Dynamic Control of Metabolism

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Abstract

Metabolic engineering reprograms cells to synthesize value-added products. In doing so, endogenous genes are altered and heterologous genes can be introduced to achieve the necessary enzymatic reactions. Dynamic regulation of metabolic flux is a powerful control scheme to alleviate and overcome the competing cellular objectives that arise from the introduction of these production pathways. This review explores dynamic regulation strategies that have demonstrated significant production benefits by targeting the metabolic node corresponding to a specific challenge. We summarize the stimulus-responsive control circuits employed in these strategies that determine the criterion for actuating a dynamic response and then examine the points of control that couple the stimulus-responsive circuit to a shift in metabolic flux.

INTRODUCTION

Metabolic engineering reprograms microbial cells to convert renewable or inexpensive feedstocks to value-added products, including compounds from pharmaceuticals to biofuels. These microbial synthesis processes take advantage of cellular machinery to express endogenous and heterologous genes encoding enzymes that carry out chemical conversions to produce desired products. Enzymatic reactions result in a highly specific product pool, simplifying downstream separation procedures, and fermentation processes occur under environmentally friendly conditions. However, cost efficiency relies on achieving high yield, titer, and productivity criteria, which has proven to be difficult for many products for a variety of reasons. In this review, we focus on the subset of those challenges that can be addressed through dynamic

regulation of metabolic fluxes.

Dynamic metabolic flux regulation is one potential method of balancing competing cellular objectives that are beneficial to achieving high titer, yield, and productivity. For example, conditions that achieve high reaction rates on a per-cell basis may result in burdened growth and, thus, low productivity. This conflict can result from diversion of cellular resources to production pathway–related processes in which the limiting resources could be general, such as ribosomes, or pathway specific, such as metabolites involved in both endogenous and production pathways. Additionally, burdened growth can result from toxicity of production pathway metabolites at high concentrations.

Microbial cells naturally face similar trade-offs and manage them by dynamically regulating gene expression. In low-nutrient conditions, survival relies on expression of metabolic pathways that do not benefit fitness in high-nutrient conditions in which essential metabolites can be scavenged from the environment. Cells employ dynamic control approaches to address this and similar situations. Zaslaver et al. (1) analyzed dynamic transcription trends in amino acid biosynthesis systems in *Escherichia coli* to show that the presence of amino acids in the media leads to decreased transcription of the corresponding pathway genes. They saw that there is temporal control within amino acid biosynthesis pathways such that transcription of upstream genes is upregulated before that of downstream ones.

The observation of dynamic regulation in natural systems raises the question of whether synthetic regulatory systems could be utilized to advance the goals of metabolic engineering (i.e., to increase titer, yield, and/or productivity) in recombinant organisms. Several computational studies exploring the impact of dynamic regulation in production systems suggest that the optimal dynamic regulation scheme at the appropriate metabolic node can improve production over static control at that point. Gadkar et al. (3) conducted two production case studies *in silico* that are each subject to a trade-off between high growth rate and high production pathway flux. In the first example, glycerol production relies on diversion of metabolic fluxes from glycolysis to the production pathway. The second example considers that an *ackA* knockout improves ethanol production at the expense of ATP generation, resulting in a growth defect. Their bi-level optimization algorithm predicts that dynamic control at the relevant metabolic node to switch from growth to production phases increases the final glycerol and ethanol production by more than 30% and 40%, respectively. Anesiadis et al. (4, 5) incorporated the circuit dynamics of a

toggle switch into their model to show that production improvements can be achieved with a more gradual transition from growth to production phases.

Based on the predicted production improvements and studies of natural microbial systems, researchers have constructed and implemented regulatory circuits to dynamically control metabolic fluxes in experimental studies. In this review, we compare and contrast dynamic control methods and summarize the experimental studies that have demonstrated significant production benefits through implementing dynamic control. A set of metabolic challenges are best addressed by dynamically regulating metabolic flux (**Figure 1*a***). Once the metabolic node associated with the challenge has been identified, a dynamic control scheme can be designed from the following considerations: (*a*) What stimulus will trigger the change in metabolic flux (**Figure 1*b***), and (*b*) what point of control will couple the stimulus response to a shift in metabolic flux (**Figure 1*c***)? This review provides an overview of challenges that are well suited to a dynamic metabolic flux regulation intervention and then explores the options for the two questions above; we summarize the types of control circuits, categorized by stimulus type, before examining the points of control by which metabolic fluxes can be altered.

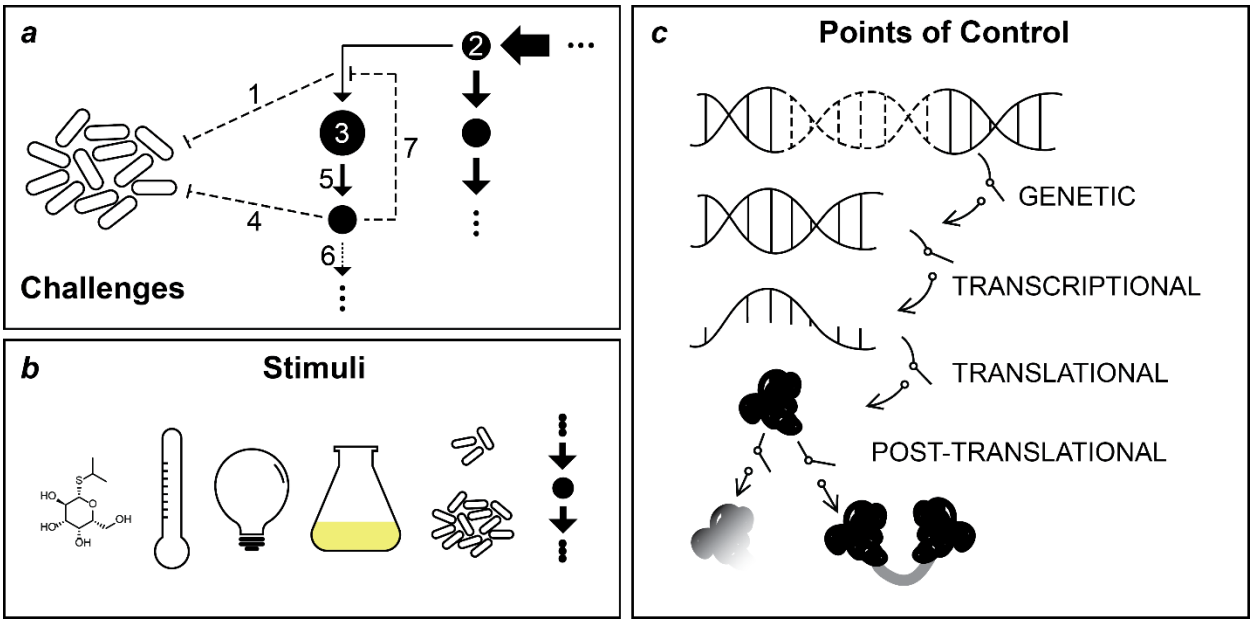

Figure 1 Overview of dynamic regulation design components. (*a*) Metabolic engineering challenges that are best addressed by dynamic regulation of metabolic flux. The numbers

correspond to the challenges listed above. (*b*) Stimuli that can trigger a shift in metabolic flux include chemical inducers, temperature, light, environmental factors within a fermentation, cell density or physiology, and metabolites. (*c*) The points of control where stimulus response is coupled to a shift in metabolic flux are at the genetic, transcriptional, translational, or posttranslational levels.

