JBEI Performance Metric for FY16: Develop an improved metabolic engineering method for modifying microorganisms for biofuel production from cellulosic sugars

Q2 Metric: New methods to modify or control regulation of engineered pathways for biofuel production

1. BACKGROUND

In recent years, we have synthesized and engineered many metabolic pathways in microbial hosts to produce useful biofuel molecules. Frequently, we have observed that the titer and yield are limited by the inefficient use of cellular resources and metabolic imbalance. Expression of pathway genes at adequate levels is essential for efficient conversion of starting sugars to final fuel products. There have been extensive studies to engineer the strength of promoters and ribosomal binding sites (RBSs) to regulate gene expression. Combinatorial libraries of promoters, RBSs, and intergenic regions have been used to tune the expression level of several genes in heterologous pathways to optimize the production of isoprenoids. These static control strategies have been used to regulate the biofuel pathways. On the other hand, biofuel-producing cells can sense nutritional resources, temperature, and pH in their environment, and more importantly, the status of their biosynthetic pathways. They can adapt their transcription, translation, and enzymatic catalysis in response to these conditions, so that genes are only transcribed and expressed when they are needed and in proper amounts, and metabolic intermediates are converted to downstream products immediately after synthesis.

In this report, we describe some new methods for various biofuels pathway regulation recently developed at JBEI.

2. ENGINEERING FATTY ACID-DERIVED BIOFUEL PATHWAYS WITH FATTY ACID-RESPONSIVE REGULATORS

A valuable synthetic biology tool that has been used at JBEI is the Dynamic Sensor-Regulator System (DSRS), whereby a metabolite-responsive transcription factor is used to regulate selected pathway genes based on concentrations of the cognate metabolite. This can be a more efficient means of regulation than, for example, use of inducers like IPTG (isopropyl β-D-1-thiogalactopyranoside), because the DSRS will upregulate pathway genes *only* when important metabolites are being produced, as opposed to a more non-specific regulation scheme that does not account for optimal timing of pathway gene expression. Here, we present an example of using DSRS in the context of fatty acid ethyl esters (FAEE), a renewable diesel substitute.

2.1. **FadR as a regulator of fatty acid biosynthesis and metabolism**

Regulation of fatty acid biosynthesis and β-oxidation is complex, as described elsewhere (Handke et al., 2011; Janssen and Steinbuchel, 2014; Magnuson et al., 1993). One important regulatory element in *E. coli* is the transcription factor, FadR. FadR is a negative transcriptional regulator of the *fad* (fatty acid degradation, or β-oxidation) operon. When fatty acyl-CoAs are at very low intracellular concentration, FadR binds to promoters of all β-oxidation genes and blocks access for RNA polymerase, thus preventing transcription. Conversely, when acyl-CoAs are present, they bind with FadR and it is released from its binding site on the promoter, thus allowing (de-repressing) transcription. FadR can also act as a positive regulator (transcriptional activator) of certain fatty acid biosynthesis genes, such as *fabA* and *fabB*.

2.2. **Using synthetic FadR promoters and DSRS to efficiently regulate FAEE biosynthesis**

JBEI researchers have developed and used the DSRS approach to successfully increase production of the fatty acid-derived FAEEs (Zhang et al., 2012). In this study, certain modules of the FAEE biosynthetic pathway were placed under the regulatory control of synthetic hybrid promoters that included FadR-binding sites as well as LacI-binding sites. The concept behind the hybrid promoters was to make them responsive to fatty acyl-CoAs (which are key metabolites in the FAEE pathway) as well as to IPTG, which introduced additional stringency, as endogenous fatty acids could cause leaky expression if only FadR-binding sites were present. A schematic of the approach is shown in Figure 1a. As shown, synthetic hybrid promoters containing FadR-binding sites were used to

control expression of genes in Module B (*pdc* and *adhB*, catalyzing ethanol production) and Module C (*fadD*, an acetyl-CoA synthetase, and *atfA*, a wax-ester synthase that produces FAEEs from acyl-CoAs and ethanol). As shown in Figure 1b, the approach was highly successful, resulting in a 3- to 4-fold increase in FAEE titers to attain a best titer of 1.5 g/L (Zhang et al., 2012). Not only did use of the synthetic promoters increase titers, but plasmid stability in this 3-plasmid system also increased dramatically.

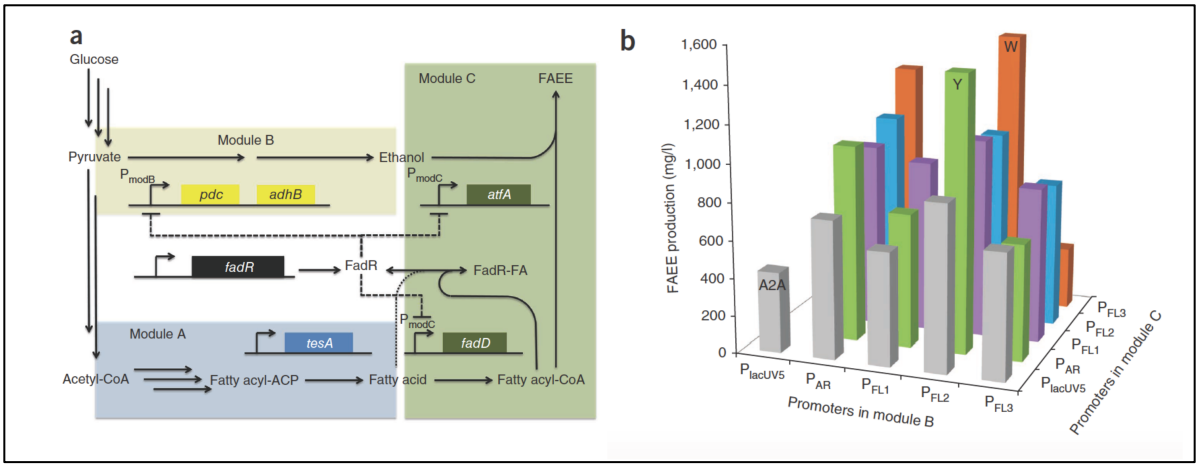

Figure 1. Use of a Dynamic Sensor-Regulator System (DSRS) to improve FAEE titers. From Zhang et al. (2012).

