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Tunable riboregulator switches for post-transcriptional control of gene expression.

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Riboregulators can be used to finely tune gene expression.

Because the output of a metabolic reaction is directly proportional to the enzyme concentration ($d[cp]/dt \sim kcat[ce]$), by far the most straightforward approach to altering the flux through a particular metabolic step is to increase or decrease the concentration of the enzyme catalyst. Current strategies for engineering gene expression are limited to increasing the expression of a particular gene by placing it under the control of a stronger transcriptional promoter or using one of several strategies to knock down or knock out a wasteful gene. To finely tune expression of enzyme concentrations, we have developed riboregulators that has the potential to independently control the concentration of each enzyme in an engineered metabolic pathway. A unique approach to rationale design and directed evolution of the ensemble of riboregulators was used to design and optimize the activity of the riboregulators. The overall architecture of the riboregulators is designed using Watson-Crick base pairing stability. In fact, many tunable small regulatory RNAs with well-defined performance characteristics have been designed using this method. The architecture will then be refined using state-of-the-art structural modeling and molecular simulation of RNA. Directed evolution will next be utilized on a portion of each riboregulator. In this manner, a set of riboregulators (one for each gene) will be evolved simultaneously to optimize the set of enzyme concentrations to maximize flux.

Graphical abstract

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1. Introduction

Recent progress in systems biology and metabolic engineering approaches has enabled the engineering of microbes for production of high-value products with important medical and biotechnological applications (Keasling, 2010; Keasling, 2012; Nielsen et al., 2014; Peralta-Yahya et al., 2012). Specifically, there is a great deal of attention on engineering functional metabolic pathways for production of advanced biofuels in microbial hosts including photoautotrophic and heterotrophic bacteria. One of the key challenges in engineering of new metabolic pathways is the maximization of flux for achieving maximal yields of the desired product. Though metabolic flux can be potentially altered by over or under-expression of an enzyme in a pathway, tunable regulation of gene expression is a highly desirable feature for physiologically relevant protein production (Callura et al., 2012; Farmer and Liao, 2000; Jensen and Hammer, 1998a; Jensen and Hammer, 1998b; Morris et al., 2014; Mutalik et al., 2013; Nowroozi et al., 2014; Solomon et al., 2012; Zhang et al., 2012). Current strategies to modulate enzyme expression include use of strong transcriptional promoters for enhanced expression or gene knockdown strategies to reduce expression of an undesired gene(s). These approaches however may not necessarily yield the optimal concentration of enzyme in a pathway for maximizing flux. A need exists for more precise control of gene expression (Dong et al., 1996). In addition, engineering a microbe to direct production of a particular metabolite may often lead to deleterious effects, as metabolites serve several other functions in the cell. On the other hand, deletion or under-expression of genes to maximize flux may decrease growth rates or affect cell density. To overcome the above challenges, synthetic biology approaches have enabled the design of tunable synthetic genetic circuits with the potential to maximize flux in a heterologous pathway (Green et al., 2014; Mutalik et al., 2013; Nowroozi et al., 2014; Woolston et al., 2013). Approaches utilizing post-transcriptional control will be very valuable for microbial engineering for production of high value products (Goldfless et al., 2012; Isaacs et al., 2004; Mutalik et al., 2012; Topp and Gallivan, 2010).

Small non-coding RNAs (sRNA) in bacteria represent an interesting mechanism for more precisely controlling gene regulation (Storz et al.). Regulatory RNAs ('riboregulators') control genes, multiple genes or operons of important systems where a coordinated cellular response is required. These include quorum sensing, virulence and various stress responses. Riboregulators possess functional characteristics ideally suited to synthetic biology, including the ability to modulate expression of multiple targets, excellent dynamic range, linear response and low noise (Levine et al., 2007). Importantly, their operation is based on principles that are well understood and amenable to manipulation (*e.g.*, gene targeting and dynamic response tuning). These regulatory elements can enable "dialed in" expression levels of each enzyme allowing optimum metabolic flux. RNA possesses the key advantage over proteins in that the sequence space of small regulatory RNAs may be substantially sampled using *in vivo* selection (directed-evolution). This is exemplified by the reported

functional selection of natural sRNA, DsrA, which regulates the *rpoS* stress response gene (Liu et al., 2005a). Here a randomly diversified library based on DsrA was selected for the ability to activate expression *in vivo*. The results were a rapidly selected set of riboregulators that spanned a wide range of activation from 0 to ~4 fold greater than wildtype DsrA. Basing this system on well-studied examples such as DsrA/*rpoS* can drastically limit the amount of sequence space required to produce a wide dynamic range of expression.

