Sensitive cells: enabling tools for static and dynamic control of microbial metabolic pathways

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Natural metabolic pathways are dynamically regulated at the transcriptional, translational, and protein levels. Despite this, traditional pathway engineering has relied on static control strategies to engender changes in metabolism, most likely due to ease of implementation and perceived predictability of design outcome. Increasingly in recent years, however, metabolic engineers have drawn inspiration from natural systems and have begun to harness dynamically controlled regulatory machinery to improve design of engineered microorganisms for production of specialty and commodity chemicals. Here, we review recent enabling technologies for engineering static control over pathway expression levels, and we discuss state-of-the-art dynamic control strategies that have yielded improved outcomes in the field of microbial metabolic engineering. Furthermore, we emphasize design of a novel class of genetically encoded controllers that will facilitate automatic, transient tuning of synthetic and endogenous pathways.

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Introduction

Metabolic pathways are broadly defined as intracellular collections of chemicals, or metabolites, which are inter-converted by complementary sets of enzymes. For at least two decades, metabolic engineers have sought to exert control over cellular pathways to perturb metabolism toward a defined objective, such as overproduction of a specified metabolite [1]. With growing economic and societal concern over dwindling resources, environmental issues, and sustainable practices, metabolic engineers have transformed microbes into cellular factories for conversion of renewable resources into an impressive suite of valuable chemicals, often to replace or supplement natural sources.

Overwhelmingly, simple static control strategies—permanent genetic modifications that impose gene expression constraints that are agnostic of variable intracellular conditions, like gene deletions and constitutive or inducible overexpressions—have been used to achieve these goals [2], but increasingly complex, dynamically controlled systems have gained prevalence in recent years [3,4]. In contrast to static control systems, dynamic pathway control requires the presence of a sensor that enables automatic, genetically encoded response to transient changes in the concentration of a metabolite or chemical cue. It is important to note that dynamic control strategies are not always preferential to static control strategies. In fact, static control will likely maintain an important biotechnological role for certain tasks, such as bioconversions that do not require co-option of limiting host cell metabolites to anabolize a product of interest. When engineered biosynthetic pathways involve metabolites whose accumulation causes toxicity, feedback inhibition, or decreased cell growth, however, tight dynamic control over production and consumption of these intermediate metabolites can prevent their accumulation, ameliorating such negative effects, driving increased flux through the pathway, and consequently enabling higher titer, yield, or productivity. Although conventional chemical induction of gene expression is sometimes described as dynamic control, we consider such one-time OFF–ON chemical induction by an external inducer (IPTG, arabinose, *etc.*) to be a static strategy in the context of this review; we instead define dynamic control as engineered genetically encoded systems that automatically and dynamically respond to the concentration of specified intracellular metabolites.

Many recent reviews have detailed traditional static control approaches [5]; thus, we begin by describing new genome engineering tools for deletion and integration of DNA (genes and pathways) that have been utilized to

facilitate static control. We then emphasize contemporary genetic regulators that have been engineered for static transcriptional and translational control. Components required for dynamic pathway control are discussed, and recent dynamic metabolic engineering examples are highlighted. We close by contrasting the roles that static and dynamic control will play in the future of metabolic engineering, and we highlight a class of highly tailorable regulators that can be adapted for dynamic control and that will likely be critical for engineering continuous automatic control of microbial metabolic pathways in the coming years.

Genome engineering tools facilitate static control of metabolic pathways

Aside from episomal expression of metabolic pathways [6], one of the most commonly employed mechanisms to engineer endogenous metabolism is alteration of the chromosome by sequential deletion and insertion of genes. While these strategies have proven effective in many cases, achieving multiple deletions or integrations in a single strain is cumbersome. Therefore modern strategies have aimed at reducing labor and time required to engineer microbes with multiple genome manipulations [7]. Multiplex Automated Genome Engineering (MAGE) and its derivatives MO-MAGE (Microarray oligonucleotide-MAGE) [8] and CoS-MAGE (Coselection MAGE) [9] allow directed or adaptive replacement of DNA at multiple loci with either defined or degenerate templates, resulting in strains with significantly altered phenotypes.