3. ALCOHOL (1-BUTANOL) SENSORS

Directed evolution, in which a non-natural selective pressure is applied to a diverse pool of target genetic sequences to evolve a desired trait, is a hallmark of metabolic engineering efforts to improve biological function. The success of any directed evolution strategy is contingent upon the effectiveness of two key steps: first, generating large, diverse genotypic libraries and second, efficiently identifying the desired phenotype from a heterogeneous population.

Our ability to generate genotypic diversity far outstrips the ability to efficiently and effectively interrogate the resulting library. *In vitro* methods for incorporation of either random or targeted mutations into user-specified plasmid DNA sequences are now numerous and well developed. The potential for genetic diversity to yield process improvements, however, is realized only when the target phenotype can be screened or selected for. For this reason, advances in directed evolution approaches within the context of metabolic engineering have been almost exclusively applied to improving host resistance to a toxic product, such as the growth-coupled improvement in tolerance to olefins (Mingardon et al., 2015) or overproduction of a natural chromophore, such as lycopene (Wang et al., 2009). In contrast, the majority of small molecules being targeted for overproduction today, including fatty acids, diols and diamines, and short-chain alcohols among others, cannot be directly screened or selected for. In nature, the need for sensitive, specific, small-molecule detection and response has been addressed, in part, through evolution and selection for ligand-responsive transcription factors and their cognate promoters. We developed a generalized approach to screen or select for improved small-molecule biosynthesis using transcription factor-based biosensors (Dietrich et al., 2012). Using a tetracycline resistance gene downstream (3′) of a small-molecule inducible promoter, we coupled host antibiotic resistance, and hence growth rate, to either small-molecule concentration in the growth medium or a small-molecule production phenotype. We constructed biosensors for alcohols, using transcription factor-promoter pairs derived from *Pseudomonas putida*, *Thauera butanivorans*, or *E. coli*. Transcription factors were selected for specific activation by 1-butanol, and we demonstrated product-dependent growth in *E. coli* using this compound. The 1-butanol biosensor was applied in a proof-of-principle liquid culture screen to optimize 1-butanol biosynthesis in engineered *E. coli*, identifying a pathway variant yielding a 35% increase in 1-butanol specific productivity through optimization of enzyme expression levels. Lastly, to demonstrate the capacity to select for enzymatic activity, we applied the 1-butanol biosensor as synthetic selection, coupling *in vivo* 1-butanol biosynthesis to *E. coli* fitness. We observed a 120-fold enrichment for a 1-butanol production phenotype following a single round of positive selection (Figure 2).

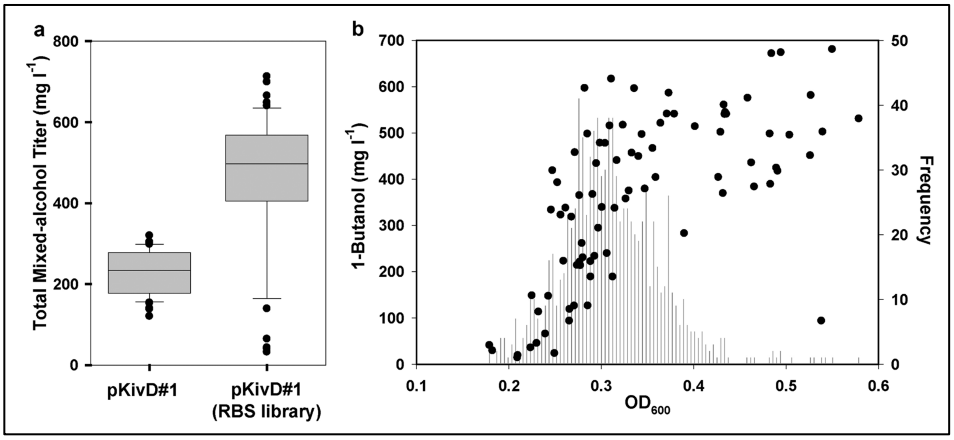

Figure 2. BmoR-P_{BMO} biosensor screen for improved 1-butanol biosynthesis. (a) The mean total mixed alcohol titer in *E. coli DH1* Δ*adhE* harboring pKivD#1 was significantly lower (t test; unpaired, $p = 1 \times 10^{-11}$) as compared to a heterogeneous population containing mutated *kivD* and ADH6 ribosome binding site (RBS) sequences, a result suggesting that the initial RBS was non-optimal. The RBS library population produced a broad range of alcohol titers ($n = 50$; box and whisker plot depicts 10th, 25th, median, 75th, and 90th percentiles) suitable for characterization of the high-throughput screen. (b) The biosensor response (OD_{600}) to spent production medium from a 960-member library of mutated *kivD* and ADH6 RBS sequences was distributed around $OD_{600} = 0.31$. GC-MS was used to confirm 1-butanol titers for 10% of the sample population, demonstrating a positive correlation between biosensor response and 1-butanol titer. From Dietrich et al. (2012).

