of poikilotherms or plants occurs widely at low temperatures. Here we chilled the simplest circadian oscillator reconstituted in a test tube and found a reduction in amplitude abrogated rhythmicity, which transitioned into a damped circadian rhythm oscillator at approximately 19°C. Physicists in the field of dynamical systems refer to this phenomenon as the

Hopf bifurcation. Further, diminished amplitude was restored by resonating with temperature cycles. These results suggest that the amplitude of the cyanobacterial circadian rhythm is sufficiently maintained during periods of cold temperatures through resonating with a periodical environment.

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Introduction

Circadian rhythms are constantly repetitive physiological processes with an approximately 24 h period in most organisms. The three diagnostic characteristics of circadian rhythms are conserved between species as follows: (i) persistence under constant conditions with self-sustained oscillation, (ii) entrained by repetitive environmental changes of light intensity or temperature, and (iii) periodicity does not change significantly in the range of temperatures that support life (temperature compensation). Further, circadian rhythms can be nullified at low temperature conditions in numerous poikilotherms and plants. For example, circadian rhythms are undetectable at 11.5°C in the marine dinoflagellate *Gonyaulax* (1, 2), at 8°C in the photosynthetic dinoflagellate *Lingulodnium polyedrm* (3), at 17°C in the roache *Leucophaea* (4), at 20°C in *Drosophila* (5), at 10.5°C in the filamentous fungus *Neurospora* (6), at 12°C in the duckweed *Lemna gibba* (7), and at 4°C in the tomato *Lycopersicon esculentum* Mill. (8), the European chestnuts *Castanea sativa* (9), and *Arabidopsis* (10). The loss of physiological rhythmicity that occurs in plants is reflected by global gene expression levels (10).

Loss of rhythmicity of a self-sustained oscillator is an example of a qualitative change to a dynamical system. A sudden qualitative change induced by a small change in the parameters of a system is referred to as “bifurcation,” which is intensively investigated in the field of nonlinear dynamics. Bifurcation theory can be applied to understand the loss of circadian rhythms by exposing organisms to reduced temperatures. Lowering ambient temperatures changes numerous parameters of the core circadian oscillator, such as rates of transcription and translation or the rate

of enzymatic reactions. The changes of these temperature-dependent parameters should lead to bifurcation of the loss of rhythmicity.

According to bifurcation theory, the Hopf and the saddle-node on an invariant circle (SNIC) bifurcations describe distinct, typical scenarios that explain the loss of rhythmicity of a self-sustained oscillator (11–13) (Fig. 1). The Hopf bifurcation involves a decrease in the amplitude of self-sustained oscillations as the temperature decreases, and when the amplitude reaches zero, the system becomes a damped oscillator. SNIC bifurcation involves the arrest of oscillations at a specific phase. Further, altering the parameters of the oscillatory system can create an attractive phase in the rhythm. The notable differences between the two bifurcations are the response of amplitude and the period required to a control parameter around a critical value at which bifurcation occurs. When the control parameter is varied, the amplitude is significantly altered in the Hopf bifurcation but not the period. In contrast, the period significantly changes in the SNIC bifurcation but not the amplitude. These qualitative differences between these bifurcations are subtle when the control parameter is far from a bifurcation point. It is therefore difficult to identify the type of bifurcation by observing the self-sustained oscillation regime. Thus, attention must be focused on the behavior of the oscillatory system near a bifurcation point. Bifurcation theory has been applied to several biological oscillatory systems to identify the mechanisms of the qualitative transitions. For example, certain resting neurons can generate an action potential in response to an applied current. When the strength of the current increases, the neurons can fire repetitively (bursting). These distinct processes are linked through the SNIC bifurcation. Thus, coalescence of stable and unstable fixed points can lead to the transition of a resting neuron to an oscillatory state. Moreover, spiking of certain neurons can be blocked by injection of a strongly depolarizing current (excitation block). As the injected current increases, the amplitude of the spiking decreases and rhythmicity disappears, indicating that Hopf bifurcation leads to this transition (12). Further, the cell cycle can be arrested at the G1/M transition when the concentrations of cyclins are lower than the threshold. The arrested cell can

enter mitosis if the concentrations of cyclins are sufficiently increased. The transition occurs when fixed points disappear through SNIC bifurcation (14).

