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Construction of an Oscillator Gene Circuit by Negative and Positive Feedbacks

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Synthetic oscillators are gene circuits in which the protein expression will change over time. The delay of transcription, translation, and protein folding is used to form this kind of behavior. Here, we tried to design a synthetic oscillator by a negative feedback combined with a positive feedback. With the mutant promoter P_{LacC} repressed by LacIq and P_{Lux} activated by AHL-bound LuxR, two gene circuits, Os-LAA and Os-ASV, were constructed and introduced into LacI-deleted *E. coli* DH5α cells. When glucose was used as the carbon source, a low level of fluorescence was detected in the culture, and the bacteria with Os-ASV showed no oscillation, whereas a small portion of those carrying Os-LAA demonstrated oscillation behavior with a period of about 68.3 ± 20 min. When glycerol was used as the carbon source, bacteria with Os-ASV demonstrated high fluorescence value and oscillation behavior with the period of about 121 ± 21 min.

Keywords: Oscillator, regulation, lactose operon, quorum-sensing system

Introduction

Synthetic biology is a new biological field where natural or artificial biodevices are used to construct new biological systems or to imitate natural systems for study or application [2]. In the past decades, different gene circuits such as genetic toggle switches [12], genetic counters [8], and various oscillators [5, 7, 9, 11, 18, 20, 22] have been designed and constructed. These devices have shown potential applications in many fields, including biofuel production, disease diagnosis, and gene therapy [16]. Among them, the synthetic oscillators are simplified models of a biological rhythm network, so that the mechanism and factors influencing on biological rhythm can be studied indirectly with the help of such gene circuits [7]. Moreover, oscillators can also be used as controllers of more complicated genetic circuits to adjust the expression of different genes [21].

The simplest oscillator is a negative feedback composed of a self-inhibited gene, where the oscillation is generated by the delay of transcription, translation, and folding of the gene, and its corresponding mRNA and protein, respectively, [22]. The Goodwin oscillator is a typical oscillator model in which a repressor is used to repress its own gene to generate oscillation [13]. Such an oscillator was successfully constructed with P_{Lac} and its repressor protein LacI [18]. This kind of oscillators is not robust and the condition for oscillation is rigorous. For example, if P_{Lac} and LacI were replaced with P_{Tet} and TetR, respectively, no oscillation could be observed [3]. This might be associated with the different times needed for transcription repression. As the activated form of TetR is a dimer, whereas the activated form of LacI is a tetramer, LacI needs more time to repress the P_{Lac} promoter [4].

Alternatively, multiple genes can be used for oscillator construction as well. The repressors LacI, TetR, and cI, together with the promoters regulated by them, have been used to construct a so-called repressilator, a gene circuit that generates oscillation by repressing the expression of one another [7]. The main principle for a multigene oscillator is that the finally expressed protein should repress the transcription of the first gene in order to form a negative feedback loop, so that an odd number of negative feedbacks should be used for such a purpose [19]. Furthermore, positive feedbacks can also be found in oscillator construction.

Theoretically, oscillators constructed by negative feedbacks combined with positive feedbacks are more robust, and are more insensitive to parameter changes [21].

Hybrid promoters had been used in most oscillators constructed by negative feedbacks combined with positive feedbacks. For example, by using a hybrid promoter activated by AraC and repressed by LacI, an oscillator with the oscillating period of 40 min was constructed [22]. In this study, we tried to use promoters that only response to one regulator, activator or repressor, to construct and analyze a genetic oscillator.

Materials and Methods

Strains, Plasmids, and Chemicals

Escherichia coli DH5α competent cells were bought from TransGen Biotech. Plasmid pUC19 was bought from Takara. T4 ligase and Fastdigest restriction enzymes were the products of Thermo Scientific. PrimeStar Max and Hi-FI DNA polymerases were bought from Takara. Low melting agarose, casamino acid, and thiamine were bought from Solarbio Life Sciences. Acyl-homoserine lactone (AHL) was brought from Sigma-Aldrich. L-Arabinose was bought from Shanghai Qianchen Biological Technology Co., Ltd. Other reagents were analytically pure.