Success has been achieved engineering small regulatory RNAs that achieve a specific end. In one example, an RNA was engineered to control chemotaxis in bacteria, forcing the bacteria to follow concentrations of theophylline (Topp and Gallivan, 2007). Tunable regulatory RNAs have also been engineered with well-defined performance characteristics (Bayer and Smolke, 2005; Isaacs et al., 2006; Winkler and Breaker, 2005). RNAs have been evolved using *in vitro* selection (directed evolution) to bind to over 80 user-specified ligands and proteins (Aquino-Jarquin and Toscano-Garibay; Stoltenburg et al., 2007).

A common mechanism for many classes of riboregulators is to increase translation by the interaction of a trans-activating riboregulator with the target mRNA. This mechanism has been found to be amenable to manipulation through rational design, selection and tuning to create new synthetic riboregulators and modify native riboregulators (Callura et al., 2010; Isaacs et al., 2004; Liu et al., 2005b; Sakai et al., 2014). Synthetic riboregulators have been engineered to control posttranscriptional gene expression utilizing highly specific RNA-RNA interactions. These kinds of riboregulators have previously been engineered to precisely regulate a variety of systems (Carter et al.; Valverde, 2009). They consist of a cis-repressor RNA (crRNA) element within the 5'-untranslated region (5'-UTR) of the messenger RNA (mRNA) of the desired gene and a trans-activating riboregulator element (taRNA) that can specifically interact with the crRNA element of the target mRNA and regulate gene expression. This approach allows highly specific and stable RNA-RNA interactions to be exploited, providing dynamic range of protein expression. Furthermore, riboregulators have the potential for tunability of protein translation, fast response and can be used to regulate multiple genes with minimal leakage.

In this study, we describe a synthetic biology approach using riboregulators to fine tune gene expression with potential application in metabolic engineering. These designer riboregulators are effective analog switches, where each sequence produces a distribution of ON/OFF states. Here, the thermodynamic stability of competing structures within and between the crRNA and taRNA elements determines the degree of expression (Fig. 1). Randomizing the taRNA element produces a continuum of activation levels for future use in directed pathway evolution experiments.

Utilizing *E. coli* as a model organism, we have developed cis-repressor elements and trans-activating riboregulator elements to control the translation of mRNAs of interest. Importantly, unlike previously reported riboregulators, we have shown that we can achieve up to >200 fold difference in activation of genes even with physiologically relevant concentrations of riboregulators in cells. Importantly, we show that the repressed gene has activity similar to its absence and the fully trans-activated gene is analogous to having no

riboregulation. In a complete metabolic pathway, optimal enzyme expression is needed at each step to maximize flux. This requires the development of a range of orthogonal riboregulators. We have shown that orthogonal targeting of genes is possible using a ~34 bp targeting sequence, specific for the interaction of a cis-repressor – trans-activator pair. This complements other sequence and structure-based efforts by identifying novel small RNAs that can be utilized for controlling *in vivo* protein production.

2. Materials and Methods

2.1. Materials.

Luria Broth (LB) culture media, Sucrose and Chloramphenicol were purchased from Fisher Scientific. Carbenicillin, D-glucose and L-arabinose were obtained from Sigma Aldrich. Gibson assembly kit, Instant Sticky-end Ligase Master Mix, One *Taq* DNA polymerase and restriction enzymes were purchased from New England BioLabs. Kits from Qiagen were used for purifications of plasmid DNA, PCR products, and enzymatic digestions. All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