Little is known about the dynamics of a core circadian clock at low temperatures, at which rhythms can be abolished. Moreover, to our knowledge, the mechanism of nullification of circadian rhythms has never been investigated according to bifurcation theory. The theory can serve to predict unidentified properties of circadian oscillators at cold temperatures through identifying the type of bifurcation.

Here, we focus on loss of core circadian oscillation of at low temperatures. Measuring bioluminescence produced by a luciferase reporter is generally a standard method used to observe circadian rhythms (15, 16). However, bioluminescence is an output that is processed through genetic regulation and metabolism, such as the expression of luciferase and the hydrolysis of ATP, the activity of which is also considered to depend on the ambient temperature. Direct observation of the core circadian oscillator using a temperature-independent method is essential because the absolute value of the amplitude is required to identify the type of bifurcation. In cyanobacteria, the oscillations of the ratio of phosphorylated to total KaiC act as a pacemaker (17, 18) that drives circadian rhythms of global gene expression through a response regulator (19–21). The core biochemical oscillator can be reconstituted in vitro in the presence of ATP by mixing three recombinant clock proteins, KaiA, KaiB, and KaiC (17). The reconstituted KaiC phosphorylation rhythms are the most appropriate circadian oscillations applicable to bifurcation analysis because the absolute amplitude of the core oscillator can be directly detected with high precision, and the Kai mixture can sense a change in ambient temperature (22).

Here we show that the reconstituted KaiC phosphorylation rhythm can be nullified at low temperature through the Hopf bifurcation. Moreover, we investigated the physiological significance of the damped oscillator that emerges below the critical temperature.

Results

Decreased amplitude of KaiC phosphorylation rhythms at low temperatures. We investigated the period and amplitude of in vitro KaiC phosphorylation rhythms close to the critical temperature to identify the bifurcation leading to arrhythmias induced by low temperatures. To exclude variations in period and amplitude caused by the recombinant protein preparation, we first mixed the cyanobacterial clock proteins KaiA, KaiB, and KaiC, divided the mixture into separate tubes, and exposed each to a temperatures gradient. The temperatures varied at approximately 1-degree intervals from 17°C to 29.9°C because in vitro rhythms are nullified at 20°C (23). Further, the period of in vitro rhythms is temperature-compensated, although its amplitude varies above 24.8°C (Fig. 2A) (17, 24). Oscillations of KaiC phosphorylation were not detected at ambient temperatures ≤18.6°C (Fig. 2A). The periods and amplitudes of the rhythms are shown in Fig. 2B. As the temperature decreased, the period of the rhythms remained relatively constant, although amplitude declined monotonically. The critical temperature where a bifurcation occurred was approximately 19°C because the amplitude approached zero. We used a different Kai protein preparation and reproduced these results (Fig. S1), suggesting that in vitro KaiC phosphorylation rhythm is nullified through Hopf bifurcation at low temperatures. Moreover, the critical temperature was slightly influenced by the preparation of Kai proteins, and we therefore kept using the Kai proteins prepared to create Fig. 2 for further experiments.

The temperature insensitivity of free-running period at low temperature was reproduced in vivo (Figs. 2C and S2). Several peaks of bioluminescence were detected between 20°C and 30°C, and the period was approximately 24 h in this range. At 18°C, rapid damping of bioluminescence rhythm occurred, and only one peak was detected.

