Dynamic metabolic control: towards precision engineering of metabolism

Dynamic metabolic control: towards precision engineering of metabolism

Di Liu[1†], Ahmad A. Mannan[2†], Yichao Han[1], Diego A. Oyarzún[2*], and Fuzhong Zhang[1*]

[1] Department of Energy, Environmental & Chemical Engineering, Washington University in St. Louis, St. Louis, Missouri 63130, United States

[2] Department of Mathematics, Imperial College London, London SW7 2AZ, United Kingdom

[*] Corresponding authors: d.oyarzun@imperial.ac.uk Tel: +44 20 7594 8533

fzhang@seas.wustl.edu Tel: +1 314-935-7671

[†] Equal contribution.

Abstract

Advances in metabolic engineering have led to the synthesis of a wide variety of valuable chemicals in microorganisms. The key to commercializing these processes is the improvement of titer, productivity, yield, and robustness. Traditional approaches to enhancing production uses the "push-pull-block" strategy that modulates enzyme expression under static control. However, strains are often optimized for specific laboratory set-up and are sensitive to environmental fluctuations. Exposure to sub-optimal growth conditions during large-scale fermentation often reduces their production capacity. Moreover, static control of engineered pathways may imbalance cofactors or cause the accumulation of toxic intermediates, which imposes burden on the host and results in decreased production. To overcome these problems, the last decade has witnessed the emergence of a new technology that uses synthetic regulation to control heterologous pathways dynamically, in ways akin to regulatory networks found in nature. Here we review natural metabolic control strategies and recent developments in how they inspire the engineering of dynamically regulated pathways. We further discuss the challenges of designing and engineering dynamic control and highlight how model-based design can provide a powerful formalism to engineer dynamic control circuits, which together with the tools of synthetic biology, can work to enhance microbial production.

Keywords: Dynamic metabolic control, genetic circuits, biosensors, synthetic biology, model-based design

§ 1. Introduction

Microbial production of valuable chemicals provides an attractive alternative to petroleum-based synthesis routes. A wide variety of chemicals such as biofuels, pharmaceuticals, and nutraceuticals have been successfully produced in microbial hosts by assembling and optimizing metabolic pathways [71,47,48]. Typically, the expression of pathway enzymes is either constitutive or under the control of inducible promoters that are tuned to balance the pathway flux to maximize titers, productivities, and yields. Static overexpression of enzymes can impose a load onto the cell by competing for native resources from metabolism and draining resources such as ribosomes, ATPs and chaperones [23]. The extra load to the host cell also makes it challenging to dynamically balance resource allocation between cell growth and the engineered pathway. In addition, the obtained strains are often optimized under certain laboratory conditions and are not as robust in large bioreactors, where environmental fluctuations (e.g., nutrient concentration, temperature, dissolved oxygen, etc.) can subject cells to suboptimal conditions and lead to decreased production. Deviation from the optimal condition may divert carbon to byproducts or lead to the accumulation of toxic intermediates that attenuate cell growth [42]. Furthermore, engineered strains often suffer from stability issues where genetic mutations may arise during fermentation that deactivate the pathway activity. By comparison, natural cells maintain robust growth and withstand environmental fluctuations by dynamically adjusting cellular metabolism through complex regulatory networks. These regulatory networks govern the distribution of cellular resources and sustain homeostasis in fluctuating environments.

The study of how natural regulatory networks enable cells to grow robustly has been a focus in systems biology. Diverse regulation mechanisms have been identified to dynamically control metabolism in response to varying environmental conditions and intracellular metabolic status [29,39,30,16]. These mechanisms sense environmental signals such as nutrient

concentration, pH, and light, as well as intracellular metabolite concentrations and cell density. The sensed signals are then coupled to transcriptional, translational or post-translational processes to control protein expression or activities for efficient carbon usage. Taking the concept of dynamic regulation, synthetic biologists have designed genetic circuits to dynamically regulate engineered pathways for optimal biochemical production [73,24,18,76,37,22,70,68].

In this review we discuss dynamic control strategies found in nature and how they inspire engineering efforts to increase bioproduction, with a particular focus on the design of control architectures. We further discuss some of the key challenges to designing dynamic control for enhancing biochemical production and highlight the utility of mathematical models to help address these. We conclude with an outlook that integrating design principles learned from natural control systems and model-based design into the metabolic engineering workflow can facilitate the design of dynamic metabolic control, towards the development of robust and efficient microbial cell factories.

§ 2. Natural strategies for dynamic control of metabolism

Dynamic regulation of metabolic pathways is ubiquitous in nature. Spanning from simple microbes to multicellular animals, all forms of life depend on complex regulatory networks to coordinate metabolism to maintain cellular activity and adapt to environmental changes. To achieve this, cells use a variety of strategies that involve the interplay between DNAs, RNAs, regulatory proteins, enzymes and metabolites. Transcriptional regulation represents a significant level of control that is responsive to a wide variety of molecules and exhibits versatile regulatory architectures. In *Escherichia coli*, 577 interactions have been identified between transcription factors and their regulated operons [55], and this number is still growing. These complex

interactions are made up of network motifs with different architectures that give rise to different functions [1].