2.2. Plasmid Construction and Bacterial Transformations.

All plasmids were constructed using standard molecular biology techniques either using Gibson assembly cloning or standard ligation using instant sticky end-ligase master mix. All riboregulated constructs were constructed using pETcoco-2 system, which combines the replication elements of a single-copy genomic cloning vector and a medium-copy plasmid, with the expression elements of pET vectors (Wild et al., 2002). Two constitutive T7A1 promoters were used to drive the RNA expression of the crRNA and taRNA on the same plasmid. Plasmids were verified by DNA sequencing at ACGT, Inc. or Genewiz, Inc. before bacterial transformations. NEB 5-alpha Competent *E. coli* (High Efficiency) from New England BioLabs were used for all bacterial manipulations and genetic engineering. Plasmids were introduced by standard heat shock transformation protocol and selected using carbenicillin (50 µg/ml). Unless otherwise mentioned, all experiments were performed in the single-copy state in *E. coli* by controlling the oriS origin of replication, repE gene and parABC partition determinants of the vector expressing the riboregulated gene and trans-activator, by propagating the pETcoco2 vector in LB medium in 0.2% D-glucose and carbenicillin (50 µg/ml) at 37°C. Sequences of crRNAs and taRNAs used and construction of all plasmids is detailed in Supplemental Information.

2.3. Gene Expression Assays.

E. coli cells transformed with appropriate constructs were grown overnight in LB media supplemented with 0.2% D-glucose and carbenicillin (50 µg/ml) at 37°C with shaking in culture tubes. Aliquots of a 1:10–1:20 dilutions were used to inoculate LB media containing 0.2% D-glucose and carbenicillin (50 µg/ml) to a final volume of 200 µl and containing increasing concentrations of chloramphenicol or kanamycin in a 96 well plate (Costar 96 well assay plates). The culture plates were shaken at 37 °C and OD600 was measured at regular intervals of time using SpectraMax M5 multimode reader from Molecular Devices. Growth studies were conducted in triplicate and data are represented as mean (SD). The OD

of each strain at a given time point was subtracted from the initial zero hour OD of that strain.

2.4. Luminescence Assays.

E. coli cells transformed with appropriate constructs were grown overnight in LB media supplemented with 0.2% D-glucose (for single copy plasmid) or 0.01% L-arabinose (for medium copy plasmid) and carbenicillin (50 µg/ml) at 37°C with shaking in culture tubes. Aliquots of a 1:20–1:40 dilutions were used to inoculate LB media containing 0.2% D-glucose and carbenicillin (50 µg/ml) to a final volume of 200 µl. Luminescence and OD600 were measured at regular intervals of time using SpectraMax M5 multimode reader from Molecular Devices.

3. Results and Discussion

3.1 Modular design of riboregulators

In our riboregulator system (Fig. 1) both the cis-repressor (crRNA) and trans-activator RNAs (taRNA) each have modular structures composed of targeting (blue) and regulatory (red) sequences. The crRNA is within the 5' UTR of the mRNA and naturally folds to a structure that sequesters the ribosome-binding site (RBS) preventing translation of the downstream gene. The taRNA is transcribed in trans and the binding and subsequent structural transition between these two regulatory RNA elements dictates whether or not the transcribed mRNA will be translated into the protein product (Fig. 1). The targeting sequences are complimentary and target a particular taRNA to a specific crRNA (Fig. 1b). Depending on the relative stability of the structural elements in the regulatory regions of the crRNA and the taRNA, the complex can rearrange to free the RBS turning on translation (Fig. 1c). Since the extended duplex between the elements has a much greater potential stability, tuning expression is accomplished by altering the availability of sequence within the taRNA. Availability is determined by weakening intramolecular base-pairing of the taRNA and adjusting the intermolecular interactions in a common spacer region (Fig. 1c). By tuning the relative stability between the regulatory helices of the crRNA and the taRNA and that of the extended crRNA/taRNA duplex, it is possible to alter the equilibrium between repressed (Fig. 1b) and activated (Fig. 1c) states and achieve intermediate translational control (Fig. 1d).

In our systems, translation of both the crRNA:gene fusion and the taRNA are driven independently by strong constitutive promoters. To achieve our overall goal of a wide dynamic range of riboregulator control of translation it was necessary to demonstrate riboregulators with the following characteristics: 1) create a modular system with the capacity to regulate multiple genes independently and consistently; 2) design crRNA regulatory sequences that sequester the RBS and completely block translation (crRNA OFF); 2) identify taRNA regulatory elements that free the RBS from the crRNA OFF state and turn back on translation to the fullest extent possible; 3) demonstrate that we can design orthogonal targeting sequences that can be used to direct a taRNA to its specific partner crRNA; 4) demonstrate intermediate levels of translation by altering the regulatory sequence in the taRNA.