Recovery from low temperature-induced arrhythmia. Circadian clocks stop at low temperature, resume after the return to the normal temperature, and the resumed rhythm tends to begin from circadian time (CT) 12, the beginning of the subjective night (7, 25). We analyzed the

effect of temperature shifts from low temperatures (4–16°C) to 30°C on in vitro KaiC phosphorylation rhythm. KaiC phosphorylation resumed oscillating at temperature step-up to 30°C (Fig. 2D). The phase of the KaiC phosphorylation rhythm upon resumption was estimated according to the phase of the rhythm after the shift to 30°C. We set CT 16 to the phase at the peak of the phosphorylation rhythm for consistency with the results of in vivo experiments (22) and CT $16 - 24t/\tau$ to the phase at which the rhythm restarted, where t is the time of appearance of the first peak after elevating the temperature, and τ is the free-running period at 30°C. Thus, we estimated that the resumed rhythm began from CTs 18, 21, 0, and 1 at 4, 8, 12, and 16°C, respectively. Further, the rhythms began from CT 22–3 when the temperature was increased from 16°C to 22–30°C (Fig. S3A). Unlike the response in vivo, the phase of the rhythm in vitro after increasing the temperature was not consistently arrested at a specific phase but depended on the combination of temperatures before and after step-up stimulus. This could be explained by the temperature-dependent position of the stable fixed point created through Hopf bifurcation. Note that it is also possible that the phase after recovery through SNIC bifurcation depends on the chilling temperature if the temperature shifts the position of the stable fixed point created by the bifurcation. Details are described in the legends of Fig. S3B and C.

Damped oscillation of KaiC phosphorylation below the critical temperature. According to the theory of Hopf bifurcation, a self-sustained oscillator acquires a stable fixed point and becomes a damped oscillator below the critical value of the parameter (Fig. 1A). If the Hopf bifurcation was adopted, damped oscillation of KaiC phosphorylation would occur below the critical temperature. We found that a 30°C pulse was effective for inducing damped oscillation (Figs. 3A and S4A), and following a 30°C pulse for 12 h, the KaiC phosphorylation ratio was approximately constant after two or three peaks at 18.8°C and only one peak at 16.1°C. Overdamping was observed when the mixture was chilled to 10°C. Above the critical temperature, the KaiC phosphorylation rhythm persisted for at least four cycles with or without pulse treatment.

Thus, the 30°C pulse induced damped oscillation of KaiC phosphorylation from 10°C to the critical temperature, and the decay rate of the amplitude was higher at lower temperatures. Moreover, the period of damped oscillation was prolonged at lower temperatures (Fig. S4B). The period of damped oscillation was approximately 30 h below critical temperature. In particular, the observation that the oscillation periods just above and below the critical temperature are almost the same strongly support Hopf bifurcation rather than other types of bifurcation.

Forced oscillation of KaiC phosphorylation induced by periodic 30°C pulses. Damped oscillation of KaiC phosphorylation simulates a pendulum with friction. A pendulum with friction swings for a while when kicked out and continues to swing when subjected to an external force with an appropriate period. We hypothesized therefore that the level of KaiC phosphorylation might oscillate below the critical temperature in response to periodic 30°C pulses. We tested this possibility by exposing the mixture to temperature cycles of 12 h at 30°C and 12 h at 18.7°C (12H12L), and found that the former forced the oscillation of KaiC phosphorylation (Fig. 3B). Further, in vitro KaiC phosphorylation gradually oscillated at a temperature cycle of 3 h at 30°C and 21 h at 18.7°C (3H21L). KaiC phosphorylation changed rhythmically with a period of 24 h even below the critical temperature, although periodic pulses of 30°C would be unrealistic in natural environment.

Resonance of the KaiC phosphorylation rhythm during low temperature cycles. We used more realistic temperature cycles of 16.7°C and 18.7°C. At a constant temperature of 16.7°C or 18.7°C, KaiC phosphorylation decreased to each equilibrium state (Fig. 4A), and temperature cycles of 12 h at 16.7°C and of 12 h at 18.7°C caused fluctuation in KaiC phosphorylation (Fig. 4B). We next varied the period of the temperature cycle T (Figs. 4B and S5). Forced oscillations were detected when the temperature cycles were ≥24 h (Fig. 4B). When we measured the amplitudes of the KaiC phosphorylation rhythm as a function of T, the amplitude of the forced

oscillation reached a maximum after approximately 30 h (Fig. 4C). KaiC phosphorylation did not fluctuate and the amplitude was low when the period was <16 h.

