We propose a revisited version of the in vivo model of the cyanobacterial circadian clock. Our aim is to address the lack of robustness predicted for the mutant cyanobacteria without transcriptional regulation of the original model. For this, we rely on an in vitro model of the clock describing explicitly the hexameric structure of the core protein of the clock. Our model is able to reproduce oscillatory behavior for the mutant, as observed experimentally, without finely tuned parameters.

INTRODUCTION

Circadian clocks are endogenous oscillators allowing organisms to synchronize their physiological activities and behavior with the time of the day [1,2]. They play a role in most living organisms from bacteria to humans. Cyanobacteria possess one of the simplest known circadian clock and serve as one of the model organisms to study mechanisms leading to endogenous oscillations of about 24 hours. One particularly beautiful property of the cyanobacterial circadian clock is that it can be reconstituted in a test tube. If one mixes adenosine triphosphate (ATP) with the three key proteins of the clock, namely KaiC, KaiB and KaiA, autonomous oscillations with a period of about 24 hours are observed during several days [3-6].

The mechanism of the clock is essentially an ensemble of phosphorylation/dephosphorylation reactions of KaiC which is the core protein of the clock. The phosphorylation state of KaiC contains the information about the phase of the clock. More specifically, the structure of KaiC is a homo-hexamer in the shape of a double-doughnut which can be phosphorylated on two residues on each monomer. The role of KaiA is to promote phosphorylation while KaiB, when bound to KaiC, inhibits phosphorylation by sequestering KaiA. Those phosphorylation/dephosphorylation reactions can be reproduced in the test tube. An additional mechanism comes at play in vivo as KaiC autoregulates its own production via a negative feedback on its own mRNA production. There are therefore two oscillatory processes underlying the functioning of the clock, the post-translational regulation (PTR) which is observed in vitro, and the transcriptional translational regulation (TTR) consisting of the negative feedback on mRNA production.

A mathematical model of the in vitro clock relying on careful experiments measuring all kinetic rates and concentrations of proteins involved was proposed in [6]. This simple model beautifully reproduces oscillations with a period of about 24 hours, without any parameter space exploration as all parameters have been measured.

In [7], we proposed an extension to the in vivo case. This model showed that PTR regulation is sufficient to generate oscillations, as observed in the experiments with mutant cyanobacteria lacking the TTR regulation. It also showed that the transcriptional regulation helps maintaining synchrony in a population of growing cyanobacteria. However, the model requires finely tuned parameters to lead to oscillatory behavior of the mutant cyanobacteria lacking the transcriptional regulation. The in vitro model is based on did not explicitly described the hexameric structure of the KaiC protein. More recently, a more realistic model of the in vitro clock has been proposed in [8]. It describes explicitly the hexameric nature of KaiC and the binding of KaiB to KaiC. We propose here to build an in vivo model based on this more realistic in vitro model. Our model is able to produce robust oscillations for the wild type cyanobacteria as well as for the mutants lacking transcriptional regulation.

The structure of the work is as follows. We first briefly introduce the in vitro model of [8] for completeness and to introduce notations. Then we describe our in vivo model of the cyanobacterial circadian clock. We analyze the robustness of our model with respect to parameter variations, and compare it with the model proposed in [7]. We end with a short discussion on the role of transcriptional regulation.