For this study, the riboregulated mRNA and the tRNA were transcribed using the strong constitutive T7A1 promoter and T7 terminator by cloning suitable sequences into the pETcoco2 plasmid backbone. pETcoco2 combines the replication elements of a single-copy cloning vector and a medium-copy plasmid. Initially, we used the riboregulators to control translation of the chloramphenicol acetyl transferase (*cat*) as a reporter, which allowed phenotype characterization by measuring the growth rate of *E. coli* transformants in the presence of varying concentrations of chloramphenicol. *E. coli* transformants were characterized by culturing in medium containing D-glucose. Under these conditions, each cell contains a single copy pETcoco2 ensuring that changes in growth phenotype were a result of changes in gene expression and not a result in changes in the plasmid copy number.

3.2 Evolution of the *cis*-Repressor

We initially optimized the *cis*-repressor element in the absence of a tRNA to determine the sequence and structure of the *cis*-repressor element required for complete repression of mRNA translation. Competent *E. coli* cells were transformed with pETcoco2 vectors encoding *cat* under the control of various *cis*-repressor elements. For our initial study, we chose four crRNA sequences CR-1, CR-2, CR-3, and CR-4 (Fig. 2a). To optimize translation, the regulatory sequences were designed with the Shine-Dalgarno sequence (AAGGAG) followed by an eight-nucleotide spacer and the start codon. We estimated the thermodynamic stability of each of these stem loop structures using the M-fold algorithm (Zuker, 2003) as follows: CR-1, -34.3 Kcal/Mol; CR-2, -40.0 Kcal/Mol; CR-3, -45.9 Kcal/Mol; CR-4, -57.5 Kcal/Mol.

We measured bacterial growth of the transformants using chloramphenicol as a sensitive, titratable marker for translational leakage (Fig. 2b). In our experimental conditions we found the growth of wild-type *E. coli* DH5 α was completely inhibited in culture medium containing 30 μ g/mL chloramphenicol. Because our initial design CR-1 showed a significant growth phenotype to chloramphenicol concentrations of 120 μ g/ml, we developed *cis* regulatory elements with the helix extended by eight base pairs (CR-2, Fig. 2a) and then further increased its stability by systematically removing mismatch bulges (CR-3 and CR-4). As expected, translation was dependent on the thermodynamic stability of the RBS occluding helix. Complete occlusion of RBS and minimal leakage of translation was possible only when the *cis*-repressor was designed to form a stem loop that has a nearly perfect double helical structure (CR-4, Fig. 2b). Significantly, subtle changes in sequence near the RBS, with single base pair mismatches, resulted in increased translation of *cat* gene (as seen for CR-2 and CR-3), indicating that even minimal structural breathing around the RBS allowed ribosome access and translation of *cat* imparting chloramphenicol resistance.

3.3 Riboregulated translation

We chose CR-4 as the regulatory element for further experiments as this sequence/structure represented the maximal OFF riboregulated state (crRNA OFF) and provided minimal leakage of translation and protein expression. Our next goal was to identify tRNA regulatory elements that free the RBS from the crRNA OFF and turn back on translation to the fullest extent possible. The tRNA contains a targeting sequence (TS1) that can recognize a specific *cis*-repressor RNA through Watson-Crick base pairing and a regulatory

element that can hybridize with the crRNA regulatory element in a fashion that can expose the RBS leading to translation. To test the taRNA, we designed four constructs diagrammed in Figure 3a. In the Maximum ON construct, expression of *cat* is driven by the pT7A1 with no translational control. The transcription of the crRNA:*cat* fusion construct is also driven by pT7A1; however, as described above, translation of *cat* is fully attenuated by a crRNA with the CR-4 regulatory element. In the Regulated ON construct, transcription of the crRNA:*cat* fusion and the taRNA are driven independently by identical pT7A1 promoters; taRNA is designed to hybridize with the crRNA in a fashion to free the RBS and modulate the translation of *cat* (Fig. 1c). The sequence of the taRNA-ON variant provides for minimal structure, facilitating the formation of an extended duplex with the crRNA. In addition, the spacer is fully complementary to the crRNA, facilitating taRNA strand invasion and RBS release (Fig. 1c). In the Regulated OFF construct, the stability of the secondary structure of the taRNA and lack of complementary spacer nucleotides prevents rearrangement of the regulatory complex (Fig. 1b) leaving the RBS occluded; thus, blocking translation.

