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ACTIVATION AND REPRESSION OF GENE EXPRESSION USING THE QUAS/QF SYSTEM IN E. COLI

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***Abstract*— *Objective:* This work explores the utility of a eukaryotic transcription system in bacteria. *Methods:* Genetic components were combined into modules that control gene expression. These modules, termed genetic circuits, contain elements of the QUAS/QF system (hereafter as Q system). The activating sequence, QUAS, was tested at various positions upstream and downstream of a T7 promoter. Circuits were tested in BL21(DE3) bacteria, and gene expression was evaluated via flow cytometry. *Results:* QUAS inhibits expression upstream of the promoter when QF is absent. The presence of QF activates gene expression. A two-fold increase of expression compared to the control was observed when QUAS was ten base pairs upstream of the promoter. At positions downstream of the promoter, QUAS greatly amplifies gene expression in the presence of QF. A seven-fold increase in expression compared to a positive control was observed when QUAS was fifteen base pairs downstream of the promoter. *Conclusion:* The Q system introduces new genetic tools to repress or activate gene expression in bacteria. When placed upstream of a T7 promoter, QUAS inhibits gene expression. At locations downstream of the promoter, QUAS and QF increase gene expression *Significance:* The Q system is an easy-to-use activation/repression system for the T7 promoter in bacteria and the first known example of a eukaryotic system used to control gene expression in a bacterial host.**

***Index Terms*— synthetic biology, genetic programming, QUAS/QF, T7 promoter**

I. INTRODUCTION

DIABETES afflicts 9.3% of the United States population, requiring 6 million people to use insulin [1]. This life-saving therapeutic is produced by bacteria. Bacteria have become modern-day workhorses in the production of therapeutics. Production, however, is limited by the current set of tools to control gene expression. Expanding the toolbox for bacterial synthetic biology may improve production of insulin and novel therapeutics.

Several systems for genetic control have been well-characterized. The lac and tet systems are commonly used in synthetic biology [2]–[4]. The lac operon regulates lactose catabolism, and the tet operon regulates tetracycline resistance [2], [5], [6]. Both the tet and lac systems function by repressing gene expression (fig. 1). They allows control of gene expression without disrupting native processes [7]–[10]. This method has been used to produce various genetic circuits that function as oscillators [11], toggle switches[12], and Boolean logic gates [13], [14]. Other applications involve the production of useful proteins such as antibodies [15].

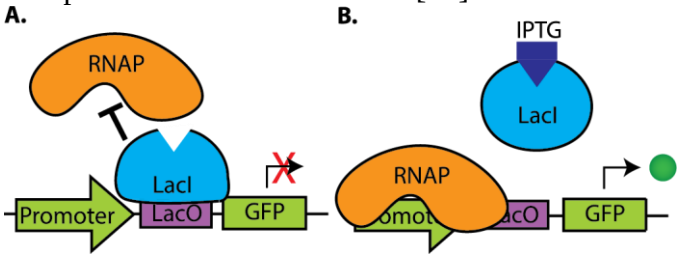

Fig. 1. The Lac system functions by blocking RNA polymerase (RNAP) from initiating transcription. A. A repressor protein (LacI) binds to its operator (LacO). This blocks RNAP from transcribing green

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fluorescent protein (GFP). Expression is off. B. Isopropyl β- d-1-thiogalactopyranoside (IPTG) binds to LacI, preventing its binding to LacO. Expression is on.

The QUAS/QF system (hereafter referred to as the Q system), found natively in the fungus *Neurospora crassa*, is a relatively new tool in synthetic biology. The Q system is comprised of an upstream activating sequence (QUAS) and an activating protein (QF). When QF binds to QUAS, gene expression is activated. In the absence of QF, expression is off. The Q system is compatible with the lac system to create novel genetic circuits in mammalian cells [16]. While the tet and lac systems function by repressing gene expression, the Q system is capable of activating, or increasing, gene expression (fig. 2).

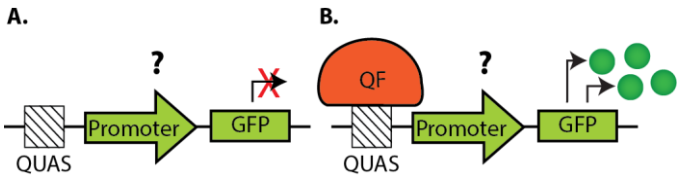

Fig. 2. A. When the QUAS is placed upstream of the promoter, green fluorescent protein (GFP) is not transcribed. Expression is off. B. QF binds to the QUAS and GFP is transcribed at a rate much higher than basal levels. Expression is activated. The Q system's exact mechanism is unknown in bacteria.