One major function of dynamic regulation is to allocate resources efficiently. This is mostly achieved by transcriptional control to avoid high cost of protein synthesis. At the transcriptional level, the expression of enzymes is often controlled by transcription factors that can sense either an intermediate or product of a pathway, generating different regulation architectures. For example, in the lysine biosynthesis pathway in *Saccharomyces cerevisiae*, the transcription factor Lys14 is activated by an intermediate alpha aminoadipate 6-semialdehyde (αAAS), which activates all the seven genes in the pathway. Similarly, enzymes in the arginine biosynthesis pathway of *E. coli* are repressed by ArgR, which is in turn activated by the end product arginine. Experimental analyses and cost-benefit models for enzyme expression have uncovered links between regulatory architecture and the timing of gene expression in unbranched pathways [44,15,72,17], revealing unique patterns of timing and promoter activity for efficient enzyme expression.

In addition to the transcriptional level, many cellular activities are modulated at the translational and post-translational levels, and oftentimes interplay among them. Translational regulation, usually through controlling translation initiation rate or mRNA stability, only respond to a small number of metabolites due to the limited chemical diversity of nucleic acids. Post translational regulation is abundant in metabolic pathways and controls enzyme activities in response to environmental stimuli or metabolite concentrations. For example, enzymes in *E. coli* central metabolism are heavily regulated at the post-translational level to tightly maintain constant metabolic flux under small environmental perturbations [50]. In addition, product allosteric inhibition of the first enzyme in metabolic pathways is commonly observed to rapidly turn down the metabolic flux through the pathway, allowing for immediate saving on carbon

usage. By comparison, transcriptional or translational regulation, though responding at a slower time scale due to slow protein synthesis and dilution, can drastically shift the distribution of metabolic flux and enable cells to save resources in the long run. Among different levels of regulation, transcriptional regulation offers a variety of traits desirable for engineering applications, including versatility in regulation architecture, chemical diversity of the sensed molecules, and tunability of the regulatory parameters. Indeed, transcriptional regulation is the most widely used control in metabolic engineering. Overall, understanding natural regulatory mechanisms provides us a wide variety of tools and design principles to develop synthetic dynamic control, which can be applied in metabolic engineering [36].

§ 3. Engineered strategies for dynamic control of metabolism

A synthetic dynamic control circuit typically consists of a biosensor and a genetic controller. The application of biosensors [75,39,35,74] and genetic control circuits [58,10] have been extensively reviewed. A variety of signals can be sensed, such as intracellular metabolites, quorum signal molecules (AHLs), exogenous stimuli (inducers and lights), environmental signals (pH, oxygen, and temperature), and molecules that reflect cellular growth status (exponential growth v.s. stationary growth, etc.). These signals can be used to repress or activate enzyme expression and thus regulate flux of a pathway. One primary design objective for dynamic control of metabolic flux is to balance the growth of the cell and production of the target molecule. Next, we discuss different types of design strategies from input signals to output regulations that attempt to address this objective.

The basic method to dynamically regulate the flux distribution is adding exogenous inducers or nutrients at a time point during fermentation (Fig. 1a). Xie et al. constructed a

glucose-dependent regulatory system in *S. cerevisiae* to control the flux from branch point farnesyl diphosphate (FPP) to ergosterol biosynthesis (an essential component in yeast membrane) or to the carotenoid pathway [69]. Squalene synthase (erg9), the first gene from FPP to ergosterol pathway, was placed under the HXT1 promoter, which was induced at high glucose concentration, while the carotenoid pathway was controlled by glucose-repressible GAL promoters so that the production pathway was turned on after glucose was partially replaced by glycerol as an alternative carbon source. Dynamic regulation by exogenous inducers is straightforward and effective, but requires addition of inexpensive and environmentally-friendly inducers. These limitations can be overcome by introducing feedback control of enzyme expression to respond to signals produced by the cell itself.

An example of autonomous control is that on growth flux through negative feedback by a quorum sensing (QS) system (Fig. 1b). Soma and Hanai employed a QS system to autonomously redirect acetyl-CoA from the TCA cycle to the isopropanol pathway at a given cell density [60]. In a recent application, QS was used to downregulate phosphofructokinase-1 (*pfk-1*) in the upper glycolysis pathway [24]. Lower *pfk-1* activity channeled more carbon flux from the interconverting branch points G6P and F6P to the glucaric acid pathway, thus turning on product synthesis while inhibiting cell growth.

One key function of dynamic control in a biosynthetic pathway is to avoid accumulation of toxic intermediates or overexpression of toxic enzymes. Inhibiting an upstream pathway that generates the toxic intermediate and activating a downstream pathway that converts it are common control strategies (Fig. 1c). One of the pioneering works in dynamic pathway regulation was demonstrated for biodiesel production from free fatty acids [73]. In the pathway, accumulation of two intermediates ethanol and acyl-CoA is harmful to cell growth. The authors developed a dynamic regulatory system to activate ethanol production and the conversion of

ethanol and acyl-CoAs to final products only when fatty acyl-CoAs are sufficient. In another example, promoters responsive to FPP (toxic to cell) accumulation were used to repress the mevalonate pathway that produces FPP and to activate amorphadiene synthase that consumes FPP. Such regulatory topology dynamically stabilized the FPP concentration below its toxic level, while increasing amorphadiene production [18]. Similar control topologies can be constructed using transcription-factor-based sensors as demonstrated in the fatty acid pathway to optimize cellular malonyl-CoA pool [70]. In addition, synthetic inverters can be used to switch regulation between repression and activation, achieving a desired control topology [37].

