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Dynamic Pathway Regulation: Recent Advances and Methods of Construction

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Abstract

Microbial cell factories are a renewable source for the production of biofuels and valuable chemicals. Dynamic pathway regulation has proved successful in improving production of molecules by balancing flux between growth of cells and production of metabolites. Systems for autonomous induction of pathway regulation are increasingly being developed, which include metabolite responsive promoters, biosensors, and quorum sensing systems. Since engineering such systems are dependent on the available methods for controlling protein abundance in the desired host, we review recent tools used for gene repression at the transcriptional, post-transcriptional and post-translational levels in *Escherichia coli* and *Saccharomyces cerevisiae*. These approaches may facilitate pathway engineering for biofuel and biochemical production.

Introduction

Microorganisms are promising hosts for the production of valuable chemicals, such as fuel alternatives[1,2], flavors[3], and polymer[4] and pharmaceutical[5] precursors. The immense diversity in the physiology of different microbes offers tremendous potential in using microbial cell factories given a desired product, starting material or growth condition. For example, the yeast *Saccharomyces cerevisiae*'s native ability to produce ethanol efficiently from glucose has rendered it the ideal host for bioethanol production[6]. *Pseudomonas putida* that naturally degrades aromatic acids is a suitable host for using lignocellulosic biomass as a starting material[7], while methanotroph bacteria provides a biological means to the upgrading of methane[8], which is the least expensive source of carbon. For growth at a desired condition, environmental strains can also be isolated and further engineered, such as a *Bacillus megaterium* strain capable of growing at sterile supercritical CO_2 conditions[9]. With the abundance of microorganisms to choose from, the limitation then lies in the availability of genomic sequence-to-function information for the microorganism and molecular biology methods for host DNA manipulation. Once the desired host has been chosen, metabolic engineering of the microorganism for production of molecules then typically involves pathway engineering and host engineering. Pathway engineering includes expressing the required enzymes, whether native or heterologous, for the conversion of substrate to product. Host engineering involves manipulation of the rest of the cell, such as removing competing or regulatory pathways to increase flux to the desired product. Often times, these methods consist of static changes, such as constitutive overexpression of pathway enzymes and deletion of competing enzymes, both of which are also generally optimized separately. Although these conventional strategies have proven to be successful in improving titers and yields, these methods are constrained by the interconnectivity of pathways in cells and a finite amount of cellular resources. To that end, dynamic regulation of pathways has emerged as a preferred method to better balance flux between growth of the cell and production of the desired molecule (Figure 1). Here, we report recent developments in applying dynamic pathway regulation to improve production of molecules in bacteria and yeast. Then, we review current methods used to engineer pathway regulation and address their limitations. These methods show promise for improving the productivity of microbes for synthesis of biofuels and bioproducts.

Dynamic Pathway Regulation by Exogenous Inducers

Regulation of pathways can be broadly divided into two categories: upregulating pathway enzymes to direct flux into the production pathway and downregulating competing enzymes that draw flux into native cell metabolism. Upregulation of pathway enzymes is well established and typically achieved by using inducible promoter systems. In downregulating competing enzymes,

an effective flux control point is first identified. This control point is typically an endogenous enzyme that catalyzes an irreversible reaction in cell metabolism that is also located where the production pathway intersects with cell metabolism. Placing this enzyme under dynamic control then enables flux redirection from cell metabolism into the production pathway at intermediate times in the fermentation by addition of an exogenous inducer or carbon source. Recent applications of dynamic regulation include the production of isopropanol in *E. coli*, where a toggle switch was designed to simultaneously increase flux through the isopropanol pathway and decrease flux into the TCA cycle, leading to a 4-fold improvement in isopropanol titers upon induction with IPTG [10]. In the yeast *S. cerevisiae*, native carbon source responsive promoters are typically used for inducible expression or repression of genes. For example, HXT and GAL promoters were used to control expression of pathway genes to increase flux of farnesyl pyrophosphate (FPP) for carotenoid production[11]. The same approach could provide yield improvements for isoprenoid-derived biofuels. The HXT1 promoter that is induced at high glucose concentrations and repressed at low glucose concentrations was also used to improve fatty alcohol production by regulating free fatty acid and acyl-CoA pools[12]. The SUC2 sucrose responsive promoter was used to induce RNA interference system for gene repression[13]. Since these promoters are responsive to glucose, the application of these methods are restricted by carbon source. To circumvent this limitation, synthetic promoter systems such as the doxycycline responsive tetracycline transactivator protein (tTA) has been used to control glucose flux into glycolysis, leading to improved yields of gluconic acid and isobutanol from glucose by 50-fold and 3-fold[14].

Autonomous Induction of Pathway Regulation

Although successful in improving production of molecules of interest, the aforementioned examples involve addition of an exogenous inducer at a pre-determined time to enable dynamic regulation. A further extension of pathway regulation is an autonomous system, where enzyme levels are controlled without manual addition of inducers. Autonomous induction can be engineered by utilizing metabolite responsive promoters or biosensors, or integrating quorum sensing systems that are dependent on cell density (Figure 2). Metabolite responsive promoters that are used to control gene expression are often derived from native promoters that are first identified by screening with the desired metabolite, followed by optimization for dynamic range by manipulating promoter strength. One advantage of using such promoters is that it involves only a promoter replacement without the need to express an additional transcription factor. An example in *E. coli* is in the improvement of flux through the mevalonate pathway, where levels of the intermediate FPP were controlled by regulating expression of the downstream amorpha-4,11-diene synthase by an FPP-responsive promoter, which was identified by mining whole-genome transcript arrays[15]. In yeast, native ergosterol responsive promoters