Preliminary work demonstrates the functionality of the Q system in bacteria [17]. When the Q system's operator is placed upstream of the promoter, gene expression is off. Placement of the QUAS downstream of the promoter in the presence of QF yields a four-fold increase in gene expression compared to the current gold standard for protein production. The mechanism of these behaviors is not understood.

We hypothesize that the relative placement of the QUAS with respect to the promoter affects gene expression. Genetic circuits were constructed with the QUAS at various positions upstream and downstream of the promoter; the circuits were tested with and without QF. Gene expression was measured via flow cytometry. This work lays the foundation for enhanced production of insulin and other therapeutics produced by bacteria.

II. Background

Synthetic biology aims to combine genetic modules to reliably control gene expression. A combination of modules that performs a specific function is called a genetic circuit. Genetic circuits are used to create memory [18], detect disease [19], and administer therapeutics [20], [21]. Characterizing new modules is a critical aspect of developing new applications. Recent advances in bacterial synthetic biology include methods to control population phenotypes [22] and engineer bacteria in the gut [14]. More fine-tuned applications will be realized as the toolset to control gene expression is expanded.

Gene expression is controlled by promoters. Promoters are specific sequences of DNA that signals cells to transcribe DNA into RNA. Transcriptional activity is modulated by activators and repressors. A repressor is a protein that inhibits transcription at a promoter; activators increase transcription. Repressors and activators often function by binding to a specific DNA sequence called an operator. Both repressors and activators are common in eukaryotic organisms where they help regulate complex gene regulatory networks. On the other hand, prokaryotes almost exclusively use repressors.

Repression of the T7 promoter, a common promoter in bacterial systems, is commonly achieved through using the lac system. In this system, the lacI protein blocks initiation of transcription by binding to a lacO operator site adjacent to and downstream of the promoter [23], [24]. When isopropyl β- d-1-thiogalactopyranoside (IPTG) is present, lacI is released from its operator site, and transcription can resume. The tet system also functions by inhibiting transcription [2]. Addition of anhydrotetracycline prevents tet repressor proteins from binding to DNA and allows transcription to occur. In both systems, addition of a specific chemical relieves repression and restores baseline transcription.

The current set of genetic devices have limitations [25]. The lac and tet systems on their own are unable to increase gene expression above baseline levels. One system (lux system) has demonstrated some activating behavior in bacteria, but it requires special promoters and autoinducing molecules, limiting its

versatility [26]. Systems that increase gene expression are necessary to create positive feedback, a fundamental element in control systems. A bacterial activator may also improve the efficiency of recombinant protein production.

The Q system uses an activator to control gene expression. This system occurs naturally in the fungus *Neurospora crassa* [27]; it is comprised of an upstream activating sequence (QUAS) and an activating protein (QF). When QF binds to the QUAS, gene expression is activated; in the absence of QF, gene expression is off [28]. The Q system has been used in eukaryotic systems such as *Drosophila* and mammalian cells [16], [29]. It has not been studied in bacteria due to its eukaryotic origins.

Synthetic biologists typically move genetic systems from simple organisms to more complex organisms (i.e., prokaryotic system to a eukaryotic host). We combined elements of the Q system with bacterial genetic circuits. Investigation of the Q system in bacteria represents a reverse approach (fig. 3). Further studies using this approach may yield useful discoveries in bacterial synthetic biology such as additional tools for genetic control.

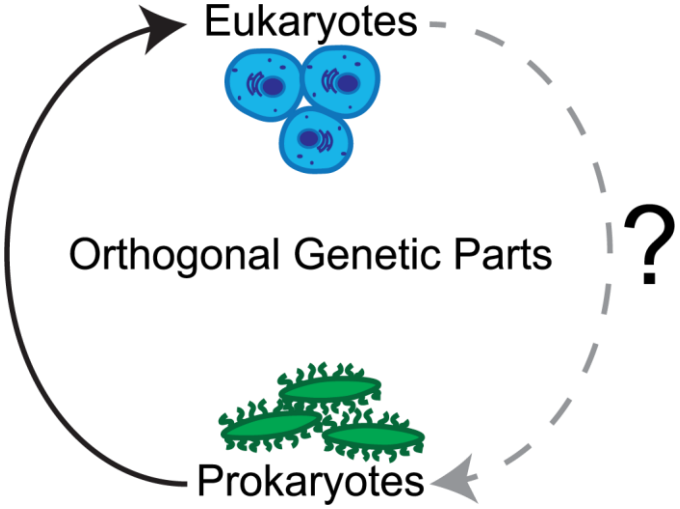

Fig. 3. Genetic parts are typically moved from simple to complex organisms (prokaryote to eukaryote). This investigation explores the reverse approach: moving eukaryotic, genetic parts into prokaryotes.