ΔP_{Lac} Plasmid Construction

The original P_{Lac} promoter was deleted from pUC19 by inverse PCR to construct the pUC19- plasmid. The following primers with *Afl*II restriction sites (underlined) were used: sense: 5′- CGGAAC TTAAGAAAGAACATGTGAGCAAAAGGCCAG-3′; antisense: 5′-CGGAACTTAAGAAGCTTGCATGCCTGCAGGTC -3′.

After restriction, ligation, and transformation, the colonies were selected and the pUC19 plasmid was extracted for further construction.

Oscillator Plasmid Construction

The design of the gene circuit and the structure of the oscillator plasmid are shown in Figs. 1A and 1B, respectively. For this purpose, the P_{LacC} promoter was amplified from pUC19 with a point mutation (T14C) incorporated in its operator, by PCR. The repressor gene *lacIq* was obtained from *E. coli* BL21 (DE3). The P_{Lux} promoter and *luxR* were synthesized according to the sequences reported (GenBank: Y00509.1 and M19039.1, respectively). The reporter gene *egfp* was preserved in our laboratory. The RBS (underlined) and its downstream sequence AAGGAGATATACAT were obtained from pET28a. Terminator *rrnBT1T2* from pBV220 was used to separate promoters and the genes they controlled. Different *ssrA*-mediated tags were added to the C-termini of EGFP and LacIq to accelerate their degradation. Among them, EGFP was fused with *AAV* tag (5′-GCAGCAAACGACGAAAACTAC GCTGCAGCAGTT-3′) with a medium half-life period, while LacIq was fused with two different tags. When *ASV* tag (5′- GCAGCA AACGACGAAAACTACGCTGCATCAGTT-3′) with a long half-life period was added, the circuit was designated as Os-ASV, and *LAA* tag (5′-GCAGCAAACGACGAAAACTACGCTTTAGCAGCT-3′) with a short half-life period was added in the circuit designated as Os-LAA [1].

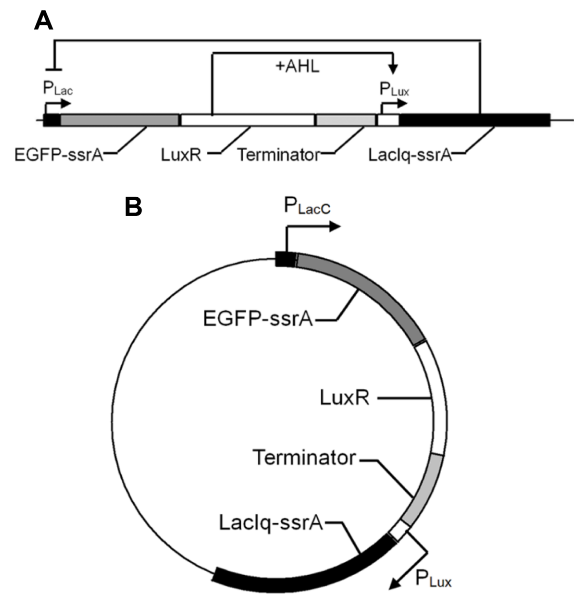

Fig. 1. Oscillator gene circuit design (**A**) and construction (**B**).
(**A**) Expression of EGFP and LuxR under the control of P_{LacC} leads to the increase of fluorescence signal and accumulation of LuxR. When the concentration of LuxR reaches a threshold, it can turn on the P_{Lux} promoter together with AHL added separately in the system. Accumulation of LacIq, in turn, represses the P_{LacC} activity, resulting in the decrease of fluorescence signal and decay of LuxR. With the decline of LuxR concentration, P_{Lux} returns to its nonactive state and the synthesis of LacIq is inhibited. Consequently, the system returns to its initial state with the degradation of LacIq. Repetition of the cycle will form an oscillation behavior of fluorescence. (**B**) P_{LacC} controls the transcription of *EGFP-ssrA* and *luxR*, while P_{Lux} controls the transcription of *lacIq-ssrA*. ssrA represents the *ssrA*-mediated tags, which can reduce the half-life period of target proteins. Terminator *rrnBT1T2* was used to separate the two promoters and the genes they controlled.

