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Model-based Design of Bistable Cell Factories for Metabolic Engineering

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ABSTRACT

Motivation: Metabolism can exhibit dynamic phenomena like bistability due to the presence of regulatory motifs like the positive feedback loop. As cell factories, microorganisms with bistable metabolism can have a high and a low product flux at the two stable steady states, respectively. The exclusion of metabolic regulation and network dynamics limits the ability of pseudo-steady state stoichiometric models to detect the presence of bistability, and reliably assess the outcomes of design perturbations to metabolic networks.

Results: Using kinetic models of metabolism, we assess the change in the bistable characteristics of the network, and suggest designs based on perturbations to the positive feedback loop to enable the network to produce at its theoretical maximum rate. We show that the most optimal production design in parameter space, for a small bistable metabolic network, may exist at the boundary of the bistable region separating it from the monostable region of low product fluxes. The results of our analysis can be broadly applied to other bistable metabolic networks with similar positive feedback network topologies. This can complement existing model-based design strategies by providing a smaller number of feasible designs that need to be tested in vivo.

Availability: http://lmse.biozone.utoronto.ca/downloads/
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1 INTRODUCTION

Living cells are complex systems that possess dynamic properties by virtue of the numerous interactions that occur within the cell and govern their behaviour (Angeli et al., 2004; DasGupta et al., 2007; Siegel-Gaskins et al., 2009; Cornelius et al., 2013; Kolch et al., 2015). Among these interactions, metabolism has relatively fast dynamics compared to either the transcriptional regulatory network or the signaling network. Owing to the fast dynamics, most contemporary models of metabolism are constructed under a pseudo-steady state assumption (Maia et al., 2016; Yilmaz and Walhout, 2017).

These pseudo-steady state models of metabolism are extensively used to predict steady state cellular responses to both environmental and genetic perturbations, and the predictions are subsequently used in design for metabolic engineering applications (Maia et al., 2016).

Typically, designs are obtained through growth coupling of the product fluxes. However, the pseudo-steady state models, that solely rely on reaction stoichiometry, fail to account for the dynamics of metabolic networks (Srinivasan et al., 2015; Costa et al., 2016). Hence, these models cannot be used to study network properties that are responsible for the wide variety of dynamic characteristics exhibited by the cell. Consequently, they cannot reliably predict the impact of dynamics on the design outcomes. Bistability is one of these dynamic characteristics that is widely seen in biological networks. For instance, bistability is seen in the mitogen activated protein kinase (MAPK) signaling pathway (Nguyen et al., 2013), in mitochondrial respiration through the electron transport chain (Selivanov et al., 2009), as well as in the regulation of the lac operon in Escherichia coli (Ozbudak et al., 2004).

There is ample evidence that supports the idea that bistability confers an evolutionary advantage to enable survival of cell populations by facilitating phenotype switching in isogenic cell populations (Lyons et al., 2014b; Kotte et al., 2014; van Heerden et al., 2014; Patra and Klumpp, 2015; Oyarzun and Chaves, 2015). Kotte et al. (2014) demonstrate an example of phenotypic diversity in a single population of E. coli grown on acetate after a substrate shift from glucose. Their work shows the presence of both growing and non-growing sub-populations within the same population. They contend that the ability to switch between phenotypes, due to a bistable metabolic network, enables the cells to survive in environments where substrate availability varies significantly.

However, bistability in metabolism, and the difference it causes in the production characteristics between the stable steady states in a bistable strain, has not been widely studied from the perspective of model-based design. Insights into the bistable characteristics exhibited by metabolic networks using kinetic models have largely focused on the presence of unstable or failure modes in metabolism (Vital-Lopez et al., 2006; Lee et al., 2014; van Heerden et al., 2014). These studies point to the existence of unstable regions wherein the biological system ceases to function and the cell dies. They accordingly emphasize the need to modulate enzyme expression away from these failure modes during design. Recent studies have also utilized the presence of these unstable regions to identify the maximum yields that can be attained in the stable operating region of a metabolic network (Lafontaine Rivera et al., 2017).