4. ISOPRENOID PATHWAY REGULATION

The regulatory mechanisms of the isoprenoid production pathways have been most extensively investigated in eukaryotes, especially in plants. In bacteria, relevant genes for a specific function are generally clustered together and transcribed collectively as an operon in the genome. However, the genes annotated as a part of the non-mevalonate methylerythritol 4-phosphate (MEP) pathway are dispersed in the *E. coli* genome. In addition, no transcription factor has been found to induce or to repress the MEP pathway genes in *E. coli*. Therefore, transcriptional regulation of the bacterial MEP pathway has not been well-characterized and the engineering of the MEP pathway regulation has not been successful so far. Although transcriptional regulation of the MEP pathway is still obscure in prokaryotes, the regulation mechanisms of the mevalonate (MVA) pathway have been reported in eukaryotes at the transcriptional, translational, and post-translational levels. We hypothesized that this information could be applicable to other microbial production hosts containing either an endogenous or heterologous MVA pathway.

4.1. **Early work on mevalonate pathway regulation**

As an early example, microbial auxotrophy has been used to monitor growth-limiting small molecules. In this early work, we had reported a novel biosensor strain for detection and quantification of mevalonate, a key intermediate in the biosynthesis of isoprenoids *via* the MVA pathway (Pfleger et al., 2007). By deleting the native pathway for the production of two universal precursors of isoprenoids, IPP and DMAPP, that are necessary for growth, and incorporating the mevalonate-utilizing pathway (by which exogenous mevalonate can be converted into IPP and DMAPP), the mevalonate sensor was developed to measure the concentration of mevalonate in mevalonate-producing cultures through simple growth monitoring. The biosensor strain was an *Escherichia coli* mevalonate auxotroph that expresses the green fluorescent protein (GFP) and reports on the mevalonate concentration in the growth medium through a change in growth rate.

Another example is a method for tuning the expression of multiple genes within operons by generating libraries of tunable intergenic regions (TIGRs), recombining various post-transcriptional control elements, and screening for the desired relative expression levels (Pfleger et al., 2006). TIGRs can vary the relative expression of two reporter genes over a 100-fold range and balance expression of three genes in an operon that encodes a heterologous mevalonate biosynthetic pathway, resulting in a seven-fold increase in mevalonate production.

4.2. **Dynamic regulation of the mevalonate pathway**

The isoprenoid pathway produces intermediates that are toxic to the cell. The accumulation of these toxic intermediates can lead to a stress response, and dynamic control of the pathway could be applied to prevent the accumulation of toxic metabolites. Such a strategy requires sensors that can detect and respond to the metabolite, but such sensors are largely unknown. We reasoned that the host's native stress response system would respond when toxic metabolites accumulated. Transcript profiling, using cDNA microarrays or RNAseq, offers a convenient way to evaluate the cell's transcriptional response to the accumulation of toxic metabolites, creating a list of candidate promoters that could be used to respond to intermediate toxicity. Using such promoters to regulate pathway expression in response to the intermediates creates a link between the cell's metabolic state and the expression of the metabolic pathway. We applied this approach to regulate toxic farnesyl diphosphate (FPP) accumulation in the isoprenoid biosynthetic pathway in *E. coli* (Dahl et al., 2013). We designed two strains with a heterologous mevalonate pathway for the production of the sesquiterpene, amorphadiene (Figure 3). One of the strains accumulated FPP due to a mutation that inactivated the sesquiterpene synthase, while the other expressed a functional sesquiterpene synthase that did not allow FPP accumulation because it efficiently metabolized FPP. Microarray analysis identified those genes either up-regulated or down-regulated by the accumulation of FPP, and a library of 35 promoters was chosen and screened for the ability to control production of the FPP-derived sesquiterpene, amorphadiene. We introduced the negative feedback to the FPP synthesis pathway and positive feed-forward to downstream FPP-consuming enzyme (terpene synthase). Therefore, promoters that are down-regulated in response to FPP accumulation tuned expression of FPP-producing enzymes, whereas up-regulated promoters controlled the FPP-consuming enzyme. The highest yield using these FPP-responsive promoters was obtained using a combination that weakly up-regulated FPP consumption, and weakly down-regulated FPP production, and the resulting self-regulated FPP production resulted in yields that were at least 2-fold higher compared to strains that used inducible or constitutive promoters. Moreover, this dynamic system eliminated the need for expensive inducers, reduced acetate accumulation, and improved growth.