Dynamic regulation can also be implemented by sensing signals that reflect the growth status of the host and using them to control production. In one of the first examples of dynamic regulation, acetyl phosphate served as the signal for excess glycolytic flux to regulate the rate-limiting enzymes in lycopene pathway [22] (Fig. 1d). Recently, a biosynthetic pathway was controlled by a two-layered circuit, which acted as an AND gate that senses both the cellular growth status and the pathway precursor availability [38]. The first enzymatic step was not turned on until stationary phase and downstream steps were activated by the intermediate from the first step (Fig. 1e), which reduces burden from the engineered metabolic pathway. Synthetic control can also be designed to sense production flux and regulate growth (Fig. 1f). Xiao et al. described a strategy that uses metabolite product to activate cell growth via expression of an antibiotic pump, TetA [68]. This ensured that high producing cells would tolerate the antibiotic treatment, and thus facilitated the selection of high producing phenotypes at the population level. Without selection, a wide variation in biosynthetic performance was observed in the whole population. With selection, only the high-performing cells could survive, thus increasing total production.

Despite a growing number of success stories, engineering dynamic control remains extremely challenging. Current implementations require multiple iterations between construction of part libraries, testing of different control architectures, and characterization of system performance. This lengthy design cycle is the result of multiple challenges that need to be addressed if the field is to move towards precision engineering of metabolism.

§ 4. Challenges for dynamic control and benefits of model-based design.

Current challenges for dynamic control include the construction and tuning of genetic parts, the assembly of parts into functional circuits, the interplay between circuit and host, and the control of population diversity (Fig. 2). Some of these challenges are particularly relevant for the success of dynamic control in industrial applications. For example, in large fermenters the level of intracellular metabolites and the environmental conditions can vary. Because biosensors are typically designed to function in model organisms under controlled laboratory conditions, their sensing ability may be impaired in industrial hosts with highly variable conditions. Control circuits also need to function robustly during long periods of fermentation, which in turn requires a good understanding of the host-circuit interactions that drive the allocation of resources within the host. Lastly, in large bioreactors there are often increased cell-to-cell variations [68], and the challenge is how to control the product distribution to shift the population to achieve higher percentage of high-producers.

Mathematical modelling is an ideal framework to integrate different design layers and explore the design space in a rational manner. Next, we discuss some of the key challenges ahead and outline how modelling can help overcome them.

§ 4.1. Construction of tunable parts

Metabolite biosensors are a key component of dynamic metabolic control. Their function is to control the expression of pathway enzymes in response to metabolic signals such as the concentrations of metabolic intermediates or other physicochemical cues. Growth conditions may shift metabolite concentrations to ranges that fall beyond the detection range of biosensors, thus impairing dynamic control and resulting in a static system unable to regulate enzyme expression. Tunability of biosensors is therefore essential for dynamic control systems to appropriately function in industrial conditions. Biosensors must respond with the appropriate sensitivity and actuate the response at the right signal threshold, according to the growth conditions. Biosensor function can be captured in the dose-response curve, which relates the concentration of the sensed metabolite to the enzyme expression (Fig. 2), and its shape can be modified through experimentally tunable parameters such as the metabolite binding affinity or the sequence of target promoters [40]. Some of the successful implementations of dynamic control have demonstrated that tuning the biosensor dose-response curve can affect performance significantly and increase production [37,70,68]. The question of how to design dynamic control is thus critical for developing production strains, especially for application in industrial settings. Much work has focused on developing new biosensors, but the precise calibration of their dose-response curve remains poorly understood [3] and leads to lengthy iterations between biosensor construction and characterization.

Common biosensors in dynamic control are transcriptional riboswitches [7] and transcription factors [75]. Progress in RNA engineering has led to a growing number of riboswitches that respond to specific metabolites [66,26]. Studies have shown that RNA sequences shape the sensitivity and threshold of riboswitch dose-response curves [52,5], yet the precise tuning of riboswitch function remains a significant challenge. Computational methods

221 have proven powerful for the design of RNA devices [13] and mathematical modelling has
222 revealed insights on the tunability of the riboswitch function in terms of biophysical parameters
223 [6]. Integration of sequence design algorithms with mathematical models may facilitate the
224 discovery of new metabolite-responsive riboswitches, and thus expand the repertoire of pathways
225 in which dynamic control can be used [7].

226 In the case of transcription factors, dose-response curves can be tuned with promoter
227 engineering [40] or protein engineering to modify metabolite binding kinetics [63]. There are
228 many natural transcription factors that respond to specific metabolites in their native host, which
229 can be repurposed as biosensors in a production host of interest. Detailed biophysical models
230 have revealed relations between sequence-dependent promoter binding affinities and protein
231 expression [9]. Moreover, mathematical models have uncovered fundamental design constraints
232 of dose-response curves, and revealed strategies for orthogonal control of biosensor dynamic
233 range and threshold [40].

234 **§ 4.2. Assembling parts to design control circuits**

235 To increase production, dynamic control circuits must achieve multiple design objectives
236 simultaneously [46]. The goal is to construct control circuits that adapt pathway activity to
237 varying bioreactor conditions, ensuring efficient expression of enzymes, minimizing the impact
238 of pathway bottlenecks or accumulation of toxic intermediates, and ultimately maximize yield,
239 titer or productivity at industrial scales. Achieving all these objectives demands the availability
240 of a wide repertoire of control circuit architectures, but in reality architectures are severely
241 constrained because well-characterized metabolite biosensors exist only for few relevant
242 compounds [75]. Key questions for architecture design are which pathway metabolite should be
243 sensed, and which enzymatic steps to implement dynamic control. Mathematical modelling can

244 be a powerful tool to explore such design space and assess performance of architectures that
245 would otherwise be infeasible or too costly to test experimentally.