III. Methods

A. Plasmid Construction

Genetic circuits were constructed in plasmids using restriction enzyme cloning techniques in E. coli DH5α competent cells (ThermoFisher Waltham, MA). Response circuits were constructed by placing the QUAS upstream or downstream of a T7 promoter in a backbone containing an ampicillin-resistance gene and the ColE1 origin of replication. Green fluorescent protein (GFP) was placed downstream of the T7 promoter. An ssrA degradation tag (GFPDAV) was added to the C-terminus of GFP to allow measurement of expression dynamics. A separate circuit was constructed consisting of QF under the control of a T7-LacO promoter in a backbone containing a kanamycin-resistance gene and the p15A origin of replication.

B. Gene Expression Experiments

Plasmids were transformed into OneShot BL21(DE3) E. coli (ThermoFisher, Waltham, MA) and grown overnight at 37 °C. This strain of E. coli expresses T7 RNA polymerase (RNAP) upon induction with isopropyl β-D-1-thiogalactopyranoside (IPTG). The T7 promoter is exclusively transcribed by T7 RNAP, and T7 RNAP does not exhibit off-target effects.

Bacterial colonies were picked in triplicate and grown overnight in 1 mL of Luria broth (LB) and antibiotics. Cultures were grown with carbenicillin (100 μg/mL) and/or kanamycin (50 μg/mL) according to resistance genes in the culture's plasmids. The following morning, fresh LB was inoculated at a 1:50 ratio with the overnight culture and induced with IPTG at ~ 0.2 optical density at 600 nm wavelength (OD600). A Synergy HTX Reader (Biotek Winooski, VT) was used to measure OD600. The final concentration of IPTG in the induced culture was 0.5 mM.

C. Flow Cytometry

GFP expression was measured via flow cytometry every hour following induction using a DXP or CytoFLEX S flow cytometer (Cytek Biosciences Fremont, CA). A plasmid containing GFPDAV under the control of a T7 promoter and lac operator was used as a positive control. BL21(DE3) cultures without genetic circuits were used as a negative control. Mean Fluorescence Intensity (MFI) was

calculated using FlowJo software (FlowJo LLC Ashland, OR), and plots were generated using MATLAB (MathWorks Natick, Massachusetts). All results were normalized to fluorescence of the positive control at hour one.

IV. Results

QUAS exhibited repressive and activating behavior when placed upstream of the T7 promoter (QUAST7). Gene expression was repressed in the absence of QF and increased when QF was present (fig. 4). Expression of QUAST7 in the presence of QF was maximal when QUAS was placed ten base pairs upstream of the promoter. Repression of gene expression was approximately equivalent in all constructs.