The enhancement of amplitude under temperature cycles can be interpreted as resonance because it depends on the period of the external cycles. We performed a numerical simulation to check if a previously proposed model of the in vitro Kai oscillator exhibited resonance. We adopted the model introduced by Hatakeyama and Kaneko (26) because it explicitly includes the temperature dependence of the reaction rate (see Materials and Methods). This model exhibited Hopf bifurcation when the temperature was reduced (Fig. S6A). We chose a pair of temperatures at which damped oscillations of in vitro KaiC phosphorylation could be reproduced. A periodic orbit was detected by switching the two temperatures with a period of T (Figs. 4D and S6B). The amplitudes of the orbits varied significantly depending on T, suggesting that the resonance of phosphorylation rhythms was caused by temperature cycles (Figs. 4E and S6C).

We next evaluated a less complex model to extract the essential conditions for resonance. The Stuart–Landau equation describes the normal form of the oscillator near the Hopf bifurcation point truncated to the third order (13) and can exhibit damped oscillation. Any oscillator near a Hopf bifurcation point can be transformed using the Stuart–Landau equation through variable changes in a good approximation. Moreover, our experiments indicated that when the ambient temperature was lowered below critical temperature, the phosphorylation of KaiC at equilibrium depended on the temperature (Figs. 4A and S1A). Therefore, we investigated the behavior of a damped Stuart–Landau oscillator with fixed points that periodically shift (see Materials and Methods). A closed orbit was observed and the amplitude depended on the external period, indicating that resonance was observed in the simpler oscillatory model (Fig. S7A-H). Some theoretical work has been also devoted to the Stuart–Landau oscillator subjected to a weak periodic force, which revealed that the oscillator can generally exhibit resonance (27–29). Therefore, any damped oscillators undergoing Hopf bifurcation may generally exhibit resonance when periodically driven. In addition, we numerically confirmed that a model in which oscillation

disappears through SNIC bifurcation shows forced oscillation but does not resonate with a periodic force (Fig. S7I-L). Therefore, the resonance of in vitro KaiC phosphorylation rhythms also supports occurrence of Hopf bifurcation.

In both the realistic and simpler models, the amplitude became higher when the frequency of the external force approached the approximate natural frequency of the system. The Hatakeyama–Kaneko model resonated with temperature cycles with a 31 h period (Fig. 4E), and the period of damped oscillation of the model at the temperature was 30 h. The agreement between the periods of a sufficient external force and intrinsic damped oscillations is generally observed in resonance, although the nonlinearity of the system can slightly shift the resonant period (30).

Discussion

Bifurcation theory provides a new perspective on the classification of circadian arrhythmia. We focused here on low-temperature-induced arrhythmias of the cyanobacterial clock and found that KaiC phosphorylation rhythms lost rhythmicity because the oscillation amplitude vanished, consistent with a scenario of Hopf bifurcation rather than SNIC bifurcation, in which the period approached infinity because of arrest at a specific phase. This death of a biochemical oscillation can be further classified as a supercritical Hopf bifurcation because the oscillation amplitude continuously diminished (Fig. 2B) and we did not detect an abrupt decrease in amplitude that is characteristic of a subcritical Hopf (11).

Discussions of whether a circadian clock is a harmonic oscillator or a relaxation oscillator have been ongoing for approximately the last 50 years (31, 32). This question could be associated with the type of bifurcation. If a system approaches a Hopf bifurcation point, the system more closely resembles a harmonic oscillator (13). In contrast, near the SNIC bifurcation point, the system exhibits very slow dynamics around a certain phase that is observed in a relaxation oscillator. Our present data lead us to conclude that the cyanobacterial circadian clock resembles a harmonic oscillator at low temperatures. Further, the waveforms of the KaiC phosphorylation rhythms

resembled a sinusoidal curve near the critical temperature, which is observed for harmonic oscillations (Figs. 2A and S1).