THE IN VITRO HEXAMERIC MODEL

The hexameric model of the clock describes the interconversions of the different phosphoforms of KaiC as well as the binding and unbinding of KaiC to KaiB. When KaiC is bound to KaiB, it forms the KaiB.KaiC complex which can undergo to same (de)phosphorylation reactions as KaiC. The nonlinearity in the system comes from the fact that the reaction rates depend on the state of the system. When KaiA is bound to KaiC, it enhances the auto-kinase activity of KaiC. Although KaiA is not
described explicitly, it impacts the (de)phosphorylation rates as KaiC binds differentially to KaiA in its different phosphoforms. Similarly, KaiB is implicitly taken into account and antagonizes the effect of KaiA in a KaiC phosphoform dependent manner. Essentially, when KaiC subunits are in the S form, KaiB will bind to KaiC and sequester KaiA, thereby repressing phosphorylation. More details are provided in the appendix. A scheme of the model is depicted in Fig. [1]. Each subunit of KaiC undergoes independent autokinase and autophosphatase reactions at two sites, serine 431 (S) and threonine 432 (T) which can be phosphorylated or not. Therefore, each subunit can be in four forms, namely: the phosphorylated form at S, the phosphorylated form at T, the doubly phosphorylated form at S and T (denoted D), and the U form which is the non-phosphorylated form. The different phosphoforms are denoted by KaiC\textsubscript{i,j,k} where \textit{i} represents the number of subunits phosphorylated at T only, \textit{j} represents the number of phosphorylated subunits at S only and \textit{k} represents the number of doubly phosphorylated subunits. By construction, the variables \textit{KaiC\textsubscript{i,j,k}} which are physical have indices obeying \textit{0 < i, j, k < n} and the number of unphosphorylated subunits is given by \textit{n - (i + j + k)}. For the hexamer model \textit{n = 6}, but to study theoretically the effect of changing the number of KaiC subunits, the model was constructed with an arbitrary number subunits denoted by \textit{n}. For instance, the unphosphorylated KaiC is \textit{KaiC\textsubscript{000}} and the fully doubly phosphorylated form is \textit{KaiC\textsubscript{006}}. One should note that the dynamical variables constructed by assuming that the different subunits of the hexamer are indistinguishable. KaiC can undergo potentially (de)phosphorylation reactions which will increase or decrease the values of \textit{i, j, k} by one unit. Additionally, \textit{KaiC\textsubscript{i,j,k}} can bind to KaiB, forming the complex \textit{KaiB.KaiC\textsubscript{i,j,k}} which undergoes the same (de)phosphorylation reactions. The model is given by Eqs. (1). The dependence of the reaction rates on the KaiC phosphoforms are given in the appendix, see Eqs. (1 and 2).

\[ \frac{d\text{KaiC}_{i,j,k}}{dt} \text{ \textit{in vitro}} = k^{i-1,j,k}_U(n + 1 - (i + j + k))\text{KaiC}_{i-1,j,k} + k^{i,j-1,k}_S(n + 1 - (i + j + k))\text{KaiC}_{i,j-1,k} \]

\[ + k^{i+1,j,k-1}_T(i + 1)\text{KaiC}_{i+1,j,k-1} + k^{i+1,j,k}_T(i + 1)\text{KaiC}_{i+1,j,k} \]

\[ + k^{i-1,j,k+1}_D(k + 1)\text{KaiC}_{i-1,j,k+1} + k^{i,j-1,k+1}_D(k + 1)\text{KaiC}_{i,j-1,k+1} \]

\[ + k^{i,j+1,k-1}_S(j + 1)\text{KaiC}_{i,j+1,k-1} + k^{i,j+1,k}_S(j + 1)\text{KaiC}_{i,j+1,k} \]

\[ - ((n - i - j - k) (k^{i,j,k}_U + k^{i,j,k}_S) + i k^{i,j,k}_T + i k^{i,j,k}_D + k^{i,j,k}_S + j k^{i,j,k}_D + j k^{i,j,k}_S) \text{KaiC}_{i,j,k} \]

\[ - k_{\text{on,B}} F^i_{B} \text{KaiC}_{i,j,k} \]

\[ + k_{\text{off,B}} \text{KaiB.KaiC}_{i,j,k} \]

\[ \frac{d\text{KaiB.KaiC}_{i,j,k}}{dt} \text{ \textit{in vitro}} = k^{i-1,j,k}_U(n + 1 - (i + j + k))\text{KaiB.KaiC}_{i-1,j,k} + k^{i,j-1,k}_S(n + 1 - (i + j + k))\text{KaiB.KaiC}_{i,j-1,k} \]

\[ + k^{i+1,j,k-1}_T(i + 1)\text{KaiB.KaiC}_{i+1,j,k-1} + k^{i+1,j,k}_T(i + 1)\text{KaiB.KaiC}_{i+1,j,k} \]