246 Unlike in static control, where genome-scale models can be used for strain design
247 [11,57], model-based approaches for dynamic control are still in early stages. Mathematical
248 models have revealed design principles to improve biofuel production through control of efflux
249 pumps [21] and have provided conditions on the parameter design space to avoid accumulation
250 of toxic intermediates [46]. Genome-scale models have been employed to determine which
251 enzymes to control, which when coupled with dynamic modelling showed higher production as
252 compared to static control [2]. A particularly promising use for modelling is the exploration of
253 circuit architectures. Models have been used to search for architectures that efficiently trade-off
254 production flux against toxicity effects by metabolic intermediates [61], to explore circuit
255 architectures that function robustly in the face of environmental or genetic perturbations [25], or
256 to discover new useful architectures, such as a bistable metabolic switch that filters out
257 fluctuations in nutrient availability [43].

258 Control engineering has been tremendously successful in designing regulation systems
259 for diverse disciplines such as aerospace, bioprocessing, and information technologies [4].
260 Principles from control engineering have gained ground in synthetic biology [19] and optimal
261 control ideas have revealed design principles in natural metabolic systems [65,72], but their
262 broader application to dynamic pathway control remains less explored. A potential area for
263 future development is the use of mathematical optimization for circuit design [53] coupled with
264 detailed kinetic models of metabolism [32,49,41,14,27]. Optimization of control architectures
265 also faces significant computational challenges, as the sheer number of circuit designs and
266 tunable parameters may lead to optimization problems that cannot be solved in feasible time.

Trade-offs between circuit size and computation time needs to be considered and the development of scalable optimization methods poses multiple opportunities for further research.

§ 4.3. Host-circuit interactions

As metabolic pathways and control circuits become larger and more complex, their footprint on their host can become a major limiting factor on function. Engineered systems draw resources from the host, which can disrupt homeostasis and cause growth defects that lead to poor or even altered functionality [62,12]. A key source for host-circuit interactions is the competition for cellular resources such as ribosomes, RNA polymerases, and amino acid pools [8]. This competition affects cell growth and ultimately may result in impaired circuit function, leading to suboptimal production that is economically impractical at industrial scale.

Mathematical models can give a systems-level understanding of the relationship between circuit function and the physiology of the host where they reside. To this end, Weiße and colleagues developed a mechanistic model for bacterial growth, based on a coarse-grained partition of the proteome and its interaction through metabolism, transcription and translation [67]. The model predicts growth defects caused by gene circuits and provides a quantitative platform to assess the impact of growth defects on circuit function. A recent extension to this work includes more detailed mechanisms of the different host-circuit crosstalks and proved useful for circuit design [34]. Models for host-circuit interactions do not yet allow the inclusion of dynamic pathway control, but the use of dynamic control to manage host load and increase production is promising, especially in light of recent evidence showing that feedback control can mitigate the impact of resource coupling [56].

§ 4.4. Control of population heterogeneity

289 Phenotypic heterogeneity is ubiquitous in cellular populations. In microbes, heterogeneity
290 has been extensively studied as a product of stochasticity in gene expression and the resulting
291 variation in protein levels [51], and recent work has focused on variability on metabolic
292 phenotypes and growth [59,31,28]. Though phenotypic variability in natural systems can serve as
293 a population survival strategy, variability amongst strains engineered for production can lead to
294 suboptimal performance. Phenotypic variability may also result from fluctuations in growth
295 conditions, and the inhomogeneities in growth media can be further exaggerated when scaling-up
296 to industrial level production. In strains engineered for chemical production, phenotypic
297 variability manifests itself as wide distributions of metabolic production [54,20]. Such variability
298 has been exploited to increase production by designing control that couples the concentration of
299 product to growth, and thereby selects for high producers [68].

300 Mathematical modelling can provide novel insights on the sources and control of
301 metabolic variability. For example, the integration of genome-scale models with single-cell
302 proteomics datasets revealed the emergence of a bimodal growth distribution in *E. coli* [33], and
303 the emergence of bimodal phenotypes was also explored with dynamic models [64,31]. A
304 seminal stochastic modelling work on enzymatic reactions revealed conditions for a dynamic
305 control circuit to amplify or attenuate the variability of a metabolic product [45].

306 The emergence of mutants and genotypic heterogeneity pose a significant problem for
307 long term biochemical production, and can plague the implementation of production strains at
308 industrial scales. Over long time scales of fermentation mutations may impair the control circuit
309 or result in the emergence of non-producing, faster growing strains that will dilute out production
310 strains. This is a key area for future development to help sustain long-term bioproduction in
311 industrial settings.

312

§ 5. Final remarks

Dynamic control of metabolism is a powerful mechanism for cells to survive and adapt to environmental perturbations. In natural systems, dynamic control shifts metabolic activity between various operating regimes. Metabolic engineering can harness similar control strategies to increase production in varying and often unpredictable bioreactor conditions. In this paper we outlined some of the natural strategies for dynamic control together with recent successful implementations on metabolic production pathways.

Dynamic control has vast potential to enhance production at the industrial scale, enabling autonomous control of pathway activity without the cost of inducers and auto-adapting production and cellular demands according to fluctuating or changing fermentation conditions. Challenges for this technology are manifold and cover several layers of complexity, from tunable control parts, to functional circuits, accounting for host physiology and demands of the cell, and sustaining production in the face of phenotypic and emergence of genotypic heterogeneity. In this paper we discussed the challenges at these levels and how they affect the application of strains engineered with dynamic control to industrial scale bioproduction. Although a few recent studies have demonstrated the computation-guided tuning of biosensor response, the reliable determination of the intracellular metabolite concentration remains a challenge to providing accurate inputs to the model. In addition, the application of dynamic control in industrially relevant hosts has been limited, which entails tools and efforts to transfer the technology into those hosts [76]. Robust controls need to use the host resources efficiently and optimize the balance between growth and production. This is a challenging objective to achieve, and one where the metabolic engineering community can learn valuable lessons from natural systems. Systems biology has revealed fundamental design principles by reverse-engineering the regulation of natural metabolic systems, thanks to the combination of mathematical modelling

and wet-lab experimentation. Natural design principles and model-based methods integrated into the metabolic engineering workflow could institute the forward-engineering of control circuits and hail a new era in which dynamic control becomes the key technology for optimizing chemical production.

