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Engineering metabolism through dynamic control

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Metabolic engineering has proven crucial for the microbial production of valuable chemicals. Due to the rapid development of tools in synthetic biology, there has been recent interest in the dynamic regulation of flux through metabolic pathways to overcome some of the issues arising from traditional strategies lacking dynamic control. There are many diverse implementations of dynamic control, with a range of metabolite sensors and inducers being used. Furthermore, control has been implemented at the transcriptional, translational and post-translational levels. Each of these levels have unique sets of engineering tools, and allow for control at different dynamic time-scales. In order to extend the applications of dynamic control, new tools are required to improve the dynamics of regulatory circuits. Further study and characterization of circuit robustness is also needed to improve their applicability to industry. The successful implementation of dynamic control, using technologies that are amenable to commercialization, will be a fundamental step in advancing metabolic engineering.

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Introduction

Metabolic engineering of microorganisms has proven crucial for improving their ability to produce valuable products, such as pharmaceuticals [1,2], nutraceuticals [3], fuels [4] and commodity chemicals [5–7]. The field of metabolic engineering has been accelerated with the introduction of new computational and experimental methods for the design and construction of plasmids [8–15] and strains [16–20]. Furthermore, synthetic biology has improved our understanding of genetic circuits [21,22] and their applicability to industry [23], while providing new tools, such as rapid and cost effective gene synthesis and sequencing, to expedite the design-build-test cycle.

To produce commercially viable strains, three crucial variables must be optimized: yield, titer and productivity. Heuristic targets proposed by Van Dien are 80% of theoretical maximum yield, 50 g/L and 3 g/(L h), respectively, for products near $1.00/lb [24]. Of these metrics, yield has classically held high priority, and this is particularly exemplified in the development of strain design algorithms (Box 1). These classical metabolic engineering methods have been reviewed at length [25].

One of the first papers to discuss the merits of titer and productivity, early in the strain design workflow, was presented by Zhuang *et al.* [26•]. They demonstrated that a strain designed for optimal yield results in suboptimal productivity, and discussed the inherent trade-off between the two. To overcome this issue, they developed an algorithm that takes into account productivity and titer, in addition to yield (DySSCo). However, this algorithm, along with most others, assumes that enzymes will not be dynamically controlled. We refer to this as *static metabolic engineering*.

In most of these strategies, product pathway flux is maximized. Since these pathways can drain metabolites used in biomass synthesis, this leads to a trade-off between growth and the production of the desired compound (Figure 1). Hence, most strategies to improve yield will also result in strains with low volumetric productivity, due to impaired growth rates. Slow growth rates can also be a result of poor cofactor balance [27], the accumulation of toxic intermediates [28], or an inefficient metabolic network resulting from the elimination of byproducts [29]. In strains with near optimal theoretical yield, optimization of the metabolic network alone is insufficient to significantly improve growth rate, as all substrate is diverted to product; this effect is evident when drawing from an early glycolytic metabolite [30,31•]. Slow growth rates can result in low volumetric productivities and high capital costs for commercial plants, and this must be addressed to ensure commercial viability.

Although growth rates can also be improved using adaptive evolution [29], such a strategy cannot be applied in all cases; the growth rate may not improve to acceptable levels or the initial growth rate can be too low for successful adaptive evolution. Another approach to overcoming the

Glossary
Capital cost: the cost associated with building a commercial fermentation plant.
Deregulation: the modification of native gene regulation through methods such as deletion, attenuation or overexpression.
Dynamic metabolic engineering: the practice of engineering microorganisms to respond to a changing intracellular (e.g. metabolite concentration) or extracellular (e.g. inducer concentration) environment.
Continuous control: control strategies which measure and control variables continuously.
Flux: the flow of metabolites through metabolic pathways (mmol/(gdw h)).
Growth stage: the stage in a batch fermentation in which optimal growth (wild-type growth rate) is targeted.
On–off control: control strategies which are implemented as either fully on or fully off, with no intermediate states (e.g. inducer addition).
Production stage: the stage in a batch fermentation in which maximal flux of the product is targeted.
Productivity: overall rate of production for the entire batch, the concentration of product per unit time (g/(L h)).
Static metabolic engineering: the practice of engineering microorganisms with genetic circuits lacking engineered dynamic control elements.
Titer: concentration of product at the end of a batch ($g_{product}$/L).
Yield: mass of product formed per mass of substrate consumed ($g_{product}/g_{substrate}$).

deleterious effects of genetic modifications is the use of two-stage dynamic control, whereby fluxes can be restored to wild-type distributions to improve growth rates in a growth stage, followed by a production stage with maximal flux through the product pathway [32,33]. To demonstrate the benefit of dynamic control, we have simulated and compared the productivity for static strategies and two-stage dynamic control using dynamic flux balance analysis [34]. These results show that the use of a dynamic strategy has the potential to further improve the productivity for any hypothetical strain designed using the previously described DySSCo strategy (Figure 2).

In this review, we focus on applications of dynamic metabolic engineering strategies and the different approaches for their implementation. We also highlight some challenges in the context of metabolic and regulatory network dynamics.

Restoring the wild-phenotype in mutant strains

To successfully implement two-stage fermentation and address low growth rates, we require a wild-type flux distribution in the growth stage, and maximal flux through the product pathway in the production stage [32,33]. In order to take advantage of the benefits of each phase, biomass can be quickly generated in the growth phase, before switching to a production phase. To restore a wild-type flux distribution in a mutant strain, an efficient strategy is to eliminate the effect of any genetic modifications. This restoration can be achieved through wild-type level expression of any native genes that have been deregulated, and repression of any heterologous pathways. Following the growth stage, the genetic manipulations must be restored in order to maximize the production rate.

Alternatively, in some cases, growth can be improved by modifying environmental conditions, including dissolved oxygen concentration, inducer concentration, and pH. In these scenarios it is possible to improve productivity by implementing dynamic control at the process level by controlling fermentation conditions; most of the early attempts to implement dynamic control utilized process level methods. More recently, owing largely to progress in synthetic biology, fluxes can be regulated dynamically by controlling the expression of key enzymes using genetic circuits. These methods will be described in the following section.