These tools can excel over rational, *a priori* strategies because of unanticipated factors that are difficult to account for at the design stage. MAGE has enabled enhanced lycopene biosynthesis in *E. coli* by evolving ribosome binding sites to tune translation of enzymes in the deoxyxylulose-5-phosphate pathway, while CoS-MAGE has improved indigo and indirubin production through replacement of promoters to alter transcription of aromatic amino acid biosynthetic pathways, both static strategies resulting in increased carbon flux toward a metabolite of interest. Genetic manipulations achieved by MAGE in distinct genomic loci of independently engineered strains can now be iteratively combined through transfer to a single strain using Conjugative Assembly Genome Engineering (CAGE) [10], a technique that was first demonstrated by genome-wide codon replacement achieved through collapse of 32 MAGE-modified *E. coli* genomes into a single genome using hierarchical conjugation [11].

A genome editing strategy rapidly increasing in prevalence is clustered regularly interspaced palindromic repeats (CRISPR)/Cas9-mediated gene deletion and integration, a technique that is accelerating the pace of genome engineering due to ease of use [12]. Cas9 is an RNA-guided endonuclease that binds a target sequence through Watson–Crick base-pairing of a 20 bp spacer sequence at the 5′ end of a small guide RNA (sgRNA), creating a double stranded break (DSB) that can be used to delete or introduce DNA. CRISPR/Cas9-mediated chromosomal engineering has enabled simultaneous deletion [13] of up to 5 genes from the genome of *Saccharomyces cerevisiae*, yielding 41-fold improvement in mevalonate production [14], and simultaneous gene integration and deletion in diverse industrial yeast strains for improved lactic acid production [15]. More recently, simultaneous Cas9-mediated integration of fifteen DNA parts into three distinct loci of the *S. cerevisiae* genome was demonstrated for carotenoid production, followed by integration of ten genetic parts in two distinct loci in *S. cerevisiae* for improved tyrosine production [16]. An important feature of Cas9 genome engineering in organisms like *S. cerevisiae* with highly efficient homologous recombination systems is that multiplexed interventions can be achieved without the use of selective markers.

Cas9-mediated chromosomal engineering is less efficient in organisms with low endogenous homology-directed repair (HDR) activity, presumably due to toxicity of unrepaired Cas9-induced DSBs; however, expression of λ Red machinery to increase recombination rate allows repair of Cas9-mediated DSBs and has enabled simultaneous deletion of up to 3 genes from the genome of *E. coli* [17,18]. Simultaneous combinatorial deletions have proven low efficiency so far in *E. coli*. Rather than suffer reduced editing efficiency by targeting multiple sites simultaneously, a CRISPR-Cas9 based iterative recombineering strategy recently enabled near 100% editing efficiency against a single target in each of multiple successive editing rounds, allowing assessment of up to 33 genomic modifications for dramatic improvement of β-carotene production [19]. Cas9-mediated gene deletions have also been demonstrated in other prokaryotes, such as four *Streptomyces* species [20,21], *Streptococcus pneumoniae* [18], and *Tatumella citrea* [17], exhibiting the tractability of Cas9 for genome editing and metabolic engineering in diverse chassis.

Genetic regulators and applications in static pathway control

Another common strategy for pathway control involves expression of transcriptional, translational, and post-translational regulators designed to tune expression of genes in a metabolic pathway or switch them between on and off states. When their expression is controlled by constitutive or externally inducible promoters, these regulators act in a static fashion, although it is theoretically possible to drive transcription of these regulators dynamically as discussed later. Significant recent progress has been made in harnessing natural regulatory systems to impose pathway control, primarily due to increased understanding of the natural systems and derivation of design principles.

In this section, we describe transcriptional and translational regulators in the context of static pathway control.

Transcriptional regulators

As the initial gatekeeper of gene expression, transcription regulation can be engineered to encompass an incredibly large dynamic range, specified as the ratio between maximum and minimum expression. Traditionally, transcriptional control has been achieved with DNA-binding proteins, or transcription factors (TFs), that bind a promoter region in DNA to occlude RNA polymerase. While natural TF-promoter pairs have been engineered to control gene expression at the transcriptional level, control of transcription at multiple loci in the chromosome is hampered by the necessity to insert a TF operator site at each location. An alternative to protein-based control of transcription is non-coding RNA (ncRNA)-mediated transcriptional regulation. Modeled on small RNA (sRNA) from natural bacterial systems, *cis*- and *trans*-acting sRNA regulators have been designed for transcriptional and translational control of a wide range of metabolic pathways [22–25] as reviewed in detail elsewhere [26]. More recently, small transcription activating RNAs (STARs) have been engineered to enable ncRNA-mediated gene activation (de-repression, in fact) in bacteria and *in vitro*, a new paradigm for ncRNA transcriptional regulation [27]; however, activation of host genes above endogenous levels has not been demonstrated with STARs and is likely not tenable. Despite increasing ability to design ncRNAs, synthetic metabolic pathway control is predominantly achieved with protein-based transcriptional repression.