The expression cassettes diagrammed in Figure 3a were cloned into pETcoco2 plasmids. In each case the taRNA expression cassette was encoded downstream of the crRNA regulated *cat* gene. Each of the constructs were used to transform competent *E. coli*. The growth rate of the transformants was measured at increasing concentrations of chloramphenicol (Fig. 3b). Because there is no translational control in the Maximum ON construct this should represent the maximum *cat* expression driven by the T7A1 promoter/T7 terminator. Transformants harboring the Maximum ON construct showed significant growth at concentrations of chloramphenicol up to 1mg/ml. Transformants harboring Maximum OFF and the Regulated OFF constructs showed the same growth phenotype as wildtype *E. coli* DH5 α . All three grew well in the absence of chloramphenicol but did not grow at any of the chloramphenicol concentrations tested. Cells transformed with the Regulated ON construct grew at chloramphenicol concentrations up to 750 μ g/mL indicating that regulated ON taRNA restored translation to a very significant extent.

The chloramphenicol resistant phenotype caused by expression of the Regulated ON taRNA shows that it is capable of freeing the RBS on the cis-repressed mRNA, resulting in translation of *cat*. In contrast, expression of the Regulated OFF taRNA did not activate translation and its phenotype was essentially identical to the Maximum OFF state, indicating the structure within the taRNA and mismatches in the 4 nucleotide spacer effectively prevented the formation of an translation-activating extended duplex

In order to demonstrate this system with other reporters and develop a more direct measure of gene expression, we replaced the *cat* gene with LuxCDABE operon to determine if bioluminescence can be riboregulated. As shown in Figure 5d, we observed that *E. coli* cells expressing the cis-repressed Lux operon showed very high levels of luminescence in the presence of taRNA-ON and negligible luminescence was seen in the presence of taRNA-OFF, which is not capable of exposing the RBS for translation of the Lux C gene in the operon.

Significantly, this >300-fold difference between riboregulated OFF and ON states for the *cat* or Lux reporters was achieved in a single plasmid copy state. Therefore this design of

ribo regulators has great potential for controlling gene expression at the chromosomal level and therefore will be highly valuable for engineering new metabolic pathways in the organism's genome to modulate protein translation for maximal flux. Furthermore, as the maximal ON achievable in this system without the riboregulators is higher than riboregulated ON, our trans-activator sequence has further potential to be evolved by modifying the trans-activator sequence to obtain higher or lower activation levels, indicating the tunability of this system for controlling protein expression.

3.4 Targeting of the taRNA to specific crRNA regulated genes

To optimize flux through an entire metabolic pathway it may be necessary to independently control the expression of all of the enzyme catalysts in the pathway, which will require multiple crRNA/taRNA pairs. In our modular design the targeting sequence is used to create orthogonal riboregulator pairs. The targeting sequences on the crRNA and taRNA are complementary. It is essential that formation of the targeting helix drives the recognition of a particular crRNA by its partner taRNA and that the targeting sequences minimize cross talk between various cis-trans riboregulator pairs. We investigated the specificity of two targeting sequences TS1 and TS2 (Fig. 5a) using antibiotic resistance reporter genes. First we tested a TS2-based crRNA/taRNA pair's ability to regulate translation. *aphA1* (kanamycin resistance) was placed under control of a crRNA composed of TS2 and CR-4. We monitored the cell growth rate as a function of kanamycin concentration (Fig. 5b). Transformants expressing the taRNA-ON riboregulator with TS2 targeting sequence showed enhanced kanamycin resistance (translation of *aphA1*). In contrast, transformants expressing the TS2-based taRNA-OFF riboregulator did not grow in the presence of kanamycin. As expected, substitution of TS1 for TS2 does not effect the behavior of the riboregulator pair; fusion of the TS2-based crRNA to the 5'-end of *aphA1* gene blocks its translation, which is restored by expression of the TS2-based taRNA-ON.

We determined how much cross-talk occurred between TS1- and TS2-based riboregulator pairs. In separate constructs *cat* was placed under translation control of TS1:crRNA and TS2:crRNA. We determined the growth rate of *E. coli* transformants harboring plasmids encoding either TS1:crRNA:*cat* and TS1:taRNA ON or TS2:crRNA:*cat* and TS2:taRNA ON (Fig. 5c). In both cases the organisms show significant resistance to chloramphenicol indicating translation of *cat*. In contrast, transformants harboring plasmids encoding TS1:crRNA:*cat* and TS2:taRNA showed significantly greater sensitivity to chloramphenicol indicating minimal crosstalk between TS1 and TS2.

