nature portfolio

Peer Review File

Rewiring native post-transcriptional global regulators to achieve designer, multi-layered genetic circuits

Corresponding Author: Professor Lydia Contreras

This manuscript has been previously reviewed at another journal. This document only contains reviewer comments, rebuttal and decision letters for versions considered at Nature Communications.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors report the design of synthetic circuits in which gene regulations were obtained by rewiring the components of the endogenous Csr system in E. coli. They show a Buffer gate discussing its tunability, as well as other 1- to 3-input logic gates and a pulse generator; then, multi-gene regulation and portability to other microbes are reported. The authors highlight different advantages over traditional transcriptional regulators, such as: the Csr system can be engineered by exploiting the endogenous CsrA protein and only designing proper 5'-UTR that are targeted by inducible csrB expression; the Csr system is conserved among bacteria, thus promoting expression systems portability to other chassis; given a logic function, the size of DNA encoding Csr-based logic gates can be significantly reduced compared with transcriptional regulators-based circuits.
A potential issue is the relatively low fold-activation/repression of the proposed system, but the authors demonstrate experimentally that the post-transcriptional gates can be successfully interconnected with transcriptional regulators to form higher-order circuits with relatively high fold-activation.
The text is well organized and the topic is of interest to audiences working on different aspects of synthetic biology.
Compared with the previous submission, many issues have been addressed. However, a number of shortcomings are still present that should be addressed before considering the manuscript for publication.

Major comments

1) The only point raised in the previous submission which was not properly addressed in the last version of the manuscript is the source of the transcriptional regulators (lacI, tetR and araC). In particular the source of lacI is very important and needs to be clear in the text, as most fold-changes described in the manuscript depend on PLlacO inducibility by IPTG. One important part of the manuscript is the portability of the Buffer gate across different species, in which the source of lacI needs to be clarified. In most of these species, the authors observe a low but statistically significant fold-activation (also qualitatively confirmed by PLlacO-gfp in Suppl.Fig.S10) which needs to be discussed in light of the source of lacI in these organisms, for which an endogenous lacI gene may not be present.

2) A discussion on the modularity of the post-transcriptional systems should be provided. The authors demonstrate that the csr system can be successfully interfaced with transcriptional regulators yielding a functional circuit (e.g., in Fig.5E). However, the limitations in the engineering of logic functions only made of csr-based logic gates, multiplexed regulations and orthogonality need to be mentioned in the discussion section.

3) The part describing a pulse generating circuit should be clarified in different aspects.
i) The pulse generating circuit described in this manuscript is a two-input network. Even though the two input molecules are added simultaneously, I think that the herein constructed circuit is not directly comparable with the previously proposed ones (included in some of the cited works, relying on a one-input incoherent feedforward loop); this design difference should be mentioned.
ii) The authors call the circuit "genetic pulse", which should be better described as a "pulse-generating circuit".
iii) The authors state in the discussion section that the circuit has a faster response than previously implemented circuits with

a similar function. Do the authors refer to the rise or fall phase, or to the total duration of the pulse?
iv) These indexes are tuned by different parameters, some of them not dependent on the csr system, such as the activation dynamics of the input promoters, the kinetic constants and the abundance of activator and repressor, and the dilution dynamics of the reporter protein due to cell division (as there is no fast-degradation tag in the used gfpmut). To fully support the discussed features in Lines 423-429, additional experimental and/or modeling work would be needed to decouple all these effects and focus on the specific contribution of the csr system. For instance, a comparison of the response time between post-transcriptional vs transcriptional regulation may be studied by comparing circuits with the same inputs and outputs. To address this point, I would recommend a revision of the discussion section in Lines 423-429 to smooth the performance comparison with previous circuits, unless the authors provide a demonstration that the csr system is the main responsible of the fast dynamics.

Minor comments

Line 30. "natively" should be "native"
Line 58. "throught" should be "through" or "throughout"
Line 64. please uniform the notation (italics/non-italics, uppercase/non-uppercase) of the sRNAs
Line 111-112. Please check the sentence for the English language.
Line 112-114. Please specify that this sentence on the secondary structure is based on the prediction by a computational tool (it is clear only by reading the supplementary caption), and please specify the tool.
Line 123. It seems that the rise time to reach saturation in fluorescence time profiles is 40-60 min only in this experiment. In the other ones described in the manuscript the time is larger. Please discuss this difference when presenting the data of the subsequent experiments.
Line 126. Do the authors use the wild type Plac promoter or is it PLlacO? Please check.
Line 134. This is the first sentence specifying that the work is being done in E. coli. Please specify it earlier in the text.
Line 140. Please specify which activation index was considered for improvement. Fold-activation or strength in the induced state?
Line 202. "at least" should be "up to".
Line 342. If I understood correctly, "lacI-gfpmut3" should be "PLlacO-gfpmut3".
Line 367-369. The sentence should be revised.
Line 420-421. (optional) In addition to the number of parts compared with traditional logic gates, the authors could consider emphasizing the reduction in nucleotides of a genetic circuit implementing the same logic function.
Line 529. Check the text to remove parts regarding the mevalonate pathway, which is not a topic of this manuscript.
Fig.1 caption. Please check the letters of the panels for duplicates.
Fig.5 caption. Fluorescence is spelled wrongly.
Supplementary file p.4. Please specify which plate reader was used for the small-scale fluorescence assays.
Supplementary Fig.S9. It seems that the fitting of the curve has not been done properly. Data should be captured by a steep Hill equation, but the reported curve is far from the data trend. Please check the procedure.