Although genome engineering should not be discounted as a metabolic engineering strategy, externally imposed control of endogenous metabolism from a plasmid based circuit can limit design complexity and expedite circuit design. A clear solution for regulating transcription of any target site with an external controller is to use a synthetic transcription factor (sTF), or a protein that can be addressed to bind any user-defined DNA sequence and thereby repress any promoter or gene of interest (Figure 1). This strategy enables regulation of genomic transcription without alteration of the endogenous promoter sequence. Zinc fingers (ZFs) and transcription activator-like effectors (TALEs) are two sTF classes that have been successfully engineered to bind a wide range of sequences, enabling both transcription repression and activation [28–30]. However, each ZF and TALE protein is designed to target only a single sequence. Therefore, while the sequence space that can be explored with a library of these transcription factors is greater than the existing space from a defined pool of natural TFs, a single ZF or TALE protein suffers the same inability of a natural TF to precisely target disparate operator sequences. Furthermore, TALE and ZF design and construction can be somewhat unpredictable

and laborious, and simultaneous expression of multiple TALEs (>100 kDa) or ZFs (approximately 30–40 kDa) to target distinct sites could lead to increased metabolic burden or toxicity as seen when expressing multiple distinct natural TFs in the same cell [31,32].

The most attractive candidate for regulating transcription at multiple endogenous loci is an engineered CRISPR system. The prototypical CRISPR sTF is known as dCas9, or nuclease deficient Cas9, an RNA-guided DNA binding protein. Simultaneous dCas9-mediated repression of multiple genes with a CRISPR array or multiple sgRNA transcripts has been shown in many organisms, including *E. coli* [33**,34], *S. cerevisiae* [35,36**], and mammalian cells [37]. The major advantage of dCas9 is the capacity for a single protein to readily target multiple distinct sites, while the primary targeting constraint is the presence of a NGG trinucleotide known as the protospacer adjacent motif (PAM) at the 3′ end of the target sequence, a feature found ubiquitously in the genomes of most commonly engineered hosts. Further expanding the targeting sequence space of engineered CRISPR systems, other CRISPR systems composed of orthologs of Cas9 [38] with distinct PAM sequence requirements have been engineered for orthogonal transcriptional repression, and *S. pyogenes* Cas9 has recently been engineered for altered specificity toward non-natural PAMs [39*].

CRISPR interference (CRISPRi, also referred to as dCas9-mediated transcriptional repression) for metabolic engineering has only recently been reported. The first application of dCas9-mediated repression of endogenous targets for metabolic engineering demonstrated improved production of the phytochemical naringenin in *E. coli* through multiplex repression of genes competing for the limiting cofactor malonyl-CoA [33**]; this work illustrated dysregulation of the entire FadR regulon through *fadR* repression as a strategy to improve malonyl-CoA availability, and it also exemplified the ease of targeting essential genes for partial repression with dCas9, an advantage that dCas9-mediated repression holds over other gene deletion. In an earlier report, carbon flux was combinatorially diverted to distinct products of the exogenous violacein pathway in *S. cerevisiae*, illustrating the ability to control multiple nodes in a highly branched biosynthetic pathway by simultaneous transcriptional activation and repression using CRISPR RNA scaffolds, or an extended sgRNA encoding RNA structures that recruit RNA-binding modules fused to effector domains [36**]. Other research showed that dCas9 can be readily implemented for partial repression of synthetic lethal pairs, or sets of genes that support growth when deleted individually but cause lethality when deleted together, leading to improved 4-hydroxybutyrate production in *E. coli* [40]. Finally, a distinct Type I-E CRISPR system endogenous to *E. coli* K-12 was engineered to repress