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Bistable phenotypes are characterized by distinct concentrations and fluxes in different stable or unstable steady states (Kotte et al., 2014; Vital-Lopez et al., 2006; Lee et al., 2014; van Heerden et al., 2014). Therefore, it is possible for bistable metabolic networks to produce at two different levels: a high production level at one steady state and a low production level at the other steady state (Xiao et al., 2016). Theoretically, it would be possible to control the transition between the steady states in bistable systems, although, the requisite experimental tools may be lacking. Recent studies have explored this idea within the context of cell differentiation, cancer therapeutics and other complex dynamical networks (Cornelius et al., 2013; Wells et al., 2015; Sootla et al., 2016; Xiao et al., 2016).

Due to the complex nature of metabolic and gene regulatory networks, perturbations executed as part of realizing a cell factory design can have an adverse impact on the bistable production characteristics of the cell. These perturbations can be made either to enzyme expression or to enzyme regulation. However, so far no analyses have been done on the impact of these perturbations on the bistable production characteristics of the metabolic network.

The ability to control the transition between steady states, by understanding of factors that cause bistability, will enable engineers to bias design outcomes towards high production phenotypes using model-based design. Therefore, in this paper, our goal is to assess the impact of various design decisions on the capacity of the cell to express one of the two bistable phenotypes. In doing so, we hope to gain insight into the role that bistable metabolic networks can play in cell factory designs.

As the basis for this study, we look at the kinetic model-based design space for a small, representative metabolic network that was experimentally demonstrated to be bistable by Kotte et al. (2014). We analyze the impact of network perturbations on bistability, and show that the most productive cell factory phenotype always occurs on the boundary separating the monostable low production steady state from the bistable steady states of the network. We then propose metabolic network designs that remove network bistability and push the cell either into the low production phenotype, or the high production phenotype.

Fig. 1. The two stable phenotypes for acetate consumption through the gluconeogenic model. a) The high production phenotype which has a high flux throughout the network as a result of a high pep concentration that reduces the inhibition on acetate uptake and b) The low production phenotype that is characterized by a relatively low pep concentration and consequently increased inhibition on the acetate uptake as a result of higher fdp levels.

2 METHODS

2.1 Kinetic network model for gluconeogenesis

Kotte et al. (2014) developed a small scale representation of gluconeogenesis for growth under acetate to elucidate the bistable nature of metabolism in E. coli. We have used a modified form of this network to further study the impact of bistable behavior on metabolic engineering design for production. The original network has four reactions. The uptake of acetate and its subsequent conversion to pep is modeled as a single reaction catalyzed by a super enzyme E (v1 in Figure 1). pep is consumed and converted to fdp through reaction v2 and finally fdp is consumed through fructose biphosphatase (FBP, v3), a positively regulated reaction. In addition to these metabolic fluxes, the model also has two additional reactions for the production and degradation of enzyme E, respectively. The reaction producing E is inhibited by fdp. We have modified this network by adding a reaction v4 to export pep as a target for overproduction (Figure 1). We provide the corresponding kinetic model from Kotte et al. (2014) for completeness.

All occurrences of metabolite or enzyme names in the following mathematical treatment of the network (Figure 1) should be treated as their corresponding concentrations. pep, fdp and enzyme E are dynamically governed by Equations (1-3). The inhibition of E by fdp is incorporated in Equation (3).

\[
\frac{d}{dt} \text{pep} = v_1 - v_2 - v_4 
\]

\[
\frac{d}{dt} \text{fdp} = v_2 - v_3
\]

\[
\frac{d}{dt} E = v_{\text{max}} \left( \frac{1}{1 + \left( \frac{\text{fdp}}{K_{\text{fdp}}} \right)^n} \right) - dE
\]

The consumption of acetate through v1 and the conversion of pep through v2 are expressed in Equations (4) and (5) respectively, using Michaelis-Menten kinetics. The acetate flux through v1 is also governed by the quantity of available enzyme E.