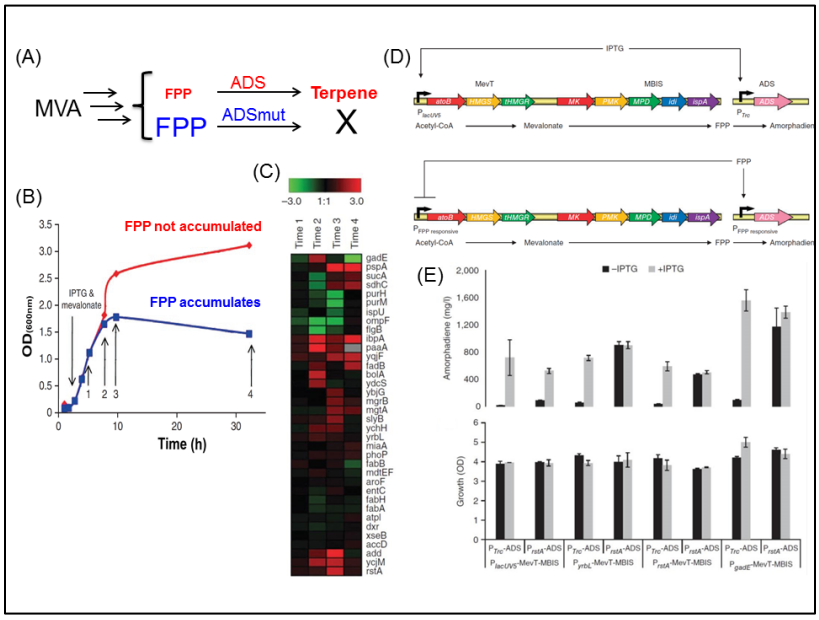

Figure 3. FPP-responsive dynamic pathway for terpene production. (A) Strategy to generate FPP stress. ADS, amorphadiene synthase; ADSmut, inactive mutant of ADS. (B) Time course of OD by FPP stress. Sampling time points for microarray analysis are numerically marked. (C) Microarray results of selected promoter's gene expression under FPP stress (ADSmut strain/ADS strain). (D) IPTG-inducible vs FPP-responsive terpene production pathway. (E) Terpene production and growth of strains using either inducible or FPP-responsive promoters. From Dahl et al. (2013).

4.3. **IPP toxicity and development of an IPP sensor**

Previous studies have implicated prenyl diphosphate (e.g., isopentenyl diphosphate, or IPP) accumulation as a cause of reduced growth and decreased glucose uptake. The strong correlation between glucose consumption and IPP accumulation suggests that glucose uptake may be inhibited, though this may be a nonspecific consequence

of reduced membrane integrity or inhibited cell metabolism (George et al., 2014). Further investigation to interrogate membrane integrity is an important first step towards addressing this uncertainty. Increasing the expression of the reductase NudB facilitated an improvement in isopentenol titer and partially relieved IPP toxicity. Even though the mechanism of IPP-related toxicity is currently unknown, we hypothesized that sensing intracellular IPP level would be a good lever to dynamically regulate the IPP toxicity of the production strain and improve isoprenoid production.

Adaptation is a behavior that allows cells to survive and thrive under constantly changing environmental conditions, and is characterized by rapid genetic change leading to rare beneficial mutations. Models and experimental data of the adaptive process suggest that a 'variable mutation rate' strategy is one of the strategies used by nature to evolve traits, where a period of high mutation rate increases the genetic diversity of populations with initially low phenotypic diversity, and the mutation rate decreases with increased genetic diversity in the population. Directed evolution strategies that generate mutant libraries *in vitro* are limited by the ligation efficiency, and those that use mutator strains with unregulated, high mutation rates to generate mutant libraries *in vivo* suffer from the accumulation of deleterious mutations that eventually lead to cell death. Although adaptation has proven useful for evolving certain phenotypes, its application has been limited to traits that are directly tied to growth. Therefore, a method capable of changing the mutation rate *in vivo* according to a particular phenotype, independent of whether it is linked to growth, could circumvent the constraints set by ligation inefficiencies, deleterious mutations, and assay availability.

We developed an adaptive-control system that increases the mutation rate in order to generate diversity in the population, and decreases the mutation rate as the concentration of a target metabolite increases (Chou and Keasling, 2013) (Figure 4). This system is called Feedback-Regulated Evolution of Phenotype (FREP), and we implemented it with a sensor to gauge the concentration of a metabolite and an actuator to alter the mutation rate. As shown in Figure 4B, the sensor activates the promoter that controls *mutD5* expression in the absence of the ligand IPP, and RFP acts as a reporter gene. MutD5 increases the mutation rate and mutations accumulate on the chromosome with each successive generation. Some of the mutations lead to increased IPP production, which decreases the ability of the sensor to activate expression of *mutD5* and *rfp* in those cells. The mutations that increase IPP production are fixed into successive generations by passaging those cells with decreased *rfp* expression. We assembled synthetic transcription factors (TFs) using metabolic enzymes and constructed four different sensors that recognized IPP in bacteria and yeast, as shown in Figures 4C and 4E. A synthetic TF consists of three parts: Part1 binds the target ligand, Part2 converts the binding signal into a change in RNA polymerase binding to the target promoter, and Part3 is an amino-acid linker fusing Part1 and Part2 together. Here, a sensor with synthetic TF IA includes Idi as Part1 and AraC's DBD and linker as Part2 and Part3, respectively. In yeast, the synthetic TF consists of Idi as Part1, GAL4's AD and DBD as Part2, and a 19-amino-acid linker as Part3 (Figure 4E). We verified FREP by evolving increased isoprenoid production.

