Leading Edge
Essay

Cell

Engineering Static and Dynamic Control of Synthetic Pathways

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DOI 10.1016/j.cell.2009.12.029

Maximizing the production of a desired small molecule is one of the primary goals in metabolic engineering. Recent advances in the nascent field of synthetic biology have increased the predictability of small-molecule production in engineered cells growing under constant conditions. The next frontier is to create synthetic pathways that adapt to changing environments.

Introduction

Metabolic engineering is the genetic manipulation of intracellular, enzyme-catalyzed, chemical reactions for the production of a desired molecule. For relatively simple products that require few gene manipulations to effect their production, natural or slightly modified control systems (such as promoters or ribosome-binding sites) have been widely used to control the expression of heterologous pathways or deregulate native genes. Most of these non-native controllers are static; that is, the level of gene expression is set without sensing changes in pathway output or cellular environment. Generally, these systems are optimized only for a particular environment and any deviations away from that set of conditions are likely to result in suboptimal productivity.

Metabolic engineering is being called upon to tackle increasingly demanding challenges, like the production of complicated pharmaceuticals and nutraceuticals that require very long metabolic pathways or the convergence of multiple heterologous pathways. For example, the pathway from acetyl-CoA to β-carotene has been implemented with 12 enzymatic steps, and the production of the anti-malaria drug precursor artemisinic acid from acetyl-CoA uses 10 enzymatic steps (Yoon et al., 2009; Ro et al., 2006). As heterologous pathways become larger and more complicated, optimizing them becomes increasingly difficult such that piecewise optimizations, where a subset of the pathway is optimized at a time, are utilized (Martin et al., 2003). Because segments of pathways are not independent, performing pathway optimization in sections and then combining the sections together can result in the final state of the cell being different from its state during the earlier optimizations. The ability to sense the output of each pathway segment and control of the levels of expression of pathway enzymes (or activities of pathway enzymes) would allow each pathway segment to optimize itself relative to the other pathway segments and the cell's native metabolism.

Another difficult, and timely, challenge in metabolic engineering is the production of molecules with a low profit margin (such as commodity chemicals and fuels), where the need to maximize yield and productivity are essential for economic viability (Zhang et al., 2008; Nakamura and Whited, 2003). The ability to sense critical metabolic intermediates and control levels of pathway gene expression could eliminate pathway bottlenecks or the accumulation of potentially toxic intermediates. Most of these molecules are produced by cells grown in large bioreactors where heterogeneities in the nutrient concentrations, oxygen levels, or pH are often the norm and markedly different from the conditions under which the strains were originally constructed and optimized (that is, in the uniform environment of the shaker flask or small fermenter) (Amanullah et al., 2004; Schmidt, 2005). In applications such as these, the ability to sense the environment in which a particular cell finds itself and respond could vastly improve production by the entire culture. Additional improvements in yield or productivity could be achieved if the pathway has the capacity to monitor and respond to the cell's growth phase and density.

In contrast to the static control engineered into most heterologous pathways, native metabolic pathways generally utilize dynamic regulatory networks to compensate for changing conditions by altering fluxes. That is, the turnover rate of molecules by an enzyme is altered, often by allosteric inhibition or by a negative feedback loop regulating expression of the enzyme. As the environment outside the cell changes (such as a change in nutrient availability or pH), the cell modulates its metabolic pathways dynamically to adjust fluxes so that required metabolic intermediates are delivered at the appropriate levels and times to optimize growth. Coupling sensory inputs with control devices allows for pathways to be dynamically controlled so that resources can be more efficiently utilized and productivity gains realized. By sensing cellular conditions and adjusting fluxes in a feedback loop,

the engineered cell can remain near the optimal productivity that corresponds to each set of conditions encountered.

The lack of dynamic controls in metabolic engineering is due in part to the complexity of designing and implementing pathways that both sense the environment and control the formation of product. Dynamic controls have the added requirements of a sensing component, a method of modulating flux, and a connection between those two elements. An additional complexity of dynamic control is the requirement for more parameters to determine transient behavior than would be required for static behavior. Thus, the benefits of dynamic control may not be perceived to outweigh the additional time and money required for implementation and testing.