\[
v_1 = k_{\text{v1}}^\text{max} E \frac{\text{acetate}}{\text{acetate} + K_{\text{acetate}}} \]

\[
v_2 = V_{\text{v2}}^\text{max} \frac{\text{pep}}{\text{pep} + K_{\text{pep}}} \]

\[
v_3 = V_{\text{v3}}^\text{max} fdp \left( \frac{1 + fdp}{1 + \left( \frac{\text{fdp}}{K_{\text{fdp}}} \right)^4} \right) \]

The consumption of fdp through FBP (v3) is allosterically regulated and is expressed in Equation (6) using the Monod-Wyman-Changeux (MWC) model for allosterically regulated enzymes. Here fdp refers to the ratio of fdp to its allosteric binding constant $K_{\text{fdp}}$. The export flux $v_4$ for pep is expressed as a linear function of pep in Equation (7).

\[
v_4 = V_{\text{v4}}^\text{max} \text{pep} \]

All parameter values are taken from Kotte et al. (2014) with the value of $V_{\text{v4}}^\text{max}$ initially fixed at 0.2 a.u.

2.2 Continuation and visualization of manifolds for kinetic models

In this section, we give some preliminary mathematical definitions and explain terminology that is used throughout this paper. We provide most of the numerical algorithms used for our analysis in the Supplementary Information.

We modeled our network (Equations 1-3) in MATLAB 2014a (MathWorks, Massachusetts, USA), and used numerical pseudo-arc length
continuation techniques for nonlinear differential equations, facilitated through the command line version of the dynamical systems analysis toolbox MATCONT (Dhooge et al., 2003) in MATLAB to characterize our system. The model Jacobians were calculated using algorithmic differentiation through the ADMAT Toolbox for Automatic Differentiation (Cayuga Research, Waterloo, ON, Canada).

The bifurcation exhibited by our current model is known as a saddle node bifurcation. Saddle node bifurcations are characterized by the presence of at least three equilibrium points or steady states, two of which are stable ($Re(\lambda) < 0$). The unstable equilibrium point or steady state value for our 3-D system has at least one eigen value ($\lambda^s$) such that $Re(\lambda^s) > 0$ and the other eigen values(s) ($\lambda^u$) wherein $Re(\lambda^u) < 0$. As per the Stable Manifold Theorem (Seydel, 2009; Lyons et al., 2014a), the locus of all points whose trajectories reach the saddle node ($x_\nu$) upon forward integration of the model is known as the unstable manifold ($W^u(x_\nu)$). Similarly, the locus of all points whose trajectories will reach the saddle node upon reverse integration of the model is called the stable manifold ($W^s(x_\nu)$). Mathematically, these statements can be written as

$$W^u(x_\nu) = \{ x \in \mathbb{R}^3 | \lim_{t \to +\infty} \phi^t(x) = x_\nu \}$$

$$W^s(x_\nu) = \{ x \in \mathbb{R}^3 | \lim_{t \to -\infty} \phi^t(x) = x_\nu \}$$

where $\phi^t(x)$ refers to the solution trajectory of the system in phase space.

If a system of ordinary differential equations (ODE) can attain more than one steady state, it is said to exhibit more than one mode of behavior. These modes can be distinctly identified in the phase plane diagram. The unstable manifolds are trajectories that separate the two modes of behaviour and divide the phase plane into two distinct basins (or domains) of attraction corresponding to the two stable steady states (Seydel, 2009). These attraction basins dictate the movement of the system from initial value to final steady state as governed by the ODEs in Equations (1-3). Thus, depending on their orientation with respect to the unstable manifold, randomly selected initially values $x(t = 0)$, not on the stable manifold $x(t = 0) \notin W^s(x_\nu)$, can reach either of the two possible steady states. We briefly describe the theory behind the calculation of the manifolds along with the algorithms required to numerically calculate them in the Supplementary Information.