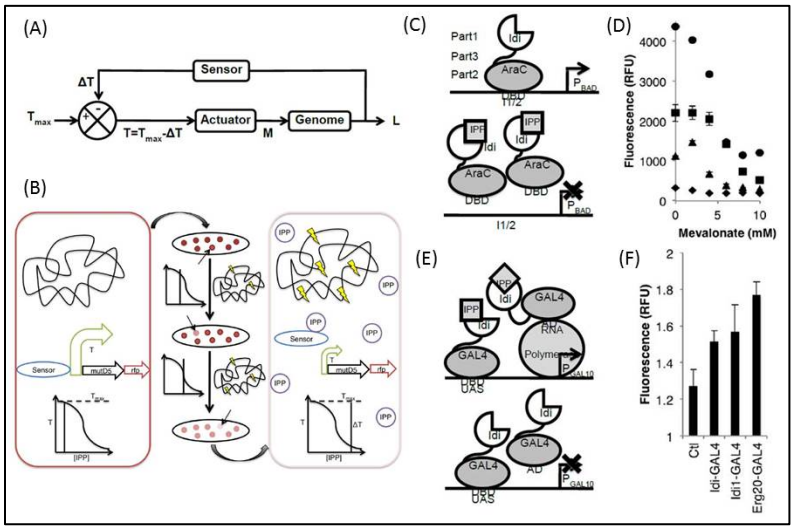

Figure 4. FREP design and synthetic transcription factors respond to IPP. (A) FREP implementation of the variable mutation strategy using an adaptive control system. The sensor controls the change in transcriptional level (DT) in the system. The actuator converts the transcriptional level (T) into a mutation rate (M) that modifies the genome to produce the target phenotype gauged by L. As L increases, the sensor increases DT, which causes the actuator to decrease M. (B) Evolution of increased IPP production using FREP. (C) A synthetic transcription factor (TF) consisting of three parts. One model for how IA regulates P_{BAD} is IA binds the DNA sequence I_1I_2, activating transcription from P_{BAD} in the absence of IPP (top), and IPP-bound IA dimerizes, preventing binding to I_1I_2 and activation of P_{BAD} (bottom). (D) Output of four sensors, each with a different TF, to changing IPP concentrations in *E. coli* HC175 monitored with mcherry. Solid diamonds represent AC, solid triangles IA32, solid squares IA and solid circles IA44. The error bars represent one s.d.

Each data point represents the average of three replicates. (E) A sensor for detecting IPP in *S. cerevisiae*. One model for P_{GAL10} regulation is that Idi dimerizes when bound to IPP, bringing the upstream activation sequence (UAS)-bound GAL4 DBD in close enough proximity with the GAL4 AD to activate transcription (top). In the absence of Part1 dimerization, there is no transcription from P_{GAL10} (bottom). (F) P_{GAL10} output from three sensors with synthetic TFs in *S. cerevisiae* MO219 induced with galactose. Output was monitored with the fluorescent protein yEcitrine and normalized to fluorescence in the absence of galactose. The error bars represent one s.d. Each data point represents the average of three replicates. From Chou and Keasling (2013).

5. SYNTHETIC TRANSCRIPTIONAL FACTOR-PROMOTER SET FOR THE YEAST METABOLIC ENGINEERING TOOLBOX

The majority of engineering in *S. cerevisiae* continues to rely on a handful of native promoters, where a few constitutive or galactose-inducible promoters remain the staple of the yeast genetic engineer's toolbox. In cases where promoter inducibility is desired, galactose induction is particularly problematic because of the limitations it imposes on the types of carbon sources that can be used for cultivation. As a potential solution to this challenge, the modular architecture of both the promoter sequences and the transcriptional factors (TFs) has been used to modify them for altered response and transcriptional regulation profiles. In particular, the native Gal4 TF and the corresponding promoters has been the target of many efforts to develop synthetic TF-promoter combinations towards desirable changes in inducer molecules, control, and dynamic range (Khalil et al., 2012; Louvion et al., 1993; McIsaac et al., 2014; Ottoz et al., 2014). At JBEI, we built upon these advances to develop a system that will be useful for engineering heterologous metabolic pathways, for finding optimum levels for each gene product in a given pathway, and for expressing multiple genes.

We constructed a library of hybrid promoters that are regulated by a synthetic TF (Figure 5). The hybrid promoters are composed of native *S. cerevisiae* promoters, where the operator regions have been replaced with sequences that recognize the bacterial LexA DNA-binding protein. Correspondingly, the synthetic TF are composed of the DNA-binding domain of the LexA protein fused with the human estrogen-binding domain (hER) and the viral activator domain, VP16. The resulting system avoids transcription of any native *S. cerevisiae* genes, and the gene of interest driven by the hybrid promoters can be induced using estradiol, a compound with no detectable impact on *S. cerevisiae* physiology. Using combinations of one or more sequence repeats and a set of native *S. cerevisiae* promoters, we designed a series of hybrid promoters that can be induced differently and to different levels with the same synthetic TF and a given level of exogenous estradiol. Using a combination of the j5 DNA assembly design software (j5.jbei.org (Hillson et al., 2012)) and the Pr-Pr laboratory automation platform (prpr.jbei.org (Linshiz et al., 2014)), both developed at JBEI, and the gene-synthesis capability at DOE's Joint Genome Institute, a library of 240 promoter sequences were designed, constructed, and tested.

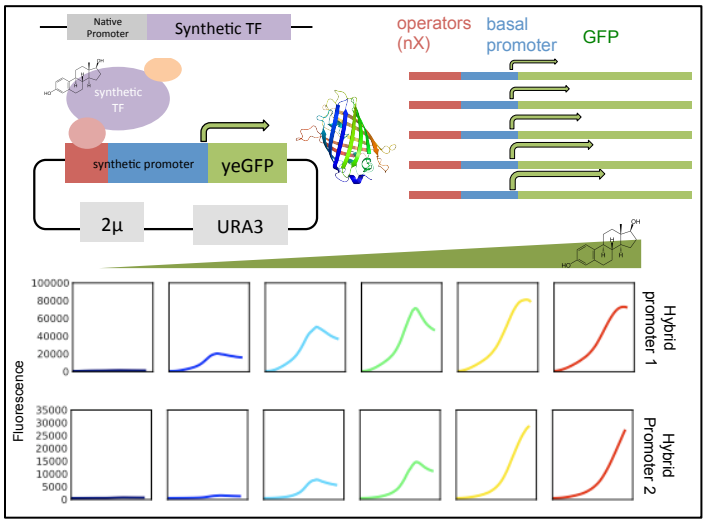

Figure 5. Synthetic Transcriptional Factor-Promoter set for the yeast metabolic engineering toolbox. The chromosomally encoded synthetic TF can be coupled with a library of modified promoters to regulate genes with different inducibility and responsiveness in the same strain using the same level of an inducer compound.

