Biological oscillations without genetic oscillator or external forcing

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Oscillators play a crucial role in biological systems, enabling complex behaviors such as cell division and circadian cycles. As a result, researchers have been studying both natural and synthetic genetic oscillators. Interestingly, oscillating cellular behaviors are often linked to biological oscillators. In a previous study [1], we discovered sustained oscillations related to phenotypic switching in different cellular systems and gene circuits, without any external forcing. These oscillations were associated with the induction of slow-growing phenotypes. In this paper, we aim to understand when and why such phenotypic instabilities can arise, leading to oscillations in cell populations. To achieve this, we developed a simple mathematical model of a stress phenotype and solved it analytically to determine the range of operating conditions in continuous culture for which the gene expression should not reach a stable equilibrium. This range of conditions is expected to exhibit an unstable biological behavior, which we verified in Bacillus subtilis cultures. Our findings demonstrate that oscillations can occur in the absence of genetic oscillators or external forcing, departing from the classical Monod model. While we explain this phenomenon for the stress response in continuous culture, it is likely that similar unexpected behavior can occur in any long-term culture where there is environmental feedback connecting the inducer and the cellular system considered.

Oscillating behaviors are ubiquitous through biology, appearing at multiple scales from single cells to multicellular organisms and controlling a sizable portion of their metabolism [2] [3]. Their study yielded the discovery of natural gene circuits acting as biological oscillators, e.g., the circadian clock of cyanobacteria [4] or the *E.coli* cell cycle[5]. These discoveries were complemented by the creation of synthetic oscillators [6], such as the repressillator, involving three repressor proteins to generate spontaneous oscillations in gene expression[7]. Oscillations in gene expression can also be generated based on external forcing. So far, biological oscillations can be produced based either on specific gene circuits [7], external forcing [8], or a combination of both [9].

Previous examples in the field have thus shown that, typically, when cells cultured in stable conditions exhibit oscillating gene expression, a biological oscillator is the cause[7]. However, we previously observed that highly burdensome gene circuits can oscillate when grown in a simple chemostat [1], a continuous cultivation device with stable operating conditions designed to promote stable conditions for the cells to grow in [10]. Notably, the two gene circuits that exhibited this behavior, the T7 expression system in *E.coli* BL21, and the sporulation of *B.subtilis*, do not have gene network architectures prone to generating oscillations. To understand the cause of these unstable behaviors, we focus our attention on the latter sytem because it can be reduced to 1) two defined phenotypes, i.e., growth and no growth, 2) one trigger, glucose, also acting as the carbon source.

To this end, we first developed a simple mathematical model of the stress response under a stable nutrient influx (a chemostat), where stress is induced when the nutrient concentration is too low. This model shows that even under stable operating conditions, there is a range of dilution rates for which no stable population profile can be found. To verify these predictions, we performed experiments on *B.subtilis* and observed that sporulation can exhibit oscillating profiles, observed as successive bursts of sporulation in the population.

A mathematical model predicts instabilities in gene expression for a specific range of dilution rate

As a first step toward the understanding of the observed oscillations, we designed an ODE model of stress response taking into account the dynamics of two phenotypes i.e., the non-stressed biomass X1 and the stressed biomass X2 (Eq. 1-5).

The stressed phenotype may not grow as fast as its counterpart, and its maximum growth rate was described as $\mu_{max,2} = k\mu_{max,1}$ where $k \in [0,1]$. A step function $H_1(S)$ was used to account for the phenotypic switch from X_1 to X_2 when the cells are stressed (Eq. 5). Accordingly, the transition rate is considered a constant H below K_I and none existing above. Since the stressed phenotype X_2 grows slower and the proteins responsible for stress are mainly diluted by growth, the opposite transition from X_2 to X_1 was neglected.