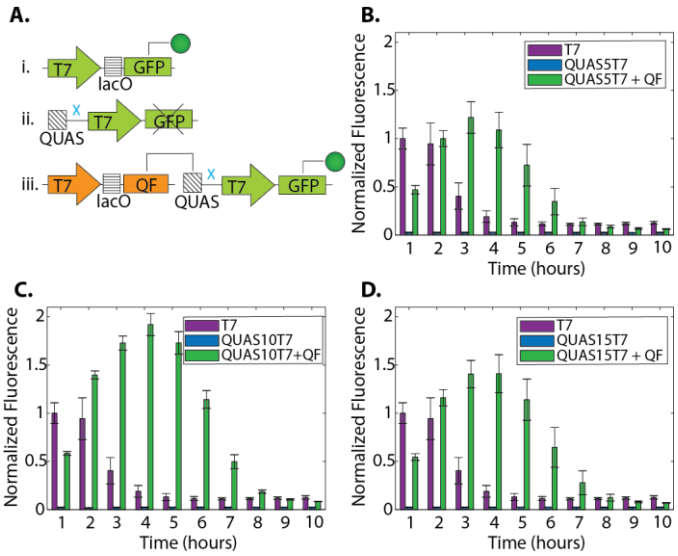

Fig. 4. A. Circuit diagrams of genetic circuits containing parts from the Q system. i. The T7 promoter drives expression of GFP for the positive control (purple bars). ii. QUAS upstream of the promoter inhibits transcription in the absence of QF (blue bars). iii. QF activates expression, allowing GFP to be transcribed (green bars). B. Expression with QUAS 5 base pairs upstream of a T7 promoter. C. Expression with QUAS 10 base pairs upstream of a T7 promoter. D. Expression with QUAS 15 base pairs upstream of a T7 promoter. Fluorescence was measured via flow cytometry. Error bars display standard deviation. n=3.

Placement of QUAS downstream of the promoter increased activator effects of QF, but repressive behavior of QF was not maintained. Genetic circuits in the absence of QF with QUAS at a position five or fifteen base pairs upstream of the promoter exhibited behavior less than or equal to the positive control (fig. 5). Circuits with QF showed large increases in gene expression compared to the control. The greatest activation was seen in constructs containing QUAS fifteen base pairs downstream of the promoter; expression was approximately seven times greater than the control.

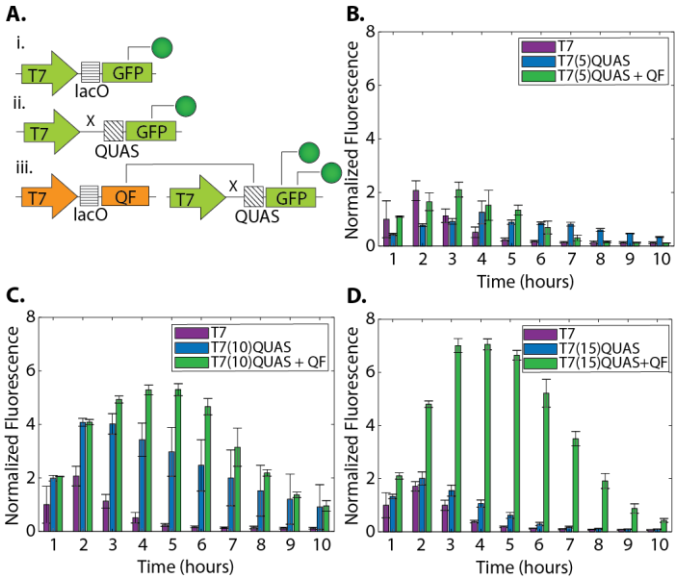

Fig. 5. A. Circuit diagrams of genetic circuits containing parts from the Q system. i. The T7 promoter drives expression of GFP for the positive control (purple bars). ii. QUAS downstream of the promoter permits transcription in the absence of QF (blue bars). iii. QF activates expression, greatly increasing GFP expression (green bars). B. Expression with QUAS 5 base pairs downstream of a T7 promoter. C. Expression with QUAS 10 base pairs downstream of a T7 promoter. D. Expression with QUAS 15 base pairs downstream of a T7 promoter. Fluorescence was measured via flow cytometry. Error bars display standard deviation. n=3.

V. Discussion

Bacteria offer promising avenues for producing environmentally friendly biofuels and lifesaving therapeutics. Improving these processes requires more genetic tools. This work investigated the utility of the Q system in bacteria by altering the position of

its upstream activation sequence (QUAS) relative to a T7 promoter. When QUAS was placed upstream of the promoter, gene expression was tightly off. Addition of the Q system's activating protein, QF, amplified gene expression beyond baseline values (fig. 4). Downstream placement of QUAS resulted in constructs that exhibited little to no repression in the absence of QF and up to seven-fold increases of gene expression when QF was present (fig. 5). These findings may support enhanced production of important therapeutics.

The Q system's repressive properties in bacterial expression occur when QUAS is placed upstream of the T7 promoter. In this configuration, QF's activating behavior was maintained, allowing the circuit to display either strong activation or strong repression. Of the three positions tested, QUAS at ten base pairs upstream of the promoter yielded the greatest activation (fig. 4B). This placement of QUAS likely optimizes interactions between transcriptional proteins and those that bind to QUAS. The DNA double helix makes a half turn every five base pairs. It is possible that affinities exist between QF and T7 RNA polymerase (T7RNAP). Interactions between the proteins may stabilize T7RNAP as it binds to the promoter. In this case, the placement of QUAS would affect steric hinderance between QF and T7RNAP. The rotational position of QF with respect to the promoter may expose regions of the QF protein that encourage T7RNAP to initiate transcription.