CHALLENGES ADDRESSED BY DYNAMIC REGULATION OF METABOLIC FLUX

The temporal nature of some challenges requires dynamic responses to alleviate and overcome them. Oftentimes, static disruption of certain metabolic fluxes that benefit production would be detrimental to the host cell. In other instances, the specific nature of a pathway intermediate or enzyme requires delaying its utilization or generation. Each application of dynamic metabolic flux regulation presented herein overcame specific metabolic challenges, the quantified outcomes of which are summarized in **Table 1** (see **Supplemental Table 1** for a comprehensive list). The broad categories of those challenges, as well as some broader applications of dynamic regulation, are listed below (**Figure 1*a***):

1. Biomass accumulation before production
2. Branch point metabolites
3. Accumulation of intermediate metabolites
4. Toxic intermediates
5. Balancing fluxes
6. Instability of downstream enzymes
7. Product inhibition of upstream enzymes
8. Population heterogeneity in large-scale vessels

For each of these challenges, and in each specific application, there are one or more candidate metabolic nodes at which a dynamic intervention strategy can be implemented to bestow production benefits. The sections below detail the stimuli that can induce a change in metabolic flux and the points of control that can act on the metabolic node to attain said benefits.

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CONTROL CIRCUIT STIMULI

Dynamic control circuits actuate changes in metabolic fluxes by regulating enzyme levels in

response to a change in some relevant condition or stimulus. Selection of the control circuit determines several key characteristics, such as the switching dynamics, tunability, and exogenous control requirements. In the following sections, we detail control circuits that respond to externally controlled factors, changing environmental factors, growth-phase transitions, and changing metabolite profiles, highlighting unique properties, key studies, and recent developments (**Figure 1*b***).

Externally Controlled Factors

The earliest dynamic control circuits responded to chemical inducers that are exogenously added to the culture media. Common chemical inducers include isopropyl β-D-1-thiogalactopyranoside (IPTG) and anhydrotetracycline (aTc). Each of these systems contains three main components: (*a*) a protein repressor that binds to a specific DNA sequence, (*b*) an inducible promoter that contains the DNA sequence that binds to the repressor, and (*c*) the chemical inducer that binds to the protein repressor. Upon binding, the chemical inducer causes a conformational change in the repressor protein to prevent DNA binding, allowing transcription from the inducible promoter (6–13). These and similar circuits have been applied to controlling gene expression in several contexts and organisms.

The chemically inducible regulation systems, along with many others, are derived from natural regulation systems, which may not exhibit desired induction curve characteristics. To address these limitations, groups have mutated or evolved circuit components to increase the dynamic range of the circuit and improve the specificity of the response to the desired chemical inducer (14, 15). For example, Meyer et al. (15) developed a generalizable dual-selection directed evolution scheme to identify regulator proteins and inducible promoters with improved characteristics, such as a larger dynamic range and lower half-induction concentrations. Implementation of these evolved parts can result in greater tunability, leading to more precise control of metabolic fluxes and production gains, or lower inducer concentrations, reducing inducer costs. Another approach to improving the response of chemically inducible control circuits is to assemble two circuits in a toggle switch architecture in which genes encoding regulator proteins mutually inhibit their corresponding target promoters (16). Under this arrangement, induction of genes controlled under the regulated promoters is bistable such that gene expression can be stably activated by a transient chemical inducer. Additionally, these circuits display a more switch-like response to inducer addition rather than a graded response of

an individual control circuit (16–18).

Although chemically inducible systems have been instrumental tools for experimentally demonstrating the potential benefit of dynamic control, this approach is not generally industrially feasible, as the chemical inducers can add significantly to material costs, potentially rendering a process cost prohibitive. In one analysis, IPTG was by far the most expensive component of a defined medium, accounting for more than half of the materials cost (18a). This limitation has motivated the development of circuits that respond to other extracellular factors, such as light and temperature. Temperature-dependent dynamic control systems build on the discovery of the temperature-sensitive mutant of the cI repressor, cI1857 (19), which represses expression from the lambda p_R and p_L promoters only at temperatures below 30°C. This circuit has been applied to dynamically repress gene expression by placing the target gene under control of a lambda promoter and typically shifting the temperature of the fermentation from 37–42°C to 28–30°C (20–22).

Synthetic light-responsive systems take advantage of proteins and DNA sequences found in naturally occurring systems that display light-inducible behavior. One such example is the EL222 regulator from *Erythrobacter litoralis*, which contains a light-oxygen-voltage (LOV) domain linked to a helix-turn-helix (HTH) DNA-binding domain. When stimulated with energy from blue-light photons, the formation of a covalent bond in the LOV domain results in a conformational change that enables DNA-binding activity (23). Zhao et al. (24) applied this gene expression regulation system in *Saccharomyces cerevisiae* by constructing fusion proteins containing the LOV and HTH domains for EL222 linked to an activator or a repressor domain. When the fusion contains an activator, stimulation with blue light results in localization of the activator domain to the promoter to upregulate transcription, whereas the repressor-containing system displays the opposite response.

In addition to regulating gene expression through controlling light-responsive DNA binding, groups have built regulatory circuits that apply other light-responsive mechanisms. The pDawn and pDusk systems control gene expression by using a light-responsive kinase, YF1, that phosphorylates a regulator, FixJ, to enable its activator behavior (25). YFI is a fusion kinase constructed by replacing the chemosensor domain of a light-inert histidine kinase with a photosensor domain and exhibits a ~1,000-fold increase in kinase activity with light activation (26). Another class of light-responsive systems that have been applied to regulating gene

expression are ones that employ light-responsive protein–protein binding properties. The Vivid protein of *Neurospora crassa* transitions from its monomeric to its homodimeric form upon activation with blue light (27). With the goal of engineering the Vivid system to improve its applicability to controlling molecular processes, Kawano et al. (28) modified Vivid pairs such that members recognize each other based on electrostatic interactions. This system can then be applied to controlling gene expression by tethering halves of a split protein to the Magnet domains. Baumschlager et al. (29) constructed a pair of fusion proteins that each contain a Magnet domain linked to complementary halves of T7 RNA polymerase (RNAP) such that light activation results in dimerization of the complete T7 RNAP. Sheets et al. (30) tethered the Vivid and Magnet domains to two portions of split Cre recombinase such that dimerization results in recombinase-mediated removal of a terminator block and expression of a target gene.