Dynamic control strategies

Control of fermentation conditions

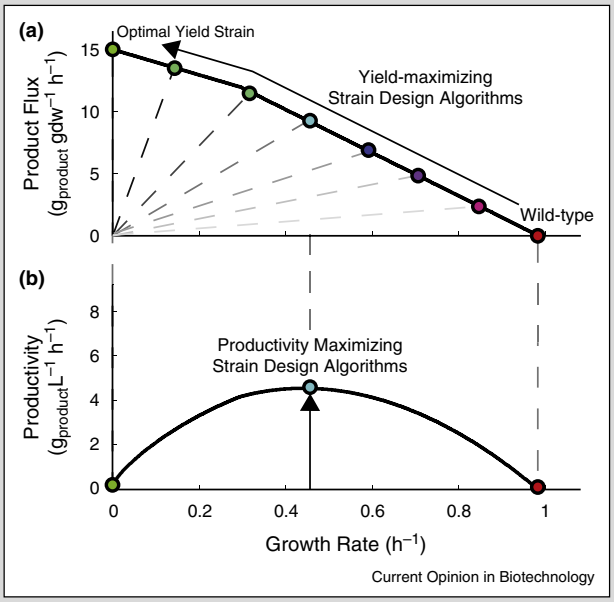
One common implementation of two-stage fermentation at the process level is to follow an aerobic growth stage by an anaerobic production stage. This strategy has been applied for lactic acid production, where single-stage anaerobic strategies (using minimal media) have productivities ranging from 0.27 to 0.33 g/(L h) [35], and can be improved approximately 10-fold using a two-stage strategy, reaching a productivity of 3.32 g/(L h) [36]. More recently, process level control has been applied for 1,4-butanediol production; cells were grown aerobically to an OD_{600} of approximately 10 before switching to microaerobic conditions and inducing pathway gene expression using isopropyl-1-β-thiogalactopyranoside (IPTG) [7].

An alternative is to use pH for the control of the growth and production phases. For example, α-ketoglutarate was produced in *Yarrowia lipolytica* using two-stage fermentation; a pH of 5.0 and 50% air saturation (aerobic conditions) was used for the growth phase, while a switch to pH 3.8 and 10% air saturation (microaerobic conditions) was used for the production stage [37]. A two-stage strategy is also common in the recombinant protein production industry, where protein production can significantly reduce the growth rate of the production host. Thus, the production stage is induced with IPTG, or other inducers, after reaching optimal cell density [38].

Two-stage fermentation has proven successful for anaerobic products and high-value proteins; however, it has not been thoroughly explored for lower-value products, or for products with pathways which are difficult to link to a process level parameter. In these cases, it is cost prohibitive to use inducers, such as IPTG, and it may not be possible to use oxygen concentration, or pH, as a trigger to switch between states. Furthermore, aerobic growth of strains designed for anaerobic production will not necessarily restore wild-type growth rates, especially when heterologous pathways draw significant flux from biomass

Box 1 Strain design algorithms

Metabolic engineering strategies can be guided by strain design algorithms [85,86]. These algorithms have placed most emphasis on the improvement of yield, with little discussion of productivity. In yield-maximizing strain design algorithms, genetic engineering strategies are proposed to improve product yield, at the expense of growth rate (a, solid arrow). These algorithms suggest manipulations which can be implemented using static techniques, such as static gene overexpression or deletion; therefore, no dynamic control of pathways will be present. More recently, algorithms which consider productivity have been developed [26•]. These algorithms use dynamic flux balance analysis (dFBA) [34] in order to estimate product concentrations throughout a fermentation batch. Using this strategy, many operating points can be established to generate hypothetical strains with a defined product flux and growth rate (a, dots). These strains are then evaluated for productivity, yield and titer, to find an optimal strain maximizing an objective which considers these three metrics (b).



precursors. To overcome these issues, dynamic control strategies have been proposed to allow for two-stage fermentation and dynamic control of gene expression [39].

Genetic circuits for on-off control of two-stage fermentation

Dynamic metabolic control relies largely on advances in synthetic biology to create genetic sensors and actuators [40]; this has led to diverse strategies for its implementation. Constraint-based metabolic models of *Escherichia coli* and dynamic flux balance analysis (dFBA) [41] were used to assess the applicability of two-stage fermentation, for the specific cases of ethanol and glycerol production. Using this strategy, it was shown that controlling gene expression in two-stages has the potential to improve productivity [32].

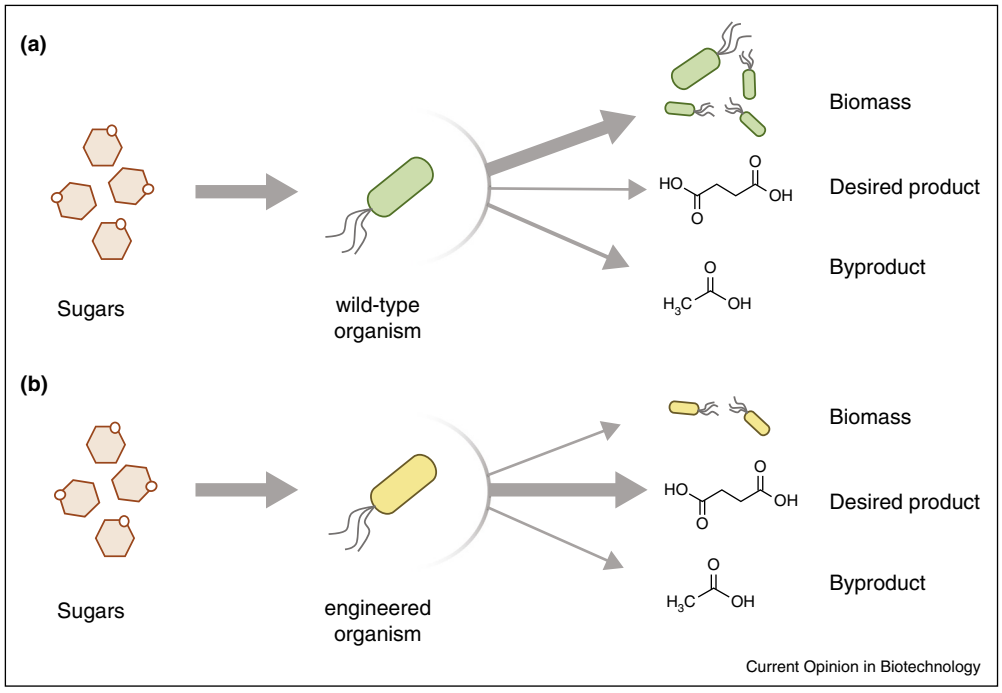
To implement this system, Anesiadis *et al.* proposed using quorum sensing (Figure 3a) [33], as described by Kobayashi *et al.* [42], and demonstrated the feasibility of this system *in silico*. Another quorum sensing system, using a pheromone and RNA interference (RNAi), was developed for para-hydroxybenzoic acid (pHBA) production in *Saccharomyces cerevisiae*. Production pathway genes are repressed, while chorismate mutase (ARO7), a gene required for efficient growth, is expressed. When the crucial cell density is reached, ARO7 is knocked down using RNAi, while production pathway genes are expressed (Figure 3b) [43].

