Cyanobacterial biofactories: combining evolved and synthetic genetic regulatory mechanisms to yield carbon-neutral bioproducts

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Abstract While cyanobacteria hold the promise of producing useful bioproducts from little more than sunlight and carbon dioxide, their economic feasibility hinges on developing better mechanisms to manipulate gene expression in order to divert photosynthate towards product formation. A recent report demonstrates how combining simple feedback loops can improve the dynamic range of target gene expression while utilizing progressively lower concentrations of chemical inducers. As an extension of this work, we propose that utilizing regulatory elements that respond to changing temperatures could circumvent the need for exogenous chemical inducers, maintaining low background expression under non-induced conditions, and enhancing dynamic range via temperature induction of cyanobacterial cultures on an industrial scale.

Keywords: synthetic biology, cyanobacteria, asRNA, RNA thermometer, bioproduct formation.

Kurt Vonnegut once summarized the great complexity of nature by proclaiming “Evolution is so creative. That’s how we got giraffes.” Synthetic biology can be said to have the goal of recreating that beautiful natural intricacy, albeit by engineering genetic pathways to generate useful compounds rather than the morphological traits they produce in tall ungulates. The full potential of this goal has not yet been attained in cyanobacterial systems. While many genetic modules such as switches and time-delayed circuits have been designed, the primary objective lies in combining modules to produce functional networks for the purpose of bioproduct generation [1]. In a recent publication, Higo, et al., [2] demonstrated proof of principle that the introduction of an inducible positive feedback loop increases expression of a reporter gene while simultaneously broadening the dynamic range, thereby allowing for expression levels to be more finely modulated via inducer concentration. This insight marks a notable advancement in the potential for utilizing cyanobacteria for biotechnological applications.

The advantages of using photosynthetic organisms to produce commercial products include both the input cost of the raw materials required for their growth, sunlight and CO_2, and their net zero effect on atmospheric CO_2 levels [3]. In particular, photosynthetic prokaryotes are distinguished from higher plant-based systems by virtue of their rapid growth rate, higher efficiency in converting solar energy to biomass, and potential for industrial-scale implementation on non-arable land. Nevertheless, control of gene expression could be further optimized to allow cyanobacteria to become a more economically viable source of bioproducts, especially at an industrial scale. Indeed, a primary goal is to utilize regulatory elements that minimize the background expression of biosynthetic genes under non-inducing conditions. This is crucial as many biosynthetic products, including biofuels, are harmful to cyanobacteria [4]. Introduction of a chemical or alteration of growth conditions would rapidly promote the expression of biosynthetic genes, generating dynamic regulation of bioproduct formation. Commonly identified obstacles