### 2.3 Design perturbations for metabolic engineering

A double negative interaction underlies the positive feedback architecture of the network. We provide a complete picture of this architecture in the Supplementary Information. Altering the enzyme regulation parameters for FBP ($v_3$), $K^{L_3}$ and $K^{L_3P}$, changing the transcriptional regulation of E by fdp ($K^{fure}_d$), or perturbing the enzyme expression parameters for the fluxes $v_1$ ($k^{L_1}_v$), $v_2$ ($V_{\text{max},v_2}$), $v_3$ ($L_3$) and $v_4$ ($V_{\text{max},v_4}$) can bring about changes to the positive feedback loop behaviour. These perturbations can consequently affect the steady states exhibited by the network. We provide a list of the perturbed numerical values and other details regarding the perturbed parameters in the Supplementary Information.

### 3 RESULTS

#### 3.1 Growth-coupled designs cannot be obtained for competing objectives

In contemporary metabolic engineering, strains are engineered to overproduce a specific target metabolite by coupling metabolite production to cell growth. Typically, growth-coupling is also accompanied by the deletion of pathways that are competing with the production related pathways for resources. These overproducing strains are obtained through genetic perturbations (e.g., deletions, over expression or repression of genes) to the metabolic network.

Although this may not always be the case, in bistable metabolic networks such as the one shown in Figure 1, the objectives of cell growth and metabolite production may be mutually exclusive. The mutual exclusivity of objectives can be determined through the elementary flux modes (EFM) of a stoichiometric network (Wortel et al., 2014). In the present case, the EFMs of the stoichiometric model of the network (Figure 1) correspond to either the production of pep-derived products ($v_4$), or to the flow of flux through FBP ($v_3$), which we have assumed to be entirely responsible for growth. Neglecting the fact that these EFMs do not take the regulatory interactions into account, we find our objectives to be competing with each other. The small size of the network notwithstanding, which might be an impediment to the implementation of reaction deletions, we show through a coupling analysis (Klamt and Mahadevan, 2015) that FBP ($v_3$) and $v_4$ cannot be either strongly or weakly coupled to each other (see Supplementary Figure S1). Hence, optimal production of pep-derived products through growth coupling is not possible.

Given the inability to couple fluxes through the deletion of reactions, one might be motivated to explore the possibility of using pseudo-steady state models to determine enzyme over expressions and repressions that might provide an optimal production strain. However, we show in the following sections how failure to account for the regulatory interactions, in studying even the steady state behavior, can lead to unforeseen design outcomes in bistable metabolic networks.

#### 3.2 Perturbations show the presence of an optimal operating point for a bistable cell factory

Studies have pointed to the presence of specific network motifs or regulatory structures within biological networks that can be used to describe the characteristic response of the entire network under certain well defined conditions (Alon, 2007; Angeli et al., 2004; DasGupta et al., 2007). The positive feedback loop is one such motif that can result in bistability (Oyarzán and Chaves, 2015; Angeli et al., 2004; Alon, 2007). As a result of the positive feedback loop present in the network (Figure 1) (see Methods and Supplementary Information), the pep levels, and consequently the production flux ($v_4$) can have two distinct stable steady state values: a high value corresponding to the high production steady state, or a low value corresponding to the low production steady state.

By perturbing some of the interactions that comprise this positive feedback loop, we are able to gain insight into the different designs through which bistable metabolic networks can produce at the highest possible steady state.

One obvious option to increase the production flux is by over expressing the enzyme(s) for $v_4$. In Figure 2a we show the monostable and the bistable regions (for pep concentrations) as a function of both $V_{\text{max},v_4}$ and the acetate levels. From the perspective of Figure 2a it is easy to suggest that designs be based on perturbations selected from within the large monostable high region (blue in Figure 2a). However, the steady state pep concentration and the corresponding flux $v_4$ for different design values of $V_{\text{max},v_4}$, shown in Figures 2b and c, reveal the possibility of obtaining a suboptimal strain that does not produce at the highest possible steady state.

The blue (squares) and the pink (circles) curves in Figures 2b and c are the loci of all possible steady states. The bistable region, with two possible stable steady states, is illustrated by the presence of both the blue and the pink steady state loci that do not coincide. Regions where these loci are coincident indicate the presence of a
either a single high or a single low steady state. We briefly point out some of our observations from this figure.