Figure 1
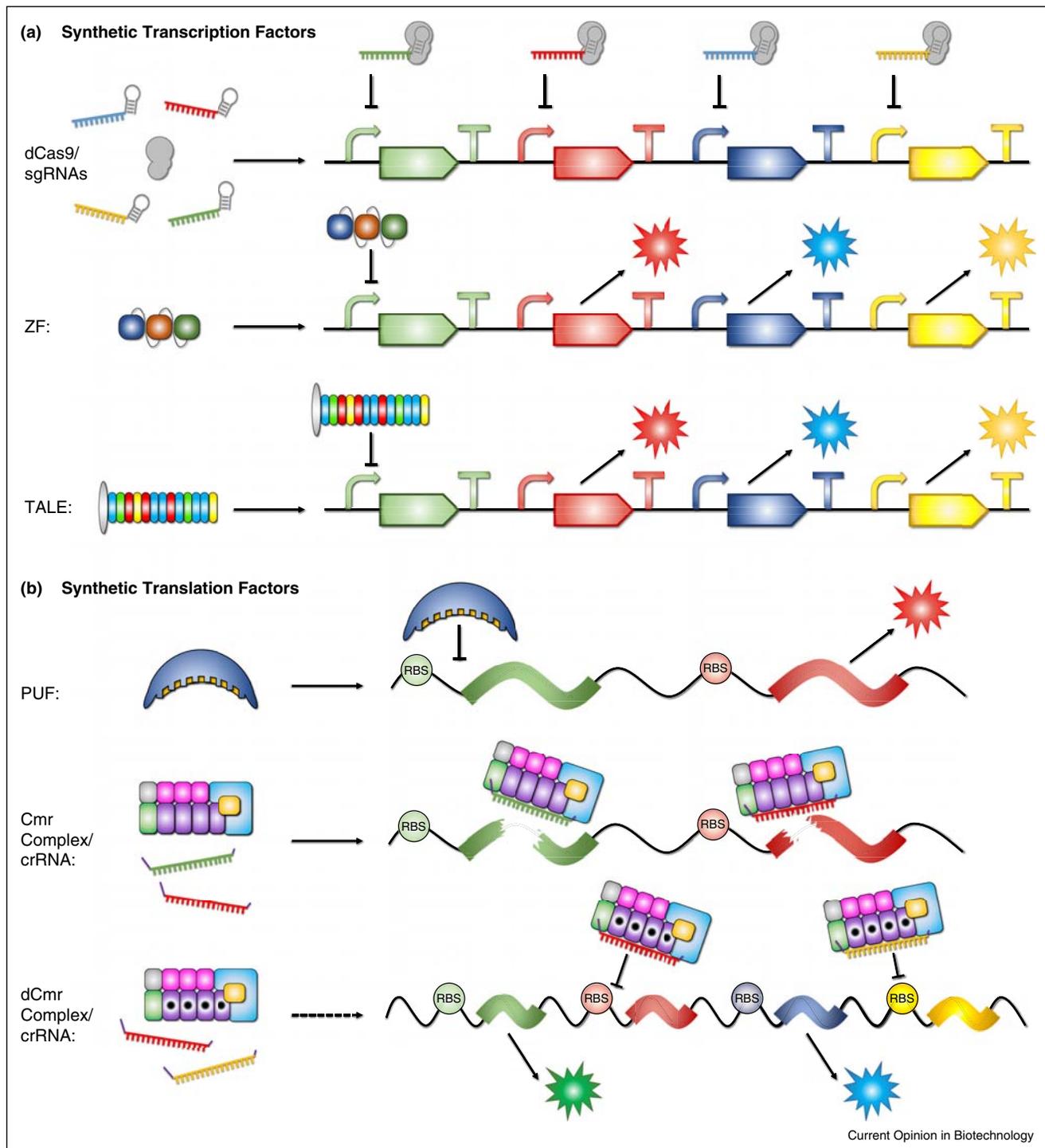
Advances in synthetic regulators of genetic transcription and translation. **(a)** Synthetic transcription factors are DNA binding proteins that modulate transcription by occluding RNA polymerase from the promoter or by roadblock repression if targeted downstream of a promoter. Zinc fingers (ZFs) and transcription activator-like effectors (TALEs) are designed to target a single site at a time, which limits their utility for regulating endogenous targets. dCas9 is capable of multiplex repression when guided to distinct promoters simultaneously by a pool of sgRNAs or processed CRISPR RNA (crRNA):*trans*-activating crRNA (tracrRNA) duplexes (see [33,69] for details on crRNA:tracrRNA). Each of these sTF classes has been shown amenable to fusion with effector domains to, for example, activate transcription at a downstream promoter in eukaryotic cells. **(b)** Synthetic translation factors modulate translation by occluding the ribosome from RBS or by degrading RNA. PUF domains have recently been shown to enable repression of an upstream gene in an operon without repressing the downstream gene and without degrading the mRNA transcript, but