were screened and used to down-regulate expression of ERG9, diverting flux of FPP away from squalene and into amorphadiene production, leading to 5-fold improvement in titers[16]. A second method to engineer autonomous induction involve biosensors that are typically derived from transcription factors that bind DNA upon interaction with a metabolite. Depending on the host, substantial engineering of the transcription factor and binding site on the promoter are often required to achieve the desired dynamic range[17,18]. Most biosensor applications in metabolic engineering have revolved around its used as a screen[19,20]; however, an example in dynamic pathway regulation is with the transcriptional repressor FapR. FapR has been engineered into a malonyl-CoA biosensor and used to improve fatty acids production in *E. coli* by autonomously balancing flux of malonyl-CoA production and consumption[21]. This FapR/malonyl-CoA biosensor has also been ported into yeast to dynamically induce expression of a heterologous malonyl-CoA reductase for the production of 3-hydroxypropionic acid[22]. In addition, a biosensor responsive to hydroxycinnamic acids has been developed in *E. coli* and used to improve production of vanillin and *p*-hydroxybenzoate[23], compounds that could potentially be derived from lignocellulosic biomass in an integrated biorefinery.

Quorum sensing systems provide another method of autonomous induction that is triggered when a certain cell mass has accumulated. The well-studied LuxR system from *Vibrio fischeri* consists of a LuxI synthase that produces acyl-homoserine lactone (AHL), which interacts with the LuxR transcription factor. Placing the gene of interest under LuxR control then enables cell density dependent gene expression upon AHL accumulation. The switching time when gene expression is triggered can be varied by manipulating the expression levels of *luxI* and *luxR*. Examples of utilizing the LuxR system for dynamic pathway regulation include late induction of pathway enzymes in the production of 1,4-butanediol from xylose [24] and redirection of flux from the TCA cycle into isopropanol production by induction of a toggle switch[25] in *E. coli*. Another quorum sensing system, the Esa QS system from *Pantoea stewartii*, has been engineered for both transcriptional activation and repression by manipulating EsaR binding sites on the promoter[26]. Building upon the Esa QS transcriptional repression constructs enabled direct repression of phosphofructokinase in *E. coli*, leading to the improvement in glucaric acid production by dynamically diverting flux away from glycolysis[27]. In yeasts, applications of quorum sensing systems for metabolic engineering remain limited. Porting LuxR and Esa QS systems that consist of bacterial transcriptional repressors into yeast proves challenging due to the nature of transcriptional repression in eukaryotes that requires chromatin remodeling. Thus, native quorum sensing systems such as the pheromone cell-to-cell communication in yeast have been engineered[28] and used to increase production of para-hydroxybenzoic acid (PHBA)[29], by inducing RNA interference (RNAi) to deplete pyruvate kinase. This down-regulation diverts flux of phosphoenolpyruvate from glycolysis into the shikimate pathway for PHBA production.

Tools to Engineer Dynamic Regulation

Engineering dynamic regulation of a pathway involves engineering the ability to control the abundance of an enzyme in an inducible manner. Upregulation of pathway enzymes would require methods of inducible gene expression and downregulation of competing enzymes would necessitate tools for protein depletion. Successful engineering of dynamic regulation then hinges on the availability and efficiency of such systems in the desired host. Enzyme abundance can be manipulated at the transcriptional, post-transcriptional or post-translational level (Figure 3). Numerous methods exist to regulate protein abundance at these different levels, each with its own advantages and limitations. In general, intervention at the transcriptional level elicits a faster response but the protein of interest is still susceptible to native post-transcriptional and post-translational regulation, if any. On the other hand, regulating at the post-translational level directly controls the protein abundance but is more wasteful as resources are used to produce the protein, only to be degraded. Notably, the efficiency of the different methods is very much host dependent and the ideal method would also depend on the intended application. Inducible gene expression systems in bacteria typically function at the transcriptional level using repressor/promoter systems such as TetR/P_{tet}, LacI/P_{lac}, and AraC/P_{BAD}, which are useful for late induction of pathway enzymes that may be beneficial for flux balancing or maintaining enzyme stability. In yeast, inducible promoter systems are relatively limited to native carbon source dependent promoters, such as the glucose/galactose responsive GAL promoters, and doxycycline responsive tetracycline promoter[30]. Since these induction systems are well established, the remainder of this review will concentrate on tools for protein downregulation and their recent applications in pathway regulation.

In bacteria, gene repression at the transcriptional level requires combining multiple known promoter systems since most widely used bacterial promoter systems turn transcription on upon interaction with an inducer. One example of such a synthetic promoter is the inverter, which combines P_{tet} and P_{lac}, and was used to downregulate glucokinase to improve gluconate yields in *E. coli* by diverting flux away from glycolysis[31]. In recent years, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) interference system, developed by engineering a nuclease-null Cas9 from *S. pyogenes* that is recruited to the target DNA by expressing a single guide RNA (sgRNA), has served as the primary method of gene repression in bacteria. In *E. coli,* the CRISPR interference (CRISPRi) system has been characterized to provide up to 100-fold repression of RFP fluorescence that is expressed from the genome[32]. In dynamic regulation of pathways, the CRISPRi system has recently been used to improve flux through the mevalonate pathway in *E. coli* for isoprene production. A 70% decrease in mRNA levels of *ispA* was achieved that led to 2.6-fold increase in isoprene titers[33]. In the cyanobacterium *Synechococcus sp*., repressing glutamine synthetase I with CRISPRi improved lactate production, by increasing