All the fragments were digested separately and ligated consequently to obtain the full-length sequences. After validation by DNA sequencing, the pUC19- plasmid carrying the gene circuit Os-ASV or Os-LAA was transformed into the *E. coli* DH5α Δ*lacI* strain for further analysis.

lacI Knockout Strain Construction

In order to avoid the possible interference caused by LacI expressed by the host strain, the Red recombination system from λ phage was used to construct an *E. coli* DH5α Δ*lacI* strain. A DNA fragment containing the kanamycin resistance gene and LacZ′

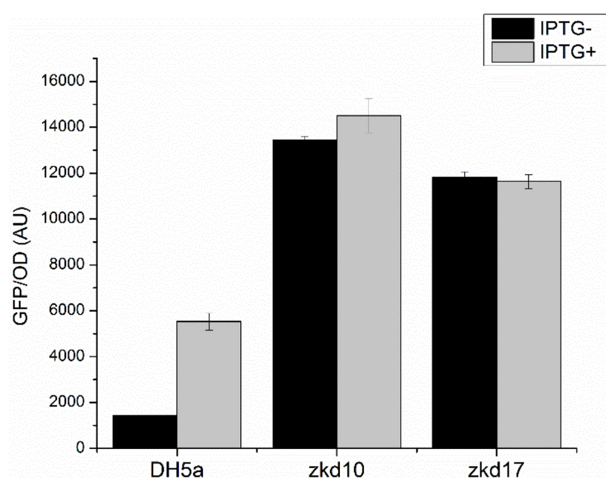

Fig. 2. Fluorescence in Δ*lacI* and wild-type *E. coli* DH5α strains.

Wild-type *E. coli* DH5α (DH5α), and *lacI* knockout strains zdk10 and zdk17 were transformed with plasmid pUC19- harboring *egfp* under the control of P_{LacC}. The fluorescence in bacteria was analyzed in the absence (IPTG-) or presence (IPTG+) of 800 μM IPTG.

was amplified by PCR with the following primers: sense: 5′-GTATGGCATGATAGCGCCCGGAAGAGAGTCAATTCAGGGTGGTGAATGTGGCGCAACGCAATTAATGTGAGTTAG-3′; antisense: 5′-TGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGTTAGAAA AACTCATCGAGCATCAAATG-3′. The resultant sequence was flanked by a 50 bp homologous arm (underlined sequence in the primers) of *lacI* gene at either side and used as a knockout linear fragment.

The recombination procedure was carried out in *E. coli* DH5α with plasmid pKD46 as previously described [18]. The blue colonies that grew in media with kanamycin, X-Gal, and IPTG were screened by PCR using the following primers complementary to the cross-sites of chromosome and the inserted fragment: sense: 5′-GGTGAATGTGGCGCAACGC-3′; antisense: 5′-GCTTTCCAG TCGTTAGAAAAACTC-3′.

Observation and Data Processing

The bacteria containing the oscillator plasmids were grown in M9 culture (0.1% casamino acid, 200 μM thiamine, 0.4% glucose or 0.5% glycerol, 10 μM IPTG, 100 μg/ml ampicillin) at 37°C until the exponential phase. The cells were washed twice with fresh medium without IPTG, and then resuspended and concentrated at room temperature in 100 μl of fresh medium containing 30 nM AHL and 0.5% low-melt agarose, which was previously maintained at 55°C. The concentrated culture was dropped on single-well depressed slides with 2% agarose pad with 30 nM AHL and solidified. The slides were then covered by cover glasses and sealed to prepare the samples for observation.