The predisposition against working on system dynamics is a problem that extends beyond synthetic biology. The majority of molecular biology and biomedical research also focuses on steady-state conditions, and this is likely due to the increased costs and difficultly of analyzing dynamic properties. Growing interest in implementing dynamic controls will result in the development of new experimental and computational procedures. These new techniques could lead to a synergy between synthetic biology and molecular biology that rapidly advances research on dynamic systems. Recent work on the dynamics of native metabolic pathways demonstrates how molecular biology can provide insights into how to measure and optimize their responses (Chin et al., 2008).

Although there are barriers to the adoption of dynamic controls, advances in synthetic and systems biology will continue to lower these barriers and make dynamic controls more attractive. In particular, progress in modeling, computer-aided design, quantitative gene expression, and DNA synthesis and assembly will play important roles in enabling dynamic controls. And the development of advanced biological devices (for example, logic gates, bi-stable switches, counters, and ring oscillators) has resulted in the availability of a large number of well-characterized biological components that can be used to construct dynamic controllers. Many of these devices make use of standardized parts or interfaces that simplify functional composition and allow for rapid reuse of devices in new contexts. Hopefully these community-driven standards will result in a lowering of the costs involved in building complex systems. Not only will these well-characterized and standardized components aid the development of dynamic controls, they will also further static control systems, which are likely to be used extensively for controlling metabolic pathways long into the future.

Static Controls

Static controllers of flux are genetically encoded components that play a role in determining the level of flux through a pathway but do not sense cellular conditions and modulate the pathway flux based on the sensed information. There are many parameters that can be used to change the static control of a particular flux. Examples of such parameters are strength and type of constitutive promoter, ribosome binding site strength, or copy number of the vector.

There is a vast literature on promoters and transcription initiation. However, much of the application of promoters in metabolic engineering involves the use of natural or slightly modified, inducible promoters or constitutive promoters of different strengths. Precise quantitative data on promoter activities from relevant culture conditions are required for predicting gene expression levels to alter flux. Recently, there has been an effort to standardize a technique for measuring relative promoter strengths (Kelly et al., 2009). In this method, the fluorescence level from green fluorescent protein from a standard reference plasmid is compared to a plasmid with only the promoter changed. The ratio of the two expression levels is expected to remain constant if the measurements are repeated with a different host strain or growth condition. Therefore, if the fluorescence from the standard plasmid is measured under a unique set of conditions, then the transcription rate can be predicted for any promoter that has been previously measured in another context along with the reference promoter.

One major challenge in specifying the level of gene expression is designing the appropriate levels of translation. In native systems, the folding of mRNA near the ribosome-binding site (RBS) can strongly contribute to the gene expression level. Because the portion of the mRNA containing the RBS can interact with the gene-coding region, it is not trivial to predict how an RBS-gene pair will express based on their individual behavior in other contexts. The energetics of translation initiation in prokaryotes has been extensively studied. However, many of the existing models have an unacceptable failure rate or are difficult to use when designing systems. A thermodynamic model of translation initiation has been developed by Salis et al. (2009) to predict the relative level of translation initiation for a given mRNA. Using the nucleotide sequence of a gene and a relative translation initiation rate, the algorithm generates a corresponding RBS sequence that when incorporated into the construct will provide the desired level of gene expression. Use of such a tool reduces the unintentional introduction of inhibitory RNA secondary structures and allows for levels of gene expression to be easily and predictably adjusted.

Another method of achieving predictable translation levels is to use bicistronic operons, which have long been employed to ensure high levels of expression from arbitrary genes (Schoner et al., 1986). The existence of mRNA secondary structure between the second RBS and downstream gene does not cause translational inhibition, and therefore the translation initiation of the second gene is independent of its coding sequence (Kimura et al., 2005). The first gene can be a truncated protein that only exists to affect the translation of the second gene. Use of synthetic bicistronic systems has been limited to achieving high protein levels. However, there is the potential to create libraries of bicistronic static controllers of flux that cover a broad range of translation initiation rates. It has not yet been demonstrated that bicistronic constructs can give predictable and precise expression of the second gene; however they are likely to outperform pairings of genes with independently characterized RBSs.