First, we note that any high steady state (based on Figure 2a) for the chosen acetate concentration (0.786 a.u.) produces pep-derived products at the lowest possible rate through $v_4$ (Figure 2b and c). Second, choosing any design value of $V_4^{max}$ from the high steady state region (depicted in blue in Figure 2a), corresponding to any other acetate concentration that has three possible steady states (two stable and one unstable steady state), will not provide the maximum possible theoretical $v_4$. However, the $v_4$ that is obtained corresponds to the theoretically highest pep concentration at steady state as seen from Figures 2b and c.

In contrast, operating within the bistable regime of $V_4^{max}$ (based on Figure 2) can potentially lead to optimal levels of pep and E that are required to maintain the high production phenotype (Figure 1a) and produce at the theoretically maximum possible rate. We find this optimum at the edge of the bistable region, marked as a red circle in Figures 2b and c. At this point, the network functions at the optimal high production state. Visually, the optimal operating point that occurs at the boundary of the bistable region in Figure 2a is on the edge of a cliff in Figures 2b and c. Any movement away from this edge, due to changes in the design parameter, could result in a precipitous drop to the low production steady states resulting in reduced process productivities.

This illustrates that the position of an optimal operating point in the parameter space of a bistable metabolic network can be determined through a systems analytic approach to metabolic engineering.

Say we refer to a cell operating at the high steady state a success, and a cell operating at the low steady state a failure. Accordingly, the desired operating region lies close to the boundary between success and failure, and the question of system robustness needs to be addressed (Figures 2b and c).

Cellular function has numerous stochastic interactions. This stochasticity is especially prevalent in the transcription and translation machinery that controls gene and protein expression (Klumpp and Hwa, 2014; Lyons et al., 2014; Oyarzúen and Chaves, 2015; Xiao et al., 2016). Since the perturbed design parameter in this case is associated with enzyme expression, a cell operating at the edge of the cliff leading down to the low production state is unlikely to be robust.

We propose that a robust design would make use of the information available on noise in gene and protein expression. Knowing the probability distribution of expression noise, one can estimate the variability (standard deviation) of the noise observed within the cell. A feasible region for the high production phenotype can be estimated on the basis of this standard deviation such that the edge of the feasible region remains at the edge of the cliff, and designs can be selected such that the resulting steady state stays within this feasible region. An illustration of the robust operating space that is within the feasible region is shown as a yellow dot in Figures 2b and c where the dashed line outlines the expected enzyme variability in the operating space. Operating within this region will...
ensure that there is a very low probability that the system will move to the low production state. This is one method to engineer robust strains with bistable metabolic networks. Next, we study how perturbations to the transcriptional regulatory network can change the bistable characteristics of the network.

This interaction is part of the positive feedback loop we mentioned earlier (see Methods, Section 3.2 and Supplementary Information). Hence, this presents an opportunity for design perturbations that will enhance the production of pep-derived products through \( V_4 \) while maintaining the network at the high production phenotype (Figure 1a). Here, the goal is to study the impact of changes in the inhibition constant for \( fdp \) in Equation (3): \( K_e^{fdp} \).

The kinetic model parameter space we analyzed is shown in Figure 3a. Notice that the size of the bistable region varies with changes in the acetate levels. However, unlike perturbations to \( V_4^{max} \) (Figure 2b and c), \( v_4 \) increases monotonically with increase in \( pep \) for increasing values of \( K_e^{fdp} \) (Figures 3b and c).