In addition to being a potentially more cost-effective option compared with chemical induction, light-based induction systems are also attractive for the ease by which the stimulus can be reversed. Zhao et al. (24) took advantage of this feature to switch between growth and production phases, following the initial switch to production phase, and found that periodic switches back to growth phase were key to achieving significant production improvements. Despite these advantages, one major critique of optogenetic regulation is that implementation at large scales may not be feasible owing to concerns about uniform light exposure. For this reason, many of the light-responsive gene control systems have been inverted, such that the gene of interest is expressed in the dark (24, 25, 31). With this regulation scheme, expression can be repressed with light exposure at relatively small seed-culture scales.

Environmental Factors

The remaining sections focus on autonomous control systems that employ cells programmed to self-actuate the desired dynamic switch when some criterion or set of criteria is reached. These regulation schemes benefit from mitigating the requirement for human supervision, eliminating chemical inducer cost, and addressing the challenge of a response criterion that is difficult or costly to measure in real time.

Conditions that trigger a response can be environmental factors that generally change during a fermentation, such as oxygen, pH, and nutrient levels. Many studies that regulate expression based on these factors take advantage of the natural response circuitry present in their host strain. For example, the *DAN1* gene of *S. cerevisiae* has been found to be expressed in anaerobic

conditions. By appending a target gene to the *DAN1* promoter, Nevoigt et al. (32) dynamically controlled expression based on the dissolved oxygen level of the culture. Similar applications have been investigated using pH-, glucose-, and phosphate-responsive promoters (17, 33–36). Although it is possible to improve the responses of these systems, studies thus far have only investigated modifications of the responsive promoter, possibly owing to the global implications of modifying regulatory components or owing to the complexity of the underlying regulatory system. Nevoigt et al. (32) performed directed evolution on the *DAN1* promoter to identify a mutant that activates transcription in microaerobic conditions, making its utilization more industrially feasible, and Moreb et al. (37) obtained different response curves and media-dependent characteristics by using different phosphate-responsive promoters in *E. coli*.

In cases where the regulation mechanism is well understood, groups can realize a greater level of control by importing a heterologous circuit or by integrating multiple circuits. Immethun et al. (38) used the fumarate and nitrate reduction (FNR) system from *E. coli* to achieve oxygen-dependent control in *Synechocystis* sp. PCC6803, a cyanobacterium that lacks a homologous circuit. The FNR system is composed of an FNR protein that dimerizes in the absence of oxygen to activate transcription of genes necessary for anaerobic growth and repressing those for aerobic growth (39). In the context of *Synechocytis*, the authors could then modulate the dynamic range of the oxygen-dependent response of the FNR-activated promoter by varying the expression level of the gene encoding the FNR protein (38). Although the environmental parameters driving these dynamic responses typically monotonically increase or decrease over the course of a fermentation, Moser et al. (40) showed that circuits that individually respond to independent environmental parameters can be combined in logic gates to achieve more complex response dynamics. Such a control scheme could be advantageous in addressing challenges that display multiple distinct shifts over the course of a fermentation.

Autoinduction

Autoinducible circuits dynamically control gene expression by responding to changes in an organism's physiological state. For example, some circuits up- or downregulate gene expression when the culture reaches a critical cell density, whereas others trigger expression changes as the cells begin to transition to stationary phase. Similar to regulatory systems that respond to common environmental factors, autoinducible systems are attractive because they are autonomous and have shown early promise for generalizability across different pathway contexts

(41–43). Here, we divide autoinducible circuits into circuits that respond during growth phase and those that respond during the transition to stationary phase.

Many regulatory systems that respond during growth phase employ quorum-sensing (QS) circuits. QS is a natural bacterial mechanism for controlling gene expression in a cell density–dependent manner. These systems are similar to chemically inducible ones in that they are composed of a regulator protein that changes conformation when bound to a small molecule and one of the conformations enables DNA-binding activity. The key difference is that the small, or signaling, molecule is produced via a pathway within the cells such that they are self-induced (44–46). Additionally, whereas the chemically inducible circuits mentioned previously all employ a regulator protein that acts as a repressor in the absence of the inducer molecule, some QS circuits contain regulator proteins with different roles. The most widely studied category of QS circuits are *lux*-type systems, which contain a regulator protein that activates transcription from its cognate promoter when bound to its signaling molecule (47, 48). Examples of *lux*-type QS systems are the *lux* system from *Vibrio fischeri* (44, 47–49) and the *las* and *rhl* systems from *Pseudomonas aeruginosa* (45, 46, 50), each of which use a unique acyl-homoserine lactone (AHL) compound as the signaling molecule.

To apply AHL-based QS circuits to dynamically control gene expression in *E. coli*, genes encoding the regulator protein and AHL synthase, responsible for producing the AHL, are expressed under constitutive promoters, such that the signaling molecule concentration increases with cell density. Expression of the gene of interest is dynamically controlled by placing the gene of interest under the control of the cognate QS promoter (41, 51, 52). Rather than importing a heterologous QS circuit, groups have alternatively rewired the native *E. coli* QS system that employs a repressor, LsrR, that is released from the *lsrR* promoter when bound to its cognate signaling molecule. This QS system includes many other proteins involved in the synthesis, transport, and modification of the signaling molecule (53). Similar approaches have been executed in *S. cerevisiae*—both importing a heterologous QS circuit (54) and rewiring a native circuit (55).

In some contexts, the cell density that corresponds to the threshold concentration of the signaling molecule is an important parameter that must be finely tuned to achieve production improvements. To modulate the switching time, Gupta et al. (41) constructed an AHL synthase expression level ladder. Here, stronger promoter-ribosome binding site (RBS) variants result in

strains that achieved the threshold AHL concentration at lower cell densities. Dinh & Prather (42) showed that the switching cell density of *lux*-type QS systems can also be controlled by varying the expression level of the gene encoding the regulator protein, and Soma & Hanai (51) demonstrated control of the switching time when the AHL synthase and regulator protein expression levels are varied in a coupled manner. As with chemically inducible circuits, the regulator-signaling molecule binding affinity can be altered by evolving the regulator protein. In this context, improved affinity corresponds to a lower threshold concentration of the signaling molecule and, thus, switching at lower cell densities (14, 56).

Another category of growth phase–induced systems couples gene expression to cellular growth rate by using native promoters known to be active during log-phase growth. For example, because ribosomes are essential components to protein synthesis, the number of ribosomes per cell is proportional to the cellular growth rate (57). Characterization of the expression of the genes encoding ribosomal RNAs and ribosomal proteins has revealed that their promoters are repressed under unfavorable nutrient conditions (58) and that individual promoters display varying dynamics (59). Hou et al. (43) applied several of these promoters to regulating expression of either degradation-tagged target genes or repressors. Regulation of the target gene directly results in downregulation as the growth rate decreases, whereas regulation of a repressor results in upregulation of the gene under the control of the repressor's promoter pair.