concentrations of α-ketoglutarate that activates catabolic genes involved in lactate synthesis from glycogen[34]. In cases where multiple genes need to be repressed simultaneously, expression vectors to construct multiple sgRNAs with repeat sequences have been developed that employ USER or Golden Gate cloning methods[35]. Up to five sgRNAs were expressed simultaneously to divert succinate semialdehyde away from the TCA cycle and into 4-hydroxybutyrate (4HB), leading to improvements in 4HB mol% in poly(3-hydroxybutyrate-co-4-hydroxybutyrate)[36]. An alternative to expressing multiple sgRNAs is to express a trans-activating CRISPR RNA (tracrRNA) with an array of precursor crRNA (pre-crRNA) that contains spacers (targeting sequences) uniformly dispersed within repeat sequences. Characterization of this method by targeting GFP expressed from a plasmid resulted in up to 10-fold repression in fluorescence. Using a crRNA array targeting three different genes, *sucA, fumC* and *scpC*, a 2.5-fold increase in naringenin titers was achieved[37]. One benefit of using the CRISPRi system over promoter replacement methods is that genome editing of the target gene is not required, enabling its application in hosts where genome editing remains a challenge. Although the off-target effects of Cas9 have been well characterized in mammalian systems[38], the extent of off-target effects are less established in bacteria. Nonetheless, the first 12bp sequence of the target sgRNA being most important for targeting with *S. pyogenes* Cas9 remains universal[32,39]. Additionally, off-target effects for gene repression could be less detrimental than for genome editing. The more relevant limitation of the CRISPRi system is therefore in the toxicity of *dCas9* expression in certain hosts[40,41] that would affect growth of pathway-expressing cells that typically already suffer from growth defects. CRISPRi systems have also been developed in yeast, where repression domains that recruit chromatin remodeling complexes, such as Mxi1, are fused to dCas9[39]. Since transcription in eukaryotes can be initiated by recruiting transcriptional activators, the CRISPR system has also been engineered for inducing gene expression by fusing dCas9 to known activating domains, such as VP64. Diverting flux with a combination of activating and repressing dCas9 led to accumulation of different products in the violacein pathway in *S. cerevisiae*[42].

Applications that employ post-transcriptional and post-translational methods for dynamic regulation remain relatively limited. At the post-transcriptional level, tools to down-regulate mRNA levels include anti-sense RNA (asRNA) in bacteria and RNA interference (RNAi) systems in yeast. Anti-sense RNA involves expressing short ~100bp complementary mRNA, encompassing the ribosome binding site (RBS) and/or open reading frame (ORF), that hybridizes with the target mRNA to prevent translation or cause mRNA degradation[43]. Its application in metabolic engineering has mostly involved screening for gene targets whose downregulation improves metabolite production[44,45]. An example of using asRNA for pathway regulation is in downregulating *fabD* in *E. coli*, where an 80% interference efficiency was achieved, leading to a 4.5-fold increase in intracellular malonyl-CoA concentrations[46]. RNAi systems in eukaryotes similarly involve expressing a complementary mRNA; however, expression of Dicer and

Argonaute proteins are also required, where Dicer cleaves the dsRNA to smaller RNAs that are then recognized by Argonaute for degradation[47]. This system has been optimized for gene repression in *S. cerevisiae*[48] and recently used to divert flux of phosphoenolpyruvate from glycolysis into the shikimate pathway for the production of PHBA[29]. Downregulating protein abundance at the post-translational level involves adding a signal peptide to the protein of interest and expressing the required degradation machinery. In *E. coli*, the native ClpXP degradation system that recognizes the SsrA signal peptide is often utilized. To engineer inducibility, the expression of SspB that tethers the target protein to the ClpXP machinery can be placed under control of an inducible promoter in a *ΔsspB* strain[49]. More recently, an N-terminal based degradation system that relies on the N-end rule has been developed[50] and is inducible in a *ΔclpP* strain. For a completely orthogonal system in *E. coli*, a protein degradation system from *Mesoplasma florum* has been engineered that also utilizes a similar SsrA degradation tag named Pdt, but that is not recognized by *E. coli*'s native machinery[51]. For dynamic regulation of pathways, the SspB/ClpXP system has been shown to be effective in downregulating competing enzymes for improved production of glucaric acid[52] and medium-chain fatty acids[53] in *E. coli*. For glucaric acid production, phosphofructokinase was depleted to divert flux of glucose-6-phosphate away from glycolysis and into the glucaric acid pathway. In the production of medium-chain fatty acids, the FabB ketoacyl synthase was downregulated to prevent further elongation of octanoic acid. In yeast, the polypeptide sequence rich in proline (P), glutamic acid (E), serine (S) and threonine (T), called the PEST sequence, serves as a proteolytic signal on cyclins involved in cell cycle regulation[54]. Attaching PEST sequences to the C-terminal of otherwise stable proteins has been used as a protein degradation method; however, given that it is recognized by native proteolytic machinery, protein depletion is not inducible. Nonetheless, use of the common PEST sequence from CLN2 to degrade ERG9 in *S. cerevisiae* that competes for FPP for squalene production has led to the improvement of nerolidol titers[55].

Conclusions

Microbial cell factories provide a renewable source for the production of biofuels, fine and commodity chemicals. In improving production of these molecules, dynamic pathway regulation has been shown as a successful method that balances flux between growth of cells and production of metabolites. To eliminate use of exogenous inducers, systems for autonomous induction of pathway regulation are increasingly being developed that include metabolite responsive promoters, biosensors, and quorum sensing systems. Engineering such systems are restricted by the available tools for controlling protein abundance in the desired host. Controlling gene expression at the transcriptional level has been widely adopted, especially with recent developments of CRISPRi systems. Nonetheless, control methods that function at the post-transcriptional and post-translational levels are useful alternatives. Ultimately, the ideal control

system for dynamic pathway regulation would be application and host dependent. These approaches may facilitate pathway engineering for biofuel and biochemical production.

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References

1. Choi YJ, Lee SY: **Microbial production of short-chain alkanes.** *Nature* 2013, **502**:571–4.

2. Sheppard MJ, Kunjapur AM, Wenck SJ, Prather KLJ: **Retro-biosynthetic screening of a modular pathway design achieves selective route for microbial synthesis of 4-methyl-pentanol.** *Nat. Commun.* 2014, **5**:5031.