Acknowledgements: This work was funded by the Human Frontier Science Program through a Young Investigator Grant awarded to D.O. and F.Z. (Grant no. RGY0076-2015) and the US National Science Foundation (MCB1453147) to F.Z.

References

1. Alon U (2007) Network motifs: theory and experimental approaches. Nat Rev Genet 8:450-461.

2. Anesiadis N, Cluett WR, Mahadevan R (2008) Dynamic metabolic engineering for increasing bioprocess productivity. Metab Eng 10:255-266.

3. Ang J, Harris E, Hussey BJ, Kil R, McMillen DR (2013) Tuning response curves for synthetic biology. ACS Synth Biol 2:547-567.

4. Aström KJ, Murray RM (2010) Feedback Systems: An Introduction for Scientists and Engineers. Princeton University Press,

5. Baird NJ, Kulshina N, Ferré-D'Amaré AR (2010) Riboswitch function: flipping the switch or tuning the dimmer? RNA Biol 7:328-332

6. Beisel CL, Smolke CD (2009) Design principles for riboswitch function. PLoS Comput Biol 5:e1000363.

7. Berens C, Groher F, Suess B (2015) RNA aptamers as genetic control devices: The potential of riboswitches as synthetic elements for regulating gene expression. Biotechnol J 10:246-257.

8. Borkowski O, Ceroni F, Stan G-B, Ellis T (2016) Overloaded and stressed: whole-cell considerations for bacterial synthetic biology. Curr Opin Microbiol 33:123-130.

9. Brewster RC, Jones DL, Phillips R (2012) Tuning promoter strength through RNA polymerase binding site design in Escherichia coli. PLoS Comput Biol 8:e1002811.

10. Brophy JAN, Voigt CA (2014) Principles of genetic circuit design. Nat Methods 11:508-520.

11. Burgard AP, Pharkya P, Maranas CD (2003) Optknock: a bilevel programming framework for identifying gene knockout strategies for microbial strain optimization. Biotechnol Bioeng 84:647-657.

12. Cardinale S, Arkin AP (2012) Contextualizing context for synthetic biology--identifying causes of failure of synthetic biological systems. Biotechnol J 7:856-866.

13. Chappell J, Westbrook A, Verosloff M, Lucks J (2017) Computational design of Small Transcription Activating RNAs (STARs) for versatile and dynamic gene regulation.

14. Chassagnole C, Noisommit-Rizzi N, Schmid JW, Mauch K, Reuss M (2002) Dynamic modeling of the central carbon metabolism of Escherichia coli. Biotechnol Bioeng 79:53-73.

15. Chin C-S, Chubukov V, Jolly ER, DeRisi J, Li H (2008) Dynamics and design principles of a basic regulatory architecture controlling metabolic pathways. PLoS Biol 6:e146.

16. Chubukov V, Gerosa L, Kochanowski K, Sauer U (2014) Coordination of microbial metabolism. Nat Rev Microbiol 12:327-340.

17. Chubukov V, Zuleta IA, Li H (2012) Regulatory architecture determines optimal regulation of gene expression in metabolic pathways. Proc Natl Acad Sci U S A 109:5127-5132.

18. Dahl RH, Zhang F, Alonso-Gutierrez J, Baidoo E, Batth TS, Redding-Johanson AM, Petzold CJ, Mukhopadhyay A, Lee TS, Adams PD, Keasling JD (2013) Engineering dynamic pathway regulation using stress-response promoters. Nat Biotechnol 31:1039-1046.

19. Del Vecchio D, Dy AJ, Qian Y (2016) Control theory meets synthetic biology. J R Soc Interface 13.

20. Delvigne F, Zune Q, Lara AR, Al-Soud W, Sørensen SJ (2014) Metabolic variability in bioprocessing: implications of microbial phenotypic heterogeneity. Trends Biotechnol 32:608-616.

21. Dunlop MJ, Keasling JD, Mukhopadhyay A (2010) A model for improving microbial biofuel production using a synthetic feedback loop. Syst Synth Biol 4:95-104.

22. Farmer WR, Liao JC (2000) Improving lycopene production in Escherichia coli by engineering metabolic control. Nat Biotechnol 18:533-537.

23. Glick BR (1995) Metabolic load and heterologous gene expression. Biotechnol Adv 13:247-261

24. Gupta A, Reizman IMB, Reisch CR, Prather KLJ (2017) Dynamic regulation of metabolic flux in engineered bacteria using a pathway-independent quorum-sensing circuit. Nat Biotechnol 35:273-279.

25. Huang B, Lu M, Jia D, Ben-Jacob E, Levine H, Onuchic JN (2017) Interrogating the topological robustness of gene regulatory circuits by randomization. PLoS Comput Biol 13:e1005456.

26. Jang S, Jang S, Xiu Y, Kang TJ, Lee S-H, Koffas MAG, Jung GY (2017) Development of Artificial Riboswitches for Monitoring of Naringenin In Vivo. ACS Synth Biol.