include improving carbon fixation efficiency, increasing tolerance to

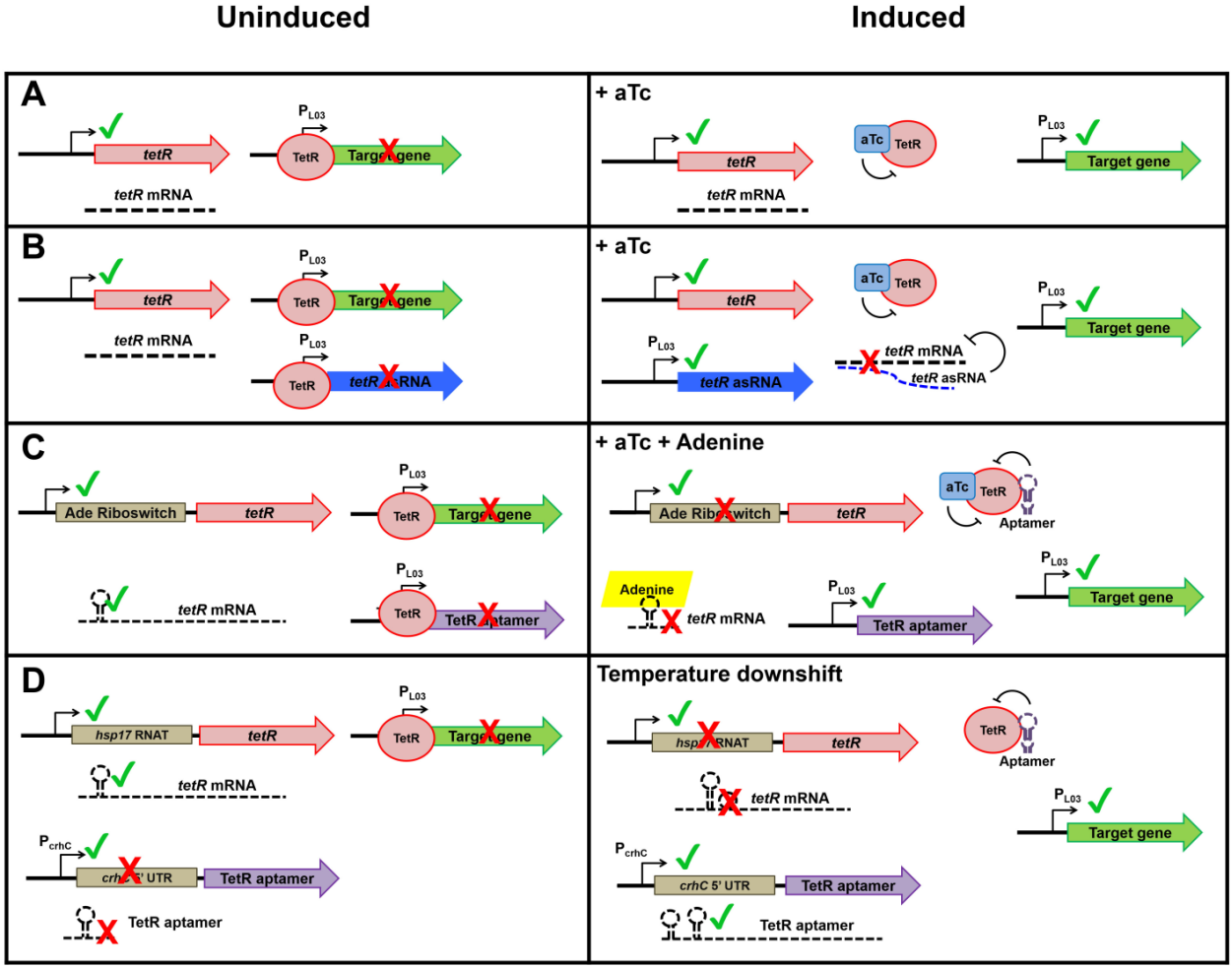

Figure 1. Regulation of cyanobacterial gene expression using modular genetic elements. A) A one component regulatory system. Expressed from a constitutive promoter, the transcriptional repressor TetR binds P_{L03} to prevent target gene expression [2]. The inducer aTc binds to and inhibits TetR binding, allowing transcription of the target gene. B) A two component regulatory system. Scenario A is elaborated by a TetR-regulated *tetR* asRNA transcribed from P_{L03}. The *tetR* asRNA represses translation of the transcript, resulting in a feedback loop that lowers the rate of *de novo* TetR expression, thereby increasing target gene expression. C) A triple component regulatory system. The aTc regulation shown in scenario A is extended by regulation of *tetR* transcription by an adenine (Ade) riboswitch that allows attenuation of *tetR* transcription in the presence of adenine [2]. TetR activity is also inhibited by aTc plus a P_{L03}-regulated TetR aptamer that displaces the repressor from P_{L03}, thus forming a feedback loop to further inhibit binding of the TetR repressor. D) A proposed abiotic stress regulatory system utilizing temperature-responsive elements to regulate target gene expression for bioproduct formation. A cyanobacterial RNA thermometer (*hsp17* RNAT) [10] will inhibit translation of *tetR* mRNA at low temperature and TetR aptamer expression from an *Anabaena* cold-induced promoter and 5' UTR (*crhC*) [11, 12] would minimize both TetR translation and activity during culture growth but allow energy and photosynthate to be directed towards bioproduct formation at low temperature where growth rates are limited. Adapted from Higo, *et al.*, 2016.