Other autoinducible systems that respond to suboptimal growth conditions employ stress-response or stationary-phase promoters. Global analyses of the relative number of transcripts at different points in the growth curve of *E. coli* cultures have yielded hundreds of promoters that upregulate gene expression during the stationary phase (60–62), and several have been individually characterized in the context of controlling expression of heterologous genes (63). Notably, RpoS is a master regulator for general stress in *E. coli*, and its promoter has been used to regulate expression of pathway genes to achieve production improvements (64, 65). Rather than identifying native promoters, Miksch et al. (66) obtained a library of synthetic stationary-phase promoters by screening a library of random promoter variants with degenerate −37 to −14 sequences. Their search yielded a set of 33 promoters that upregulate transcription at varying points between the late-exponential and early-stationary phases. Instead of using general stationary-phase or stress-response promoters, groups have instead carried out global transcriptional analyses under specific burdensome conditions that may be more relevant to the

desired application (67, 68). For example, to address a growth defect owing to accumulation of farnesyl pyrophosphate (FPP), the final intermediate of their production pathway, Dahl et al. (67) identified transcripts that were upregulated when the final enzyme was omitted from the pathway. This analysis yielded a set of promoters that could be used to dynamically control the expression of the gene encoding the FPP-consuming enzyme, alleviating the growth burden.

Metabolites

A metabolite-responsive regulation scheme is the most direct method for addressing the limitations of some metabolic pathways. For example, when aiming to minimize accumulation of a production pathway intermediate, the concentration of that intermediate, which might fluctuate over the course of the fermentation, is the most relevant indicator for how the regulation system should behave at that particular time. In this section, we summarize previous work on biosensors that detect the relevant endogenous or pathway metabolites, focusing on the studies that demonstrated application to metabolic flux control.

When a production pathway shares a common precursor with an essential endogenous pathway, it is important to maintain a balance between limiting flux through the production pathway to maintain a sustainable pool of the precursor and maximizing production. Farmer & Liao (69) addressed this challenge in the lycopene production pathway that consumes glycolytic intermediates glyceraldehyde 3-phosphate and pyruvate by developing a strategy to sense excess glycolytic flux and trigger upregulation of the production pathway. They controlled their production pathway under the Ntr regulation system native to *E. coli*, which contains the g*lnAp2* promoter that is upregulated under conditions of high acetyl phosphate levels, indicative of excess glycolytic flux. Rather than using native regulatory components from *E. coli*, Xu et al. (70, 71) imported a malonyl-CoA-responsive control circuit from *Bacillus subtilis* that contains the FapR regulator protein that exhibits DNA-binding behavior in the absence of malonyl-CoA. Additionally, they constructed two hybrid promoter variants that display opposing responses to malonyl-CoA (i.e., one ON-to-OFF and one OFF-to-ON). With these two promoters, they were able to both turn ON malonyl-CoA production genes and turn OFF fatty acid production pathway genes in low malonyl-CoA conditions and achieve the opposite responses in high malonyl-CoA conditions.

Some production pathways are subject to a challenge that is better addressed by sensing a pathway metabolite. Zhang et al. (72) developed a strategy to regulate expression of fatty acid

ethyl ester (FAEE) pathway genes based on the level of the key intermediate, acyl-CoA. To achieve this response, they used the acyl-CoA regulator FadR that binds to the FadR recognition DNA sequence in the absence of acyl-CoA and hybrid promoters that contain at least one recognition sequence in the core region. This system was used to repress expression of the ethanol biosynthesis branch of the FAEE pathway under low acyl-CoA conditions, which would otherwise be subject to toxicity from ethanol accumulation. Doong et al. (73) addressed the bottleneck caused by an unstable enzyme, MIOX, in the glucaric acid pathway by developing a biosensor that upregulates gene expression in the presence of the substrate of MIOX, *myo*-inositol. Their biosensor was imported from *Corynebacterium glutamicum* and contains a regulator protein, IpsA, which represses expression from an engineered promoter until the level of *myo*-inositol reaches a critical threshold. By controlling expression of MIOX under this promoter, the authors could synchronize periods of high *myo*-inositol and MIOX levels, resulting in production improvements. Additionally, the authors demonstrated that the switching dynamics of their biosensor can be tuned by varying the expression level of IpsA.

Although metabolite-responsive biosensors have proven to be effective dynamic regulators leading to improved production, the studies previously mentioned have used naturally evolved biosensors that may not be available for other target metabolites. In the case that a biosensor exists for a structurally similar molecule, it is feasible to evolve the biosensor components to respond to a new target molecule. Alternatively, groups have taken a modular approach to engineering novel metabolite-responsive biosensors by constructing fusion regulators composed of DNA- and ligand-binding domains taken from different proteins. Louvoin et al. (74) rewired the *S. cerevisiae* GAL4 transcriptional activator to respond to estrogen instead of galactose by replacing the native ligand-binding domain with the estrogen receptor hormone-binding domain and an activator domain. In the presence of estrogen, the hormone-binding domain dimerizes, resulting in GAL4 dimerization, DNA binding, and localization of the activator domain to the promoter region. Since then, several others have constructed fusions consisting of a DNA-binding domain and a ligand-binding domain to create a novel repressor, and additionally an activator to create a novel activator (75–77).

Others have developed novel metabolite-responsive biosensors by identifying RNA aptamers that bind to the metabolite of interest. By coupling aptamer domains with a sequence responsible for regulating expression, control responses based on ligands with no previously known binder

may be realized. RNA aptamers that bind to a molecule of interest are commonly identified through SELEX (systematic evolution of ligands by exponential enrichment), an approach that enriches for RNA or DNA with improved binding properties from a pool of sequences containing a variable region by repeated cycles of ligand binding and amplification of bound sequences (78). Riboswitches combine the aptamer sequence with an antisense domain that can repress expression of a target gene when the RNA molecule is in the ligand-bound state. To achieve this response, the RNA molecule is designed such that the antisense domain slightly favors a double-stranded state in the absence of the ligand, and a binding event in the aptamer domain renders the antisense domain accessible to downregulate expression (79). Ribozymes similarly combine a ligand-binding aptamer domain with a ribozyme domain that can be engineered to display activity only when the ligand is present or absent. Rather than acting on a separate target gene transcript like riboswitches, ribozymes are incorporated onto the target gene transcript. For example, Win et al. (80) appended the ribozyme sequence to the 3′ untranslated region of their target gene such that an active ribozyme domain results in cleavage of the poly A tail and, thus, gene silencing.

POINT OF CONTROL

The stimulus used to trigger a change in metabolic flux can interface with myriad devices at various levels of control: genetic, transcriptional, translational, and posttranslational. These devices all have the ability to sense the stimulus, either directly or indirectly, and actuate a cellular response. Each level of control has its strengths over the others. Whereas transcriptional and translational control offer the most flexibility and ease of tuning, the response timescale is quickest through posttranslational interventions. Transcription and translation each occur on the order of minutes, whereas it takes microseconds to activate or inactivate a protein (81). The slowest process is DNA recombination for genetic-level control, which occurs on the order of hours (82). The tools available for each level of control and their applications are detailed below (**Figure 1*c***).