A Nikon Ti series inverted fluorescence microscope was used for observation. The samples were incubated at 37°C by a heater. DIC and green fluorescence (FITC) images were taken every 5 min for 5–6 h.

For data processing, the fluorescence intensities of the corresponding cells in FITC images were measured by Image Pro Plus. The raw data were smoothed using adjacent-averaging in Origin.

Results

Gene Circuit Design

The gene circuit designed is shown in Fig. 1A. The transcription of *egfp-ssrA* and *luxR* is under the control of promoter P_{LacC}, whereas the transcription of *lacIq-ssrA* is under the control of promoter P_{Lux}. Terminator *rrnBT1T2* was used to separate the two promoters and the genes they controlled. In order to reduce binding between P_{Lac} and LacIq, P_{LacC}, a mutant promoter with a point mutation (T14C) in the operator, was used [15]. The *lacI* gene on the bacterial chromosome was knocked out by Red recombination to inhibit its expression. To avoid circuit distraction by protein accumulation, *ssrA*-mediated tags that can reduce the half-life period of target proteins [1, 17] were added to C-termini of all proteins in the circuit, except LuxR, which is unstable in *E. coli*. AHL was added additionally at the beginning of observation to activate LuxR and subsequently initiate the transcription from promoter P_{Lux} [6, 10].

By our design, the expression of LuxR and EGFP under the control of P_{LacC} leads to the accumulation of the proteins and detection of fluorescence signal. When the concentration of LuxR reaches a threshold, it could turn on the P_{Lux} promoter in the presence of AHL and initiate the expression of LacIq-ssrA, a repressor of P_{LacC}. Repression of P_{LacC} is followed by the degradation of EGFP-ssrA and LuxR, and the decline of the fluorescence signal. With the decrease of LuxR amount in cells, the P_{Lux} promoter is turned off and the system returns to its initial state with the degradation of LacIq. Repetition of the cycle will form an oscillation behavior of the fluorescence signal.

Validation of *lacI* Strains

The Red recombination system from phage λ was used for *lacI* knockout in *E. coli* DH5α. Blue colonies were selected and further analyzed by PCR, with primers complementary to the sequences formed only after recombination between the linear fragment and the chromosome. Among 20 colonies analyzed, only strain No. 10 and 17 (designated as zkd10 and zkd17, respectively) gave positive results (data not shown). For additional functional characterization, the