On-off genetic control was also implemented using temperature or inducers as triggers [44]. The P_r and P_L promoters from the bacteriophage λ were used to control the native lactate dehydrogenase gene (ldhA), such that growth at 33 °C repressed ldhA and was able to increase biomass yield by 10% (as compared to static strategies), while a switch to 42 °C induced expression of ldhA and increased lactate production (Figure 3c) [44]. This strategy improved productivity by 30%, when compared to two-stage strategies without genetic manipulation [36]. A similar switch was designed for isopropanol production; the growth stage was achieved by expressing citrate synthase (gltA), subject to a tetR/lacI based inverter, which repressed pathway genes and allowed for high growth rates. Upon addition of IPTG, gltA was repressed and pathway genes were expressed to redirect flux to isopropanol production (Figure 3d) [45]. Another implementation of two-stage dynamic control used engineered antisense RNA (Figure 3e), or an inverting gene circuit, to control glucokinase expression for the production of gluconate [31•]. The latter system reduced growth rate and carbon waste to acetate by 50% by redirecting flux to the heterologous pathway.

Genetic circuits for continuous control using metabolite sensors

The explicit two-stage strategies defined above are typically on-off strategies, with a single explicit switch within the batch. Alternatively, continuous control has the benefit of being able to dynamically sense the environment and control metabolism accordingly, thereby allowing cells to respond to variations within each batch and within the non-uniform environment of an industrial fermenter. The earliest such strategy was proposed by Farmer & Liao, who demonstrated the use of dynamic control to improve the yield and productivity for the production of lycopene [46]. An acetyl phosphate-activated sensor was used as a marker for excess glycolytic flux; this sensor was used to drive the expression of genes responsible for lycopene synthesis (Figure 3f). More recently, dynamic control has been used to upregulate efflux pumps upon detection of toxic metabolites, improving yield using synthetic feedback loops [47–49]. Dynamic sensors have also been discovered using transcriptome analysis, by identifying promoters that are sensitive to pathway intermediates and using them to control biosynthesis pathways (Figure 3g, [50••]; Figure 3h, [51•]). A similar

Figure 1



Substrate partitioning of wild-type and engineered organisms. The relative amount of flux for each product is depicted by the arrow thickness. **(a)** Wild-type organisms mostly produce biomass with minimal or no desired product and a variable amount of byproducts (based on the selected organism and growth conditions). **(b)** Engineered organisms generally produce a low amount of biomass and byproducts, and will mostly produce the desired product. Since the maximum total flux is limited by the substrate uptake rate, there is an inherent trade-off between flux towards biomass, and the desired product.

strategy was applied using malonyl-CoA sensitive promoters, which are based on the fapR protein from *B. subtilis* [52]. In the presence of high malonyl-CoA levels, the malonyl-CoA production pathway is downregulated while the malonyl-CoA consumption pathway is upregulated; the inverse is true for low level of malonyl-CoA (Figure 3i) [53••].

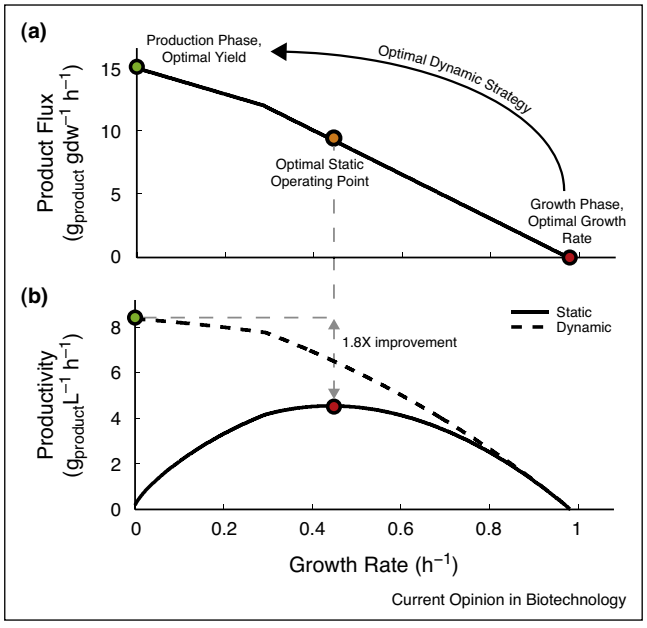
Although these dynamic strategies have proven successful, they are difficult to extend to a multitude of products. In many cases, sensors and actuators will need to be derived for each compound of interest, which can be time consuming. Two-stage fermentation is an alternative approach to feedback control strategies, and both techniques can be combined. Using two-stage fermentation subject to an on-off dynamic controller, we can explicitly define the optimal switching time based on known fermentation characteristics, such as the ideal batch time and growth kinetics of an organism. Then, continuous control can be used during the production stage to finely tune gene expression. However, optimization of the dynamic controller to regulate the expression of metabolic pathways, combined with dynamic control of the external environment, will require sufficiently detailed mathematical models [54•] and improved genetic circuits. This represents an opportunity for further research and motivates the integration of systems and synthetic biology.

Challenges in the genetic implementation of dynamic control

The regulation of gene expression in the product and growth related pathways, along with many other dynamic control strategies, can be accomplished at the transcriptional (DNA), translational (mRNA) or post-translational (protein) level. Many potential systems are discussed in a recent synthetic biology review [55]. In the simple case of eliminating flux through competing pathways to produce a compound of interest, the proteins associated with these competing pathways must be either inactive or absent. To control their activity, transcription can be reduced using well studied techniques, including inducible and repressible promoters, and translation can be controlled using several RNA regulatory mechanisms [56–62]. Alternatively, the protein can be inactivated through targeted degradation using degradation tags [63,64], or specific inactivation, such as acetylation [65,66] or phosphorylation [67,68]. A recent review describes many of these strategies in natural systems [69].