transcriptional repression and was shown capable of suppressing catabolism of disparate sugars [41[•]], a strategy that could be utilized to control sequential order and rate of carbohydrate utilization from biomass hydrolysate containing mixed carbon sources. A notable disadvantage of engineered transcriptional repression is that the fates of all genes in an operon downstream of the target gene are coupled; that is, it is not possible to repress a gene in the middle of an operon without similarly repressing all downstream genes unless there is an intervening promoter to drive transcription posterior to the repressed gene (sometimes referred to as a pseudo-operon). However, as operons typically encode genes in related pathways, repressing an entire operon is often consistent with the metabolic engineering goal achieved by repressing the single gene target [33[••]].

Translational regulators

Translational and post-translational regulators will ultimately enable more rapid response to fluctuating metabolite concentrations. Lagging design principles have hampered their broad implementation compared to transcriptional regulators, but some key examples illustrate the potential of ncRNA-based regulators for dynamic metabolic pathway control. *Trans*-acting antisense RNAs have been engineered to repress translation of fatty acid biosynthetic enzymes in *E. coli* to enhance polyphenol production [23,25] and to repress translation of glycolytic enzymes and aromatic amino acid regulatory proteins for increased production of tyrosine and cadaverine [42]. Furthermore, riboregulators (cognate pairs of *trans*-activating and *cis*-repressing RNAs) have been engineered to control flux through distinct glucose-utilization pathways [24]. In order for these systems to be readily retrofitted for automatic control, more effort is required to develop a library of functional RNA biosensors (*e.g.* synthetic aptamers), which must be obtained by brute force screens or elegant *in vitro* [43] or *in vivo* selections to expand the candidate ligand pool to encompass critical metabolic intermediates. A rigorous assessment of design principles must also be undertaken to ensure modularity and retention of function when these biosensors are incorporated into synthetic riboswitches [44]. RNA-based regulators have typically suffered from low dynamic range, but a novel class of riboregulators called toehold switches have recently been engineered to control translation with low crosstalk and a dynamic range above 400, on par with the dynamic range achieved by robust protein-based regulators [45]. While toehold switches show great promise for synthetic gene circuit design due to low metabolic cost and rapid dynamics, they must be integrated into the

genome to control endogenous pathways and thus might suffer limited adoption by metabolic engineers.

To this end, a universal strategy for protein-based modulation of translation using the Pumilio/FBF (PUF) domain, an RNA-binding protein that can in principle be engineered to bind any 8-nucleotide RNA sequence based on theoretical design rules, has been demonstrated in *E. coli* by introducing a PUF binding sequence known as the Nanos response element (NRE) between the RBS and start codon to block access of the ribosome to translational regulatory elements [46[••]]. Importantly, this work demonstrated that coding sequences in a bacterial polycistronic mRNA transcript can be independently and specifically repressed by PUF domains, although the low dynamic range exhibited in this proof-of-principle limits its utility until repression can be further improved. The capacity to independently repress genes in the middle of an operon make PUF domains a critical tool for metabolic engineering when a target gene precedes an essential or objective-incompatible gene in an operon. PUF domains are analogous to the sTF proteins of the ZF and TALE class because they non-destructively bind a single cognate nucleic acid target based on engineered protein-nucleic acid contacts, and they are also amenable to fusion with effector domains for translation activation in eukaryotes, for example, as shown in a recent report in mammalian cells [47]. Despite the potential of PUF domains, they are fundamentally limited by their ability to bind only the single cognate target RNA sequence. We herein propose that PUF domains and other proteins capable of binding a user-defined RNA sequence to modulate translation be classified as synthetic translation factors (Figure 1).