To gain a more quantitative measure of the cross-talk between TS1 and TS2 we used a cis-repressed Lux operon with the original targeting sequence (TS1) and determined the ability of the TS2-based taRNA to activate translation. Significantly, negligible luminescence was seen using an orthogonal pair (Fig 5d) indicative of minimal cross-talk between the TS1- and TS2-based riboregulators.

3.5. Trans-activator sequence can dictate extent of translation

To achieve our goal of tunable regulation it is necessary to demonstrate that by altering the regulatory sequence in the taRNA, it is possible to achieve intermediate levels of translation.

To identify tRNAs with discrete regulatory control, we performed SELEX on a tRNA selection construct (Gold, 1995; Tuerk et al., 1992). Briefly, the selection construct contained a weakened secondary structure that was found to possess negligible activation as a tRNA. This was used as a scaffold to create a small 32-member library of tRNAs by substituting nucleotides at 5 positions with two possible nucleotides. Four of the positions were in the important spacer region and one was in the base of the tRNA stem-loop. Two possible nucleotides were chosen for each position that either allowed or prevented base pair formation between the tRNA/crRNA in the spacer or within the tRNA stem-loop (Fig. 6a). Competent *E. coli* were transformed with pETcoco2 vectors harboring expression cassettes encoding the TS1 crRNA:*cat* fusion and a 32-member library. Transformants were selected by growth at increasing concentrations of chloramphenicol. Clones from each concentration were recovered and the tRNA sequenced. While the details of this SELEX experiment will be reported elsewhere, we used it to identify tRNA sequences that were enriched when grown at intermediate concentrations of chloramphenicol. Here we tested the ability of three of the evolved tRNA to regulate translation of the cis-repressed *cat* mRNA.

In separate experiments, pETcoco2 vectors harboring expression cassettes encoding the TS1 crRNA:*cat* and one of the evolved tRNA sequences were cultured at increasing chloramphenicol concentration. Figure 6b compares the growth rate of three transformants with those of the tRNA OFF and tRNA ON constructs described above (Fig. 3b). These results demonstrate that the level of *cat* translation and hence antibiotic resistance can be tuned based on subtle changes in the regulatory region of the tRNA. By using small libraries of tRNAs it will be possible to fine tune translation of multiple genes in an engineered pathway.

3.6. Comparison of riboregulator designs

Collins and coworkers have reported on cis/trans riboregulators that can be used to regulate gene expression (Callura et al., 2012; Green et al., 2014; Isaacs et al., 2004). While the riboregulators reported here are very similar in concept to those reported by Collins and coworkers, they are quite distinct in the details of their design. The difference in design was necessitated by our experimental constraints. Collins and coworkers tested their riboregulators using high copy number plasmids and fluorescent reporters. Because we wanted to mimic chromosomal engineering and be sure that changes in growth phenotype could be attributed to changes in translation and not masked by changes in plasmid copy number, we used a single copy number plasmid to test our riboregulator concepts. While fluorescent reporters have the advantage of being a direct measure of translation, we found that at the relatively low levels of single plasmid copy transcription in our system, the background fluorescence of *E. coli* cells gave an unacceptably high threshold for their detection; thus, we chose antibiotic resistance reporters. While the antibiotic resistance reporters have the disadvantage that bacterial growth is not a direct measure of gene expression, they are a very sensitive particularly to low levels of translation. In other words, *E. coli* will grow in the presence of 30 µg/mL chloramphenicol if there is any expression of *cat*. As a consequence, we found that a much more extensive and stable regulatory helix was required to completely occlude the RBS and turn off translation than any reported by Collins and coworkers. In their original designs (Callura et al., 2012; Isaacs et al., 2004), the start

codon was not part of the regulatory helix; using Mfold (Zuker, 2003) we estimated the thermodynamic stability of crRNA regulatory helices to be in the range -13.8 to -27.50 Kcal/Mol. Also, in their recent 'toehold' design (Green et al., 2014) the crRNA regulatory helix includes not only the start codon, but the three more codons (nine nucleotides) of the transcribed protein; we estimated the thermodynamic stability in the range of -22 to -30 Kcal/mol for the regulatory regions of the seven toehold designs that were reported to have an ON/OFF ratio of >200 . In our systems we found that, in general, translational leakage was inversely proportional to stability and a significantly more stable regulatory helix was required to fully occlude the RBS and turn off translation; the estimated thermodynamic stability of our regulatory helix (CR-4) of was -57.50 Kcal/Mol.