$$\frac{\mathrm{d}S}{\mathrm{d}t} = (S_{in} - S)D - \frac{\mu X_1}{Y_{X1}} - \frac{k\mu X_2}{Y_{X2}} \tag{1}$$

$$\frac{\mathrm{d}X_1}{\mathrm{d}t} = (\mu - D)X_1 - H_1(S)X_1 \tag{2}$$

$$\frac{\mathrm{d}X_2}{\mathrm{d}t} = (k\mu - D)X_2 + H_1(S)X_1 \tag{3}$$

$$\mu = \mu_{max}\frac{S}{K_S + S} \tag{4}$$

$$H_1(S) = \begin{cases} H & \text{if } S \leq K_I \\ 0 & \text{if } S > K_I \end{cases} \tag{5}$$

Where S_{in} is the sugar concentration in feed, D is the dilution rate, Y_{Xi} are the yields of each phenotype, and K_S is the sugar concentration at half maximum of the growth rate.

Solving this model analytically (see SI Appendix) showed that there is a range of dilution rates where equilibrium between the different state variables cannot be reached and the system is unstable (Fig. 1 A). Within this region, the substrate concentration S cannot get to the substrate concentration predicted by the traditional Monod curve, as that is below the switching concentration K_I. It cannot reach the expected equilibrium where cells switch to the stressed phenotype either, as that would be above K_I. Instead, the cells must constantly respond to their environment and S should approach K_I. This constant response is a negative feedback loop for the growth of the population, and, combined with a delayed response to changing conditions, the outcome is an oscillator analogous to the repressilator[7] (Fig. 1 B and C). The oscillations, their appearance, and their persistence are expected to depend on the importance of the delay and the accuracy of the step function to approximate the switch to a stressed phenotype, but the range of dilution rate in which they appear can be verified experimentally.