Increasing enzyme expression levels is an easy route to achieving high fluxes with static controls; however, other methods may make more efficient use of

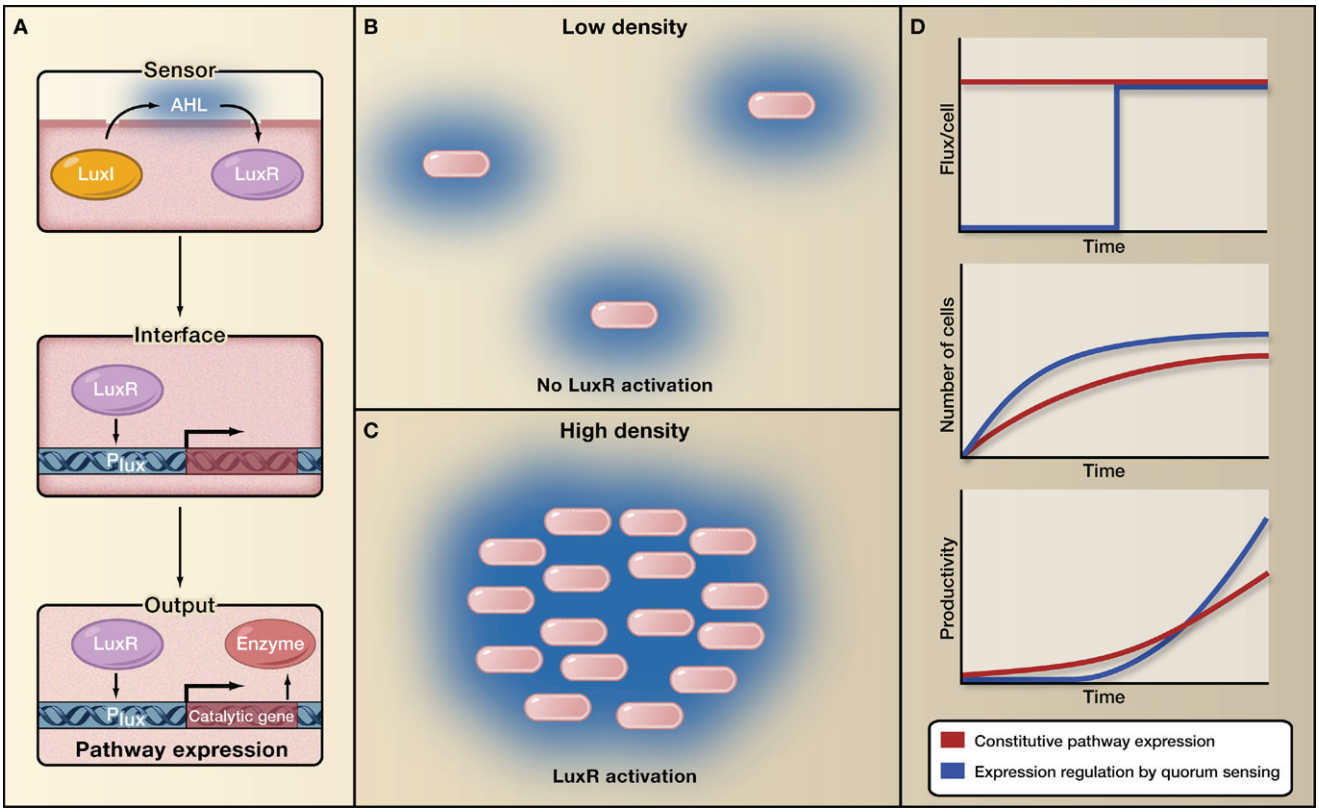

Figure 1. Dynamic Control of a Synthetic Pathway

A dynamic controller of flux can achieve higher productivity than constitutive expression of the proteins in the pathway. The dynamic system only expresses the proteins involved in the production pathway once a threshold cell density is reached.

(A) All dynamic controllers of flux contain a sensor, an output, and an interface between them.

(B) At low cell densities the diffusible molecule acyl-homoserine lactone produced by LuxI is too dilute to activate its receptor LuxR.

(C) Once a critical cell density is reached LuxR is activated and turns on expression of the pathway genes from the Lux promoter (P_{lux}).