Genetic Control

One way to control metabolic activity dynamically is to remove or introduce genes into an organism while it grows. Yamanishi & Matsuyama (83) used a galactose-induced Cre-*lox*

genetic switch to delete the floxed, endogenous gene encoding pyruvate decarboxylase and replaced it with the heterologous gene encoding lactate dehydrogenase (LDH) in *S. cerevisiae*. The recombination switched the end metabolite of pyruvate from ethanol to lactate, achieving maximum LDH activity within 6 h of induction, with significant activity increase within 3 h. The authors noted the necessity of quickly removing Cre after the recombination event, which they achieved by appending a short mRNA half-life terminator and pulsing the galactose inducer.

Transcriptional Control

Controlling the timing and strength of transcription, through the interactions of promoters, RNAPs, and transcription factors (TFs), is the most well-studied (84–87) and commonly used dynamic regulation strategy. Synthetic promoter libraries have been developed and well characterized and are readily available engineering tools (88–90). Hybrid synthetic promoters that are controlled by a TF can be engineered easily owing to the modularity of promoters and TF binding sites (91, 92). There is also a wide array of characterized metabolite-responsive TFs (MRTFs) that can be used to control expression from these promoters, and over 200 more reported in *E. coli* by various groups (87, 93), which have yet to be further tested. Synthetic MRTFs have been engineered via mutagenesis to respond to new inducers (94) and via fusing the ligand-binding domain of one to the DNA-binding domain of another (95) or to zinc finger DNA-binding motifs (76). RNAP-promoter systems from phage confer high transcription rates and function orthogonally from bacterial hosts (96).

Early applications of dynamic transcriptional control employed MRTFs to modulate gene expression in response to pathway intermediates. Zhang et al. (72) developed FadR repressor regulated synthetic promoters, using phage lambda promoter P_L and phage T7 promoter P_{A1}, containing the FadR binding site, which had 10- and 25-fold fluorescence induction in response to endogenously produced fatty acids. The promoters were employed to control expression from two out of three modules containing downstream enzymes for FAEE production in response to the accumulation of the fatty acyl-CoA intermediate. Xu et al. (71) used the malonyl-CoA-responsive FapR TF from *B. subtilis* to downregulate the native *E. coli* promoter, pGAP, expressing malonyl-CoA production enzymes and upregulate T7 with fapO, expressing the malonyl-CoA consumption pathway to make fatty acid products. The promoters exhibited 20–100% activity across the malonyl-CoA range tested. Zhou et al. (97) used temperature as the stimulus to initiate transcription by replacing the chromosomal promoter of *ldhA* in its lactate

production strain with the lambda P_R and P_L promoters. Following cell growth at 30°C, the lambda repressor was denatured at 42°C, resulting in twofold-higher LDH activity and dynamic lactate production.

Feed flux responsive dynamic transcription, using the cognate MRTF and promoter, has been employed as a proxy for cell growth, to switch to production mode from an initial growth phase. Farmer & Liao (69) repurposed the endogenous Ntr two-component regulon and its controlled *glnAp2* promoter from *E. coli* to activate pathway expression in response to acetyl phosphate, serving as a proxy for glycolytic flux. The regulon natively adapts cells to nitrogen deficiency but was previously reported to respond to acetyl phosphate when the sensor gene is knocked out. Glucose consumption was later sensed more directly with the tandem *TaraF* promoter, a CRP promoter, to express the polyhydroxybutyrate pathway. Autonomous expression from *TaraF* took up to 8 h after glucose induction (17). Soma et al. (98) interrupted the TCA cycle when switching to production mode by using IPTG to induce expression of the TetR repressor, which acted on the $P_L tetO_1$ expressing citrate synthase *gltA*, and the isopropanol pathway enzymes expressed from $P_L lacO_1$. Lo et al. (36) coupled growth and substrate availability into an AND-gate to express pathway enzymes. Glucose depletion, as a proxy for growth, activated the *E. coli* promoter P_{csiD}, which expressed a CoA ligase. The CoA ligase product was a pathway intermediate and induced the transcription of the rest of the pathway. One variant of the AND-gate circuit showed a 30-fold transcriptional increase 4 h after induction.

Direct transcriptional control induced by cell density occurs naturally through QS systems (99). Gupta et al. (41) used the *esa* QS system from *Pantoea stewartii* to dynamically divert glucose from glycolysis to their production pathway. The authors replaced the native promoter of the glycolysis flux control gene *pfkA* with P_{esaS}, which deactivates at high cell density, and linked expression to enzyme abundance by appending a strong degradation tag to Pfk-1, encoded by *pfkA*. The switching time and OD were determined by the strength of AHL synthase expression. In a separate application, the Esa knockdown strategy was applied to dynamically downregulate transcription of the shikimate kinase *aroK* to disrupt endogenous aromatic amino acid production, in order to accumulate the intermediate shikimate as a fermentation product. The cell density–induced QS regulation strategy was layered with a *myo*-inositol-responsive IpsA transcriptional repressor and hybrid promoter containing an IpsA binding site to divert glycolytic flux toward the heterologous glucaric acid pathway and couple transcription of the pathway gene

MIOX with its substrate. Characterization of the hybrid promoter showed a 16- to 55-fold increase in fluorescence depending on IpsA expression level (73). Other QS systems have also been engineered to varying levels of complexity, such as the native *E. coli* AI-2 QS system (53) and the *lux* QS system from *V. fischeri* in an AND-gate with the stationary-phase promoter P_{rpoS} (100).

In addition to TF-promoter pairs, other tools are available to enact dynamic transcriptional control. The PhoPQ two-component system that responds to Mg^{2+} depletion and its regulated promoter, P_{rstA}, were used to upregulate downstream enzyme expression in response to the toxic intermediate FPP (67). Wang et al. (101) engineered a transcriptional activator with duplex DNA thrombin aptamers inserted in the sense and antisense strands upstream of a promoter, such that the DNA bubble that formed in the presence of the ligand gave RNAP better access to the promoter. Cell-free characterization showed 1.5-fold higher activity of the expressed enzyme 4 h after thrombin addition. In *B. subtilis*, the thrombin aptamer regulatory scheme conferred up to 48-fold upregulation of reporter expression and was used to control heterologous pathway genes (102). CRISPR interference (CRISPRi) uses inactive dCas9 and single-guide RNA (sgRNA) to interfere with transcription by blocking TF or RNAP binding and/or stalling transcriptional elongation; the system is reversible and can be applied to multiple genes (103–105). Qi et al. (103) achieved up to 300-fold repression with a single sgRNA and combined two sgRNAs to achieve 1,000-fold repression of a fluorescent reporter; repression began within 10 min and reached full capacity within 4 h. Cress et al. (104) improved naringenin production with IPTG-inducible CRISPRi, used to knock down *fumC*, *scpC*, and the *sucABCD* operon to reduce TCA flux and CoA consumption for by-product formation. Gordon et al. (106) used an aTc-inducible CRISPRi strategy to slow nitrogen assimilation into *Synechococcus* sp. strain PCC 7002 by reducing endogenous *glnA* expression, effectively increasing glycolytic flux and lactate production. Dinh & Prather (42) expressed sgRNA and dCas9 from P_{lux} to autonomously knock down expression from endogenous genes that compete for the product's precursor at high cell density. CRISPR can also be used for gene activation (CRISPRa) by directing transcriptional machinery upstream of target genes (107). Dong et al. (108) applied CRISPRa with the endogenous *E. coli* SoxS activator to regulate expression of a heterologous ethanol production pathway. The activation system achieved a 50-fold increase in mRNA of the GFP reporter. The authors also demonstrated simultaneous activation and repression of fluorescent reporters by