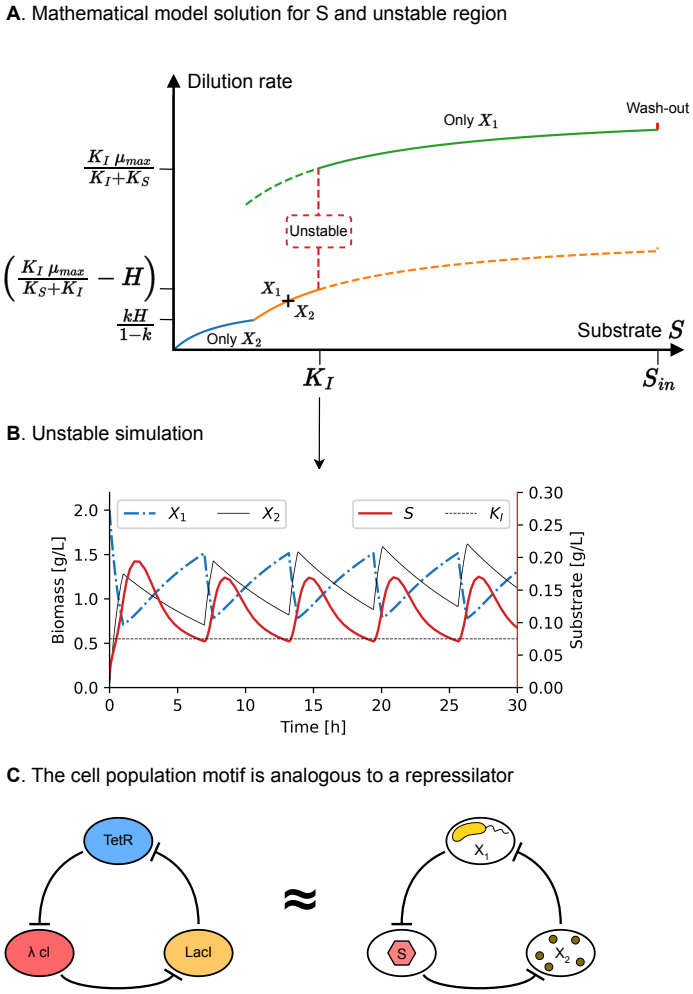

Figure 1: Representation of the solution of the mathematical model at steady state. **A**. There are two sides to the set of equations. At high dilution rates, only the non-stressed phenotype is present, as the sugar concentration is above the threshold K_I. At low dilution rates, the stressed phenotype is present as the sugar concentration is below K_I. In between those two cases, however, there is a region where no steady-state exists and oscillations are possible. **B**. As long as there is a delay in stress response, oscillations appear in silico in the unstable region. Here, a system with similar values to *B.subtilis* sporulation is represented with $D = 0.1$ h^{-1} and a delay of 0.5 h, but very small changes in parameters can have a very large impact on the results. **C**. The system, here represented schematically for bacillus sporulation (right), acts as a repressilator system (left)[7]. When sporulation or stress is induced, the amount of unstressed cells decreases. This causes the sugar concentration to increase and inhibit sporulation, letting the unstressed cells take over anew.

Experimental confirmation of instability region under multiple dilution rates in chemostat

Thus, the model predictions were challenged under continuous cultivation conditions, a type of cultivation commonly used to promote stable conditions and set the

residual substrate concentration S through the dilution rate. As a case study, *Bacillus subtilis* and one of its stress responses, sporulation, were selected. In this case, X_1 is the non-stressed biomass and X_2 is the stressed biomass, i.e., the spores. Sporulation is triggered when the residual glucose concentration drops below a concentration K_I and can be monitored with automated flow cytometry (FC) using a GFP transcriptional reporter, i.e., P_{spoIIE}*::GFP*.

Given the values of H, K_I, μ_{max} and K_S determined in batch cultivation (SI Appendix) and the absence of growth rate for the stressed phenotype, the model predicts that when D is high and the residual glucose concentration is above K_I, only the vegetative cells X_1 are present. If D is low and the residual glucose concentration should be equal or below K_I, both X_1 and X_2 can be observed with the abundance of X_2, the spores, periodically increasing. As predicted (Fig. 2), at high dilution rates, where the residual glucose concentration exceeds the switching threshold, cells do not activate the sporulation cascade, and the entire biomass consists of X_1. Upon decrease of the dilution rate, the residual concentration falls below the switching threshold, and bursts of gene expression from X_1 to X_2 appear. This temporal instability in gene expression persists for at least five retention times and gradually decreases in amplitude.