The crucial differences between these methods are the rate at which they act, and the tools available to engineer

Figure 2



Comparison of static and dynamic metabolic engineering strategies to improve productivity. **(a)** Production envelope for succinate production. **(b)** Productivity comparison between the static strategy (solid line) and the dynamic strategy (dashed line) for strains along the production envelope. Hypothetical operating points were defined along the production envelope (a, solid line), to define strains with a specific product flux and growth rate. dFBA [34] was used to estimate product profiles. The productivities for static strategies assumed a single-stage fermentation, with no dynamic gene regulation [26•]. The dynamic strategy implies two-stage fermentation, with wild-type growth rates in the first stage and a production stage with product fluxes corresponding to each hypothetical strain. Simulations were performed using a genome-scale metabolic model (iJO1366 [84]), 90 g/L starting glucose, 10 mmol/(gdw h) glucose uptake rate and succinate as the target metabolite.

them. When inducing pathway expression, transcription and translation are required and each step is on the order of minutes [70]; however, eliminating specific pathway flux requires the degradation of the associated biomacromolecules, which is much slower. Any mRNA or proteins which are present in the cell must be degraded using the natural cell machinery, and typical half-lives for mRNA are on the order of 5 min for *E. coli* [71] and 20 min for *S. cerevisiae* [72], whereas protein half-lives are on the order of hours [73–75]. Transcriptional regulation is the best studied of these three targets; however, it will exhibit the slowest response. Techniques to control translation would suffer from similar problems as with transcriptional regulation, but with slightly quicker dynamics, since transcription is not required; however, the tools to implement translational control are not as well characterized. Transcription-based dynamic control circuits are appropriate when the dynamics are on the order of hours (e.g. cell density sensing via quorum sensing circuits); however, the application of these circuits for control based on metabolite sensing can be challenging, given the fast time scales involved in metabolite turnover, on the order of 1 min [76].

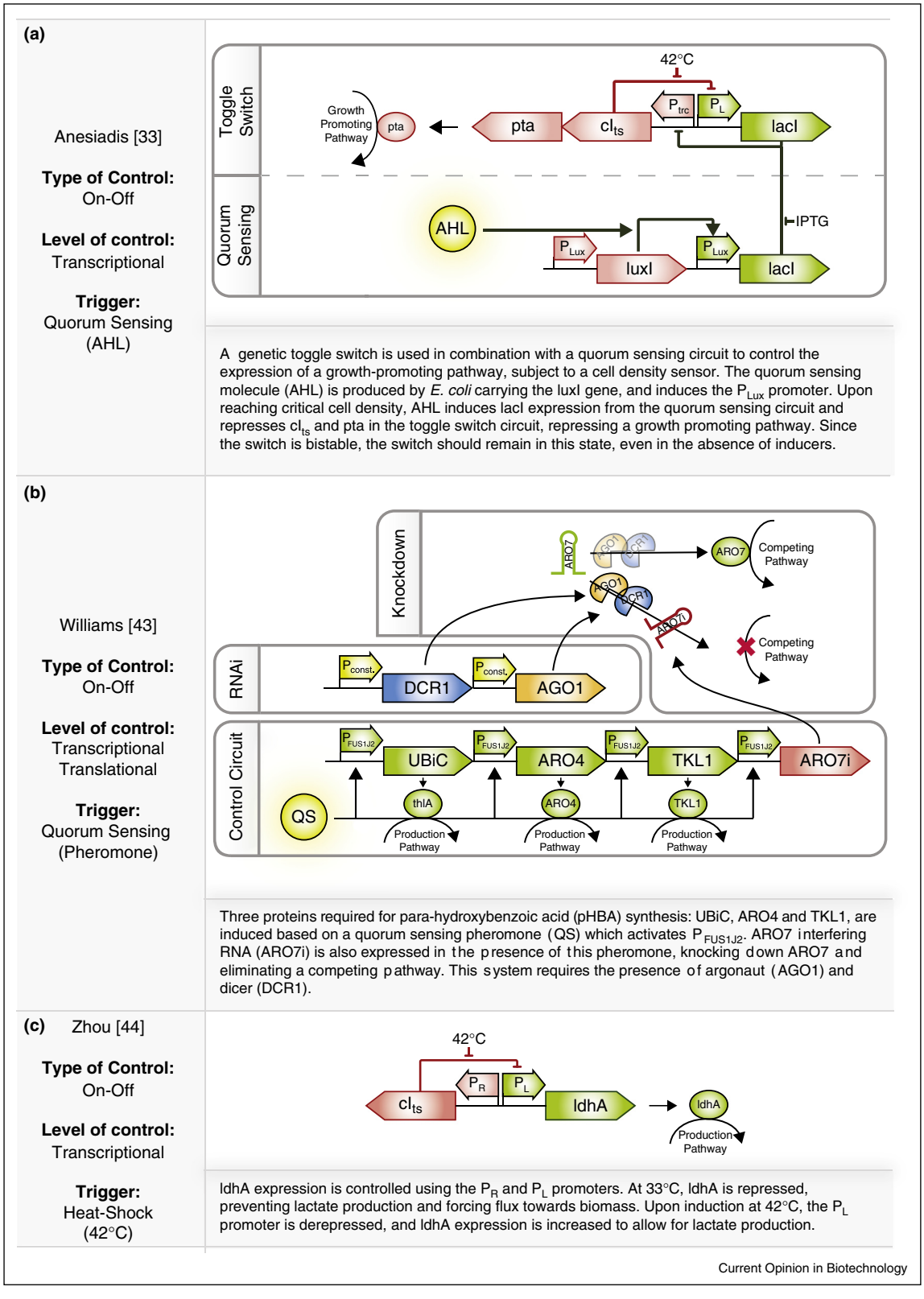
Post-translational control would exhibit fast dynamics, since proteins can be rapidly activated and inactivated, on the order of 1–100 μs [70]. Thus, they would have immediate effects on the flux through associated pathways. The speed of this system is exemplified in signaling cascades and in allosteric regulation of amino acid pathways, where a quick response is required to control the amino acid pools [77]. However, many target enzymes cannot be natively controlled at the protein level, and protein engineering is required to make them amenable to regulation. This is a more challenging task than DNA or mRNA level control since effective tools are lacking. In the case of eliminating flux through a pathway, by inactivating key pathway enzymes of interest, the dynamics can be improved by introducing degradation tags to the proteins [63]. However, this approach increases the burden on the cell due to continuous degradation and lower steady-state protein levels. To maintain steady state protein levels, transcription and translation rates must be increased, leading to a futile cycle in which proteins are continuously produced and degraded. Hence, there is a need for new tools for post-translational control and additional research to understand the metabolic burden imposed by these dynamic control circuits, especially when degradation tags are introduced.