Response to Reviewer Comments

We thank the reviewer for taking the time to review and provide very helpful feedback for our latest submission and as well as their support regarding the quality of our text. Specifically, for the acknowledgement that our work is of interest to synthetic biology audiences. We also would like to thank the reviewer for their very insightful comments to further improve clarity and impact of our manuscript. We have incorporated new sections for each of the major comments below, which we expand upon in greater detail below. We also revised our manuscript to address the minor comments.

Major comments

1) The only point raised in the previous submission which was not properly addressed in the last version of the manuscript is the source of the transcriptional regulators (lacI, tetR and araC). In particular the source of lacI is very important and needs to be clear in the text, as most fold-changes described in the manuscript depend on PLlacO inducibility by IPTG. One important part of the manuscript is the portability of the Buffer gate across different species, in which the source of lacI needs to be clarified. In most of these species, the authors observe a low but statistically significant fold-activation (also qualitatively confirmed by PLlacO-gfp in Suppl.Fig.S10) which needs to be discussed in light of the source of lacI in these organisms, for which an endogenous lacI gene may not be present.
We thank the reviewer for bringing up this point and agree that additional explanation regarding the source of our transcriptional regulators is important for clarity and repeatability.

In the updated manuscript, we added three sections (Lines 118-120 for LacI, Lines 264-266 for TetR and Lines 276-279 for AraC) that detail the source of the LacI, TetR, and AraC regulatory proteins used in the Csr-regulated and transcriptional circuits. Additionally, we added another section in our results (Lines 333-334, and 357-361) that explains we used the LacI regulator derived from *Escherichia coli* with the P_{LlacO} promoter when establishing cBUFFER and the transcriptionally regulated BUFFER Gates in *S. oneidensis, E. coli Nissle, P. putida*.

2) A discussion on the modularity of the post-transcriptional systems should be provided. The authors demonstrate that the csr system can be successfully interfaced with transcriptional regulators yielding a functional circuit (e.g., in Fig.5E). However, the limitations in the engineering of logic functions only made of csr-based logic gates, multiplexed regulations and orthogonality need to be mentioned in the discussion section.

We thank the reviewer for highlighting this point to expand our discussion. We agree that commenting on the modularity, and orthogonality of our Csr-regulated circuits will help underscore their current use, as well as opportunities for future developments. We added an additional paragraph to our discussion sections (Lines 439 - 451) that expands upon these topics.

3) The part describing a pulse generating circuit should be clarified in different aspects.
i) The pulse generating circuit described in this manuscript is a two-input network. Even though the two input molecules are added simultaneously, I think that the herein constructed circuit is not directly comparable with the previously proposed ones (included in some of the cited works, relying on a one-input incoherent feedforward loop); this design difference should be mentioned.
We agree with the reviewer and have updated our discussion section on this pulse-generating circuit to modify our comparisons to be more appropriate, as well as to highlight key differences between our system and other previously cited works (Lines 452 - 464).

ii) The authors call the circuit "genetic pulse", which should be better described as a "pulse-generating circuit".
We agree with reviewer that “pulse-generating circuit” is a much more appropriate name for this system, as such we updated the nomenclature to “pulse-generating circuit” in the latest version of the manuscript

iii) The authors state in the discussion section that the circuit has a faster response than previously implemented circuits with a similar function. Do the authors refer to the rise or fall phase, or to the total duration of the pulse?
This is a good point and we address this comment as well as the next one together in the paragraph below.