(D) Plots showing how higher productivity can be obtained by delaying expression of the pathway until a high cell density is reached.

cellular resources. By localizing tagged pathway enzymes to a scaffold using protein-protein interaction domains, Dueber et al. (2009) have been able to increase the flux 77-fold through a biosynthetic pathway for mevalonate in the bacterium *Escherichia coli*. They use an engineered scaffold, containing mouse and rat protein-protein interaction domains, to increase the local concentration of the enzymes and prevent the accumulation of pathway intermediates that may be harmful to the cells.

Ideally, one would use a model to specify the gene expression levels needed to achieve the desired flux in any one particular reaction. The existence of genome-scale metabolic models has lead to the development of new computational algorithms for improving production strains. Microbes appear to maximize their formation of biomass, which often does not lead to high yields of the desired product. By altering or eliminating expression levels of native genes it is possible to improve a strain's yield or productivity. However, the number of possible combinatorial changes is too large to test in vivo and therefore in silico methods are used to predict the best set of modifications. These predictions are generally made using flux balance analysis, which makes an assumption of steady-state conditions in order to reduce computational complexity (Llaneras and Picó, 2008).

Because flux balance analysis does not incorporate any regulatory information, these methods can only suggest changes to expression levels of catalytic enzymes but not to regulatory genes. Another drawback of flux balance analysis is the absence of any kinetic information. Despite these short-comings, flux balance analysis has proven to be a useful tool for choosing where to implement static controllers of flux. Several algorithms have been published for determining targets for altered expression levels. To simplify calculations some methods only determine genes to be knocked out. The OptKnock framework uses linear programming techniques to efficiently find combinations of knockout candidates (Burgard et al., 2003). OptReg, an extension of the OptKnock framework, can select genes that should be expressed at higher levels, lower levels, or not at all (Pharkya and Maranas, 2006).

An alternative iterative search methodology was used by Alper et al. (2005a) for optimizing lycopene biosynthesis in *E. coli*. They first identified advantageous single knockouts and then additional knockouts were introduced in the single knockout strains, which were then tested for lycopene production. Strains with single, double, and triple knockouts

were created and tested in vivo (Alper et al., 2005a). For most strains they find good agreement between in silico and in vivo results. To explore the limits of flux balance analysis, Alper et al. performed a global transposon search, which found new knockout targets, and used a convenient screening method based on colony color to rapidly locate the advantageous knockouts. Although the transposon knockouts were able to improve production, they did not exceed the production of the best strain from flux balance analysis, nor did the combination of both knockout pools result in a new maximum level of lycopene production (Alper et al., 2005b). This illustrates that developing in silico methods for determining which regulatory genes are good targets for knockouts or modification of expression level is an open area of research.

Dynamic Control

Dynamic control is a common feature of native metabolic pathways, and allosteric regulation is an important mechanism to maintain flux or limit accumulation of a metabolic intermediate through the pathway in the face of changing conditions. In general, the first enzyme or a key branchpoint of a pathway is downregulated by the pathway's product. For example, the first enzyme in *E. coli's* serine biosynthesis pathway, D-3-phosphoglycerate dehydrogenase (SerA), is allosterically inhibited by serine (Grant et al., 1996). There are few published examples of engineered dynamic control of fluxes in heterologous pathways. However, these systems will become increasingly common as engineered networks become more predictable (Figure 1).

Farmer and Liao (2000) were the first to demonstrate that engineering dynamic control of fluxes could improve yield and productivity of a heterologous pathway. Excess glucose flux and the diversion of carbon to acetate formation reduce the productivity of lycopene-generating *E. coli* cultures. An acetyl phosphate-activated transcription factor and promoter were used as a sensor of excess glucose flux. When acetyl phosphate accumulates inside the cell and is detected by the engineered system, transcription of two genes regulated by the engineered system (*pps* and *idi*) are upregulated, which diverts flux from acetate production to lycopene. The strain with dynamic control of *pps* and *idi* produces titers of lycopene that are 18-fold higher (and comparable improvement to productivity) than those from a strain with constitutive control of the genes.