Furthermore, successful implementation of regulatory circuits at the transcriptional, translational or post-translation level, will require robust performance [78–80]. This requirement for robustness necessitates a clear understanding of circuit characteristics, including their input–output relationships [21], and their behavior in the diverse environments common to metabolic engineering [81–83]. In addition, tools for the design of biological circuits with faster response times (less than 1 min) will be needed to obtain more effective dynamic control, especially in continuous control systems. A key component of these circuits will be the construction of biosensors with fast measurement dynamics, along with actuators that implement the flux changes rapidly through protein-level control.

Outlook

With recent progress in synthetic biology, dynamic metabolic engineering has become increasingly attractive. This technique has the potential to improve crucial fermentation metrics, including yield, titer and productivity. Two of the most promising current technologies are using two-stage growth conditions and using dynamic feedback control systems to control pathway flux. Both of these systems have shown potential to improve not only productivity, but also yield and titers.

Figure 3



Genetic circuits for implementing dynamic control.

Figure 3 (Continued).

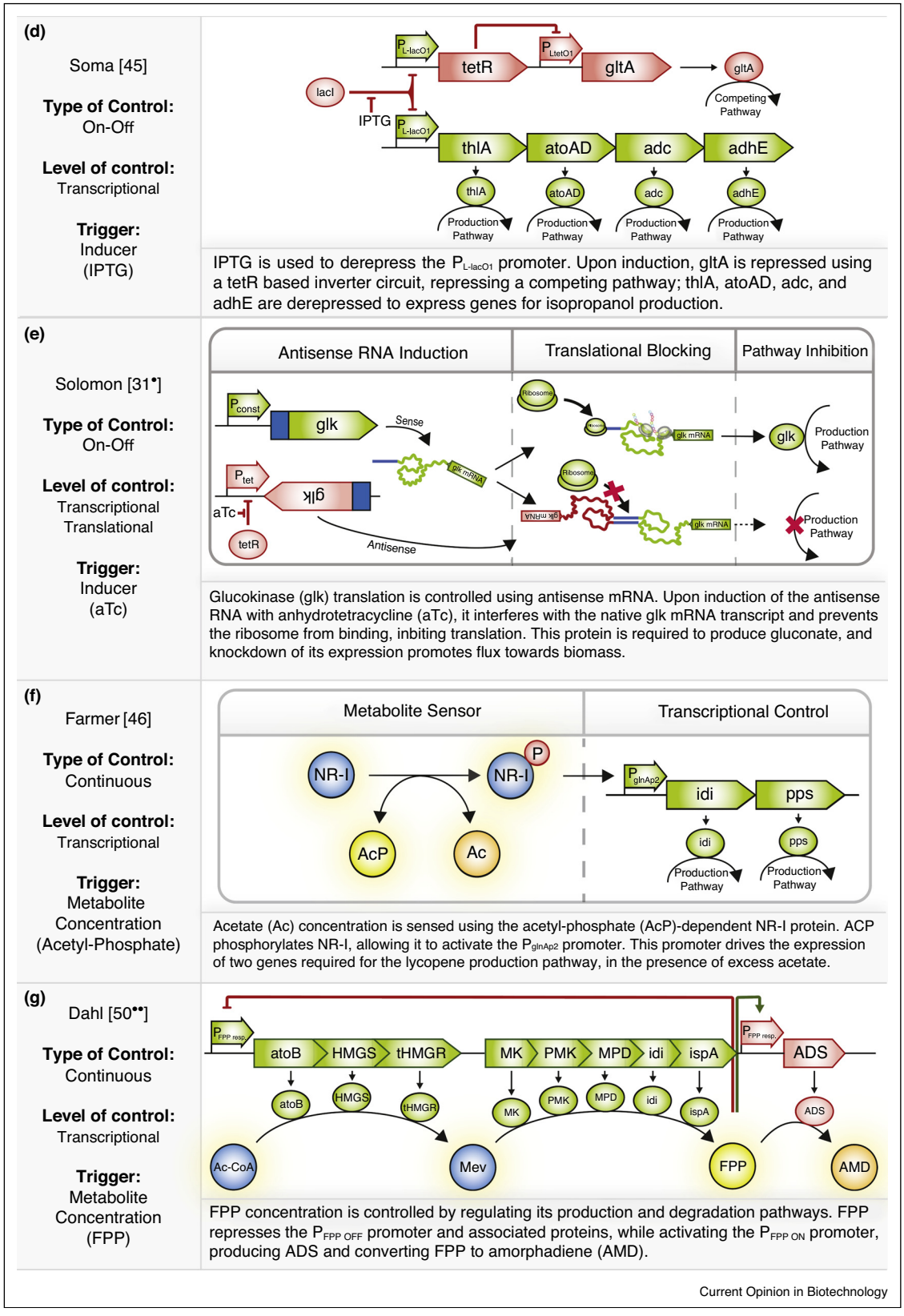
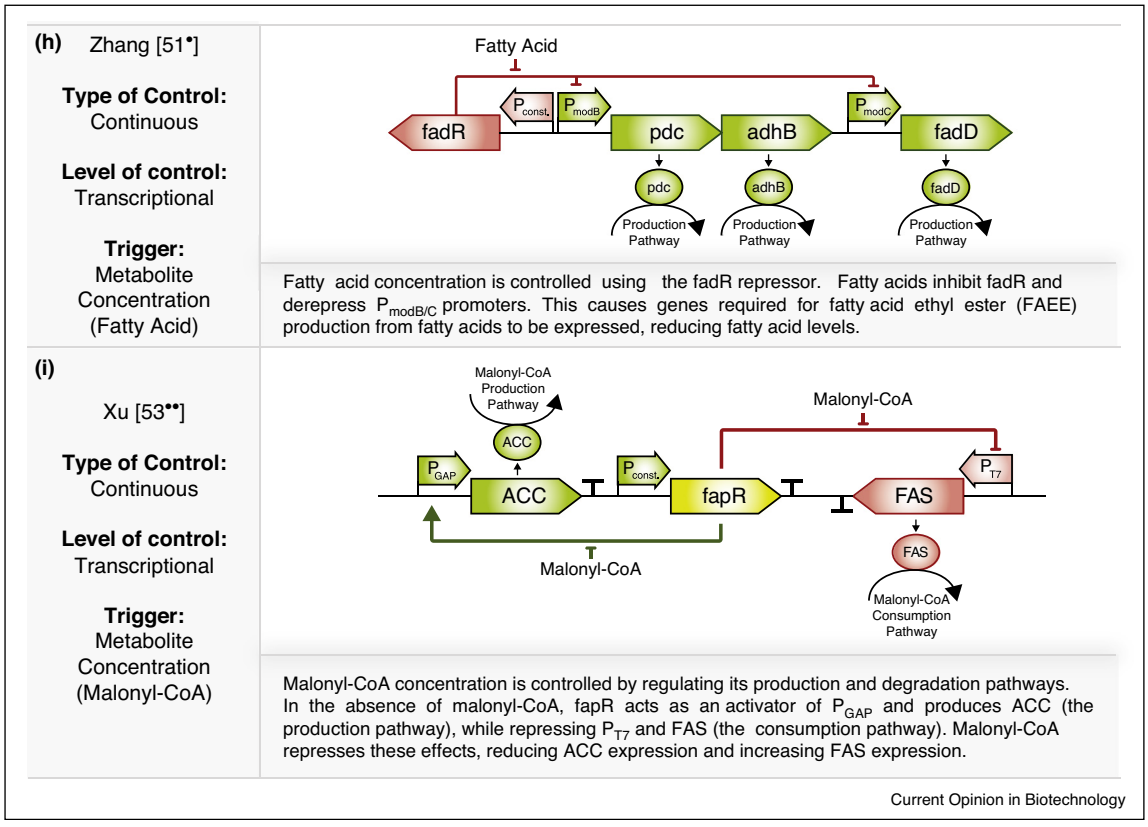