In order to easily test the large number of promoter constructs, we chromosomally integrated a native yeast promoter driven copy of the LexA-hER-vp16 TF (Figure 5). We selected a native promoter that provides a constitutive expression of the TF. For the promoter series, with the exception of a few promoter scaffolds, almost

combinations of basal promoters in two lengths (100bp and 250bp) could be built with 1x, 2x or 3x repeats of four selected operator sequences. The plasmids with these hybrid promoter:yeGFP cassettes were transformed into the yeast strain with the chromosomally encoded synthetic TF. Estradiol concentrations from 0-100 nM were tested for each variant. Parameters of interest for each promoter; inducibility and response, measured in terms of the fold change in induction from 0-100 nM, and the maximal level of induction at 10 nM estradiol. The best results were achieved with the longer promoter regions (250bp). The final library contained many promoters that displayed the range of desirable profiles required to modulate multiple to genes different levels in a given strain using the same synthetic TF and therefore the same inducing compound.. This set of promoters, in combination with our synthetic TF, can be used to regulate numerous genes or pathways simultaneously, to multiple desired levels, with the same small molecule added to the culture medium, in a given *S. cerevisiae* strain.

6. ADDITIONAL REGULATION CONTROLS

6.1. **Global regulators**

Exploiting global regulators to improve production phenotypes in *E. coli* is another approach to controlling engineered biosynthetic pathways for biofuel production. We explored the use of endogenous non-coding RNA, csrB, to alter carbon flux in *E. coli* (McKee et al., 2012). CsrA is an RNA-binding protein that alters concentrations of intracellular metabolites. We demonstrated alterations at the proteomic and metabolite level that were attributed to changes in the levels of CsrB, a non-coding RNA antagonist of CsrA, and employed these molecular variations to augment flux through engineered pathways (Figure 6). We employed this regulatory switch to improve production through the following routes: the 1-butanol pathway (from *Clostridium acetobutylicum*), the mevalonate pathway (from *S. cerevisiae*), and the native fatty acid (FA) pathway of *E. coli*. In each case we demonstrated fold-level improvements in the production of advanced biofuels or their precursors by perturbing the host strain's native Csr regulatory system.

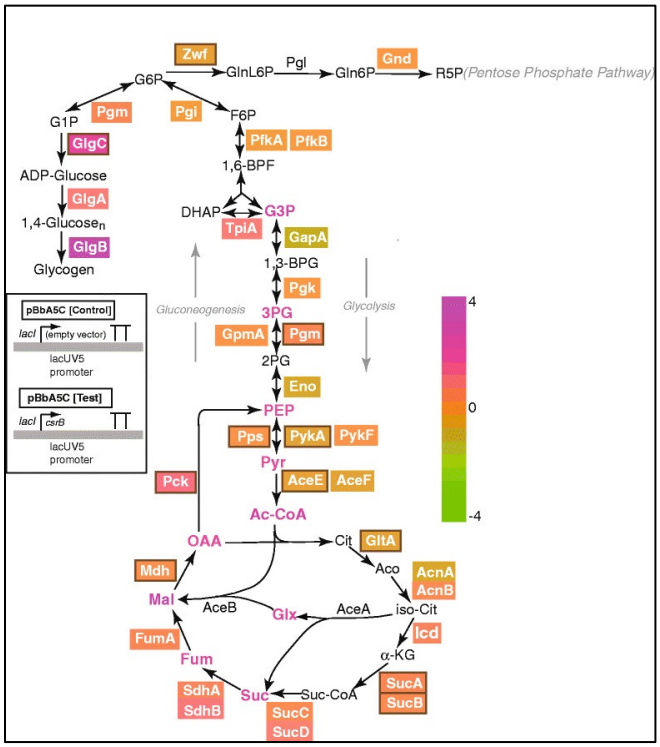

Figure 6. Extensive metabolic remodeling is achieved through CsrB manipulation. The color scale indicates average fold ratios of intracellular metabolites and proteins from central metabolism in BLR-DAJ cells bearing pBbA5C-CsrB relative to the empty plasmid (pBbA5C) alone. Certain metabolites and proteins were not analyzed (black). Fold level changes represented by colored fonts (for metabolites) or colored boxes (for proteins) corresponding to the scale shown on the right. CsrA targets with potential or known binding sites are depicted by a brown outline around the corresponding protein box. Pentose phosphate (penP), erythrose-4-phosphate, (E4P), glyceraldehyde-3-phosphate (G3P), 3-phosphoglycerate (3PG), phosphoenolpyruvate (PEP), pyruvate (pyr), acetyl-CoA (acCoA), oxaloacetate (oaa), citrate (cit), iso-citrate (i-cit), a-ketoglutarate (akg), glyoxylate (glx), succinyl-CoA (sucCoA), succinate (suc), malate (mal), fumarate (fum). From McKee et al (2012).

6.2. **RNA device to control gene expression**

Biological systems exhibit functional complexity across multiple scales, from RNA, DNA, and protein subunit interactions to interactions among genes, pathways, circuits, and cells (Hazen et al., 2007). In nature, RNA structures process cellular information and regulate genetic expression at the levels of transcription, translation, and RNA degradation (Zhang et al., 2010). Synthetic aptamers, ribozymes (rbzs), and aptazymes (aptzs) assembled into

static or dynamic ligand-responsive regulators can control gene expression in bacteria, yeast, and mammalian cells (Saito and Inoue, 2009). Given this functional potential, creating methods to rapidly assemble RNA-regulated devices with predictability should allow engineering of programmable pathway and circuit controller activities (Holtz and Keasling, 2010) and higher-order information-processing mechanisms (Benenson, 2009).