An enticing but yet unexplored paradigm for protein-mediated translational regulation involves engineered Type III-B CRISPR systems. Coupling this ssRNA-shredding protein complex—which is guided by a ~40–45 bp crRNA to a complementary target site in ssRNA with no apparent target sequence or PAM constraint [48]—with dCas9-mediated transcriptional repression should effectively abrogate leaky transcription, a valuable multi-tiered regulatory strategy for pathway control that would require no prior genome editing. In eukaryotes, such selective ssRNA cleavage might enable polycistron processing and novel strategies for CRISPR-mediated control of riboswitches and riboregulators. Furthermore, it has recently been shown that mutation of a key conserved aspartic acid residue in Cmr4 of the Type III-B Cmr complexes abrogates RNAse activity but

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PUF domains are only capable of binding a single RNA sequence at a time; PUF domains have also been successfully fused to nuclease and effector domains. Although no application has been demonstrated to date, the Type III-B CRISPR Cmr complex has been shown to degrade target ssRNA, which could be useful for degrading mRNA from leaky transcription or for processing polycistronic, structural, or actuatable RNA in synthetic circuits. Furthermore, an aspartate to alanine mutation in Cmr4 (purple with black dot indicates catalytically inactive Cmr4) abrogates RNAse activity, and we thus speculate that the Cmr complex and dCmr (catalytically dead Cmr) will be applied for multiplex translation modulation in the near future (dashed arrow).

retains RNA binding capability and specificity [49]. This exciting discovery paves the way for non-destructively binding any desired ssRNA sequence, enabling selective, independent translational modulation of coding sequences at any position within a polycistron. As with other DNA- and ssRNA-binding proteins, it is possible that fusing a series of translation effectors [50] or RNA processing domains to a protein in the Cmr complex would enable multiplex translation modulation as encoded by a program of distinct Type III-B crRNA guides.

Automatic dynamic control of metabolic pathways

A common theme with the aforementioned strategies is the static nature of their design, where the control parameters are defined *a priori* and transient feedback control of the system is not enabled. Therefore set-points must be empirically adjusted by tuning design parameters and then assessing performance. The primary disadvantage associated with these types of systems is their inability to sense perturbations and adjust unfavorable metabolic states toward the designed goal. Automatic dynamic control of biosynthetic pathways thus offers a mechanism to react to stochasticity and correct unexpected metabolic imbalances or disturbances; such tight regulation is particularly valuable when a specified metabolite is toxic, inhibits a pathway of interest, or directly competes with an important cellular function that is critical for a designed engineering outcome.

Careful consideration must also be given to controller choice with respect to the time scale on which metabolite turnover occurs [3]. It is probable that engineered protein allostery [51,52] or enzyme degradation [53] (protein half-life tuning) would exert the tightest control over pathway flux. Although likely difficult to engineer, ligand-induced reconstitution of a split enzyme [54] could be used to directly control metabolic flux through upstream and downstream pathways. However, design principles are not sufficiently established for implementing on-demand allosteric control of user-specified enzymes or for independently tuning degradation rates of multiple enzymes simultaneously. Thus regulation at the RNA level, mediated by ligand-responsive RNA sensor-actuators (*e.g.* riboswitches), is desirable for dynamic pathway balancing particularly because it avoids uneconomical transcription steps through direct control of the translation rates of pathway enzymes. As with post-translational control, however, the small number of characterized extensible natural and engineered RNA sensor-actuators [55] limits utility of translational control of metabolic pathways. Although transcriptional control exhibits the slowest dynamics for control of metabolic fluxes, well-characterized transcriptional regulators with tailorable sensors (ligand-responsive domains) and actuators (DNA-binding domains) are abundant, making this class of regulator the most frequently implemented for dynamic pathway control to date.

Before describing examples of engineered dynamic control systems, it is critical to understand the three components which must exist to exert dynamic control over metabolic pathways:

1. a biosensor capable of sensing a measured variable, such as the concentration of an environmental factor or small molecule of interest;
2. a transducer element that physically conveys this signal to an actuator, such as allosteric conformational change or split protein association;
3. an actuator device that receives the transduced signal and adjusts some parameter in the system (a manipulated variable) in response, such as degradation of the measured variable or modulation of gene expression.

Natural ligand-responsive transcription factors (LRTFs) are prominent candidates for elements within engineered feedback controllers, because they have evolved as feedback regulators and thus possess the ability to fill all three roles. LRTFs sense ligand concentration through a metabolite binding domain (MBD), transduce the allosteric signal to the DNA binding domain (DBD) through a conformational change in the TF structure, and actuate control over transcription by then binding or dissociating from the cognate operator site to exert control over the nearby promoter [51]. Depending on the class of LRTF and the location of the operator sequence with respect to the regulated promoter, this actuation can either repress or activate transcription. Furthermore, LRTFs often exist as dual transcriptional regulators, repressing some genes while activating others. These features have made LRTFs attractive as integral components in the first wave of dynamic pathway controllers described below.