biosynthetic products, and maintaining photosynthetic efficiency when scaling-up laboratory cultures to industrial-scale bioreactors [5]. Higo, et al., [2] have shown how a synthetic biology approach, combining genetic elements that simultaneously regulate expression at multiple levels, is an effective strategy to alleviate these impediments.

The theophylline riboswitch, which induces translation in the presence of theophylline, is a commonly used regulatory module in synthetic biology [6]. However, this system has a narrow dynamic range and cannot regulate the expression of small non-coding RNAs; which are highly valuable for synthetic biology due to their widespread roles including degradation or stabilization of target RNAs [2]. Instead, Higo, et al., achieved enhanced expression levels using an anhydrotetracycline (aTc)-TetR induction system (Figure 1A) in the filamentous cyanobacterium *Anabaena* sp. PCC 7120. In this "second generation" regulatory system, transcription from the P_{L03} promoter was induced by aTc (2 µg/mL), whose binding to TetR relieves transcriptional repression, thus temporally regulating the expression of a downstream reporter gene [2, 7]. Although able to regulate the transcription of non-coding RNAs, the aTc-TetR system lacked a wide dynamic range and required an elevated concentration of the expensive inducer aTc to achieve maximum induction.

Minimizing inducer input is a straightforward means by which cyanobacteria-generated bioproducts can be priced more competitively against conventional sources. To reduce inducer cost, Higo, et al., [2] generated a third *Anabaena* strain in which separate L03 promoters control expression of both a *tetR* antisense RNA (*tetR* asRNA) and a reporter gene (Figure 1B). Under inducing conditions, a feedback loop is generated in which *de novo* TetR translation and repressor activity will be reduced as aTc will prevent TetR binding to the L03 promoters while the *tetR* asRNA will reduce *tetR* mRNA translation. In this construct, a 10-fold reduction in aTc concentration (200 ng/mL) achieved expression levels equivalent to that of the second-generation strain, but still retained a narrow dynamic range.

Higo, et al., [2] then introduced two additional regulatory elements to the initial aTc strain, shown in Figure 1A, that reduced *tetR* transcription and inhibited pre-existing TetR activity (Figure 1C). aTc-TetR binding inhibits TetR repression from L03 promoters, thus inducing expression of the TetR aptamer, an RNA molecule that displaces TetR from P_{L03}. In addition, adenine attenuates *tetR* transcription by binding to the adenine riboswitch, decreasing *de novo* TetR formation. Although adenine is capable of de-repressing P_{L03} on its own, it has a far stronger effect when added in combination with aTc. In this strain, the combined effects of adenine, TetR aptamer, and aTc resulted in maximum reporter gene expression in response to the addition of only one percent of the aTc (20 ng/mL) required for the initial construct (Figure 1A) [7]. The combination of three regulatory mechanisms would be predicted to reduce TetR abundance and activity, establishing a feedback loop that further limits repression of P_{L03}, generating enhanced reporter gene expression. Unexpectedly, target gene expression did not exceed that obtained in previous constructs and had the added disadvantage of increased input cost as a result of aTc and adenine addition. The lack of increased dynamic range may result from the central role performed by aTc. While adenine inhibits *de novo tetR* transcription and induces a low level of target gene expression on its own, it is unable to repress the activity of pre-existing TetR.