Growing cultures to an intermediate or high density before inducing product formation is a common practice because it can greatly improve productivity. However using inducer adds cost and potential regulatory hurdles. An alternative is to engineer microbes to sense their own density and activate gene expression at the appropriate time (Kobayashi et al., 2004). By linking a genetic toggle switch to a synthetic quorum sensing system based on the lux system from *Vibrio fischeri*, cells activate gene expression when they reach a threshold density (Kobayashi et al., 2004). At low cell densities the protein of interest is not detectable, at intermediate densities there is a bimodal distribution with the majority of cells not expressing the protein, and at high densities all the cells express the protein. Although this system has been used in the context of protein production, it could easily be applied to metabolic engineering to upregulate an enzyme or pathway.

More recently, Gadkar et al. (2005) have shown that it is possible to use metabolic flux analysis to predict improvements in product formation resulting from dynamic pathway control. Using a metabolic model of a glycerol-producing strain of *E. coli*, the optimal induction time for the glycerol kinase gene, *glpK*, was determined so that growth is maximized in one phase and production maximized in the other phase. Introducing this level of dynamic control of enzyme expression increased biomass, leading to higher glycerol productivity.

The same modeling framework has also been applied to ethanol production by an *E. coli* strain deficient in the lactate biosynthetic enzyme lactate dehydrogenase and expressing a heterologous pyruvate decarboxylase and alcohol dehydrogenase (Gadkar et al., 2005). Knocking out the gene encoding acetate kinase, *ackA*, is known to drastically decrease the growth rate but increase the flux of carbon to ethanol production. The authors compared an *ackA* knockout to the repression of *ackA* expression at an optimal time during the process. The general results are similar to those revealed for glycerol production; a higher initial growth rate allows the culture with a modulated *ackA* to have an increased productivity in experiments of limited duration. This work provides a mathematical framework for dynamically controlling fluxes and demonstrates the advantages of doing so.

Anesiadis et al. (2008) assessed in silico the ability of the dynamic controller devised by Kobayashi et al. (2004) to modulate fluxes in strains engineered to produce ethanol or succinate. In all cases, Anesiadis et al. had the dynamic controller repress expression of one or more genes when cell density reached a threshold. The affinity between LuxR (a quorum sensing transcriptional activator) and its ligand acyl-homoserine lactone is used to tune the threshold cell density. The modulated genes were in competing pathways or consumed an upstream metabolite. With an in silico genome-scale model of *E. coli*, the authors demonstrate that a self-regulating biphasic culture could be obtained and that such a culture could result in increased productivity.

Opportunities for Dynamic Controls

The literature on dynamic control that we have presented provides examples of added layers of regulation that could be integrated with existing heterologous pathways to turn on or off genes at certain stages of the culture. This is a powerful paradigm and for the near term will likely constitute the majority of research on dynamic controls. However there are other ways of applying dynamic controls to metabolic engineering that have not yet been demonstrated.

Mimicking allosteric regulation is difficult to implement for an arbitrary product, in part because protein engineering is not yet sophisticated enough to perform such a task. However it would be easier to implement a feedback loop that modulates the expression level of an upstream enzyme. An engineered aptazyme (a fusion of an aptamer and ribozyme to form an allosteric catalytic RNA) could be used to sense product and accordingly inhibit translation of an enzyme in its metabolic pathway. Alternatively, the dynamic control could forgo

sensing the product and simply attempt to maintain an enzyme at a constant concentration by creating a fusion between the enzyme and a transcriptional inhibitor so that the enzyme autoregulates its own transcription. Such a feedback loop could reduce the noise present in gene expression to give a more constant level of enzyme (Becskei and Serrano, 2000).

One opportunity is to engineer dynamic controls that will respond to cell heterogeneities and "dead zones" present in large-scale industrial cultures. Through scale up, it can be difficult to maintain a homogeneous culture in part due to gradients of carbon source, O_2, pH, or CO_2 in large fermentation vessels (Amanullah et al., 2004). Strains with only static control of flux through the heterologous pathway cannot be optimized for production in all of these different environments. By using dynamic controls it may be possible to partially rescue the productivity of cells while they are in the undermixed regions of the bioreactor. This is a challenging proposition because time constants of the dynamic controls will need to be less than the circulation time of cells in the bioreactor.

One final application for dynamic controls is to enable piecewise optimization of a large pathway. If a strain is designed and optimized to produce a common precursor, then later work to add more genes downstream has the potential to interact with the upstream portion of the pathway and perturb it away from the optimal flux. A system that could sense and respond to the change in flux would allow downstream engineering to proceed with less concern about how additions to the strain might negate previous work.