27. Kadir T, Mannan AA, Kierzek AM, McFadden J, Shimizu K (2010) Modeling and simulation of the main metabolism in Escherichia coli and its several single-gene knockout mutants with experimental verification. Microb Cell Fact 9:88.

28. Kiviet DJ, Nghe P, Walker N, Boulineau S, Sunderlikova V, Tans SJ (2014) Stochasticity of metabolism and growth at the single-cell level. Nature 514:376-379.

29. Kochanowski K, Sauer U, Chubukov V (2013) Somewhat in control--the role of transcription in regulating microbial metabolic fluxes. Curr Opin Biotechnol 24:987-993.

30. Kochanowski K, Sauer U, Noor E (2015) Posttranslational regulation of microbial metabolism. Curr Opin Microbiol 27:10-17.

31. Kotte O, Volkmer B, Radzikowski JL, Heinemann M (2014) Phenotypic bistability in Escherichia coli's central carbon metabolism. Mol Syst Biol 10:736.

32. Kurata H, Sugimoto Y (2017) Improved kinetic model of Escherichia coli central carbon metabolism in batch and continuous cultures. J Biosci Bioeng.

33. Labhsetwar P, Cole JA, Roberts E, Price ND, Luthey-Schulten ZA (2013) Heterogeneity in protein expression induces metabolic variability in a modeledEscherichia colipopulation. Proceedings of the National Academy of Sciences 110:14006-14011.

34. Liao C, Blanchard AE, Lu T (2017) An integrative circuit–host modelling framework for predicting synthetic gene network behaviours. Nature Microbiology.

35. Liu D, Evans T, Zhang F (2015) Applications and advances of metabolite biosensors for metabolic engineering. Metab Eng 31:35-43.

36. Liu D, Hoynes-O'Connor A, Zhang F (2013) Bridging the gap between systems biology and synthetic biology. Front Microbiol 4:211.

37. Liu D, Xiao Y, Evans BS, Zhang F (2015) Negative feedback regulation of fatty acid production based on a malonyl-CoA sensor-actuator. ACS Synth Biol 4:132-140.

423 38. Lo T-M, Chng SH, Teo WS, Cho H-S, Chang MW (2016) A Two-Layer Gene Circuit for Decoupling
424 Cell Growth from Metabolite Production. Cell Syst 3:133-143.
425 39. Mahr R, Frunzke J (2016) Transcription factor-based biosensors in biotechnology: current state and
426 future prospects. Appl Microbiol Biotechnol 100:79-90.
427 40. Mannan AA, Liu D, Zhang F, Oyarzún DA (2017) Fundamental Design Principles for Transcription-
428 Factor-Based Metabolite Biosensors. ACS Synth Biol.
429 41. Mannan AA, Toya Y, Shimizu K, McFadden J, Kierzek AM, Rocco A (2015) Integrating Kinetic
430 Model of E. coli with Genome Scale Metabolic Fluxes Overcomes Its Open System Problem and
431 Reveals Bistability in Central Metabolism. PLoS One 10:e0139507.
432 42. Martin VJJ, Pitera DJ, Withers ST, Newman JD, Keasling JD (2003) Engineering a mevalonate
433 pathway in Escherichia coli for production of terpenoids. Nat Biotechnol 21:796-802.
434 43. Oyarzún DA, Chaves M (2015) Design of a bistable switch to control cellular uptake. J R Soc
435 Interface 12:20150618.
436 44. Oyarzún DA, Ingalls BP, Middleton RH, Kalamatianos D (2009) Sequential activation of metabolic
437 pathways: a dynamic optimization approach. Bull Math Biol 71:1851-1872.
438 45. Oyarzún DA, Lugagne J-B, Stan G-BV (2015) Noise propagation in synthetic gene circuits for
439 metabolic control. ACS Synth Biol 4:116-125.
440 46. Oyarzún DA, Stan G-BV (2013) Synthetic gene circuits for metabolic control: design trade-offs and
441 constraints. J R Soc Interface 10:20120671.
442 47. Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, McPhee D, Leavell MD, Tai A, Main A,
443 Eng D, Polichuk DR, Teoh KH, Reed DW, Treynor T, Lenihan J, Fleck M, Bajad S, Dang G,
444 Dengrove D, Diola D, Dorin G, Ellens KW, Fickes S, Galazzo J, Gaucher SP, Geistlinger T,
445 Henry R, Hepp M, Horning T, Iqbal T, Jiang H, Kizer L, Lieu B, Melis D, Moss N, Regentin R,
446 Secrest S, Tsuruta H, Vazquez R, Westblade LF, Xu L, Yu M, Zhang Y, Zhao L, Lievense J,
447 Covello PS, Keasling JD, Reiling KK, Renninger NS, Newman JD (2013) High-level semi-
448 synthetic production of the potent antimalarial artemisinin. Nature 496:528-532.