3. Kunjapur AM, Tarasova Y, Prather KLJ: **Synthesis and Accumulation of Aromatic Aldehydes in an Engineered Strain of *Escherichia coli*.** *J. Am. Chem. Soc.* 2014, **136**:11644–54.

4. Zhu MM, Lawman PD, Cameron DC: **Improving 1,3-Propanediol Production from Glycerol in a Metabolically Engineered *Escherichia coli* by Reducing Accumulation of sn-Glycerol-3-phosphate**. *Plasmid* 2002, **18**:694–699.

5. Ro D-K, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho K a, Eachus R a, Ham TS, Kirby J, et al.: **Production of the antimalarial drug precursor artemisinic acid in engineered yeast.** . *Nature* 2006, **440**:940–3.

6. Dombek KM, Ingram LO: **Ethanol production during batch fermentation with *Saccharomyces cerevisiae*: Changes in glycolytic enzymes and internal pH**. *Appl. Environ. Microbiol.* 1987, **53**:1286–1291.

7. Johnson CW, Salvachúa D, Khanna P, Smith H, Peterson DJ, Beckham GT: **Enhancing muconic acid production from glucose and lignin-derived aromatic compounds via increased protocatechuate decarboxylase activity** . *Metab. Eng. Commun.* 2016, **3**:111–119.

8. Henard CA, Smith H, Dowe N, Kalyuzhnaya MG, Pienkos PT, Guarnieri MT: **Bioconversion of methane to lactate by an obligate methanotrophic bacterium.** . *Sci. Rep.* 2016, **6**:21585.

9. Peet KC, Freedman AJE, Hernandez HH, Britto V, Boreham C, Ajo-Franklin JB, Thompson JR: **Microbial growth under supercritical CO2.** *Appl. Environ. Microbiol.* 2015, **81**:2881–92.

10. Soma Y, Tsuruno K, Wada M, Yokota A, Hanai T: **Metabolic flux redirection from a central metabolic pathway toward a synthetic pathway using a metabolic toggle switch** . *Metab. Eng.* 2014, doi:10.1016/j.ymben.2014.02.008.

11. Xie W, Ye L, Lv X, Xu H, Yu H: **Sequential control of biosynthetic pathways for balanced utilization of metabolic intermediates in *Saccharomyces cerevisiae*** . *Metab. Eng.* 2014, doi:10.1016/j.ymben.2014.11.007.

12. Teixeira PG, Ferreira R, Zhou YJ, Siewers V, Nielsen J: **Dynamic regulation of fatty acid pools for improved production of fatty alcohols in *Saccharomyces cerevisiae***. *Microb. Cell Fact.* 2017, **16**:45.

13. Williams TC, Espinosa MI, Nielsen LK, Vickers CE: **Dynamic regulation of gene expression**

using sucrose responsive promoters and RNA interference in *Saccharomyces cerevisiae*. . *Microb. Cell Fact.* 2015, **14**:43.

14. Tan SZ, Manchester S, Prather KLJ: **Controlling Central Carbon Metabolism for Improved Pathway Yields in *Saccharomyces cerevisiae*** . *ACS Synth. Biol.* 2015, doi:10.1021/acssynbio.5b00164.

15. Dahl RH, Zhang F, Alonso-Gutierrez J, Baidoo E, Batth TS, Redding-Johanson AM, Petzold CJ, Mukhopadhyay A, Lee TS, Adams PD, et al.: **Engineering dynamic pathway regulation using stress-response promoters.** . *Nat. Biotechnol.* 2013, **31**:1039–1046.

16. Yuan J, Ching CB: **Dynamic control of ERG9 expression for improved amorpha-4,11-diene production in *Saccharomyces cerevisiae*** . *Microb. Cell Fact.* 2015, **14**.

17. Raman S, Rogers JK, Taylor ND, Church GM: **Evolution-guided optimization of biosynthetic pathways** . *Proc. Natl. Acad. Sci.* 2014, doi:10.1073/pnas.1409523111.

18. Younger AKD, Dalvie NC, Rottinghaus AG, Leonard JN: **Engineering Modular Biosensors to Confer Metabolite-Responsive Regulation of Transcription**. *ACS Synth. Biol.* 2016, doi:10.1021/acssynbio.6b00184.

19. Rogers JK, Guzman CD, Taylor ND, Raman S, Anderson K, Church GM: **Synthetic biosensors for precise gene control and real-time monitoring of metabolites** . *Nucleic Acids Res.* 2015, doi:10.1093/nar/gkv616.

20. • Skjoedt ML, Snoek T, Kildegaard KR, Arsovska D, Eichenberger M, Goedecke TJ, Rajkumar AS, Zhang J, Kristensen M, Lehka BJ, et al.: **Engineering prokaryotic transcriptional activators as metabolite biosensors in yeast.** *Nat. Chem. Biol.* 2016, **12**:951–958.
The authors engineered a bacterial transcriptional activator into a cis,cis-muconic acid biosensor in *S. cerevisiae* and utilized the biosensor as a screen for high-producing cis,cis-muconic acid strains.

21. Xu P, Li L, Zhang F, Stephanopoulos G, Koffas M: **Improving fatty acids production by engineering dynamic pathway regulation and metabolic control** . *Proc. Natl. Acad. Sci.* 2014, **111**:1–6.

22. David F, Nielsen J, Siewers V: **Flux Control at the Malonyl-CoA Node through Hierarchical Dynamic Pathway Regulation in *Saccharomyces cerevisiae***. *ACS Synth. Biol.* 2016, **5**:224–233.

23. • Lo T-M, Chng SH, Teo WS, Cho H-S, Chang MW: **A Two-Layer Gene Circuit for Decoupling Cell Growth from Metabolite Production**. *Cell Syst.* 2016, **3**:133–143.
The authors developed a biosensor responsive to hydroxycinnamic acids to improve production of vanillin and *p*-hydroxybenzoate in *E. coli*.