4. Conclusions

To optimize engineered metabolic pathways it is necessary to independently tune the level of expression of each enzyme catalyst in the pathway. We have shown it is possible to use riboregulators to achieve translational control of gene expression over a wide dynamic range. In our system, the crRNA is encoded in the 5'-untranslated region of the mRNA and folds to block the ribosome-binding site. We have demonstrated a crRNA regulatory sequence that can completely shut off translation (CR-4, Fig. 2). The trans-activator RNA (taRNA) is expressed in trans and the targeted binding event followed by structural rearrangements between the crRNA and taRNA dictates whether or not the transcribed mRNA will be translated into the protein product. We have designed the regulatory sequence in the taRNA that can interact with the CR-4 regulatory sequence to turn back on translation to a high level. Using the bioluminescent reporter system, we demonstrated an ON/OFF ratio >300 (Fig. 3). In our riboregulator system (Fig. 1) both the cis-repressor and trans-activator RNAs are composed of targeting and regulatory sequences. The targeting sequences on a crRNA/taRNA pair are complementary and formation of the targeting helix is required for taRNA to modulate translation of a crRNA-regulated gene. We have also designed orthogonal targeting sequences with minimal cross-talk (Fig. 4). In our modular design, only the targeting sequence is changed to develop riboregulators that can independently regulate translation of many genes. By subtly altering the taRNA's regulatory helix and spacer it is possible to alter the equilibrium between repressed and activated states and achieve intermediate translational control (Fig. 5). We introduced sequence diversity at only five positions in the regulatory region of the taRNA and were able to identify taRNAs that produced intermediate levels of *cat* expression.

5. Outlook and application

The riboregulators described in this paper will be valuable for optimizing metabolic flux through engineered metabolic pathways using directed evolution approaches. We envision chromosomal genes encoding each enzyme in the pathway to be placed under independent transcription control using the same strong promoter/terminator pair for each gene. The genes will be translationally repressed by a crRNA consisting of a unique targeting sequence and the all OFF regulatory helix CR-4. Targeting sequences will specifically pair a single crRNA controlled gene with its taRNA pair. The pathway taRNAs will be plasmid encoded. By introducing diversity into a few positions in the trans-activator regulatory sequence, we

will prepare a small library of taRNAs that cover the range of translational activation. Gibson-assembly methods make it possible to prepare a random library of plasmids each containing trans-activators of varying effectiveness targeted independently to each gene in the pathway. The libraries of taRNA are small enough that it will be possible to simultaneously optimize expression of all of the enzymes in a pathway. After transformation, each cell will contain a plasmid encoding taRNAs targeted to each gene producing a unique regulatory profile for that pathway. All combinations of TA regulatory sequences are generated to create a cell population expected to have a range of metabolic fluxes. It will be necessary to develop high throughput screening or selection methods to identify cells in this population with optimal metabolic flux. The riboregulators described in this manuscript will provide the metabolic engineer a new tool to independently tune the level of expression of each enzyme catalyst in a pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

7. Acknowledgements

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6. Abbreviations

sRNA	small non-coding RNA
LB	Luria Broth
<i>E. coli</i>	<i>Escherichia coli</i>
crRNA	<i>cis</i> -repressor RNA
taRNA	<i>trans</i> -activator RNA
<i>cat</i>	chloramphenicol acetyl transferase
crRNA OFF	maximal OFF riboregulated state
TS	targeting sequence

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Figure 1: Schematic structure of the riboregulatory elements.

(a) The crRNA has modular design that includes a targeting sequence and a regulatory region that forms a stem-loop structure at the 5'-end of the gene sequestering the Ribosomal Binding Sequence (RBS) preventing translation of the mRNA; the taRNA is transcribed in trans. (b) The taRNA contains modules that are complementary to the targeting and regulatory sequences and allow it form a complex with the transcribed crRNA/mRNA. In this complex, translation of the target gene is either repressed or (c) the complex rearranges to free the RBS and activate translation. (d) It is possible to tune the relative stability of the

secondary structural elements in the crRNA and taRNA to obtain intermediate levels of translation.