As for the in vitro clock under critical temperature, only nullification of rhythms has been reported (23). In this study, we found that damped oscillation occurs below the critical temperature. Consistent with this finding, we found here that resonant oscillation occurred when the ambient temperature varied periodically, even below the critical temperature. Such forced oscillation of KaiC phosphorylation may regulate the output pathway in cyanobacterial cells and functions as a biological clock at low temperature. This hypothesis is analogous to the fact that a pendulum with friction cannot be distinguished from a self-sustained oscillator when subjected to periodic external forces. Moreover, certain organisms such as yeast or purple bacteria employ such degenerated circadian oscillators and are therefore unable to exhibit self-sustained rhythmicity under constant conditions, although rhythms are detected when exposed to an environment with daily variation. (33, 34). We further suspect that the emergence of mammalian circadian rhythms during differentiation of embryonic stem cells is mediated via Hopf bifurcation because the oscillation amplitude gradually increases during development (35). Our study raises the possibility that a functional circadian rhythm exists in natural environments more broadly than naively expected from the results of experiments conducted under constant conditions. It may be physiologically advantageous that circadian rhythmicity appears and disappears via Hopf bifurcation, because the clock is rescued by a resonance effect in a natural environment, even under conditions below the critical parameter. We therefore expect that Hopf bifurcation is widely conserved not only in bacteria but also in organisms other than bacteria and could be a novel property of circadian clocks along with the three prominent properties stated in the Introduction.

Hopf bifurcation provides a further advantage compared with the SNIC bifurcation in terms of the temperature compensation of period. As mentioned above, the oscillation period is approximately constant for the Hopf bifurcation near a bifurcation point, whereas significant slowing occurs in

the SNIC bifurcation. Given that temperature compensation across a wide range of temperature is crucially adaptive, the bifurcation type of the circadian clock should be Hopf bifurcation. The fact also implies universality of Hopf bifurcation in circadian rhythms under cold conditions.

The molecular basis of the Hopf bifurcation at low temperatures is unknown. One possibility is the desynchronization of phosphorylation cycle in KaiC monomers. KaiC molecules are assembled into a hexamer in solution and each KaiC protomer has two phosphorylation sites (S431 and T432). Phosphorylation and dephosphorylation of each site proceeds sequentially as follows: phosphorylation of T432, phosphorylation of S431, dephosphorylation of T432, and dephosphorylation of S431 (36, 37). Each KaiC protomer follows a cycle comprising the four steps in the presence of interactions among hexamer subunits and between hexamers. Therefore, the reaction rate of each step can be affected by the phosphorylation of other KaiC protomers within the same hexamer or by another KaiC hexamer through competition for free KaiA (38). If the cycle of each KaiC phosphorylation proceeds synchronously, the ratio of KaiC phosphorylation oscillates with large amplitude. Conversely, desynchronization among KaiC protomers leads to a diminishing oscillation amplitude at the protein population level. Devising a novel tool to detect the phosphorylation of KaiC molecules may facilitate developing a microscopic model of low temperature-induced arrhythmia (39).

Here we determined that the amplitude of KaiC phosphorylation rhythms was enhanced when the period of the in vitro oscillator matched that of the temperature cycles, i.e., resonance. To the best of our knowledge, this is the first experimental demonstration of resonance associated with a circadian clock (Fig. 4C). Mechanical oscillators efficiently gain kinetic energy when resonance occurs. Moreover, in biochemical oscillations, energetic merits in resonant conditions have been suggested (40, 41). Organisms might also enjoy such energetic merits. Note that the resonance period was approximately 30 h in this study. The resonance period may depend on experimental conditions such as the concentrations of Kai proteins, and clocks in vivo may have a resonance period closer to 24 h. Further, the concept of circadian resonance was proposed in

the context of enhanced growth and metabolic activity, when the period of light-dark cycles is close to that of the endogenous circadian clock (42–44). The acquisition of an optimal phase relationship between the clock and environmental rhythms when the periods are close may provide an explanation. Our finding of amplitude enhancement may represent another mechanism underlying circadian resonance.