The results obtained by Higo, et al., [2] utilize a strategy that relies on the addition of a chemical inducer, aTc, to inhibit TetR from repressing the P_{L03} promoter. The addition of a TetR asRNA (Figure 1B) or aptamer (Figure 1C) also expressed from P_{L03} allows for enhanced target gene expression and lower aTc requirements by decreasing either TetR expression or activity, respectively. Combined with an adenine

riboswitch, the latter regulatory module (Figure 1C) can simultaneously limit TetR activity and expression when aTc and adenine are supplied, yielding the broadest dynamic range of target gene induction obtained by the authors. These results demonstrate how introducing feedback loops that limit TetR repression at P_{L03} improves the dynamic range and decreases the aTc required for maximal induction.

Despite the broad dynamic range of expression derived from the constructs described by Higo, et al., [2] aTc is still required, resulting in increased input costs. The addition of adenine as a repressor of TetR expression (Figure 1C), while contributing to improved dynamic range, constitutes an additional input cost in order to decrease the amount of aTc required. As well, relying on multiple modules controlled by P_{L03} ensures that expression cannot be induced in the absence of aTc, and that TetR repression will re-occur when aTc becomes depleted.

We suggest that the dynamic range and inducer cost issues encountered by Higo, et al., [2] may be addressed by constructing regulatory networks based solely on elements that respond to environmental conditions, reducing input cost with the additional goal of optimizing resource flow to bioproduct formation rather than cell biomass [8]. To this end, we propose a hypothetical scenario which would utilize two divergently active temperature-sensitive RNA thermometers (RNATs) that will provide nearly instantaneous regulation (Figure 1D) [9]. In practice, industrial waste CO_2 could be used as a carbon source for a cyanobacterial bioreactor, in which a more heat-responsive variant of the *hsp17* RNAT, M4.4, [10] would promote expression of the TetR repressor at optimal growth temperature (32˚C), preventing bioproduct formation during initial culture growth. A subsequent temperature downshift (15˚C) would simultaneously halt *de novo tetR* translation, mediated by the *hsp17* RNAT, and induce TetR aptamer transcription from the *Anabaena* cold-inducible *crhC* 5' UTR RNAT [11, 12]. While *hsp17* is not native to *Anabaena*, its temperature-responsive effects on transcription are recapitulated in *E. coli* [10], suggesting that it could be utilized in multiple cyanobacterial species. This system will thereby decrease the repressor activity of pre-existing TetR, both mechanisms combining to provide a broad dynamic range of target gene transcription from P_{L03}. In this scenario, induction of bioproduct formation would only occur at low temperature and would promote enhanced carbon flow to bioproduct formation, rather than cellular growth, thereby optimizing yield. Additionally, while chemical inducers differentially affect gene induction based on their concentration, many RNATs demonstrate a similar effect in response to temperature change due to the progressive melting/annealing of their RNA secondary structures [9]. Thus, this allows for target gene expression to be finely tuned by modulating both the inducing and non-inducing temperatures based on the host organism and bioproduct being produced.

The regulatory system outlined in Figure 1D could be elaborated by including the *tetR* asRNA under the control of the *crhC*-5' UTR promoter, providing an inducer-free mechanism to potentially further limit background TetR abundance. Controlling biosynthetic gene expression in an environment-dependent manner would not only allow rapid modulation of biosynthetic gene expression, and thus bioproduct production, but would negate the need for expensive inducers as well as minimizing upscaling complications frequently encountered in commercial settings (4).

As described here (Figure 1D), the proposed RNAT-based regulatory circuit represents a generalized method of controlling bioproduct formation, which could be combined with other modular regulatory units based on required environmental conditions and/or chemical properties specific for each bioproduct.

In conclusion, the Hisabori lab [2] has demonstrated that introducing feedback loops to regulatory genetic circuits enhances the

expression levels and dynamic range of target genes while concurrently reducing input cost. Continued integration of disparate genetic regulatory mechanisms, providing tighter control over the temporal and dynamic range of target gene expression, will aid in overcoming the challenges facing cyanobacterial biotechnology. This approach will help to further the goal of harnessing the biotechnological potential of photosynthetic prokaryotes as a major source of renewable, environmentally friendly fuels and bioproducts for humanity's future.

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