In the archetypal implementation of automatic pathway control in *E. coli*, rate-limiting genes were placed under control of a natural promoter that, in the presence of a molecular indicator of excess glucose flux, acetyl phosphate, drives flux toward lycopene [56]. This simple case demonstrated that intracellular metabolites can be used not only to describe the state of a system, but also to take advantage of that state to improve an engineering outcome. Another significant milestone in dynamic pathway control involved construction of a dynamic sensor-regulator system (DSRS) for improved biodiesel production in *E. coli* [57]. In this system, a biosensor based on a natural acyl-CoA LRTF known as FadR was constructed to dynamically regulate enzymes involved in the biodiesel fatty acid ethyl ester (FAEE) pathway. Specifically, hybrid promoters consisting of elements from phage promoters, a natural FadR-regulated promoter, and the lac

operator were engineered for increased dynamic range and tight transcription control in response to FadR binding in absence of acyl-CoAs. In the engineered system, expression of genes responsible for toxic intermediates was only de-repressed upon accumulation of fatty acids, thus driving FAEE production only when precursor availability was sufficient. This tight regulatory control yielded 3-fold improvement in biodiesel production over the unregulated strain. Furthermore, a strategy for natural LRTF mining and DSRS construction was demonstrated for a wide-range of metabolite classes.

In related work, a transcriptome-mining strategy was used to uncover stress-responsive *E. coli* promoters that are activated in the presence of toxic pathway intermediates farnesyl pyrophosphate and HMG-CoA [58]. Candidate promoters were used to drive transcription of the intermediate consumption pathway to rapidly convert toxic intermediates into product in concert with FPP- or HMG-CoA-associated toxicity. This transcriptomics approach exemplifies one way to circumvent our inability to easily engineer *de novo* biosensor-actuators for a given ligand. A similar approach was applied in *S. cerevisiae* to impose negative regulation over side-product (ergosterol) formation through repression of the first committed gene in the pathway, squalene synthase (*ERG9*), enabling up to 5-fold improvement in the heterologous amorpha-4,11-diene pathway [59].

A series of reports also demonstrated refactoring of the *Bacillus subtilis* malonyl-CoA responsive LRTF, FapR, to improve production of malonyl-CoA derived fatty acids. Malonyl-CoA is a highly regulated metabolite involved in critical cellular functions and required for production of many valuable secondary metabolites, and it is thus an important target for dynamic pathway control. In one report, a hybrid promoter was constructed by placing the *B. subtilis fapO* operator downstream of the P*T7lac* promoter, leading to transcription activation in the presence of malonyl-CoA [60]. In the other, a negative feedback regulatory circuit was constructed to prevent accumulation of excess malonyl-CoA, leading to improved fatty acid production [61]. Recently, FapR was also utilized in one of the first examples of an artificial dual-regulatory system engineered in *E. coli*, where upstream pathway genes are repressed and downstream pathway genes are activated in response to malonyl-CoA accumulation, ultimately improving fatty acid production by 3-fold compared to the unregulated control strain [62••]. Critically, none of these strategies have attempted to dynamically regulate competing side pathways, and we expect that on-demand sTFs will soon be incorporated into such circuits to control endogenous targets.

Conclusion and outlook

Common to all of the dynamic pathway controlling circuits described so far is the centrality of LRTFs as

feedback regulators of transcription. Other dynamic pathway control implementations using ON/OFF switching schemes and external cue-responsive controllers [63–65] are detailed in an excellent recent review [3]. Despite significant progress in this field, we expect that the next decade of dynamic pathway engineering will rely heavily on multiplexable, addressable sTFs like dCas9 to interface with endogenous metabolism. Furthermore, we anticipate that the common practice of mining [66] and harnessing natural LRTFs as sensor-actuators will gradually be supplanted by rational design [67] or mutagenesis [68] of LRTFs to recognize distinct and even non-natural ligands with high specificity. Attention should be paid to the effect of natural TF redesign on allosteric signaling from the MBD to DBD, as it is not yet clear how mutations in the MBD affect conformational changes in the DBD upon ligand binding [51]. If MBD specificity can be altered in a universal manner, however, natural TF regulation will still be constrained by DBD specificity toward a cognate operator site; thus, even if design principles are elucidated to allow DBD exchange without abrogating allostery, a single engineered LRTF will not attain widespread addressability. To this end, we propose an intermediate framework for transitioning to the next generation of automatic pathway control circuits, where natural LRTFs control expression of a synthetic TF, which then imposes control over a user-defined array of endogenous pathways (Figure 2).