We demonstrate that a bifurcation-based classification offers biological insights and predictions of possible physiological behaviors. Identifying the bifurcation type may serve as a common tool for classifying biological rhythms because it can be applied regardless of species. Parameters of interest are also not limited to temperature but to controllable parameters, such as light intensity, pH, and chemical dosage. Studying the association among types of bifurcation, evolutionary advantages, and molecular mechanisms may contribute to revealing the universal property of biological rhythms across species.

Materials and Methods

Reconstitution of KaiC phosphorylation rhythms in vitro and regulation of temperature. Purification of recombinant KaiA, KaiB, and KaiC and reconstitution of the KaiC phosphorylation rhythm in vitro were performed as described elsewhere (36). Mixtures of Kai proteins and ATP were incubated with a thermal-block incubator (BI-151, ASTEC) or a temperature-gradient apparatus. Incubation temperatures were changed by quickly moving the mixtures to different incubators as described (22). All experiments were performed at least twice.

Temperature-gradient apparatus. Two heating baths (UT40U100F, Ampere) were bridged by an aluminum block (Fig. S8). By setting an arbitrary temperature of the heating baths, desirable temperatures gradient was generated with an accuracy of ±0.1°C. Fourteen samples under different temperature were simultaneously analyzed

Bioluminescence assay. *Synechococcus elongatus* PCC 7942 carrying P*kaiBC-luxAB* bioluminescence reporter and P*psbA1-luxCDE* genes (transformant of NUC42 with pAM1619) were cultured in modified BG-11 liquid medium under continuous light (LL) of 46 μmol m^{-2} s^{-1} at 30°C in a continuous culture system to maintain an optical density (at 730 nm) = 0.3. After two 12-h light/12-h dark (LD) cycles at 30°C to synchronize the clock, the cells were released into LL conditions at 18–30°C. Bioluminescence was monitored in a flow cell using a photomultiplier (H9319-11, Hamamatsu).

Simulation of KaiC phosphorylation rhythms during temperature cycles. The model developed by Hatakeyama and Kaneko (26) was used to simulate in vitro rhythms during temperature cycles. Inverse temperature β of the model was periodically altered to simulate temperature cycles. Note that higher β values indicate lower temperatures. In addition, the Stuart–Landau equation, $\dot{Z} = (\mu + ia - |Z|^2)Z$, where Z is a complex variable and $\mu < 0$ and a are parameters, was adopted as the simplest damped oscillator model near the Hopf bifurcation. We assumed that lowering the temperature contributes to shifting the position of stable fixed point, which is represented by $\dot{Z} = (\mu + ia - |Z - \alpha|^2)(Z - \alpha)$, where the complex variable α indicates the amount of shift. To simulate circadian rhythms during temperature cycles, the value of α was periodically switched between 0 and $0.5 + 0.5i$. The Runge-Kutta forth-order method was used for numerical integration of the above two models.

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Figure Legends

Fig. 1. Two alternative scenarios explaining the low temperature-induced arrhythmia of the circadian clock. Nullification of circadian rhythms by reducing the temperate can be considered the transformation of a limit cycle oscillator generated by altering the value of a system's parameters according to bifurcation theory. The loss of rhythmicity can be classified into typical scenarios based on the theory as follows. (A) In the scenario referred to as the Hopf bifurcation, the amplitude of the oscillations decreases to zero when lowering the temperature and a stable fixed point (closed circle) can be created at the critical temperature. Below the critical temperature, the limit cycle oscillator transforms into a damped oscillator that winds around the fixed point and eventually converges on the fixed point below the critical temperature. (B) In the scenario referred to as SNIC bifurcation, the velocity of angular direction becomes slower at a specific phase, causing a bottleneck. The period of oscillations approaches infinity, and a pair of stable and unstable fixed points (closed and open circles) is created at the critical temperature. Below the critical temperature, the oscillator transforms into an excitable system. Analyzing the behavior around the critical temperature allows the identification of the classification of the loss of rhythmicity because period or amplitude can be significantly changed.