Hence, from a design perspective, any large value of \( K_e^{fdp} \) may be chosen as long as the said value occurs in the monostable high region in Figure 3a. Furthermore, since the model predicts the lack of any qualitative limitations on the suitable operational values of this parameter, the fluxes and concentrations attained at the highest steady state are theoretically much higher than those predicted for perturbations to \( V_4^{max} \) (Figures 2b and c). Thus, limitations on the maximum value for the transcription factor inhibition constant in the Hill equation (see Methods) for \( fdp \) are only governed by the limits of the physical system. In the context of metabolic engineering, the above observation regarding the absence of limits on the value of \( K_e^{fdp} \) for the existence of phenotype I (Figure 1a) can also be inferred as the substitution of the Cra regulated enzyme responsible for acetate uptake \((v_1)\) with an alternative unregulated enzyme, or through the use of a constitutively expressed promoter to alleviate Cra-dependent inhibition. Such a scenario where the acetate uptake \((v_1)\) is unregulated results in a monostable system expressing phenotype I (Figure 1a).

Alternatively, either increasing the regulation on \( v_1 \) by decreasing \( K_e^{fdp} \), or switching to a more tightly regulated enzyme for acetate uptake can potentially result in the network expressing the low production phenotype (Figure 1b). This is corroborated by the fact that the currently chosen value for \( K_e^{fdp} \) (0.45 a.u.) is at the edge of the bistable region for this parameter (Figure 3a). Hence, the simplicity of this approach of substituting enzymes might be an attractive alternative for experimental implementation, compared to changing \( V_4^{max} \) through enzyme over expression.

3.4 Modifications in allosteric binding constants can also enable movement between phenotypes in bistable systems

Perturbations to enzyme expression through manipulation of gene expression levels, and changes to transcription factor binding are common in metabolic engineering. However, newer methods of perturbing metabolism through the use of protein engineering techniques offer a less cumbersome way to implement cell factory designs in vivo.

Proteins can be engineered to modify the nature of the allosteric interaction between metabolite and enzyme to affect regulation. Protein engineering methods can be used to modify regulatory binding sites on enzymes through changes to amino acid residues to bring about changes in the allosteric activity of the enzyme. In the present scenario, this would involve perturbations to the allosteric activation parameters \( K_s^{Cra} \) and \( L_3 \) for altering the relationship between \( pep \) and FBP \((v_3)\).
Fig. 4. Network response to perturbations in the allosteric activation parameters: enzyme binding parameter ($K_3^{pep}$) and the allosteric factor ($L_3$), and acetate concentration are shown. a) The region of bistability for different combinations of $K_3^{pep}$ and $L_3$, the allosteric regulation parameters that are being perturbed. b) The region of bistability for changes in both $K_3^{pep}$ and acetate concentration for a fixed value of $L_3 = 3 \times 10^3$. The high and low unstable regions are shown in blue and pink respectively.

We summarize our results on the effect of enzyme regulatory parameter changes on the bistable behaviour of the system in Figure 4. In Figure 4a, we show how the size of the bistable region is a function of both $K_3^{pep}$ and $L_3$, the allosteric regulation parameters that are being perturbed.

In Supplementary Figure S2, we show how the cell switches from the high production phenotype to the low production phenotype with increasing $K_3^{pep}$ using a bifurcation diagram (for a chosen value of $L_3$). A low affinity for pep at the allosteric site of $v_3$ (high $K_3^{pep}$) will require increased pep levels to activate FBP ($v_3$). Consequently, at low pep concentrations, the reduction in fdp consumption through FBP ($v_3$) results in increased inhibition on acetate uptake ($v_1$).

Eventually, a lower acetate uptake causes the low production phenotype to be expressed. In contrast, a higher affinity (low $K_3^{pep}$) will reduce the quantity of pep required to activate FBP ($v_3$) which is responsible for consuming fdp. This, in turn, will offset the inhibition on acetate uptake ($v_1$) and result in the high production phenotype becoming more prevalent.

In Supplementary Figure S2, we also show the changes to the bifurcation characteristics of the network in response to changes in $K_3^{pep}$. We see the saddle node bifurcations contract (movement of bifurcation points LP1 and LP2 closer to each other) (Engl et al., 2009; Lu et al., 2006) with respect to changes in the acetate concentrations for increasing $K_3^{pep}$ values.