24. Liu H, Lu T: **Autonomous production of 1 , 4-butanediol via a de novo biosynthesis pathway in engineered *Escherichia coli***. *Metab. Eng.* 2015, **29**:135–141.

25. • Soma Y, Hanai T: **Self-induced metabolic state switching by a tunable cell density sensor for microbial isopropanol production**. *Metab. Eng.* 2015, **30**:7–15.
The authors integrated the LuxR quorum sensing system for cell dependent induction of a toggle switch that diverts flux from the TCA cycle in isopropanol propoduction in *E. coli*.

26. Shong J, Collins CH: **Engineering the esaR promoter for tunable quorum sensing-dependent gene expression.** . *ACS Synth. Biol.* 2013, **2**:568–75.

27. • Gupta A, Reizman IMB, Reisch CR, Prather KLJ: **Dynamic regulation of metabolic flux in engineered bacteria using a pathway-independent quorum-sensing circuit**. *Nat. Biotechnol.* 2017, **35**:273–279.
The authors adapted the EsaR quorum sensing system for cell dependent gene repression to improve production of glucaric acid and shikimate in *E. coli*.

28. Williams TC, Nielsen LK, Vickers CE: **Engineered Quorum Sensing Using Pheromone-Mediated Cell-to-Cell Communication in*Saccharomyces cerevisiae***. *ACS Synth. Biol.* 2013, doi:10.1021/sb300110b.

29. • Williams TC, Averesch NJH, Winter G, Plan MR, Vickers CE, Nielsen LK, Krömer JO: **Quorum-sensing linked RNA interference for dynamic metabolic pathway control in *Saccharomyces cerevisiae*** . *Metab. Eng.* 2015, **29**:124–134.
The authors developed the native pheromone quorum sensing system in *S. cerevisiae* for autonomous induction of RNAi to improve production of para-hydroxybenzoic acid. This is the first quorum sensing-based dynamic regulation application in *S. cerevisiae*.

30. Garí E, Piedrafita L, Aldea M, Herrero E: **A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in *Saccharomyces cerevisiae*.** . *Yeast* 1997, **13**:837–48.

31. Solomon K V, Sanders TM, Prather KLJ: **A dynamic metabolite valve for the control of central carbon metabolism.** . *Metab. Eng.* 2012, **14**:661–71.

32. Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA: **Repurposing CRISPR as an RNA-Guided Platform for Sequence-Specific Control of Gene Expression** . *Cell* 2013, **152**:1173–1183.

33. Kim S, Hahn J-S: **Efficient production of 2,3-butanediol in *Saccharomyces cerevisiae* by eliminating ethanol and glycerol production and redox rebalancing** . *Metab. Eng.* 2015, doi:10.1016/j.ymben.2015.07.006.

34. Gordon GC, Korosh TC, Cameron JC, Markley AL, Begemann MB, Brian FP: **CRISPR interference as a titratable , trans -acting regulatory tool for metabolic engineering in the cyanobacterium Synechococcus sp . strain PCC 7002**. *Metab. Eng.* 2016, **38**:170–179.

35. Jakočiūnas T, Bonde I, Herrgård M, Harrison SJ, Kristensen M, Pedersen LE, Jensen MK, Keasling JD: **Multiplex metabolic pathway engineering using CRISPR/Cas9 in *Saccharomyces cerevisiae*** . *Metab. Eng.* 2015, doi:10.1016/j.ymben.2015.01.008.

36. • Lv L, Ren Y, Chen J, Wu Q, Chen G: **Application of CRISPRi for prokaryotic metabolic**

engineering involving multiple genes , a case study : Controllable P (3HB- co -4HB) biosynthesis. *Metab. Eng.* 2015, **29**:160–168.
The authors employed the CRISPRi gene repression system to simultaneously downregulate five genes to improve 4HB mol% in poly(3HB-co-4HB) in *E. coli*.

37. Cress BF, Toparlak OD, Guleria S, Lebovich M, Stieglitz JT, Englaender JA, Jones JA, Linhardt RJ, Koffas MAG: **CRISPathBrick: Modular Combinatorial Assembly of Type II-A CRISPR Arrays for dCas9-Mediated Multiplex Transcriptional Repression in E. coli**. *ACS Synth. Biol.* 2015, **4**:987–1000.

38. Pattanayak V, Lin S, Guilinger JP, Ma E, Doudna J a, Liu DR: **High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity.** *Nat. Biotechnol.* 2013, **31**:839–43.

39. Gilbert L a, Larson MH, Morsut L, Liu Z, Brar G a, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna J a, et al.: **CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes.** *Cell* 2013, **154**:442–51.

40. • Rock JM, Hopkins FF, Chavez A, Diallo M, Chase MR, Gerrick ER, Pritchard JR, Church GM, Rubin EJ, Sassetti CM, et al.: **Programmable transcriptional repression in mycobacteria using an orthogonal CRISPR interference platform.** *Nat. Microbiol.* 2017, **2**:16274.
The authors demonstrated that the efficiency of CRISPRi using the widely used *S. pyogenes* dCas9 is limited in mycobacteria, indicating that the utility of *S. pyogenes* CRISPRi is probably host dependent. The authors then tested other dCas9 variants and developed *S. thermophilus* dCas9 for gene repression in mycobacteria.

41. Nielsen AA, Voigt C a: **Multi-input CRISPR/Cas genetic circuits that interface host regulatory networks.** *Mol. Syst. Biol.* 2014, **10**:763.

42. Zalatan JG, Lee ME, Almeida R, Gilbert L a., Whitehead EH, La Russa M, Tsai JC, Weissman JS, Dueber JE, Qi LS, et al.: **Engineering Complex Synthetic Transcriptional Programs with CRISPR RNA Scaffolds**. *Cell* 2014, **160**:339–350.