\[ + k^{i-1,j,k+1}_D(k + 1)\text{KaiB.KaiC}_{i-1,j,k+1} + k^{i,j-1,k+1}_D(k + 1)\text{KaiB.KaiC}_{i,j-1,k+1} \]

\[ + k^{i,j+1,k-1}_S(j + 1)\text{KaiB.KaiC}_{i,j+1,k-1} + k^{i,j+1,k}_S(j + 1)\text{KaiB.KaiC}_{i,j+1,k} \]

\[ - ((n - i - j - k) (k^{i,j,k}_U + k^{i,j,k}_S) + i k^{i,j,k}_T + i k^{i,j,k}_D + k^{i,j,k}_S + j k^{i,j,k}_D + j k^{i,j,k}_S) \text{KaiB.KaiC}_{i,j,k} \]

\[ - k_{\text{on,B}} F^i_{\text{B}} \text{KaiB.KaiC}_{i,j,k} \]

\[ + k_{\text{off,B}} \text{KaiB.KaiC}_{i,j,k} \]

**THE IN VIVO HEXAMERIC MODEL**

To construct an in vivo model, we rely on the in vitro model of \textit{[5] described in the section above and add terms for production, degradation and dilution similarly.
to what is proposed in [7]. Our model is given by the Eqs. 2

\[
\begin{align*}
\frac{dK_{aiC_{ijk}}}{dt} &= \frac{dK_{aiC_{ijk}}}{dt} \bigg|_{in\ viro} - V_dK_{aiC_{ijk}} - V_mK_{aiC_{ijk}} - \frac{K_{aiC_{ijk}}}{K + K_{aiC_{ijk}}} \\
\frac{dK_{aiB.KaiC_{ijk}}}{dt} &= \frac{dK_{aiB.KaiC_{ijk}}}{dt} \bigg|_{in\ viro} \\
\frac{dK_{aiC_{000}}}{dt} &= \frac{dK_{aiC_{000}}}{dt} \bigg|_{in\ viro} - \frac{K_{aiC_{000}}}{K + K_{aiC_{000}}} \\
\frac{dmRNA}{dt} &= V_sK_i^4 + \left(\sum_{j \neq 0} K_{aiB.KaiC_{ijk}}\right)^{4} - V_mK_{mRNA}
\end{align*}
\]

Firstly, we consider explicitly KaiC mRNA as a dynamical variable. Its production term is dependent on KaiC which acts as a negative feedback on its production. As in [7], we consider only active degradation and not dilution for mRNA as it is order of magnitude larger. Secondly, we add a production term for unphosphorylated KaiC from its mRNA. Finally, all forms of KaiC are assumed to have the same linear dilution rate and active degradation rate.

As in [7], we consider on top of the wild type (WT) cyanobacterial circadian clock described above, the case of a mutant cyanobacteria lacking the transcriptional feedback. This is simply done by replacing the production term of mRNA by a constant production rate $V_{sptr}$. This mutant is referred to as post-transcriptional (PTR) mutant while the WT clock has a transcriptional translational regulation (TTR).

We analyzed the sensitivity to parameter changes of the PTR mutant and showed that model does not requires fine tuning to generated oscillations, see Fig. 2. We observe that the main change between the WT cyanobacteria and the mutants lacking the TTR circuit is that the WT bacteria are more robust to changes in the translation rate. However, the TTR circuit is not crucial for the clock to be robust against changes in the dilution rate.

**DISCUSSION**

We showed that our revised *in vivo* model of the cyanobacterial circadian clock is compatible with the robust oscillations of the cyanobacteria mutant lacking the TTR feedback. This result suggests that although the TTR feedback enhances the robustness of the clock, the effect is not as strong as initially suggested. Our conclusion is in agreement with the experiments performed with higher growth rates for (see Fig. S4 of [7]). Indeed, those experiments show that the mutants lacking the TTR regulation are able to generate oscillations in an extended range of growth rate. Although the growth rate of the bacteria is not explicitly modeled, it will impact the dilution rate. In the original model of the *in vivo* clock, the dilution rate needed to be finely tuned. The TTR regulation could actually play a more important role if the nonlinearity of the regulator terms was increased [9, 10]. Coordination of both mechanisms is key to enhance the robustness too. To sum up, we propose a revisited version of the *in vivo* cyanobacterial clock which is in agreement with current experiments. We expect that the synchronisation properties of the clock will also be enhanced by TTR regulation. In the future, we should to include the key proteins RpaA and SasA in the models, to be able to describe quantitatively how the clock’s time is read as an output [11, 12].