Conclusions

Synthetic biology has the potential to reshape how heterologous pathways are designed and controlled. Static controls for synthetic pathways are becoming more predictable, and in combination with advances in DNA synthesis, new levels of complexity in pathway design are now feasible. This additional complexity can enable new, longer pathways or can be used to add dynamic controls that can increase productivity and make strains more robust to changing conditions.

REFERENCES

Alper, H., Jin, Y., Moxley, J., and Stephanopoulos, G. (2005a). Metab. Eng. *7*, 155–164.

Alper, H., Miyaoku, K., and Stephanopoulos, G. (2005b). Nat. Biotechnol. *23*, 612–616.

Amanullah, A., Buckland, B.C., and Nienow, A.W. (2004). Mixing in the fermentation and cell culture industries. Handbook of Industrial Mixing: Science and Practice (New York: John Wiley & Sons), pp. 1071–1170.

Anesiadis, N., Cluett, W.R., and Mahadevan, R. (2008). Metab. Eng. *10*, 255–266.

Becskei, A., and Serrano, L. (2000). Nature *405*, 590–593.

Burgard, A.P., Pharkya, P., and Maranas, C.D. (2003). Biotechnol. Bioeng. *84*, 647–657.

Chin, C., Chubukov, V., Jolly, E.R., DeRisi, J., and Li, H. (2008). PLoS Biol. *6*, e146.

Dueber, J.E., Wu, G.C., Malmirchegini, G.R., Moon, T.S., Petzold, C.J., Ullal, A.V., Prather, K.J., and Keasling, J.D. (2009). Nat. Biotech. *27*, 753–759.

Farmer, W.R., and Liao, J.C. (2000). Nat. Biotechnol. *18*, 533–537.

Gadkar, K.G., Doyle III, F.J., Edwards, J. S., and Mahadevan, R. (2005). Biotechnology and Bioengineering *89*, 243-251.

Grant, G.A., Schuller, D.J., and Banaszak, L.J. (1996). Protein Sci. *5*, 34–41.

Kelly, J., Rubin, A., Davis, J., Ajo-Franklin, C., Cumbers, J., Czar, M., de Mora, K., Glieberman, A., Monie, D., and Endy, D. (2009). J. Biol. Eng. *3*, 4.

Kimura, S., Umemura, T., and Iyanagi, T. (2005). J. Biochem. *137*, 523–533.

Kobayashi, H., Kærn, M., Araki, M., Chung, K., Gardner, T.S., Cantor, C.R., and Collins, J.J. (2004). Proc. Natl. Acad. Sci. USA *101*, 8414–8419.

Llaneras, F., and Picó, J. (2008). J. Biosci. Bioeng. *105*, 1–11.

Martin, V.J.J., Pitera, D.J., Withers, S.T., Newman, J.D., and Keasling, J.D. (2003). Nat. Biotechnol. *21*, 796–802.

Nakamura, C.E., and Whited, G.M. (2003). Curr. Opin. Biotechnol. *14*, 454–459.

Pharkya, P., and Maranas, C.D. (2006). Metab. Eng. *8*, 1–13.

Ro, D., Paradise, E.M., Ouellet, M., Fisher, K.J., Newman, K.L., Ndungu, J.M., Ho, K.A., Eachus, R.A., Ham, T.S., Kirby, J., et al. (2006). Nature *440*, 940–943.

Salis, H.M., Mirsky, E.A., and Voigt, C.A. (2009). Nat. Biotechnol. *27*, 946–950.

Schmidt, F.R. (2005). Appl. Microbiol. Biotechnol. *68*, 425–435.

Schoner, B.E., Belagaje, R.M., and Schoner, R.G. (1986). Proc. Natl. Acad. Sci. USA *83*, 8506–8510.

Yoon, S., Lee, S., Das, A., Ryu, H., Jang, H., Kim, J., Oh, D., Keasling, J.D., and Kim, S. (2009). J. Biotechnol. *140*, 218–226.

Zhang, K., Sawaya, M.R., Eisenberg, D.S., and Liao, J.C. (2008). Proc. Natl. Acad. Sci. USA *105*, 20653–20658.