We formulated a design-driven approach that used mechanistic modeling and kinetic RNA-folding simulations to engineer RNA-regulated genetic devices that control gene expression (Carothers et al., 2011) (Figure 7). Ribozyme- and metabolite-controlled, aptazyme-regulated expression devices with quantitatively predictable functions were assembled from components characterized *in vitro*, *in vivo*, and *in silico*. We verified the models and design strategy by constructing 28 *E. coli* expression devices that gave excellent quantitative agreement between the predicted and measured gene expression levels ($r = 0.94$). We applied these technologies to engineer RNA-regulated controls in metabolic pathways. More broadly, we provided a framework for studying RNA functions and showed the potential for use of biochemical and biophysical modeling to develop biological design methods.

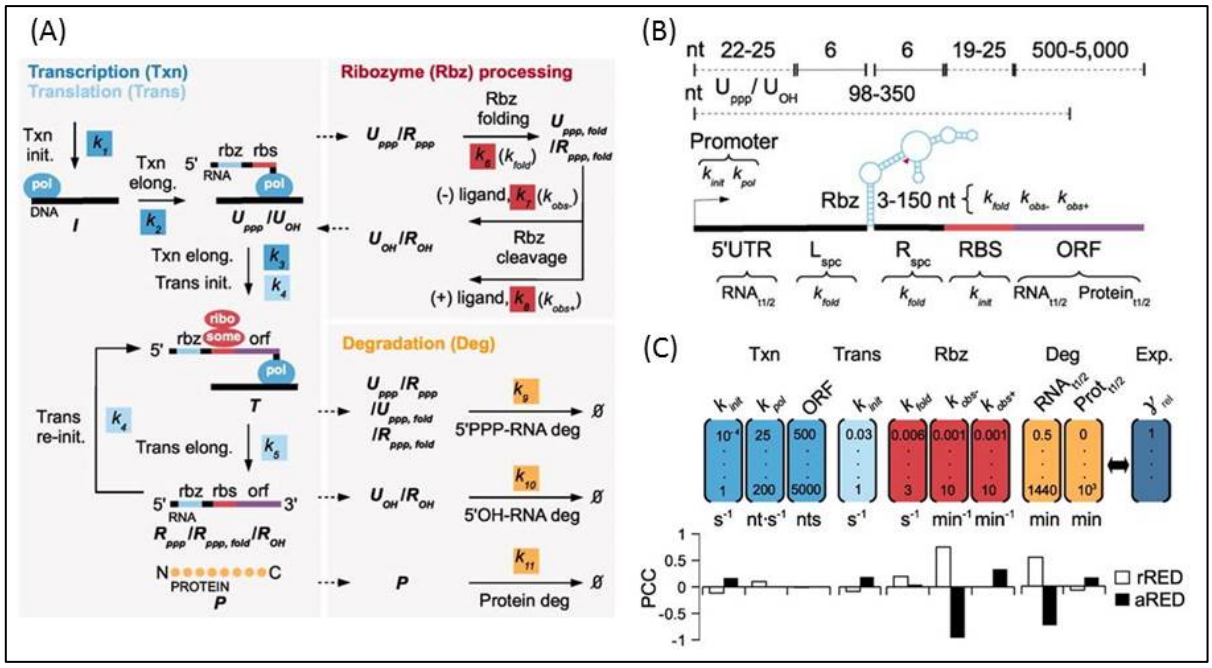

Figure 7. RNA-regulated expression devices and functional design space. (A) Functions of rREDs and aREDs were simulated with a coarse-grained mechanistic model of effective rate constants. (B) Tunable components and design variables for static rRED and dynamic aRED genetic controllers. nt, nucleotides. (C) Combinatorial design variable space was mapped to device outputs (g_{rel}) with Monte Carlo filtering. PCCs with g_{rel} measure the impact of individual design variables on device behavior. From Carothers et al (2011).

7. CONCLUDING REMARKS

JBEI has successfully used a variety of approaches to artificially regulate gene expression with the goal of more precisely modulating the expression of biofuel metabolic pathway genes and balancing metabolism. One way of accomplishing this is to engineer biofuel-producing hosts to dynamically modify gene expression in response to key intracellular or environmental conditions, so that genes are only expressed when needed and in proper amounts, and metabolic intermediates are converted to downstream products immediately after they are synthesized. For example, dynamic regulation based on metabolite-responsive transcription factors and promoters was used to improve FAEE and sesquiterpene production. We have also (1) developed synthetic transcription factor-promoter sets for yeast that provide a range of expression strength and are orthogonal to regulation of native genes, (2) used global regulators that modulate carbon flux in *E. coli*, and (3) formulated a design-driven approach that used mechanistic modeling and kinetic RNA-folding simulations to engineer RNA-regulated genetic devices that control

gene expression. Finally, we have used regulation-based approaches for strain development, for example, by (1) developing a generalized approach to screen or select for improved small-molecule (e.g., biofuel) biosynthesis using transcription factor-based biosensors and (2) using Feedback-Regulated Evolution of Phenotype (FREP), an adaptive-control system that increases the mutation rate in order to generate diversity in the population, and decreases the mutation rate as the concentration of a target metabolite (e.g., biofuel) increases.

Q2 report Authors: Taek Soon Lee, Jay D. Keasling, Harry R. Beller, and Aindrila Mukhopadhyay.

JBEI Fuel Synthesis Team: Harry R. Beller, Taek Soon Lee, Hector Garcia Martin, Nathan J. Hillson, Jay D. Keasling, Aindrila Mukhopadhyay.

REFERENCES

Benenson, Y., 2009. RNA-based computation in live cells. Curr Opin Biotech. 20**,** 471-478.

Carothers, J. M., Goler, J. A., Juminaga, D., Keasling, J. D., 2011. Model-driven engineering of RNA devices to quantitatively program gene expression. Science. 334**,** 1716-9.