Python codes to reproduce all results are available at https://github.com/sophiedeb/cyano_clock_models.

**APPENDIX : REACTION RATES DEPEND ON THE STATE OF THE SYSTEM**

To obtain the dependence of the reaction rates on the various phosphoforms of KaiC, we need to describe the different allosteric states of KaiC. KaiC can be in a state competent to bind KaiA and a state competent to bind KaiB, respectively denoted $KaiC^A$ and $KaiC^B$ (to simplify notations, we omit the $i, j, k$ indices those allosteric state should carry). From the state competent to bind KaiA, KaiC can be converted in the active form of KaiC, which we denote $KaiC^*_i,j,k$ with indices specifying the phosphoform. The allosteric reactions are considered to be at equilibrium, and one can therefore obtain the ratio of $KaiC$ in its different allosteric states. The fact that those ratios are depend on the specific $(i, j, k)$-phosphoform is key to the *in vitro* feedback mechanism.
The (de)phosphorylation reaction rates are given by:

$$k_{XY}^{i,j,k} = F_{A}^{i,j,k} k_{XY}^{A} + (1 - F_{A}^{i,j,k}) k_{XY}^{0},$$

(3)

where $F_{A}^{i,j,k}$ denotes the fraction of KaiC in the state competent to bind KaiA. The first term is dominant as KaiC autokinase activities are enhanced by KaiA. The constants $k_{XY}^{A}$ represent the (de)phosphorylation reaction rates which is given by

$$F_{B}^{i,j,k} = \frac{\text{KaiC}^{B}}{\text{KaiC}^{B} + \text{KaiC}^{A} + \text{KaiC}^{*i,j,k}}$$

$$= \frac{1}{1 + \frac{1}{K_{A}^{*i,j,k}} + \frac{\text{KaiA}}{K_{m} K_{A}^{*i,j,k}}}$$

Finally, we should note that KaiC$_{i,j,k}$ in Eqs. 1 represents the sum of all allosteric forms of KaiC in the $(i,j,k)$ state.

$$k_{m,B}$$

$0.15$ $h^{-1}$

$$\Delta G_{pT}$$

$2.0$ $k_{B}$ $T$

$$\Delta G_{pS}$$

$-1.0$ $k_{B}$ $T$

$$\Delta G_{U}$$

$1.0$ $k_{B}$ $T$

$k_{m}$

$0.43$ $\mu$ $M$

$k_{T}^{*}$

$0.0$ $h^{-1}$

$k_{T}^{D}$

$0.0$ $h^{-1}$

$k_{T}^{U}$

$0.21$ $h^{-1}$

$k_{T}^{D}$

$0.11$ $h^{-1}$

$k_{T}^{U}$

$0.21$ $h^{-1}$

$k_{S}^{*}$

$5.32$ $10^{-2}$ $h^{-1}$

$k_{S}^{D}$

$0.506$ $h^{-1}$

$k_{S}^{U}$

$0.172$ $h^{-1}$

$k_{D}^{*}$

$0.0$ $h^{-1}$

$k_{D}^{D}$

$0.9$ $h^{-1}$

$k_{D}^{U}$

$0.15$ $h^{-1}$

$k_{B}^{*}$

$3.316$ $10^{-2}$ $\mu M h^{-1}$

$k_{B}^{T}$

$2.0$ $\mu$ $M$

$k_{B}^{D}$

$0.20$ $\mu$ $M$

$k_{B}^{U}$

$2.0$ $\mu$ $M$

$k_{A}$

$1.0$ $\mu$ $M$

$k_{s}$

varied

Table I: Parameter values - taken from [5, 7, 8]
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