43. Nakashima N, Tamura T: **Conditional gene silencing of multiple genes with antisense RNAs and generation of a mutator strain of *Escherichia coli***. *Nucleic Acids Res.* 2009, **37**.

44. Na D, Yoo SM, Chung H, Park H, Park JH, Lee SY: **Metabolic engineering of *Escherichia coli* using synthetic small regulatory RNAs.** *Nat. Biotechnol.* 2013, **31**:170–4.

45. Meng HL, Xiong ZQ, Song SJ, Wang J, Wang Y: **Construction of polyketide overproducing *Escherichia coli* strains via synthetic antisense RNAs based on in silico fluxome analysis and comparative transcriptome analysis**. *Biotechnol. J.* 2016, **11**:530–541.

46. Yang Y, Lin Y, Li L, Linhardt RJ, Yan Y: **Regulating malonyl-CoA metabolism via synthetic antisense RNAs for enhanced biosynthesis of natural products**. *Metab. Eng.* 2015, **29**:217–226.

47. Drinnenberg I a, Weinberg DE, Xie KT, Mower JP, Wolfe KH, Fink GR, Bartel DP: **RNAi in**

budding yeast. . *Science* 2009, **326**:544–50.

48. Crook NC, Schmitz AC, Alper HS: **Optimization of a yeast RNA interference system for controlling gene expression and enabling rapid metabolic engineering.** *ACS Synth. Biol.* 2014, **3**:307–13.

49. McGinness KE, Baker T a, Sauer RT: **Engineering controllable protein degradation.** . *Mol. Cell* 2006, **22**:701–7.

50. Sekar K, Gentile AM, Bostick JW, Tyo KEJ: **N-terminal-based targeted, inducible protein degradation in *Escherichia coli***. *PLoS One* 2016, **11**:1–17.

51. Cameron DE, Collins JJ: **Tunable protein degradation in bacteria**. *Nat. Biotechnol.* 2014, **32**.

52. Brockman IM, Prather KLJ: **Dynamic knockdown of E. coli central metabolism for redirecting fluxes of primary metabolites**. *Metab. Eng.* 2014, **28**:104–113.

53. Torella JP, Ford TJ, Kim SN, Chen AM, Way JC, Silver PA: **Tailored fatty acid synthesis via dynamic control of fatty acid elongation.** *Proc. Natl. Acad. Sci. U. S. A.* 2013, **110**:11290–5.

54. Rechsteiner M, Rogers SW: **PEST sequences and regulation by proteolysis**. *Trends Biochem. Sci.* 1996, **21**:267–271.

55. Peng B, Plan MR, Chrysanthopoulos P, Hodson MP, Nielsen K, Vickers CE: **A squalene synthase protein degradation method for improved sesquiterpene production in Saccharomyces cerevisiae**. *Metab. Eng.* 2017, **39**:209–219

Figure 1: Dynamic pathway regulation balances flux between growth of cells and production of desired metabolites. Once sufficient biomass and cellular resources have accumulated from an initial growth phase, substrate flux can then be diverted into the production pathway. An effective flux control point at the intersection of cell metabolism and production pathway is identified and the expression of this enzyme is controlled in a dynamic manner. Autonomous induction of pathway regulation has recently been developed using metabolite responsive promoters, biosensors and quorum sensing systems. Metabolite responsive promoters are often derived from native promoters that can be implemented simply by promoter replacement of the gene of interest (GOI) without additional expression of transcription factors. Biosensors are engineered from transcription factors that bind to their associated promoters upon interaction with the metabolite of interest. Placing the GOI under this promoter then enables metabolite dependent gene expression. Quorum sensing systems, such as the LuxR system, consist of a synthase that produces acyl-homoserine lactone (AHL) that serves as a proxy for cell mass. Controlling the expression of the GOI using a repressor that is responsive to AHL then enables cell density dependent expression of the GOI.