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Figure 2: Turning off translation with the crRNA.

(a) crRNA sequences that occlude the RBS with stem loop structures of increasing stability. (b) Plot of the growth rate of *E. coli* transformants carrying a plasmid encoding a crRNA/*cat* (chloramphenicol resistance) fusion. In every case translation is driven constitutively by the T7A1 promoter/T7 terminator pair and translation is controlled by CR-1 (open circles), CR-2 (triangles), CR-3 (squares), or CR-4 (filled circles).

Figure 3: Turning on translation with the taRNA.

(a) Schematic of the plasmid encoded expression cassettes used to test the efficacy of riboregulators. In the Maximum ON construct, expression of *cat* is driven by the pT7A1 with no translational control. The Maximum OFF construct transcription of the crRNA:*cat* fusion is driven by pT7A1; translation of *cat* is attenuated by the crRNA. In the Regulated ON construct transcription of the taRNA is driven independently by pT7A1; taRNA modulates the translation of *cat*. In the Regulated OFF construct the stability of the secondary structure of the taRNA prevents rearrangement of the regulatory complex (see

Fig. 1b); translation remains blocked. (b) Plot of growth rate of *E. coli* transformants as a function of chloramphenicol concentration. The *E. coli* were transformed with the various *cat* expression vectors are plotted as follows: Maximal ON (squares), Maximal OFF (triangles), Regulated ON (open circles), Regulated OFF (diamonds) and wild-type *E. coli* DH5 α (filled circles).

Figure 4: Quantitative measure of translational control using the LUX reporter.

(a) Design of cis-repressed *Lux* operon, *Lux* gene is under the control of crRNA CR-4. In the Regulated ON construct transcription of the taRNA is driven independently by pT7A1; taRNA modulates the translation of *Lux* operon. In the Regulated OFF construct the stability of the secondary structure of the taRNA prevents rearrangement of the regulatory complex (see Fig. 1b); translation remains blocked. (b) Turning bioluminescence ON with taRNA: luminescence of *E. coli* transformants containing the crRNA regulated *Lux* operon in the presence of taRNAs as follows Riboregulated ON, (ON); Riboregulated OFF (OFF); and wild-type *E. coli* DH5α (Cont.).

Figure 5: Targeting of the taRNA to specific crRNA regulated genes.

(a) Nucleotide sequence of Targeting Sequence 1 (TS1) and Targeting sequence 2 (TS2). (b) The new targeting sequence (TS2) does not affect riboregulator activity. *aphA1* (kanamycin resistance) was placed under control of a crRNA composed of TS2 and CR-4 (Fig. 2). The plot of cell growth rate as a function of kanamycin concentration shows enhanced kanamycin resistance of *E. coli* cells expressing the Trans ON riboregulator with TS2 targeting sequence (open circles) in comparison to cells expressing the TS2 Trans OFF riboregulatory (diamonds) or wild-type controls (triangles) (c) Cross-talk determination

using chloramphenicol resistance in *E. coli*. In separate constructs *cat* was placed under translation control of TS1:crRNA and TS2:crRNA. Plotted is the growth rate of *E. coli* transformants harboring plasmids encoding: TS1:crRNA:*cat* and TS1:taRNA, (open circles); or TS1:crRNA:*cat* and TS2:taRNA, (triangles). **(d)** Cross-talk determination using luminescence reporter. In separate constructs the lux operon was placed under translation control of TS1:crRNA and TS2:crRNA. Plotted is the bioleuminescence of *E. coli* transformants harboring plasmids encoding: TS1:crRNA:*Lux* and TS1:taRNA ON; TS1:crRNA:*Lux* and TS1:taRNA OFF; TS2:crRNA and TS2:taRNA ON, and TS1:crRNA/*cat* and TS2:taRNA.

Figure 6: Intermediate levels of translational control.

(a) Nucleotide sequence of the CR-4-based crRNA:*cat* and the 32-member taRNA library. Random nucleotide substitutions at five sites were used alter the equilibrium between the repressed and activated states. (b) Plot of the growth rate of *E. coli* transformants as a function of chloramphenicol concentration. The *E. coli* were transformed with plasmids encoding the TS1:crRNA:*cat* and TS1:taRNAs of variable structure. Intermediate levels of

translational control (open symbols) fall between the TS1:taRNA OFF (filled circles) and TS1:taRNA ON (squares).

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