As in the previous analyses, we show the dependence of network bistability on the chosen parameter ($K_3^{pep}$) and acetate concentrations in Figure 4b. It is important to note that the chosen value of acetate (0.786 a.u.) does not fall within the bistable region in this figure. The fact that the steady state loci of both pep and $v_4$ do not show a bistable region (see Supplementary Figure S3), unlike the ones shown in Figures 2b and c, also supports this observation. Furthermore, we also see that the production flux ($v_4$) changes monotonically with changes in pep.

In summary, keeping $K_3^{pep}$ low can push the network into the monostable high region of the parameter space for a broad range of acetate concentrations (Figure 4b). As mentioned at the beginning of this section, proteins can be engineered to change their regulatory characteristics. However, an experimental methodology to quantify the changes required for fine tuning the allosteric binding constants on enzymes, based on levels suggested by our design, is still an open challenge that needs to be overcome. The alternative of substituting the regulated FBP ($v_3$) with an unregulated enzyme through heterologous expression of the relevant proteins leads to a monostable network expressing phenotype II (Figure 1b).

We have evaluated the effect of perturbations to the enzyme expression levels on network bistability in the Supplementary Information (see Supplementary Figure S4).

4 DISCUSSION

In this work we have analyzed the impact of bistability on the production of a desired metabolite using a small metabolic network that has been experimentally demonstrated to exhibit bistability. In order to maximize the concentration of pep and the production rate of pep-derived products, we find that certain design perturbations have the most optimal points in the high steady state close to the edge of the bistable region. Further, we have also examined different perturbations that will enable the cell to operate at either the high or the low steady state. A summary of all design scenarios that enable production at the theoretically highest possible steady state is given in Figure 5.

Specifically, we have shown that designs employing protein engineering to change either the transcriptional regulatory or the allosteric interaction in the network would be more favorable to obtain high production phenotypes than designs based on enzyme over expression or repression (Figure 5). However, their experimental implementation, and the development of relevant tools are still an area of active research. Alternatively, one could also screen for mutants wherein the relevant enzymes are not regulated. For the presented network, one could screen for possible FBP ($v_3$) mutants that are not regulated to alleviate its impact on the dynamics of the network.

On the other hand, the ineffectiveness of some of these designs is evident from their ability to completely annihilate the boundary between phenotypes through removal of the switch-like behavior and the shift towards monostable low production phenotypes (e.g., over expression of $v_2$). Our results also agree with previous observations that have shown that perturbations to different constituent components of the positive feedback loop that cause bistability can push metabolic networks into their low production states (Sootla et al., 2016; Oyarzún and Chaves, 2015).
Designing Bistable Metabolic Networks

Fig. 5. A summary of all designs proposed and analyzed in this paper increasing the productivity of a bistable metabolic network. In the top panel, we have designs based on perturbations to a) $K_{f\text{dp}}^{e}$ and b) $K_{p\text{ep}}^{e}$. In the bottom panel we have designs based on perturbations to c) $V_{3}^{\max}$, d) $V_{e}^{\max}$, e) $k_{1}^{\text{cat}}$, f) $V_{2}^{\max}$, g) $V_{2}^{\max}$ and h) $k_{1}^{\text{cat}}$, $V_{3}^{\max}$ and $V_{2}^{\max}$ together. The top panel, in blue, covers all perturbations that were proposed on the basis of using protein engineering techniques to implement them in vivo. The bottom panel, in yellow, includes all other designs that rely on changes in enzyme expression levels for implementation in vivo. The right hand side panel shows both the high and low production phenotype along with the legend for colors used in the network diagrams in the left hand side panels. The effect of changing the desired parameter on the bifurcation characteristics of the systems (with respect to acetate concentrations) are also summarized.

We believe these types of dynamic analyses will not only provide more information on the network at hand, but also enable metabolic engineers to propose better designs. Designs that enable movement between phenotypes, or restrict movement within a desired high production phenotype can be proposed by following our method of analyzing network responses to various perturbations. One example where such analyses can be applied is in the case of bistability in glycolysis observed in Saccharomyces cerevisiae (van Heerden et al., 2014). In this scenario, our methodology can be applied to design for a network wherein the imbalanced state that results in cell death does not exist.