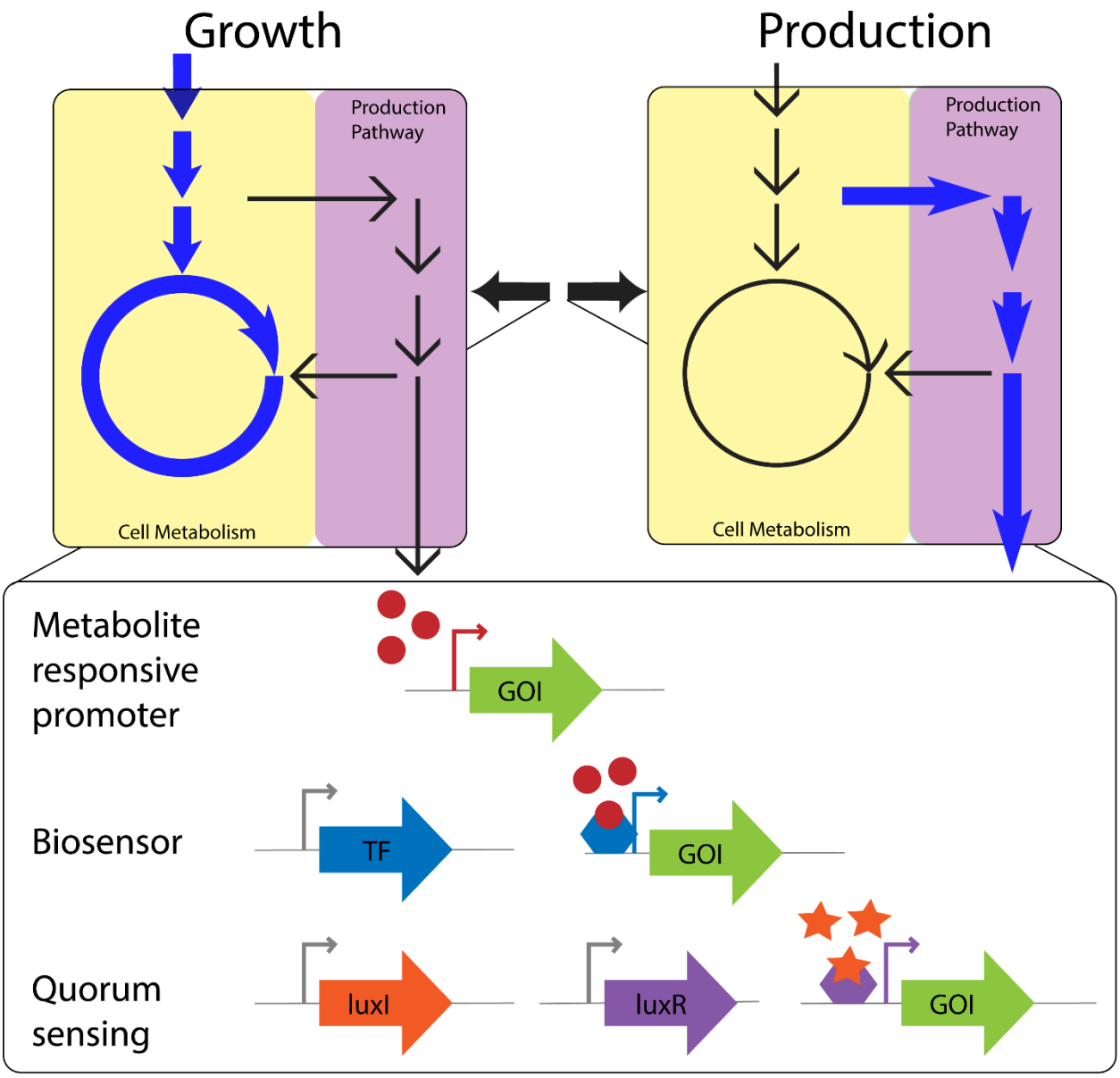
Growth
Production
Production Pathway
Cell Metabolism
Production Pathway
Cell Metabolism
Metabolite responsive promoter
GOI
Biosensor
TF
GOI
Quorum sensing
luxI
luxR
GOI

Figure 2: Examples of recent autonomous pathway regulation for improved metabolite production. (Left) The malonyl-CoA biosensor in *E. coli* [21] involves engineering the FapR transcriptional repressor to induce transcription of the upstream acetyl-CoA carboxylase (ACC, encoded by *accADBC*) and repress transcription of the downstream fatty acid synthase (FAS, encoded by *fabADGI* and *tesA'*) upon accumulation of malonyl-CoA. This balancing of flux between production and consumption of malony-coA improves fatty acid production. (Right) The Esa quorum sensing system in *E. coli* [27] was engineered to divert flux away from growth into the production of glucaric acid by repressing phosphofructokinase-1 (Pfk-1, encoded by *pfkA*) upon accumulation of sufficient biomass. The production of acyl-homoserine lactone (AHL) by AHL synthease (EsaI) serves as a proxy of biomass that activates the repression of Pfk-1 upon binding to the transcriptional repressor EsaR170V. Downregulation of phosphofructokinase enables the diversion of glucose-6-phosphate away from glycolysis and into the glucaric acid pathway.