Chou, H. H., Keasling, J. D., 2013. Programming adaptive control to evolve increased metabolite production. Nature communications. 4**,** 2595.

Dahl, R. H., Zhang, F., Alonso-Gutierrez, J., Baidoo, E., Batth, T. S., Redding-Johanson, A. M., Petzold, C. J., Mukhopadhyay, A., Lee, T. S., Adams, P. D., Keasling, J. D., 2013. Engineering dynamic pathway regulation using stress-response promoters. Nature biotechnology. 31**,** 1039-46.

Dietrich, J. A., Shis, D. L., Alikhani, A., Keasling, J. D., 2012. Transcription Factor-Based Screens and Synthetic Selections for Microbial Small-Molecule Biosynthesis. ACS Synth Biol. pp. 47–58.

George, K. W., Chen, A., Jain, A., Batth, T. S., Baidoo, E. E., Wang, G., Adams, P. D., Petzold, C. J., Keasling, J. D., Lee, T. S., 2014. Correlation analysis of targeted proteins and metabolites to assess and engineer microbial isopentenol production. Biotechnology and bioengineering. 111**,** 1648-58.

Handke, P., Lynch, S. A., Gill, R. T., 2011. Application and engineering of fatty acid biosynthesis in *Escherichia coli* for advanced fuels and chemicals. Metab Eng. 13**,** 28-37.

Hazen, R. M., Griffin, P. L., Carothers, J. M., Szostak, J. W., 2007. Functional information and the emergence of biocomplexity. P Natl Acad Sci USA. 104**,** 8574-8581.

Hillson, N. J., Rosengarten, R. D., Keasling, J. D., 2012. j5 DNA Assembly Design Automation Software. ACS Synth Biol. 1**,** 14-21.

Holtz, W. J., Keasling, J. D., 2010. Engineering Static and Dynamic Control of Synthetic Pathways. Cell. 140**,** 19-23.

Janssen, H. J., Steinbuchel, A., 2014. Fatty acid synthesis in Escherichia coli and its applications towards the production of fatty acid based biofuels. Biotechnol Biofuels. 7, 7.

Khalil, A. S., Lu, T. K., Bashor, C. J., Ramirez, C. L., Pyenson, N. C., Joung, J. K., Collins, J. J., 2012. A synthetic biology framework for programming eukaryotic transcription functions. Cell. 150**,** 647-58.

Linshiz, G., Stawski, N., Goyal, G., Bi, C., Poust, S., Sharma, M., Mutalik, V., Keasling, J. D., Hillson, N. J., 2014. PR-PR: Cross-Platform Laboratory Automation System. ACS Synth Biol. 140114162902002.

Louvion, J. F., Havaux-Copf, B., Picard, D., 1993. Fusion of GAL4-VP16 to a steroid-binding domain provides a tool for gratuitous induction of galactose-responsive genes in yeast. Gene. 131**,** 129-34.

Magnuson, K., Jackowski, S., Rock, C. O., Cronan, J. E., 1993. Regulation of Fatty-Acid Biosynthesis in *Escherichia coli.* Microbiol Rev. 57**,** 522-542.

McIsaac, R. S., Gibney, P. A., Chandran, S. S., Benjamin, K. R., Botstein, D., 2014. Synthetic biology tools for programming gene expression without nutritional perturbations in *Saccharomyces cerevisiae*. Nucleic Acids Res. 42**,** e48.

McKee, A. E., Rutherford, B. J., Chivian, D. C., Baidoo, E. K., Juminaga, D., Kuo, D., Benke, P. I., Dietrich, J. A., Ma, S. M., Arkin, A. P., Petzold, C. J., Adams, P. D., Keasling, J. D., Chhabra, S. R., 2012. Manipulation of the carbon storage regulator system for metabolite remodeling and biofuel production in *Escherichia coli*. Microbial Cell Factories. 11**,** 79.

Mingardon, F., Clement, C., Hirano, K., Nhan, M., Luning, E. G., Chanal, A., Mukhopadhyay, A., 2015. Improving olefin tolerance and production in E. coli using native and evolved AcrB. Biotechnology and Bioengineering. 112**,** 879-88.

Ottoz, D. S., Rudolf, F., Stelling, J., 2014. Inducible, tightly regulated and growth condition-independent transcription factor in *Saccharomyces cerevisiae*. Nucleic Acids Res. 42**,** e130.

Pfleger, B. F., Pitera, D. J., D Smolke, C., Keasling, J. D., 2006. Combinatorial engineering of intergenic regions in operons tunes expression of multiple genes. Nature Biotechnology. 24**,** 1027-1032.

Pfleger, B. F., Pitera, D. J., Newman, J. D., Martin, V. J. J., Keasling, J. D., 2007. Microbial sensors for small molecules: Development of a mevalonate biosensor. Metab Eng. 9**,** 30-38.

Saito, H., Inoue, T., 2009. Synthetic biology with RNA motifs. Int J Biochem Cell B. 41**,** 398-404.

Wang, H. H., Isaacs, F. J., Carr, P. A., Sun, Z. Z., Xu, G., Forest, C. R., Church, G. M., 2009. Programming cells by multiplex genome engineering and accelerated evolution. Nature. 460**,** 894-U133.

Zhang, F., Carothers, J. M., Keasling, J. D., 2012. Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. Nature Biotechnology. 30**,** 354-9.

Zhang, J., Lau, M. W., Ferre-D'Amare, A. R., 2010. Ribozymes and Riboswitches: Modulation of RNA Function by Small Molecules. Biochemistry-US. 49**,** 9123-9131.