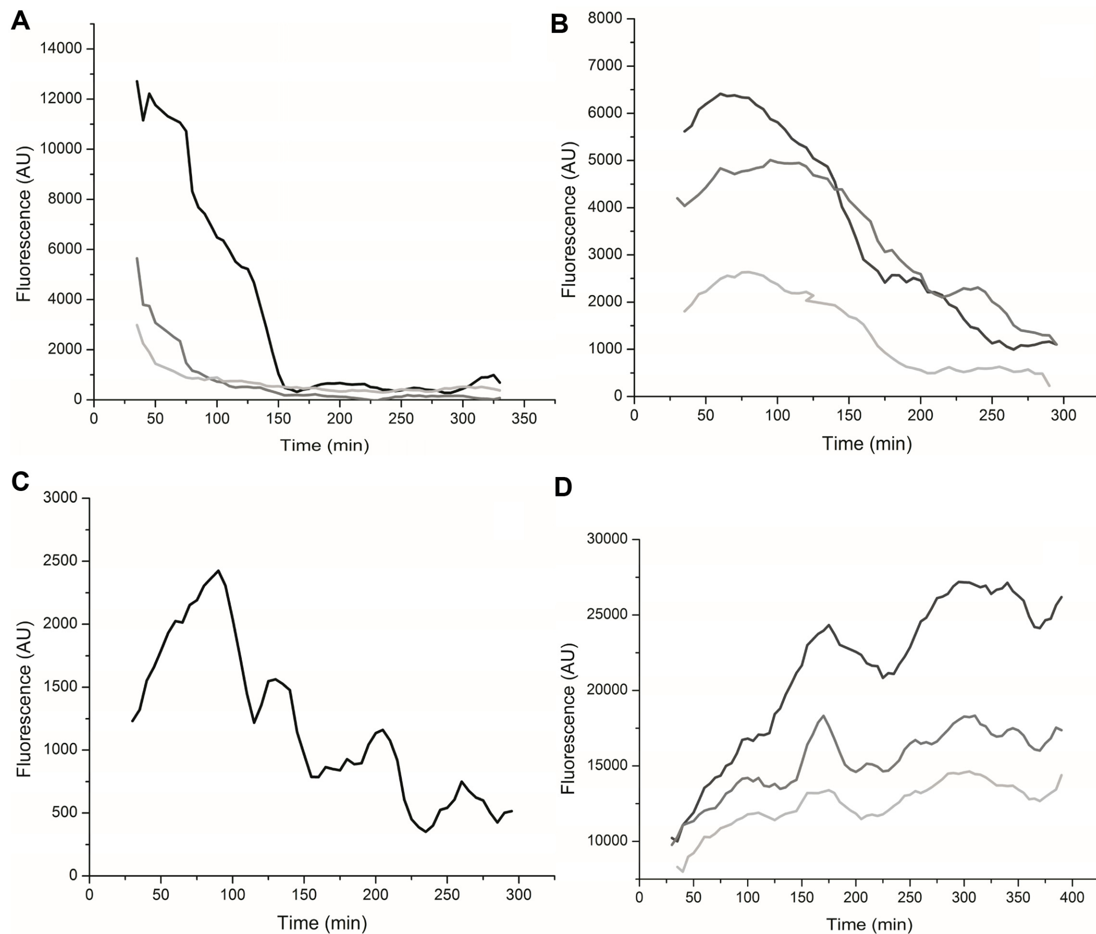

Fig. 3. Fluorescence behavior in bacteria with Os-ASV (**A** and **D**) or Os-LAA (**B** and **C**) when glucose (**A**, **B**, and **C**) or glycerol (**D**) was used as carbon source.

Bacteria were grown in M9 medium with 0.4% glucose or 0.5% glycerol and 30 nM AHL at 37°C during detection. Images were captured every 5 min and analyzed by Image Pro plus. The different lines represent fluorescence in different bacteria in the same experiment. No oscillation of fluorescence signals was detected in bacteria with Os-ASV (**A**) and in most bacteria with Os-LAA (**B**) when glucose was used as carbon source. Fluorescence oscillation with a period of 68.3 ±20 min was detected in about 10% bacteria with the Os-LAA gene circuit when glucose was used as carbon source (**C**). When glycerol was used as carbon source, oscillation in fluorescence with a period of 121 ± 21 min in bacteria with Os-ASV was recorded (**D**).

plasmid with *egfp* controlled by P_{LacC} was transformed into zkd10, zkd17, and original DH5α strains, respectively. Analysis of the fluorescence signal in the presence and absence of IPTG showed that the fluorescence in zkd10 and zkd17 was much higher than that in wild-type DH5α, and IPTG had no notable influence on EGFP expression (Fig. 2). Based on the results, zkd17 was used for further experiment.

Fluorescence Observation with Glucose as Carbon Source

Although P_{LacC} was a weak promoter when glucose was used in M9 medium as the carbon source, the growth profile of bacteria was much better than that when glycerol was added (data not shown). Recording the fluorescence signal under the conditions showed that the fluorescence of bacteria zkd17 with Os-ASV declined during the observation, and no obvious rise was noticed (Fig. 3A). Since there was no remarkable fluorescence decline (less than 21%) in the observation without AHL, this behavior was not caused by fluorescence decay but by the expression of LacIq-ASV.