Fig. 2. The cyanobacterial circadian rhythm is nullified by low temperature in vivo and in vitro. (A) Low temperature limit of the in vitro KaiC phosphorylation rhythm. Cyanobacterial clock proteins KaiA, KaiB, and KaiC were mixed and incubated at 4°C for 24 h and then at 18.6–29.9°C for 96 h. Aliquots of the mixtures were collected at every 4 h, and subjected to SDS-PAGE and Coomassie Brilliant Blue (CBB) staining. ImageJ software (NIH) was used for

densitometric analysis. The ratios of phosphorylated KaiC (P-KaiC) to total KaiC were plotted against incubation time. (B) Effect of temperature on period (pink) and amplitude (black) of the in vitro KaiC phosphorylation rhythm, which were estimated by fitting to a sinusoidal function with a linear component, $A + Bt + C\sin(\frac{2\pi}{T}t + D)$, through Levenberg-Marquardt method, where A, B, C, D, and T are fitting parameters. The asymptotic standard error for the estimated period are displayed as an error bar. We plotted the data for ≥ 19.5 ºC, where the asymptotic standard error of T is < 1.0. The blue line represents 19°C, which appears to be a critical temperature. (C) Effect of temperature on the period of bioluminescence rhythms in cyanobacteria. After two LD cycles at 30°C, cells were subjected to LL conditions at 18–30°C and bioluminescence was monitored in a flow cell. Time course of bioluminescence is shown in Fig. S2. The period was defined as the average distance from peak to peak of the bioluminescence rhythms. (D) Recovery from low temperature-induced arrhythmia. The reaction mixture containing Kai proteins was incubated at 4°C (black), 8°C (blue), 12°C (green), and 16°C (pink) for 48 h to reach equilibrium and then transferred to the 30°C heating bath. KaiC phosphorylation was monitored for 120 h and plotted against incubation time to compare the phase of the resumed rhythm.

Fig. 3. Damped and forced oscillation of in vitro KaiC phosphorylation induced by a 30°C pulse. (A) Damped oscillation of KaiC phosphorylation. After 24 h incubation at 4°C, samples were subjected to a 30°C pulse for 12 h and then transferred to 10°C–20.4°C. KaiC phosphorylation following the pulse was monitored and plotted against incubation time. (B) Forced oscillation of KaiC phosphorylation by periodic 30°C pulses. The mixture was incubated at 18.7°C for 24 h and then subjected to temperature cycles of 12 h at 30°C and 12 h at 18.7°C (12H12L), or 3 h at 30°C and 21 h at 18.7°C (3H21L). KaiC phosphorylation was monitored for 104 h.

Fig. 4. Resonance of in vitro KaiC phosphorylation under temperature cycles of 16.7/18.7°C. (A) KaiC phosphorylation at constant temperatures of 16.7°C (black) and 18.7°C (blue). Reaction mixtures of Kai proteins were incubated at each temperature and the ratios of KaiC phosphorylation were plotted against incubation time. (B) Forced oscillation by cycles of temperatures lower than the critical temperature. The mixtures were incubated at 18.7°C for 24 h and then subjected to symmetrical temperature cycles (duration of the 16.7°C and 18.7°C pulses were equal) with a period T (T = 12, 24, 30, and 36 h). KaiC phosphorylation at each temperature cycle was monitored for 104 h. (C) Resonance of the KaiC phosphorylation rhythm during the low temperature cycle. The amplitude of the KaiC phosphorylation during the temperature cycle was estimated by calculating the difference between peak and trough values (Fig. S5), and plotted against T. (D) Closed orbit of the Hatakeyama–Kaneko model during temperature cycles. The parameter of temperature included in the model is periodically switched with a period of T hours. The trajectory of the system is shown in a phase space of KaiC phosphorylation level and abundance of free KaiA. (E) Resonance of the Hatakeyama–Kaneko model. The difference between the maximum and minimum ratios of P-KaiC at steady state was measured as a function of T.