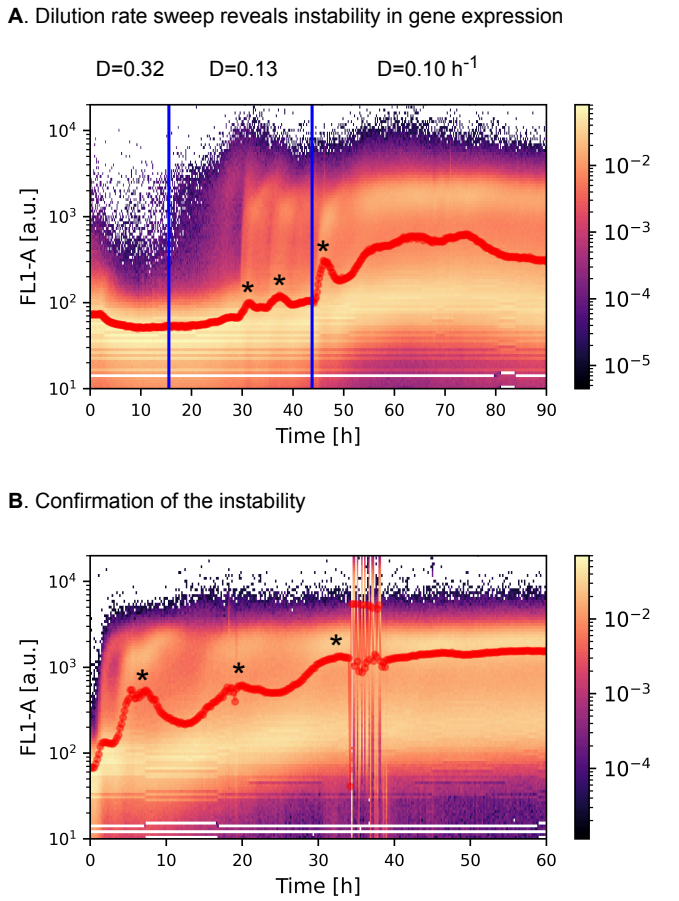

Figure 2: Mapping of cell population dynamics based on automated flow cytometry. The line indicates the 75th percentile of the fluorescence data. Large oscillations are highlighted with a '$*$'. **A**. According to the model, the gene expression dynamics depend on the dilution rate. At a high dilution rate ($D = 0.32$ h^{-1}) cells are not sporulating but once the dilution rate goes down ($D = 0.13$ h^{-1}) cells sporulate as burst that can be seen based on the activation of the P_{spoIIE}*::GFP*. The bursts gradually fade away when switching to a dilution rate of 0.1 h^{-1}. **B**. To confirm the observed instability in gene expression at a low dilution rate, we performed another chemostat ($D = 0.11$ h^{-1}) and observed similar outcome.

Conclusion and Perspectives

The results displayed in this work show how the output of a gene network without oscillating properties can nevertheless oscillate without the need for environmental forcing. This finding challenges the long-held assumption that microbial populations grown in chemostat exhibit a stable phenotype, instead suggesting that the population's characteristics can fluctuate over time. These oscillations are the result of a strong environmental feedback that could also be observed for many other phenotypic switches associated with an important reduction in growth. Eventually, oscillations fade away in the case of *Bacillus*. This deviation from the model prediction is speculated to originate from an over simplistic view of the switching that does not follow exactly a step function but more a hill one that allow for some heterogeneity in the response. Further, if a single sharp transition in phenotype causes unstable oscillations, there is no telling how multiple gene network interacting both with each other and the environment might create emergent behaviors on top of the ones predicted by current synthetic biology.

If cells can show such instabilities under stable operating conditions, then other culture conditions such as fed-batch (SI Appendix), turbidostat, or periodically refreshed cultures may be subject to the same or worse effects. In fact, similar oscillations involving stress related genetic switches have already been observed in a microfluidic where the feed was periodically interrupted [11].

Once those effects are brought to light, it may be necessary to take them into account when studying the biological system of interest. By doing so, researchers can gain a more comprehensive understanding of population dynamics and develop more effective strategies to control and manipulate cellular behavior.

Materials and methods

Additional details of the materials and methods are available in the SI Appendix, and the relevant data is available in the Supplementary Dataset.

Three sets of experiments were performed for this paper: first, the growth parameters of a *Bacillus subtilis 168* with the P_{spoIIE}*::GFP* construction were determined with a triplicate batch experiment. During this experiment, on-line flow cytometry was employed to measure the sporulation status as indicated by the *spoIIE* reporter, and samples were regularly taken to measure the OD and glucose concentration over time. Glucose was measured with a glucose assay kit (D-glucose enzymatic assay kit; Megazyme, Bray, Ireland). These results were used to approximate reasonably well the parameters needed, although the fast variations in sugar towards the end of the culture made K_I and K_S very uncertain.

Second, two chemostat experiments with successive dilution rates going from 0.3 h^{-1} to 0.1 h^{-1} were performed to observe the limits of the instability region. Just like with the batches, online flow cytometry was used to measure the sporulation state of the population. Further, samples from the 0.3 h^{-1} dilution rate were used to provide a better approximation of K_S.

Finally, an additional chemostat at 0.11 h^{-1} was performed to showcase the difference in equilibrium time between different starting points (here, a batch, in previous experiments, a continuous culture where some cells had already sporulated).

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