iv) These indexes are tuned by different parameters, some of them not dependent on the csr system, such as the activation dynamics of the input promoters, the kinetic constants and the abundance of activator and repressor, and the dilution dynamics of the reporter protein due to cell division (as there is no fast-degradation tag in the used gfpmut). To fully support the discussed features in Lines 423-429, additional experimental and/or modeling work would be needed to decouple all these effects and focus on the specific contribution of the csr system. For instance, a comparison of the response time between post-transcriptional vs transcriptional regulation may be studied by comparing circuits with the same inputs and outputs. To address this point, I would recommend a revision of the discussion section in Lines 423-429 to smooth the performance comparison with previous

circuits, unless the authors provide a demonstration that the csr system is the main responsible of the fast dynamics.
We agree with the reviewer's last two points (iii and iv) regarding response times and our original statement detailed in Lines 423-429, and have updated this section to focus the utility of having CsrD as a tunable node that can "reset" the synthetic system, as well as ways the CsrD-CsrB interactions could be tuned in the future to modulate dynamics within this Csr-regulated genetic circuit. This is specifically addressed in Lines 455-461.

Minor comments
We thank the reviewer for taking the time to provide this additional feedback to further strengthen and improve the clarity of this manuscript. We have addressed each of these minor comments and provided the line numbers of the new manuscript with the implemented changes.

Line 30. "natively" should be "native"
We have corrected this in the latest manuscript.

Line 58. "throught" should be "through" or "throughout"
We have corrected this to "throughout".

Line 64. please uniform the notation (italics/non-italics, uppercase/non-uppercase) of the sRNAs
We corrected this, and reviewed the rest of the document to make sure all notation is uniform.

Line 111-112. Please check the sentence for the English language.
This sentence has been edited to improve clarity.

Line 112-114. Please specify that this sentence on the secondary structure is based on the prediction by a computational tool (it is clear only by reading the supplementary caption), and please specify the tool.
We updated this section (Lines 111-114) now detail we utilized ViennaRNA as the secondary structure prediction software.

Line 123. It seems that the rise time to reach saturation in fluorescence time profiles is 40-60 min only in this experiment. In the other ones described in the manuscript the time is larger. Please discuss this difference when presenting the data of the subsequent experiments.

We appreciate the author for commenting on these differences and added a section (Lines 151-154) to discuss why these differences could occur.

Line 126. Do the authors use the wild type Plac promoter or is it PLlacO? Please check.
We utilized the P_{LlacO} promoter and updated the rest of the manuscript to make sure that is reflected throughout (now Line 128).

Line 134. This is the first sentence specifying that the work is being done in E. coli. Please specify it earlier in the text.
We updated the manuscript to specific our work is first established in *E. coli* in Lines 56 and 93.

Line 140. Please specify which activation index was considered for improvement. Fold-activation or strength in the induced state?
We chose to use fold-activation as the metric for improvement and updated the manuscript to ensure that comparison was utilized throughout this section (Lines 141, and 149).

Line 202. "at least" should be "up to".
We agree with the reviewer that using “up to” is much more appropriate in the sentence, and we have corrected this in the latest version.

Line 342. If I understood correctly, "lacI-gfpmut3" should be "PLlacO-gfpmut3".
The reviewer is correct, and we updated the text to say P_{LlacO}-*gpfmu3* and made sure this was consistent for the remainder of the manuscript.

Line 367-369. The sentence should be revised.
We have revised this sentence to improve clarity and flow, which is now Lines 381-382.

Line 420-421. (optional) In addition to the number of parts compared with traditional logic gates, the authors could consider emphasizing the reduction in nucleotides of a genetic circuit implementing the same logic function.
We thank the reviewer for taking the time to highlight this point, and completely agree. We added a lines that expand upon this idea in the discussion section (Lines 435-438)

Line 529. Check the text to remove parts regarding the mevalonate pathway, which is not a topic of this manuscript.
We thank the reviewer for taking the time to catch this, and we have removed all parts regarding our previous mevalonate work from the manuscript.

Fig.1 caption. Please check the letters of the panels for duplicates.
The figure caption for Figure 1 has been updated to remove duplicates.

Fig.5 caption. Fluorescence is spelled wrongly.
Spelling in Figure 5 caption has been revised to fix all misspellings

Supplementary file p.4. Please specify which plate reader was used for the small-scale fluorescence assays.
Plate reader used in these assays was a BGM Clariostar, which is now noted in the supplementary files.

Supplementary Fig.S9. It seems that the fitting of the curve has not been done properly. Data should be captured by a steep Hill equation, but the reported curve is far from the data trend. Please check the procedure.
We thank the reviewer for noticing this and we have reprocessed the data to refit the figure with an updated Hill Function.

As a final note, we would once again like to thank this reviewer for taking the time to review our original submission to Nature Chemical Biology, the updated submission to Nature Communications, and this latest draft. It is clear the reviewer spent time and care with the manuscript and was able to provide very helpful feedback, and ideas to strengthen the manuscript. The comments were incredibly thoughtful and valuable to us during the revision process. Thank you for taking the time to help us improve our work.