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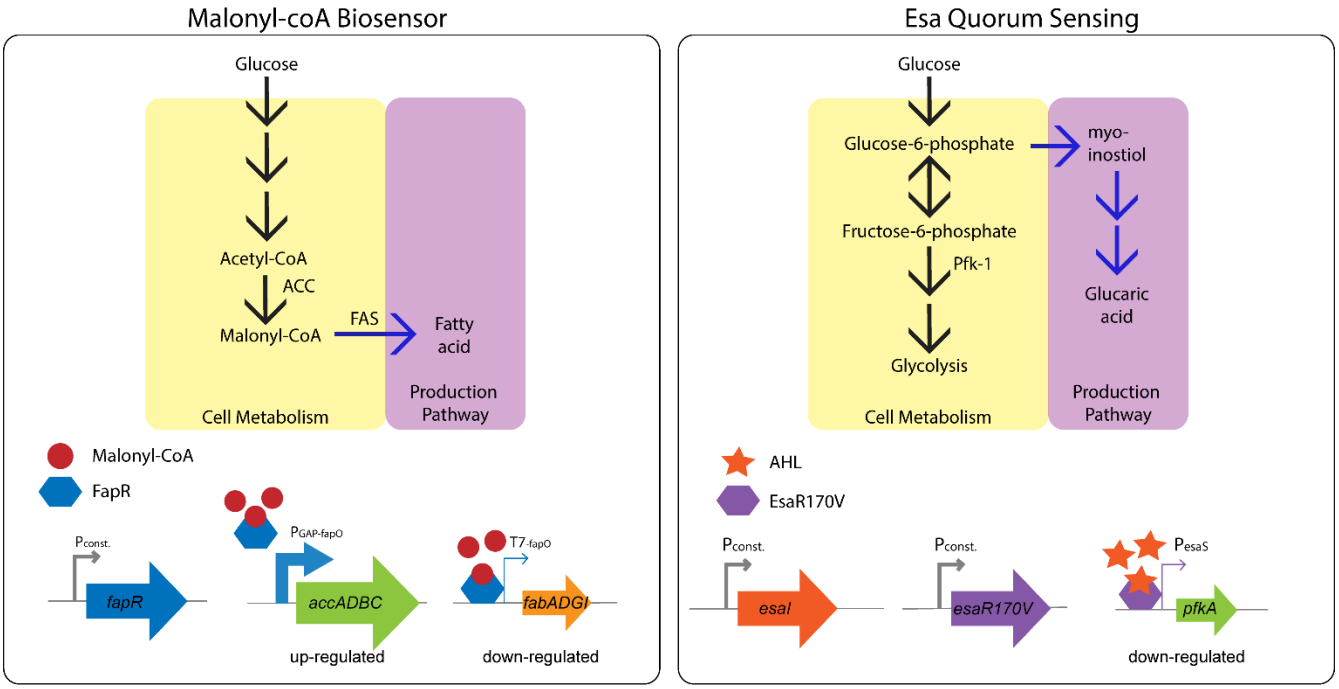
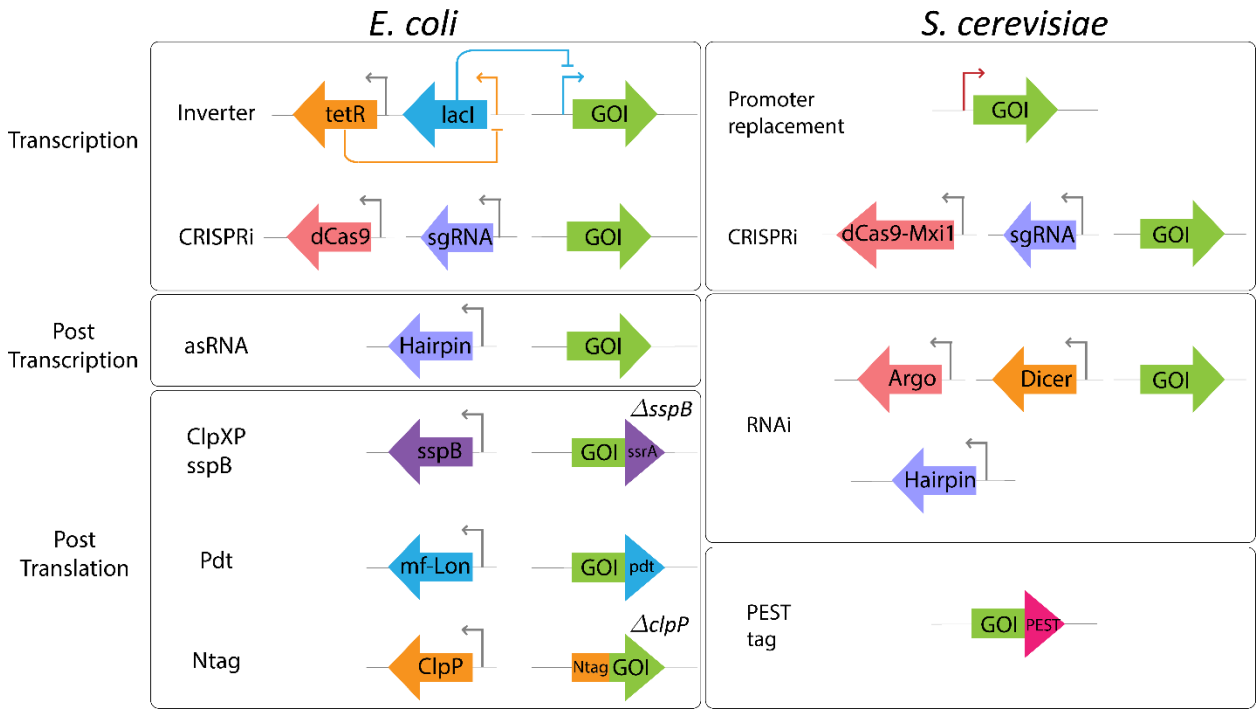

Malonyl-coA Biosensor
Glucose
Acetyl-CoA
ACC
Malonyl-CoA
FAS
Fatty acid
Cell Metabolism
Production Pathway
Malonyl-CoA
FapR
Pconst.
fapR
PGAP-fapO
accADBC
up-regulated
T7-fapO
fabADGI
down-regulated
Esa Quorum Sensing
Glucose
Glucose-6-phosphate
myo-inostiol
Fructose-6-phosphate
Pfk-1
Glucaric acid
Glycolysis
Cell Metabolism
Production Pathway
AHL
EsaR170V
Pconst.
esaI
Pconst.
esaR170V
PesaS
pfkA
down-regulated

Figure 3: Methods to deplete gene of interest (GOI) at the transcriptional, post-transcriptional and post-translational levels that were used in recent examples of dynamic pathway regulation discussed in this review. In *E. coli*, combinations of known repressor/promoter systems, such as the inverter, and CRISPR interference (CRISPRi) systems, which consist of dCas9 and a targeting sgRNA, have been used for gene repression at the transcriptional level. At the post-transcriptional level, anti-sense RNA (asRNA) involves expression of a hairpin that binds to regions of the GOI to prevent translation. Post-translational methods consist of adding signal peptides to GOI and expressing the required protein degradation machinery. Since SspB and ClpP are native to *E. coli*, inducibility of protein degradation with the ClpXP/SspB and Ntag methods can only be performed in *ΔsspB* and *ΔclpP* mutants, contrary to the Pdt system that uses an orthogonal *mf*-Lon protease. In *S. cerevisiae*, methods for gene repression at the transcriptional level include native carbon source responsive promoters and the CRISPRi system. At the post-transcriptional level, RNAi systems degrade target mRNA by expressing a hairpin alongside Argonaute (Argo) and Dicer proteins. A post-translation method that has been used for dynamic regulation is the native PEST degradation tag, which involves adding the signal peptide to the C-terminus of GOI.


E. coli
S. cerevisiae
Transcription
Inverter
tetR
lacI
GOI
CRISPRi
dCas9
sgRNA
GOI
Promoter replacement
GOI
CRISPRi
dCas9-Mxi1
sgRNA
GOI
Post Transcription
asRNA
Hairpin
GOI
RNAi
Argo
Dicer
GOI
Hairpin
Post Translation
ClpXP sspB
sspB
ΔsspB
GOI
ssrA
Pdt
mf-Lon
GOI
pdt
Ntag
ClpP
ΔclpP
Ntag
GOI
PEST tag
GOI
PEST