combining CRISPRi and CRISPRa. CRISPR sgRNAs have been fused with aptamers that change functional interactions with regulatory elements in response to their respective ligand, which acts as a direct stimulus to alter transcription (109, 110). Moser et al. (40) used CRISPRi to dynamically knock down acetate production, which is harmful to product yield and cell health. Given that *poxB* is the main acetate producer during stationary phase, whereas it is *pta* during exponential phase, the authors used a glucose and acetate AND-gate to express *poxB* sgRNA and a glucose AND NOT low oxygen gate for *pta* sgRNA, with constitutive dCas9.

Transcriptional regulation is achievable with RNA-based systems. Chappell et al. (111) developed *trans*-acting small RNAs (sRNAs) called small transcription activating RNAs (STARs), which activate expression by alleviating a hairpin terminator in the DNA upstream of a gene. STARs were used to control the expression of the four-gene deoxyviolacein pathway, and sgRNA to layer the strategy with CRISPRi. Additionally, the authors generated a STAR library that produced 37 variants with >50-fold induction, including a few variants with up to 400-fold induction; when an unstable GFP reporter was used to lower the baseline signal, 9,000-fold induction was achieved. The toxic P450 CYP725A4 that was expressed from either stress-activated promoter, P_{metN} or P_{ompF}, and activated by a *lux* QS-driven STAR produced oxygenated taxanes in *E. coli*; the transcriptional control system activated at high cell density and self-regulated the P450 toxicity (112).

Translational Control

Translational control, or posttranscriptional control, occurs naturally through *trans*-regulatory sRNA elements (113) or *cis*-regulatory elements within mRNA (114) that alter the accessibility of the RBS or destabilize the mRNA. Rational design of synthetic RNA tools is often straightforward for specific applications because only the mRNA sequence is needed. Nakashima et al. (115) employed IPTG-inducible antisense RNA, containing a paired terminus for improved stability, to silence translation of growth essential genes *aceE* and *accA* to accumulate pyruvate. The authors reported 71% and 64% mRNA silencing efficacy for two vectors tested and >99% efficacy at the protein level with both vectors. Na et al. (116) developed synthetic small regulatory RNAs (sRNAs) containing an antisense region that spanned the RBS and a scaffold for the Hfq protein that hybridizes and degrades RNA. Their synthetic sRNAs were used in 14 different strains to test combinatorial repression of four genes involved in regulation of and competition with tyrosine production. The repression of regulatory genes *tyrR* and *csrA* was

identified to be the most advantageous for tyrosine production; their respective sRNA binding energies were tuned to further increase tyrosine titer. The authors also screened of a library of 122 synthetic sRNAs that repress cadaverine-related genes to find 31 that increased production. Repression of the essential gene *murE* had the most significant impact on cadaverine production, which was maximized through binding energy tuning. Design principles for general sRNA constructs to control translation have been established (117, 118). Wang et al. (119) developed OFF-to-ON and ON-to-OFF riboregulated translation switches by employing a *trans*-regulatory RNA to expose a *cis*-repressed RBS or sequester an exposed RBS, respectively. These switches turn the succinic acid pathway genes *ecaA* and *pepC* from OFF during lag phase to ON during the fermentative logarithmic phase, when they are effective, and *mgtC* OFF from ON between lag and logarithmic phases, when it improves growth under low Mg^{2+} concentrations.

In addition to the RBS sequence itself, nearby mRNA structure also plays a significant role in translation and protein levels (120). To expand the sequence space that can be riboregulated, Green et al. (121) developed toehold switches that use a *trans*-activating RNA to expose the RBS and start codon of the switch containing mRNA while directly binding neither. A library of 13 toehold switches showed an average ON/OFF GFP fluorescence ratio of 406 3 h after induction. Toehold switches were applied to genomic uidA (β-glucoronidase) and lacZ (β-galactosidase). Because *lacZ* is regulated transcriptionally as well, the toehold switch created an AND gate for the trigger RNA and lactose or lactose analogs. Two-input AND gating of toehold switches and layering of further inhibitory regulatory elements into the toehold mRNA was explored for further regulatory complexity and control (122). Regulatory circuitry involving up to four toehold-based riboregulators has been demonstrated (123). The general translation initiation area can be effectively used to knockdown gene expression up to 99% using *trans*-acting sRNA and was demonstrated on 15 endogenous genes, including *pgi*, *glnA*, and *argF*, to improve putrescine production (124).

The eukaryotic transcriptional repression system RNA interference (RNAi) confers mRNA silencing that parallels the effect of sRNAs and Hfq in prokaryotes. Crook et al. (125) imported RNAi from *Saccharomyces castelii* into *S. cerevisiae* to perform a combinatorial test of *ade3* knockdowns for itaconic acid production. The optimized system achieved up to 93% downregulation in fluorescence when tested on genomic YFP. Williams et al. (126) used the same system under the control of a synthetic QS system for para-hydroxybenzoic acid

production. At high cell density, pathway genes and RNAi elements that arrest cell division were expressed from the QS promoter, initiating production and halting growth.