The repressive properties of upstream QUAS are likely due to interactions with native proteins in bacteria. In traditional systems, operators are ineffective at repressing gene expression when placed upstream of a T7 promoter [24], [30]. QUAS likely attracts an endogenous protein of sufficient size to block the T7 promoter from access to its RNA polymerase. When QF is present, QF preferentially binds to QUAS and activates expression. QF's affinity for QUAS is likely much greater than that of the endogenous protein.

Downstream placement of QUAS resulted in strong amplification of gene expression. Expression up to seven times that of the positive control were observed when QUAS was placed downstream of the promoter and QF was present (fig. 5D). If QF activates gene expression by attracting T7RNAP to the promoter region, then QF's downstream position allows optimal interaction with T7RNAP. QUAS without QF resulted in little to no change in gene expression compared to the control. The putative, endogenous protein discussed earlier may have a shape that does not block the promoter region when the protein interacts with QUAS downstream of the promoter. Interference must occur at the 3' end of T7 in order to prevent transcription; once T7RNAP begins transcription, it's affinity for the DNA is too great to be disrupted by repressor-operator complexes [23]. If the putative, repressor protein does not strongly block the 3' end of the T7 promoter, then transcriptional repression would be unlikely.

These findings introduce a novel approach to synthetic biology. Traditionally, genetic parts are taken from simple organisms and utilized in more complex hosts. For example, the T7 expression system, native to bacteriophage, is commonly used to control expression in bacteria [31]. The tet and lac systems are native to bacteria and are staples in mammalian genetic circuits [4], [7], [10], [16]. This project, however, takes a reverse approach. The Q system is native to the fungus *Neurospora crassa*, and its components function to control gene expression in bacteria. Bacteria are typically considered incompatible for eukaryotic systems due to their simpler, cellular machinery. Eukaryotic proteins may require post-translational modifications that are not native to bacteria. The transcription factors in bacteria are different than other cell types, too.

This work is limited in its findings regarding the Q system's mechanism of action in bacteria. Our experiments demonstrate several properties of the Q system but fails to empirically explain their cause. Due to the work's novel approach, prior literature does not describe functionality of eukaryotic parts in a bacterial host. Many aspects of the Q system are yet to be discovered and limit interpretation of this work's findings.

The Q system's versatility offers several promising applications. In the upstream position, QUAS tightly represses gene expression until QF is introduced into the system. Tight off states are difficult to achieve in bacterial systems. Some researchers aim to engineer bacteria that target tumors [32]. The Q system could be used to control production of a cytotoxic protein. Interaction with the tumor could cause expression of QF and release the toxic protein, killing cancer cells. This strategy would target cancer cells while

minimizing collateral damage to healthy cells due to leaky expression of the cytotoxic protein.

Activators in bacteria are rare and difficult to engineer. The Q system offers a simple means to increase gene expression beyond baseline levels. The Q system's activation abilities could greatly improve recombinant protein production. Bacteria are promising biological factories to produce biofuels and therapeutics. Using QUAS downstream of the promoter in conjunction with QF could amplify protein production. More efficient production would decrease production costs and may alleviate financial burden on patients. Activators can also be used to produce positive feedback. This may be useful for programming memory modules in bacteria.

Developing industrial and clinical applications will require greater understanding of the Q system. Future work will investigate the Q system's interactions within the host bacterium. Pull-down assays will be used to determine protein interactions with QUAS. Does QUAS interact with native proteins? The Q system's compatibility with other common systems will also be investigated. Other systems are confined to a narrow range several base pairs downstream of the promoter while QUAS can be placed within fifteen base pairs of either side of a T7 promoter to modulate gene expression. This leaves ample room to for multiple regulatory systems to be placed about a single promoter. The functionality of such genetic circuits will be determined in future work.

This work establishes a new set of genetic circuits using eukaryotic, genetic parts. These findings can be used to realize novel applications in recombinant protein production and novel genetic circuit design. Eukaryotic transcription factors have yet to be used in bacterial genetic circuits. Other eukaryotic transcription factors may yield useful results in a bacterial host. The Q system is an exciting expansion to bacterial synthetic biology.

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