449 48. Peralta-Yahya PP, Zhang F, del Cardayre SB, Keasling JD (2012) Microbial engineering for the
450 production of advanced biofuels. Nature 488:320-328.
451 49. Peskov K, Mogilevskaya E, Demin O (2012) Kinetic modelling of central carbon metabolism
452 inEscherichia coli. FEBS J 279:3374-3385.
453 50. Pisithkul T, Patel NM, Amador-Noguez D (2015) Post-translational modifications as key regulators of
454 bacterial metabolic fluxes. Curr Opin Microbiol 24:29-37.
455 51. Raj A, van Oudenaarden A (2008) Nature, nurture, or chance: stochastic gene expression and its
456 consequences. Cell 135:216-226.
457 52. Rode AB, Endoh T, Sugimoto N (2015) Tuning riboswitch-mediated gene regulation by rational
458 control of aptamer ligand binding properties. Angew Chem Int Ed Engl 54:905-909.
459 53. Schmid JW, Mauch K, Reuss M, Gilles ED, Kremling A (2004) Metabolic design based on a coupled
460 gene expression-metabolic network model of tryptophan production in Escherichia coli. Metab
461 Eng 6:364-377.
462 54. Schmitz AC, Hartline CJ, Zhang F (2017) Engineering Microbial Metabolite Dynamics and
463 Heterogeneity. Biotechnol J 12.
464 55. Shen-Orr SS, Milo R, Mangan S, Alon U (2002) Network motifs in the transcriptional regulation
465 network of Escherichia coli. Nat Genet 31:64-68.
466 56. Shopera T, He L, Oyetunde T, Tang YJ, Moon TS (2017) Decoupling Resource-Coupled Gene
467 Expression in Living Cells. ACS Synth Biol 6:1596-1604.
468 57. Simeonidis E, Price ND (2015) Genome-scale modeling for metabolic engineering. J Ind Microbiol
469 Biotechnol 42:327-338.
470 58. Slusarczyk AL, Lin A, Weiss R (2012) Foundations for the design and implementation of synthetic
471 genetic circuits. Nat Rev Genet 13:406-420.
472 59. Solopova A, van Gestel J, Weissing FJ, Bachmann H, Teusink B, Kok J, Kuipers OP (2014) Bet-
473 hedging during bacterial diauxic shift. Proc Natl Acad Sci U S A 111:7427-7432.
474 60. Soma Y, Hanai T (2015) Self-induced metabolic state switching by a tunable cell density sensor for
475 microbial isopropanol production. Metab Eng 30:7-15.

476 61. Stevens JT, Carothers JM (2015) Designing RNA-based genetic control systems for efficient
477 production from engineered metabolic pathways. ACS Synth Biol 4:107-115.
478 62. Tan C, Marguet P, You L (2009) Emergent bistability by a growth-modulating positive feedback
479 circuit. Nat Chem Biol 5:842-848.
480 63. Taylor ND, Garruss AS, Moretti R, Chan S, Arbing MA, Cascio D, Rogers JK, Isaacs FJ, Kosuri S,
481 Baker D, Fields S, Church GM, Raman S (2016) Engineering an allosteric transcription factor to
482 respond to new ligands. Nat Methods 13:177-183.
483 64. van Heerden JH, Wortel MT, Bruggeman FJ, Heijnen JJ, Bollen YJM, Planqué R, Hulshof J, O'Toole
484 TG, Wahl SA, Teusink B (2014) Lost in transition: start-up of glycolysis yields subpopulations of
485 nongrowing cells. Science 343:1245114.
486 65. Waldherr S, Oyarzún DA, Bockmayr A (2015) Dynamic optimization of metabolic networks coupled
487 with gene expression. J Theor Biol 365:469-485.
488 66. Wang Y-H, McKeague M, Hsu TM, Smolke CD (2016) Design and Construction of Generalizable
489 RNA-Protein Hybrid Controllers by Level-Matched Genetic Signal Amplification. Cell Syst
490 3:549-562.e547.
491 67. Weiße AY, Oyarzún DA, Danos V, Swain PS (2015) Mechanistic links between cellular trade-offs,
492 gene expression, and growth. Proc Natl Acad Sci U S A 112:E1038-1047.
493 68. Xiao Y, Bowen CH, Liu D, Zhang F (2016) Exploiting nongenetic cell-to-cell variation for enhanced
494 biosynthesis. Nat Chem Biol 12:339-344.
495 69. Xie W, Ye L, Lv X, Xu H, Yu H (2015) Sequential control of biosynthetic pathways for balanced
496 utilization of metabolic intermediates in Saccharomyces cerevisiae. Metab Eng 28:8-18.
497 70. Xu P, Li L, Zhang F, Stephanopoulos G, Koffas M (2014) Improving fatty acids production by
498 engineering dynamic pathway regulation and metabolic control. Proc Natl Acad Sci U S A
499 111:11299-11304.
500 71. Xue Z, Sharpe PL, Hong S-P, Yadav NS, Xie D, Short DR, Damude HG, Rupert RA, Seip JE, Wang
501 J, Pollak DW, Bostick MW, Bosak MD, Macool DJ, Hollerbach DH, Zhang H, Arcilla DM,
502 Bledsoe SA, Croker K, McCord EF, Tyreus BD, Jackson EN, Zhu Q (2013) Production of

omega-3 eicosapentaenoic acid by metabolic engineering of Yarrowia lipolytica. Nat Biotechnol 31:734-740.

72. Zaslaver A, Mayo AE, Rosenberg R, Bashkin P, Sberro H, Tsalyuk M, Surette MG, Alon U (2004) Just-in-time transcription program in metabolic pathways. Nat Genet 36:486-491.