When the strain zkd17 with Os-LAA was grown and observed under the same conditions, the fluorescence of most bacteria, unlike that of bacteria with Os-ASV, increased at first and then started to decline. Similar to that in strain zkd17 with Os-ASV, no obvious rise was noticed after that in most bacteria (Fig. 3B). Detailed analysis of hundreds of bacteria showed that the fluorescence fluctuation from

about 10% bacteria with Os-LAA exhibited a typical oscillating behavior during decline, with a period of 68.3 ± 20 min (Fig. 3C).

Fluorescence Observation with Glycerol as Carbon Source

As P_{LacC} is a strong promoter in medium with glycerol as the carbon source, a stronger fluorescence was expected. The fluorescence behavior of zkd17 with the Os-ASV gene circuit in M9 with glycerol was recorded in 6.5 h (Fig. 3D). The results showed that the fluorescence of most bacteria increased with fluctuation. The oscillation period was 121 ± 21 min.

Discussion

When glucose was added as carbon source, the fluorescence in bacteria with Os-ASV declined continuously (Fig. 3A), whereas that in bacteria with Os-LAA rose at first and then declined (Fig. 3B). We supposed that the different pattern might be associated with the different degradation rate of LacIq mediated by *ssrA* tags fused to the C-termini of the protein. As the half-life period of LacIq-ASV is longer than that of LacIq-LAA, the repressor would accumulate faster in bacteria with Os-ASV than that in bacteria with Os-LAA. Consequently, compared with bacteria with Os-LAA, less time would be needed for LacIq-ASV to reach the concentration threshold for P_{LacC} repression in bacteria with Os-ASV, leading to the fluorescence decline at the beginning of observation. In contrast, as LacIq-LAA degraded more rapidly, the concentration of the protein at the beginning of observation was insufficient to repress the P_{LacC} promoter in bacteria with the Os-LAA circuit, and the fluorescence continued to increase over time until LacIq-LAA reached the threshold concentration.

P_{LacC} was a weak promoter with a relatively low transcription rate when glucose was used as the carbon source. In the presence of the repressor LacIq-ssrA, the expression rate of EGFP might be lower than its degradation rate, resulting in the continuous fluorescence decline as observed in most cases. Only about 10% of bacteria with Os-LAA showed oscillation during the signal decline (Fig. 3C). We supposed that in a small fraction of bacteria with rapidly degrading LacIq-LAA, a temporary accumulation of EGFP could occur, which was responsible for the oscillation observed.

When glycerol was used instead of glucose, the fluorescence in most bacteria containing Os-ASV increased with oscillating behavior during observation (Fig. 3D). As there are two different states of oscillation, unstable (oscillating) and stable [7], once such parameters as protein and mRNA concentrations fell into the stable state range, no oscillation occurs. Based on the results observed, we supposed that the unstable range of bacteria with Os-LAA was wider than that of bacteria with Os-ASV in the presence of glucose, whereas the unstable range of bacteria containing Os-ASV with glycerol was much larger than that of bacteria with Os-LAA when glucose was used. Moreover, for the gene circuit in this study, it is more likely to oscillate when P_{LacC}, the promoter responding to a repressor, is a strong promoter, or LacIq, the repressor, degrades rapidly. Furthermore, the differences between the gene circuits involving promoter transcription rate, protein degradation rates, *etc*. have influence not only on the possibility to oscillate but also on the oscillating period. According to the results, the period in bacteria containing Os-LAA with glucose was much shorter than that of bacteria containing Os-ASV with glycerol.

It has also been noticed that the fluorescence behaviors of different bacteria in the same culture were not fully synchronous, which was especially notable when glucose was used as the carbon source. We supposed that "noises" such as the discrepancy of the microenvironment around the bacteria and the diversity in protein expression were responsible for the phenomenon.

In this study, promoters responding to only one regulator were used for oscillating gene circuit construction, and several parameters influencing the oscillation and its period were analyzed. In further studies, a mathematical model simulating the gene circuit designed should be built to systemically analyze the influence of each element on the final oscillation of the gene circuit.

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