The previous examples relied on transcriptional controllers, such as stimulus-induced or QS promoters, to express the regulatory RNA strands dynamically. Riboswitches and aptamers directly bind metabolites; thus, they can regulate translation as culture conditions change, without the need for auxiliary control elements. Zhou et al. (127) employed the natural lysine riboswitches from *E. coli* (ECRS) and *B. subtilis* in *C. glutamicum* to control the TCA cycle through *gltA* translation, which is essential but competes with lysine accumulation. The riboswitches were chromosomally integrated upstream of the *gltA* start codon such that its mRNA secondary structure sequestered the RBS and inhibited translation when bound to lysine, resulting in 30% and 43% enzyme activity compared with the parent strain. The same ECRS riboswitch was engineered into a lysine-ON riboswitch used to control the lysine secretion gene, *lysE*, to further lysine production (127). Rudolph et al. (128) noted the simplicity of translation regulation when employing only a synthetic theophylline riboswitch in *Streptomyces coelicolor* to control the heterologous β-glucuronidase reporter gene and endogenous agarase *dagA*. Qi et al. (129) fused *trans*-acting noncoding RNAs with RNA aptamers. One such fusion included an *E. coli* antisense RNA translational inhibitor sequence and a theophylline aptamer and was demonstrated to inhibit the GFP reporter fluorescence, with an 83% dynamic range, in the presence of theophylline. The placement of a vascular endothelial growth factor RNA aptamer upstream of the RBS of the target gene resulted in translation repression by ligand blockage of the RBS (101), whereas duplex RNA thrombin aptamers upstream of the RBS create a bubble when bound, exposing the RBS to ribosomes for activation (130). Aptazymes comprising aptamers fused to ribozymes confer both ligand-binding and mRNA cleavage functionalities to enact translational control. Stifel et al. (131) developed aptazyme switches with up to 33-fold OFF-switch behavior and up to 9-fold ON-switch behavior tested on GFP reporters. The ligand inactivated the ribozyme, leaving the RBS sequestered, and the ligand induced cleavage to expose the RBS in the switches, respectively.

Posttranslational Control

Although there is an abundance of readily tunable transcriptional and translational control strategies, they do not address proteins that are already present in the cell. Posttranslational control strategies have the advantage of directly altering target protein abundance and

performance. Allosteric regulation in response to a ligand is a common natural posttranscriptional control point. However, this strategy has not been adopted for metabolic enzymes owing to the inherent complexity and limitations in protein engineering. Dynamic protein degradation is another rapid control strategy but comes at the cost of wasted energy and high ATP usage (132). Protein degradation is facilitated by a peptide sequence that directs the protein to degradation machinery. In *E. coli*, transfer mRNA flags proteins for ClpXP and ClpAP protease degradation with an SsrA peptide fused to the C terminus (133). Varying the last three residues of the peptide tag changes the protein degradation rate (134). McGinness et al. (135) engineered a less-effective SsrA tag, which required the SspB adaptor to link ClpXP for hundredfold improved degradation. Degradation of target proteins, cloned with the modified SsrA tag, was activated by the addition of arabinose, as *sspB* expression was arabinose inducible. Brockman & Prather (136) appended the modified SsrA tag to Pfk-I and controlled *sspB* expression under an aTc-induble circuit to dynamically switch carbon flux from glycolysis to heterologous *myo*-inositol production. This strategy resulted in a decrease of Pfk-I activity to 35% of wild type within 1 h of *sspB* expression and 18% after 4 h. Other protease-based switches have also been employed to direct metabolic flux from growth to production pathways (43, 137, 138). Cameron & Collins (139) engineered a synthetic, inducible posttranslational control system by expressing an aTc-induced Lon protease from *Mesoplasma florum* and cloning target genes with the *M. florum* SsrA tag in *E. coli* and *Lactococcus lactis*. Tagged GFP reporter signal was reduced to 1–5% of the initial levels within 4 h of protease induction. Moser et al. (40) achieved the rapidity of *M. florum* Lon protease action with the potency of CRISPRi when the two systems were co-utilized to knock down the expression of a fluorescent reporter.

Alternatively, posttranslational control can be enacted to enhance protein abundance or functionality. Durante-Rodríguez et al. (138) engineered the first enzyme of a heterologous pathway with a hybrid NIa/SsrA tag, to be constitutively degraded, until the 3-methylbenzoate-induced viral NIa protease removed the tag to generate active enzyme. Addition of the inducer resulted in an eightfold increase in target enzyme activity, which was also 1.6-fold higher than that of the natively expressed enzyme. The 3-methylbenzoate-induced NIa system with NIa/SsrA tag was also demonstrated in *Pseudomonas putida*, where GFP was undetectable in the absence of NIa (140). Enzymes split into inactive fragments, fused to inducible dimers, can be dynamically reassembled by adding the appropriate chemical or light signal (30, 141). Haslinger

& Prather (142) tethered Cytochrome P450 to different redox partners using the PUPPET heterotrimeric DNA sliding clamp system by genetically fusing the subunits of the clamp to each enzyme. This strategy improved the titers from nonnatural redox partners with relatively low-affinity interactions. Co-localization of pathway enzymes using DNA, RNA, and protein scaffolds has been shown to improve pathway yields, likely by increasing the effective concentration of intermediates by flux channeling (143–145). Zhao et al. (146) formed light-inducible metabolic organelles by fusing pathway enzymes to the light-activated optoCluster system and to the dark-activated PixELL system. The PixELL organelle achieved an 18-fold change in the ratio of product to the spontaneously oxidized by-product. Kang et al. (147) assembled one unit of Idi to two units of CrtE using the interactions of the fused RIAD and RIDD peptides, respectively, which improved cell growth and production by aiding transfer of the toxic intermediate produced from Idi to the membrane-bound CrtE.

DISCUSSION

As the layout of this review suggests, stimuli, points of control, and strategies to overcome metabolic challenges are somewhat modular in application and can be mixed and matched if the available tools exist for each unique application. Dynamic control strategies can be layered to address multiple challenges at once or to enforce a single shift in metabolic flux. Furthermore, many approaches can be taken to address the same challenge, and even to make the same product. To illustrate this, we can compare examples of different approaches to making the same products, glucaric acid and succinic acid, and employing regulation at the same TCA cycle metabolic node for making different products.

The heterologous glucaric acid pathway branches from the glycolysis and pentose phosphate pathway intermediate glucose-6-phosphate. Both Gupta et al. (41) and Hou et al. (43) used transcriptional and posttranslational control in production strains that grew solely from glycolysis. Gupta et al. (41) used cell density as the stimulus through the *esa* QS system to downregulate the transcription of the gene encoding Pfk-1, which consumes glucose-6-phosphate in glycolysis. The authors layered on the posttranslational strategy of fusing a modified SsrA tag to Pfk-1 to ensure that the abundance of the enzyme was directly related to QS control. The production pathway was induced at inoculation. This approach resulted in a glucaric acid titer of 0.85 g/L in shake flasks. Hou et al. (43) expressed the gene encoding Pfk-1 with a growth phase–

dependent promoter (GPP) that is active until stationary phase; a modified SsrA tag was also fused to Pfk-1. The gene encoding the first heterologous pathway enzyme, INO1, was expressed from a Ptet promoter, whereas TetR was expressed from another GPP with a fused SsrA tag. Thus, the transition to stationary phase induced both knockdown of glycolytic flux and upregulation of *INO1* expression. The rest of the pathway genes were constitutively overexpressed. This strategy resulted in a titer of 1.16 g/L of glucaric acid in shake flasks, which increased to 1.56 g/L in a 5-L fermenter.