Despite the increasing prevalence of dynamic control applications for metabolic engineering over the past decade, the limited number of reports compared to static control suggests a barrier to widespread adoption. Although the reasons for this trend are not entirely clear, we provide some potential explanations. First, the additional level of complexity in engineering dynamic control systems at the genetic level can lead to poor design predictability. Integration of computational tools into the design-build-test cycle for dynamic control circuits could improve outcomes and reduce design space, while combinatorial circuit design coupled with high-throughput screening or selection—a strategy that has not yet been applied for dynamic pathway control—might prove useful for exploring parameter space and tuning circuits for defined objectives. Furthermore, dynamic strategies are likely most suitable for controlling metabolites that are toxic or highly sought-after for critical cellular processes; therefore, dynamic control of other metabolite classes might not yield production improvement over traditional static control strategies, and more work is necessary to predict when one control strategy maintains an advantage over the other. Finally, the toolbox of characterized biosensor-promoter pairs is inadequately populated, and additional biosensor mining, *de novo* design, and characterization efforts will be required to effectively monitor and respond to the intracellular concentration of metabolites in diverse biosynthetic

Figure 2

Current Opinion in Biotechnology
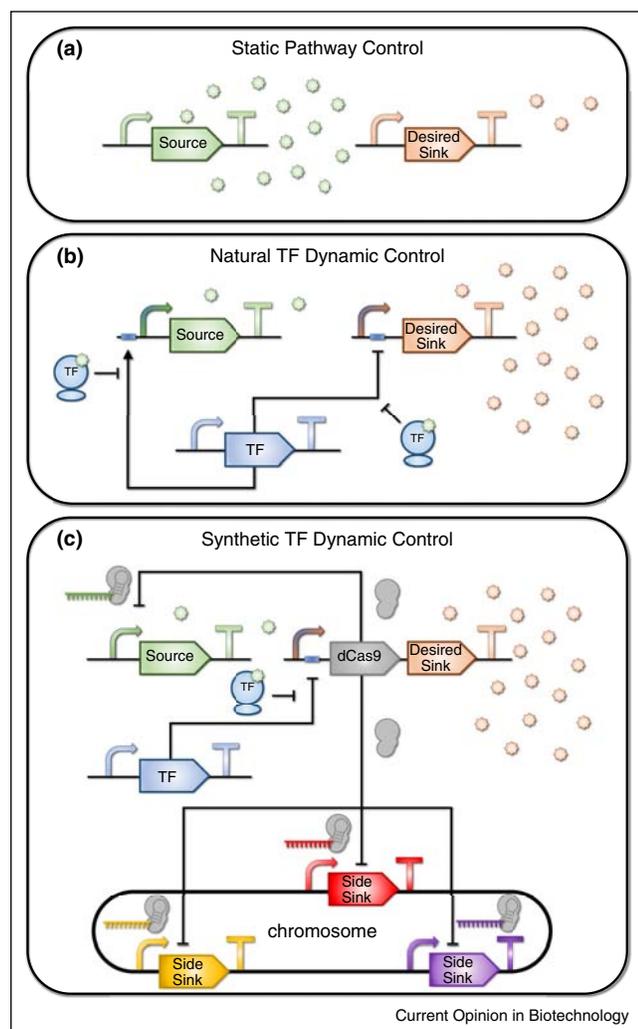
Static and dynamic control of metabolic pathways. **(a)** When pathways are controlled in a static manner, buildup of toxic or inhibitory intermediates or metabolites that draw carbon away from important cellular processes can lower productivity. **(b)** Implementation of automatic dynamic transcriptional regulation of the source and sink pathway genes for a toxic or high-demand metabolite; a natural transcription factor is used as a sensor-actuator that responds to change in concentration of the metabolite of interest, leading to improved productivity. **(c)** A hypothetical automatic dynamic control circuit based on two transcription factors. The first is a natural ligand-responsive transcription factor biosensor that de-represses transcription of dCas9 and the desired sink pathway upon accumulation of the cognate intermediate metabolite. dCas9 simultaneously represses the source pathway to prevent further accumulation, but it can also readily be targeted to endogenous pathways to dynamically tune expression as desired.

pathways. As work in these areas rapidly advances, the ability to construct sensitive cells will undoubtedly transform the interface of synthetic biology and metabolic engineering while further driving our understanding of natural metabolite control systems.

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