Figure 3 (Continued).



For dynamic control strategies to be more effective and widely applicable, two important developments are required. First, genetic circuits must become more robust, so that their function is preserved across strains and conditions. Second, the dynamics of sensing and actuation have to be rapid enough to enable effective control of metabolic pathway flux, especially in the case of continuous control. In the future, we expect that advances in synthetic biology can specifically address these issues and enable the widespread application of dynamic control in metabolic engineering and bioprocess development. Ultimately, given that dynamic metabolic engineering combines the dynamics of regulatory circuits, intracellular metabolism and the process environment, additional computational methods that integrate these aspects into a single framework will be needed to design and optimize such dynamic control strategies for the production of valuable products.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, McPhee D, Leavell MD, Tai A, Main A, Eng D *et al.*: **High-level semi-synthetic production of the potent antimalarial artemisinin**. *Nature* 2013, **496**:528-532.
2. Fossati E, Ekins A, Narcross L, Zhu Y, Falgueyret J-P, Beaudoin GAW, Facchini PJ, Martin VJJ: **Reconstitution of a 10-gene pathway for synthesis of the plant alkaloid dihydrosanguinarine in *Saccharomyces cerevisiae***. *Nat Commun* 2014, **5**:3283.
3. Santos CNS, Koffas M, Stephanopoulos G: **Optimization of a heterologous pathway for the production of flavonoids from glucose**. *Metab Eng* 2011, **13**:392-400.
4. Atsumi S, Hanai T, Liao JC: **Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels**. *Nature* 2008, **451**:86-89.
5. Nakamura CE, Whited GM: **Metabolic engineering for the microbial production of 1,3-propanediol**. *Curr Opin Biotechnol* 2003, **14**:454-459.
6. Song H, Lee SY: **Production of succinic acid by bacterial fermentation**. *Enzyme Microb Technol* 2006, **39**:352-361.
7. Yim H, Haselbeck R, Niu W, Pujol-Baxley C, Burgard A, Boldt J, Khandurina J, Trawick JD, Osterhout RE, Stephen R *et al.*:

Metabolic engineering of Escherichia coli for direct production of 1,4-butanediol. *Nat Chem Biol* 2011, **7**:445-452.

8. Salis H, Mirsky E, Voigt C: **Automated design of synthetic ribosome binding sites to control protein expression**. *Nat Biotechnol* 2009:27.

9. Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, Smith HO: **Enzymatic assembly of DNA molecules up to several hundred kilobases**. *Nat Methods* 2009, **6**:343-345.

10. Engler C, Kandzia R, Marillonnet S: **A one pot, one step, precision cloning method with high throughput capability**. *PLoS One* 2008, **3**:e3647.

11. Kok S, Stanton L, Slaby T: **Rapid and reliable DNA assembly via ligase cycling reaction**. *ACS Synth Biol* 2014, **3**:97-106.

12. Cunningham DS, Liu Z, Domagalski N, Koepsel RR, Ataai MM, Domach MM: **Pyruvate kinase-deficient *Escherichia coli* exhibits increased plasmid copy number and cyclic AMP levels**. *J Bacteriol* 2009, **191**:3041-3049.

13. Lee M, Aswani A, Han A, Tomlin CJ, Dueber JE: **Expression-level optimization of a multi-enzyme pathway in the absence of a high-throughput assay**. *Nucleic Acids Res* 2013, **10**.

14. Xu P, Vansiri A, Bhan N, Koffas MAG: **ePathBrick: a synthetic biology platform for engineering metabolic pathways in *E. coli***. *ACS Synth Biol* 2012, **1**:256-266.

15. Farasat I, Kushwaha M, Collens J, Easterbrook M, Guido M, Howard M Salis: **Efficient search, mapping, and optimization of multi-protein genetic systems in diverse bacteria**. *Mol Syst Biol* 2014, **10**.

16. Yang L, Cluett WR, Mahadevan R: **EMILiO: a fast algorithm for genome-scale strain design**. *Metab Eng* 2011, **13**:272-281.

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

18. Datsenko KA, Wanner BL: **One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products**. *Proc Natl Acad Sci U S A* 2000, **97**:6640-6645.

19. Song CW, Lee SY: **Rapid one-step inactivation of single or multiple genes in *Escherichia coli***. *Biotechnol J* 2013, **8**:776-784.

20. Zomorrodi AR, Suthers PF, Ranganathan S, Maranas CD: **Mathematical optimization applications in metabolic networks**. *Metab Eng* 2012, **14**:672-686.

21. Olson EJ, Hartsough LA, Landry BP, Shroff R, Tabor JJ: **Characterizing bacterial gene circuit dynamics with optically programmed gene expression signals**. *Nat Methods* 2014, **11**:449-455.

22. Moon TS, Lou C, Tamsir A, Stanton BC, Voigt C: **Genetic programs constructed from layered logic gates in single cells**. *Nature* 2012, **491**:249-253.

23. Moser F, Broers NJ, Hartmans S, Tamsir A, Kerkman R, Roubos JA, Bovenberg R, Voigt CA: **Genetic circuit performance under conditions relevant for industrial bioreactors**. *ACS Synth Biol* 2012, **1**:555-564.

24. Van Dien S: **From the first drop to the first truckload: commercialization of microbial processes for renewable chemicals**. *Curr Opin Biotechnol* 2013 http://dx.doi.org/10.1016/j.copbio.2013.03.002.