For succinic acid production, Wang et al. (119) and Sachdeva et al. (144) took entirely different dynamic regulation approaches, including different points of control and metabolic nodes. Wang et al. (119) relied on riboregulated translational control to enhance biomass accumulation in early fermentation by expressing a beneficial gene for lag-phase growth and delaying upregulation of the production pathway. This control strategy resulted in increases in yield and productivity over the parent strain, from 0.69 to 0.78 g/g glucose and from 1.05 to 1.16 g/L/h. Sachdeva et al. (144) used posttranscriptional RNA scaffolding to channel flux through four pathway enzymes, increasing yield over the no-scaffold control from 0.68 to 1.28 g/L.

Just as many routes can be taken to make the same product, the same metabolic node can also be useful for the synthesis of multiple products. Although Soma et al. (98) and Zhou et al. (127) engineered strategies to produce isopropanol and lysine, respectively, both products used a branch point metabolite in the TCA cycle. Soma et al. (98) knocked down the TCA cycle by enacting transcriptional repression on *gltA*, the gene encoding citrate synthase, with an IPTG-induced toggle switch. The authors achieved up to 93% reduction in citrate synthase activity, which resulted in a 3.7-fold increase in isopropanol titer (from 13.7 to 50.9 mM) (98). Zhou et al. (127) achieved the same knockdown with a lysine riboswitch that interrupted *gltA* translation, reducing citrate synthase activity to 30% and improving lysine yield by 63% (from 0.139 to 0.227 mol/mol glucose) compared with the parent strain.

Dynamic control of metabolism is a powerful strategy to balance conflicting cellular objectives that has yielded significant production improvements in numerous pathway contexts. In applying dynamic control to address a metabolic challenge, we must select a stimulus-responsive control circuit that determines the criterion for actuating a dynamic response and the appropriate point of control that couples the stimulus-responsive circuit to a shift in metabolic flux. In this review, we have outlined the stimuli and points of control that have been used to

address various production challenges, noting the key features of each approach. We note that different combinations of stimuli and control points can be used to effectively address the same challenges, and some approaches are generalizable to multiple pathways, suggesting that these options can be applied modularly. Additionally, several examples demonstrate the benefit of applying multiple control circuits and/or points of control to realize more complex regulatory responses or to control more than one metabolic flux. While several demonstrations of dynamic regulation have been reported, and some have validated performance in bench-scale reactors, to our knowledge these methods have not been demonstrated at commercial scale. Autonomously triggered switching is especially attractive as an alternative to expensive chemical inducers, as previously noted, to realize the benefits of dynamic control while avoiding the high material costs of the former in large volume reactors. Additional process development will be required to demonstrate the advantages of this approach in commercial manufacturing.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Table 1 Selected applications of dynamic regulation of metabolic flux

Stimulus	Control element	Challenge addressed	Target product	Outcome	Reference
Primary point of control: genetic					
Galactose	Cre recombinase, MIG1/ *GAL1m1*	Biomass accumulation, branchpoint metabolite	Lactate	85% yield increase over 8% in uninduced strain	83
Primary point of control: transcription					
IPTG and aTc, toggle	Ptrc/LacI and Ptet/TetR	Biomass accumulation	Lactate	55% increase in instantaneous production rate over knockout control	18
Temperature	cI857 repressor and p_R/p_L promoters	Biomass accumulation, branchpoint metabolite	Itaconic acid	48% increase in peak productivity compared with constitutive control	22
Light	Light-inducible fusion proteins	Biomass accumulation, branchpoint metabolite	Isopropanol	>Twofold increase in titer compared with constitutive control	24
pH	Pgas promoter (low-pH-induced)	Biomass accumulation, branchpoint metabolite	Itaconic acid	Fivefold increase in titer compared with constitutive control	33
Lux AHL (cell density)	LuxR and Plux	Biomass accumulation, branchpoint metabolite	Isopropanol	Up to threefold titer increase compared with IPTG-induced control	51
FPP-induced stress	PgadE promoter	Prevent accumulation of toxic intermediate	Amorphadiene	Twofold increase in titer over IPTG-inducible and >fivefold increase in titer over Pconst controls	67
myo-inositol	IpsA and engineered hybrid promoter	Unstable enzyme	Glucaric acid	Threefold increase compared with constitutive control	73

IPTG and aTc, toggle	Ptrc/LacI and Ptet/TetR	Biomass accumulation, branchpoint metabolite	Isopropanol	3.7-fold increase in titer	98
IPTG, CRISPRi	dCas9, sgRNA	Biomass accumulation, branchpoint metabolite, intermediate accumulation	Naringenin	2.5-fold titer increase over nontargeting control strain	104
Lux AHL (cell density), CRISPRi	LuxR/Plux, dCas9, sgRNA	Biomass accumulation, branchpoint metabolite	Naringenin	Sixfold titer increase over static strategies	42
Arabinose, CRISPRa	pTet, pBAD, SoxS activator, dCas9, scRNA	Biomass accumulation, branchpoint metabolite	Ethanol	Threefold titer increase over cells without CRISPRa	108
Primary point of control: translation					
Arabinose and temperature	sRNA	Knockdown of endogenous regulation (found through library)	Cadaverine	31% titer increase over base strain	116
IPTG	sRNA	Biomass accumulation	Succinic acid	51% productivity increase over group's previously engineered strain	119
Lysine	Riboswitch	Biomass accumulation, branchpoint metabolite	Lysine	63% yield increase	127
Tryptophan, QS pheromone (cell density)	ARO9 promoter, synthetic QS, RNAi	Biomass accumulation, branchpoint metabolite	Para-hydroxybenzoic acid	37-fold yield increase over base strain	126
Primary point of control: posttranslation					
aTc	Modified SsrA tag and SsrB adaptor	Biomass accumulation, branchpoint metabolite	*myo*-inositol	Fivefold titer increase over parent strain	136

IPTG, arabinose, aTc	Inducible promoters, DNA scaffold	Substrate channeling, stoichiometric enzyme balance	Resveratrol	Fivefold titer increase over random scaffold control	143
IPTG	Inducible promoters, RNA scaffold	Substrate channeling	Succinate	88% titer increase over no scaffold control	144
IPTG, arabinose, aTc	Protein scaffold	Substrate channeling, balancing enzymatic activity	Glucaric acid	200% titer increase over nonscaffolded control	145
IPTG	RIAD and RIDD peptides	Enzyme colocalization, toxic intermediate channeling	Carotenoids	5.7-fold titer increase over base strain with no assembly	147

Abbreviations: AHL, acyl-homoserine lactone; aTc, anhydrotetracycline; CRISPR, clustered regularly interspersed short palindromic repeats; CRISPRa, CRISPR activation; CRISPRi, interference; FPP, farnesyl pyrophosphate; IPTG, isopropyl β-D-1-thiogalactopyranoside; QS, quorum sensing; RNAi, RNA interference; scRNA, scaffold RNA; sgRNA, single-guide RNA; sRNA, small RNA.