By taking a dynamical systems approach to the bistable metabolic network, we have also been able to establish the dependence between initial and final steady states within the context of biological phenotypes using a kinetic model. Asymptotic stability of the steady states associated with this bistable network is only guaranteed within a small neighborhood of the respective steady states. Accordingly, it is possible to define local regions of attraction
for each of the two stable steady states (Supplementary Figure S5-S7). From a metabolic engineering perspective, these regions of attraction define the metabolomic, proteomic and the fluxomic boundary for each phenotype. Any initial phenotype, whose metabolome, proteome and fluxome falls within this boundary, will always reach the corresponding stable phenotype eventually. On the contrary, phenotypes whose states do not fall within these regions of attraction, will never reach the stable steady state they were designed for.

We also account for the uncertainty inherent in biological processes (Klumpp and Hwa, 2014; Lyons et al., 2014b; Oyarzún and Chaves, 2015) in two ways. First, based on the approximate boundaries of the regions of attraction for each production phenotype, we have shown that it is possible to establish a robust range of design outcomes that might asymptotically result in the stable high production phenotype. Second, in scenarios where parameter perturbations have to be robust to the stochastic interactions (e.g., changes to $V_i^{\text{meta}}$) to keep the cell in the high production phenotype, we have proposed establishing a robust boundary in the neighborhood of the optimal point in the parameter space. Thus, our methods for designing robust production strains based on bistable metabolic networks do not depend on changes to the environment to maintain productivity (e.g., changes in acetate to control the production regime). Rather, our in silico designs inherently account for and are theoretically robust to biological uncertainty to a certain degree.

We would also like to point out that there is abundant literature pertaining to the design of bistable systems to control their output at the desired state. One example is the proposal by Sootla and co-workers (Sootla et al., 2016) to use a pulse-based control methodology to control outputs from bistable systems seen within the context of biology (e.g., the genetic toggle switch). Another example is that of Wells et al. (2015), wherein the strategy to dictate transitions between regions of attraction in biological systems uses an optimal control methodology that takes advantage of biological noise. Methods have also been proposed to determine robust operating parameters for bifurcating systems (Lu et al., 2006; Mönnigmann and Marquardt, 2002). The design of robust production strains with bistable metabolic networks could potentially be a novel application of these methods.

Although the model used in this paper reduces the regulatory interactions involved in glycolysis (Kotte et al., 2010; Kochanowski et al., 2017; Long et al., 2017) to pep, fdp and the super enzyme E (Kotte et al., 2010, 2014), this model offers a starting point to account for the inherently complex regulatory network governing both glycolysis and gluconeogenesis. As more regulatory interactions are discovered (Enjalbert et al., 2015; Kochanowski et al., 2017), we believe future modeling efforts incorporating these interactions will not only facilitate better design, but will also uncover the various nuances of these interactions.

Furthermore, issues of scale with respect to the network size used for our analysis can be addressed through the use of motif detection (Kashan et al., 2004; Wernicke and Rasche, 2006) and network reduction techniques (Angeli et al., 2004; DasGupta et al., 2007). These methods can be applied to identify relevant network structures that contribute towards a variety of dynamic responses, and subsequently formulate small networks/motifs with the necessary topology to exhibit desired dynamic characteristics. Feng et al. (2016) and Karin and Alon (2017) are two such examples where small networks/motifs are used to elucidate the molecular nuances of larger networks. The small networks or motifs can be subject to the analyses that we have presented here.

### 5 CONCLUSION

We have shown that accounting for metabolic network dynamics and regulation in model-based design expands the scope of possible design perturbations that can be leveraged to change the bistable characteristics of the metabolic networks. Specifically, it is evident from our analyses that parameters that control the positive feedback interconnection between acetate uptake and pep provide ample areas for design modifications to control production outcomes in bistable cell factories. Broadly, our results reveal the potential existence of optimal operating points in bistable metabolic networks with similar positive feedback network topologies. Our work lays the foundation for exploring the application of different in silico methodologies for the design of bistable metabolic networks for cell factory production.

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