25. Lee JW, Na D, Park JM, Lee J, Choi S, Lee SY: **Systems metabolic engineering of microorganisms for natural and non-natural chemicals**. *Nat Chem Biol* 2012, **8**:536-546.

26. • Zhuang K, Yang L, Cluett WR, Mahadevan R: **Dynamic strain scanning optimization: an efficient strain design strategy for balanced yield, titer, and productivity. DySScO strategy for strain design**. *BMC Biotechnol* 2013, **13**:8.
To overcome some of the issues resulting from strain design algorithms which solely maximize yield, the authors introduce a new algorithm which also accounts for productivity and titer. This work is one of the first to consider yield, titer, and productivity in a strain design algorithm.

27. Charusanti P, Conrad TM, Knight EM, Venkataraman K, Fong NL, Xie B, Gao Y, Palsson BØ: **Genetic basis of growth adaptation of *Escherichia coli* after deletion of pgi, a major metabolic gene**. *PLoS Genet* 2010, **6**:e1001186.

28. Kizer L, Pitera DJ, Pfleger BF, Keasling JD: **Application of functional genomics to pathway optimization for increased isoprenoid production**. *Appl Environ Microbiol* 2008, **74**:3229-3241.

29. Fong SS, Burgard AP, Herring CD, Knight EM, Blattner FR, Maranas CD, Palsson BO: **In silico design and adaptive evolution of *Escherichia coli* for production of lactic acid**. *Biotechnol Bioeng* 2005, **91**:643-648.

30. Solomon KV, Moon TS, Ma B, Sanders TM, Prather KLJ: **Tuning primary metabolism for heterologous pathway productivity**. *ACS Synth Biol* 2013, **2**:126-135.

31. • Solomon KV, Sanders TM, Prather KLJ: **A dynamic metabolite valve for the control of central carbon metabolism**. *Metab Eng* 2012, **14**:661-671.
In this article, gluconate is produced using a heterologous pathway which draws flux from an early glycolytic metabolite. To overcome issues resulting from such a strategy, including poor biomass accumulation, the authors design a dynamic strategy for pathway expression. Using antisense RNA, or an inverting gene circuit, the authors control flux through the heterologous gluconate pathway. Gluconate production provides a clear example of a heterologous pathway which draws flux away from crucial biomass precursors, and this works shows the implementation of dynamic control to mediate these effects.

32. Gadkar KG, Doyle Iii FJ, Edwards JS, Mahadevan R: **Estimating optimal profiles of genetic alterations using constraint-based models**. *Biotechnol Bioeng* 2005, **89**:243-251.

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

34. Varma A, Palsson BO: **Stoichiometric flux balance models quantitatively predict growth and metabolic by-product secretion in wild-type *Escherichia coli* W3110**. *Appl Environ Microbiol* 1994, **60**:3724-3731.

35. Zhou S, Yomano LP, Shanmugam KT, Ingram LO: **Fermentation of 10% (w/v) sugar to D: (−)-lactate by engineered *Escherichia coli* B**. *Biotechnol Lett* 2005, **27**:1891-1896.

36. Zhou L, Zuo Z-R, Chen X-Z, Niu D-D, Tian K-M, Prior BA, Shen W, Shi G-Y, Singh S, Wang Z-X: **Evaluation of genetic manipulation strategies on D-lactate production by *Escherichia coli***. *Curr Microbiol* 2011, **62**:981-989.

37. Yovkova V, Otto C, Aurich A, Mauersberger S, Barth G: **Engineering the α-ketoglutarate overproduction from raw glycerol by overexpression of the genes encoding NADP+-dependent isocitrate dehydrogenase and pyruvate carboxylase in *Yarrowia lipolytica***. *Appl Microbiol Biotechnol* 2014, **98**:2003-2013.

38. Rosano G, Ceccarelli E: **Recombinant protein expression in *Escherichia coli*: advances and challenges**. *Front Microbiol* 2014:5.

39. Holtz WJ, Keasling JD: **Engineering static and dynamic control of synthetic pathways**. *Cell* 2010, **140**:19-23.

40. Michener JK, Thodey K, Liang JC, Smolke CD: **Applications of genetically-encoded biosensors for the construction and control of biosynthetic pathways**. *Metab Eng* 2012, **14**:212-222.

41. Mahadevan R, Edwards JS, Doyle FJ: **Dynamic flux balance analysis of diauxic growth in Escherichia coli**. *Biophys J* 2002, **83**:1331-1340.

42. Kobayashi H, Kaern M, Araki M, Chung K, Gardner TS, Cantor CR, Collins JJ: **Programmable cells: interfacing natural and engineered gene networks**. *Proc Natl Acad Sci U S A* 2004, **101**:8414-8419.

43. Williams TC, Averesch NJH, Lekieffre N, Winter G, Vickers CE, Nielsen LK, Kromer JO: **Quorum-sensing linked RNAi for dynamic pathway control in *Saccharomyces cerevisiae***. *Metab Eng* 2014.

44. Zhou L, Niu D-D, Tian K-M, Chen X-Z, Prior Ba, Shen W, Shi G-Y, Singh S, Wang Z-X: **Genetically switched D-lactate production in *Escherichia coli***. *Metab Eng* 2012, **14**:560-568.

45. 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 http://dx.doi.org/10.1016/j.ymben.2014.02.008.

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

47. Dunlop MJ, Dossani ZY, Szmidt HL, Chu HC, Lee TS, Keasling JD, Hadi MZ, Mukhopadhyay A: **Engineering microbial biofuel tolerance and export using efflux pumps**. *Mol Syst Biol* 2011, **7**:487.

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

49. Harrison ME, Dunlop MJ: **Synthetic feedback loop model for increasing microbial biofuel production using a biosensor**. *Front Microbiol* 2012, **3**:360.

50. •• 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.

Using transcriptomics, the authors identify promoters which are sensitive to the accumulation of toxic intermediates. They use these promoters to control farnesyl pyrophosphate production in an isoprene pathway, and are able to improve amorphadiene production. This strategy can be applied to many products and is an important step in implementing dynamic control.

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

This article describes the implementation of a fatty acid sensor in order to control flux for the production of fatty acid ethyl ester (FAEE). Upon high levels of fatty acids, genes responsible fatty acid consumption, including upstream pathways, are activated in order to produce FAEE.

52. Xu P, Wang W, Li L, Bhan N, Zhang F, Koffas MAG: **Design and kinetic analysis of a hybrid promoter–regulator system for malonyl-CoA sensing in *Escherichia coli***. *ACS Chem Biol* 2013, **9**:451-458.

53. •• 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.