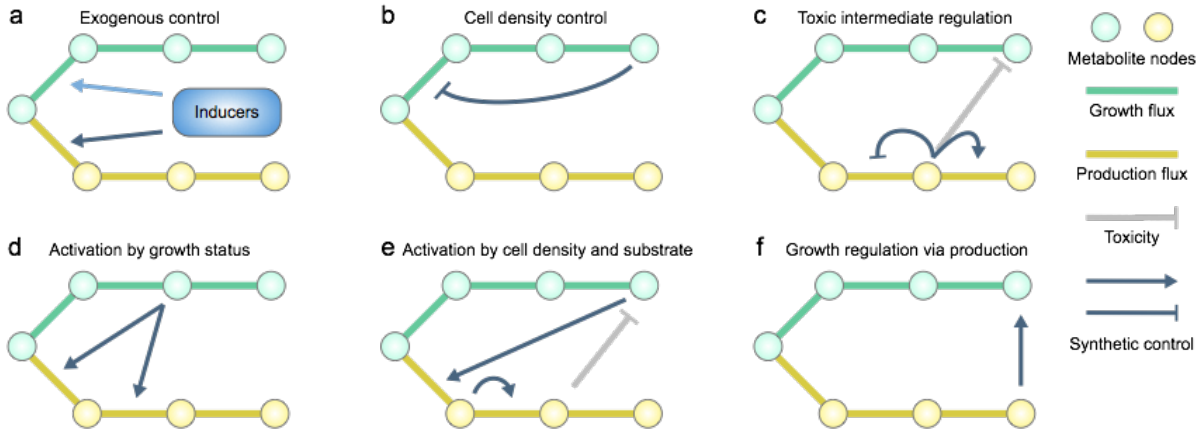
73. Zhang F, Carothers JM, Keasling JD (2012) Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. Nat Biotechnol 30:354-359.

74. Zhang F, Keasling J (2011) Biosensors and their applications in microbial metabolic engineering. Trends Microbiol 19:323-329.

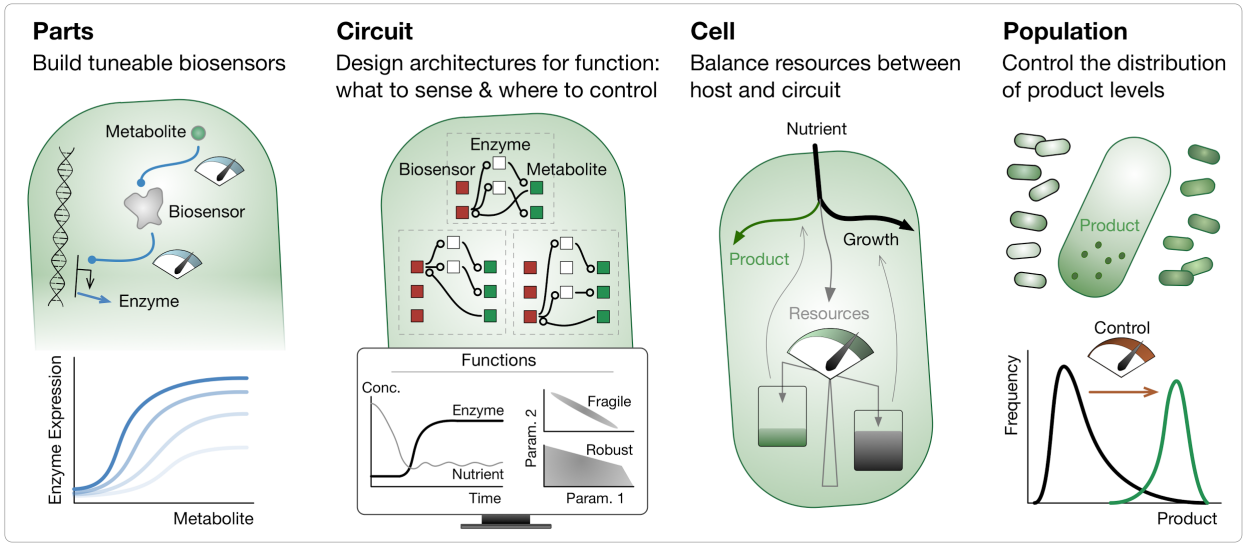
75. Zhang J, Jensen MK, Keasling JD (2015) Development of biosensors and their application in metabolic engineering. Curr Opin Chem Biol 28:1-8.

76. Zhou L-B, Zeng A-P (2015) Exploring lysine riboswitch for metabolic flux control and improvement of L-lysine synthesis in Corynebacterium glutamicum. ACS Synth Biol 4:729-734.

Figures



519 **Figure 1. Engineered control strategies of metabolic pathway in bioproduction.** To abstract
520 the designs of different dynamic control strategies, we represent cell growth and product
521 biosynthesis as two linear fluxes that branch from the same precursor metabolite, where growth
522 encompasses fluxes towards essential metabolites (e.g. TCA cycle, amino acid biosynthesis,
523 nucleotide biosynthesis, membrane biosynthesis). (a) Using inducers to control flux from the
524 branch point [69]. (b) Using QS systems to control growth flux by negative feedback circuits
525 [24,60]. (c) Using metabolite-responsive regulators to control toxic intermediate levels
526 [73,18,37,70]. (d) Using growth flux to activate the production pathway [22]. (e) Using
527 metabolite levels and growth status, which accounts for toxic effects, to regulate the production
528 pathway [38]. (f) Using product level to control survival of the cells [68].



529

530 **Figure 2. Challenges for designing dynamic control circuits at various levels.** These
531 challenges include how to tune parts to obtain desired dose-response functions, when control is
532 actuated by riboswitches [6,52] or transcription factors [40,63]; how regulatory architectures
533 affect dynamics [17,46,61] and robustness [43], as learned from models of natural control
534 systems; how to balance limited resources between growth and production, studied theoretically

535 [34,67]; and how to control cell-cell heterogeneity for sustainable and efficient production,
536 studied theoretically [45] and experimentally [68].

537

538