This article describes a strategy to control the concentration of an important intermediate metabolite, malonyl-CoA (mal-CoA). The authors use promoters which were activated by mal-CoA to induce a consumption pathway, and promoters which are repressed by mal-CoA to induce a production pathway. In this fashion, mal-CoA levels are regulated to improve fatty acid production. This is a clear demonstration of using dynamic control to regulate metabolite levels.

54. • Anesiadis N, Kobayashi H, Cluett WR, Mahadevan R: **Analysis and design of a genetic circuit for dynamic metabolic control**. *ACS Synth Biol* 2013, **2**:442-452.

This article describes the design of a bistable genetic circuit, which is activated by quorum sensing. The authors present a sensitivity analysis of this circuit for the production of serine, and identify key parameters which may affect the robust behavior of this circuit. This work presents a clear modeling framework which can be used to identify and address issues which may decrease circuit performance.

55. Khalil AS, Collins JJ: **Synthetic biology: applications come of age**. *Nat Rev Genet* 2010, **11**:367-379.

56. Mutalik VK, Qi L, Guimaraes JC, Lucks JB, Arkin AP: **Rationally designed families of orthogonal RNA regulators of translation**. *Nat Chem Biol* 2012, **8**:447-454.

57. Dixon N, Duncan JN, Geerlings T, Dunstan MS, McCarthy JEG, Leys D, Micklefield J: **Reengineering orthogonally selective riboswitches**. *Proc Natl Acad Sci U S A* 2010, **107**:2830-2835.

58. Lucks JB, Qi L, Mutalik VK, Wang D, Arkin AP: **Versatile RNA-sensing transcriptional regulators for engineering genetic networks**. *Proc Natl Acad Sci U S A* 2011, **108**:8617-8622.

59. Serganov A, Nudler E: **A decade of riboswitches**. *Cell* 2013, **152**:17-24.

60. Breaker RR: **Prospects for riboswitch discovery and analysis**. *Mol Cell* 2011, **43**:867-879.

61. Winkler WC, Breaker RR: **Genetic control by metabolite-binding riboswitches**. *Chembiochem* 2003, **4**:1024-1032.

62. Chang AL, Wolf JJ, Smolke CD: **Synthetic RNA switches as a tool for temporal and spatial control over gene expression**. *Curr Opin Biotechnol* 2012, **23**:679-688.

63. Keiler KC, Waller PR, Sauer RT: **Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA**. *Science* 1996, **271**:990-993.

64. 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**:11290-11295.

65. Wang Q, Zhang Y, Yang C, Xiong H, Lin Y, Yao J, Li H, Xie L, Zhao W, Yao Y *et al.*: **Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux**. *Science* 2010, **327**:1004-1007.

66. Zhang K, Zheng S, Yang J: **Comprehensive profiling of protein lysine acetylation in *Escherichia coli***. *J Proteome Res* 2013, **12**.

67. Ptacek J, Devgan G, Michaud G, Zhu H, Zhu X, Fasolo J, Guo H, Jona G, Breitkreutz A, Sopko R *et al.*: **Global analysis of protein phosphorylation in yeast**. *Nature* 2005, **438**:679-684.

68. Oliveira AP, Ludwig C, Picotti P, Kogadeeva M, Aebersold R, Sauer U: **Regulation of yeast central metabolism by enzyme phosphorylation**. *Mol Syst Biol* 2012, **8**:623.

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

70. Alon U: *An Introduction to Systems Biology*. Chapman and Hall/CRC; 2006.

71. Bernstein JA, Khodursky AB, Lin P-H, Lin-Chao S, Cohen SN: **Global analysis of mRNA decay and abundance in Escherichia coli at single-gene resolution using two-color fluorescent DNA microarrays**. *Proc Natl Acad Sci U S A* 2002, **99**:9697-9702.

72. Wang Y, Liu CL, Storey JD, Tibshirani RJ, Herschlag D, Brown PO: **Precision and functional specificity in mRNA decay**. *Proc Natl Acad Sci U S A* 2002, **99**:5860-5865.

73. Mosteller R, Goldstein R, Nishimoto K: **Metabolism of individual proteins in exponentially growing *Escherichia coli***. *J Biol Chem* 1980, **255**:2524-2532.

74. Larrabee K, Phillips J: **The relative rates of protein synthesis and degradation in a growing culture of *Escherichia coli***. *J Biol Chem* 1980, **25**:4125-4130.

75. Goldberg A, John ASt: **Intracellular protein degradation in mammalian and bacterial cells: Part 2**. *Annu Rev Biochem* 1976, **43**:747-803.

76. Nielsen J: *Metabolic Turnover*. 2002. eLS.

77. Jones E, Fink G: **Regulation of amino acid and nucleotide biosynthesis in yeast**. *The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression*. Cold Spring Harbor Laboratory Press; 1982, 181-299.

78. Qi L, Haurwitz R, Shao W: **RNA processing enables predictable programming of gene expression**. *Nat Biotechnol* 2012, **30**:1002-1006.

79. Lou C, Stanton B, Chen Y, Munsky B, Voigt C: **Ribozyme-based insulator parts buffer synthetic circuits from genetic context**. *Nat Biotechnol* 2012, **30**:1137-1142.

80. Bashor C, Collins J: **Insulating gene circuits from context by RNA processing**. *Nat Biotechnol* 2012, **30**:1061-1063.

81. Klumpp S, Zhang Z, Hwa T: **Growth rate-dependent global effects on gene expression in bacteria**. *Cell* 2009, **139**:1366-1375.

82. Scott M, Gunderson CW, Mateescu EM, Zhang Z, Hwa T: **Interdependence of cell growth and gene expression: origins and consequences**. *Science* 2010, **330**:1099-1102.

83. Klumpp S, Hwa T: **Bacterial growth: global effects on gene expression, growth feedback and proteome partition**. *Curr Opin Biotechnol* 2014, **28C**:96-102.

84. Orth JD, Conrad TM, Na J, Lerman JA, Nam H, Feist AM, Palsson BØ: **A comprehensive genome-scale reconstruction of *Escherichia coli* metabolism — 2011**. *Mol Syst Biol* 2011, **7**:535.

85. Lewis NE, Nagarajan H, Palsson BO: **Constraining the metabolic genotype–phenotype relationship using a phylogeny of in silico methods**. *Nat Rev Microbiol* 2012, **10**:291-305.

86. Von Kamp A, Klamt S: **Enumeration of smallest intervention strategies in genome-scale metabolic networks**. *PLoS Comput Biol* 2014, **10**:e1003378.